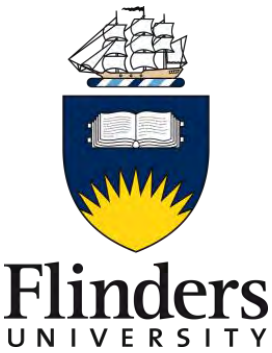

The Flinders University of South Australia

Peroxiredoxins and Glutathione Peroxidases in the Fertility of Yellowtail Kingfish (*Seriola lalandi*)

A thesis submitted in partial fulfilment of the requirements for the award of Honours Degree of Bachelor of Science in Marine Biology at Flinders University of South Australia

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Table of Contents

Declaration

Candidate:

"I certify that this document does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any University; and that to the best of my knowledge does not contain any material previously published by another person except where due reference is made in the text"

_____

Andrew Scholefield

Supervisor:

"I believe that this thesis is properly presented, conforms to the specifications for the thesis and is of sufficient standard to be, *primafacie*, worthy of examination"

_____

Associate Professor Kathy Schuller

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Finally, to my God and my saviour Jesus Christ, without whom I would have nothing, and from whom I have been gifted with the privilege to study and the abilities to achieve all that I have.

Summary

This study investigated the distribution of the expression of selected members of the peroxiredoxin (Prx) and glutathione peroxidase (GPx) antioxidant enzyme families in yellowtail kingfish (YTK, *Seriola lalandi*), an important finfish aquaculture species in South Australia. The purpose of investigating the expression of these enzymes was to determine if they played a role in male fertility in fish, as both these families play a role in male fertility in mammals and individual members of these protein families were shown to be highly conserved between mammals and fish.

It was shown that multiple Prx proteins could be detected in YTK they and were discovered to be almost ubiquitously expressed in various organs. A specific sub-class of Prx proteins, Prx 4, was detected in abundance in YTK liver and also showed expression in YTK intestine. GPx 4 was detected in YTK liver but the results for GPx 4 were inconsistent and no firm conclusions could be drawn.

Further analysis was conducted for the Prx proteins found that two monomeric forms at 22 kDa and 25 kDa could be detected in brain, liver, kidney, testes and gill of a YTK male. Furthermore two monomeric Prx 4 proteins were detected at 25 kDa and 29 kDa in liver and the latter size was also detected in intestine, testes and gill. Since two different sized isoforms are known to occur in male gonad tissue in mammals, these findings indicate that Prx 4 could play a role in male fertility in fish, as is seen in mammals, but is likely to have a more diverse role in fish.

This study has made a significant contribution to our understanding of the expression of Prx and GPx antioxidant proteins in YTK, a commercially important aquaculture species in South Australia and it will provide a strong basis for future research into the roles of these important proteins in fertility in fish and other vertebrates.

Abbreviations

Cys	Cysteine
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
FMC	Flinders Medical Centre
GPx	Glutathione Peroxidase
H_2O_2	Hydrogen Peroxide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
kDa	Kilodalton
MEGA	Molecular Evolutionary Genetics Analysis
NCBI	National Centre for Biotechnology Information
NKEF	Natural Killer Enhancer Factor
$O_2^{\cdot-}$	Superoxide Radical
$\cdot OH$	Hydroxyl Radical
$\cdot OOH$	Hydroperoxyl Radical
Prx	Peroxiredoxin
PUFA	Polyunsaturated Fatty Acid
ROOH	Lipid hydroperoxide
ROS	Reactive Oxygen Species
SBT	Southern Bluefin Tuna
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
Sec	Selenocysteine
Trx	Thioredoxin
YTK	Yellowtail Kingfish

Chapter 1 - Introduction

1.1 Overview

Life in the oxygen-rich environment of the Earth has been forced to evolve protection against the potential toxicity of reactive species derived from molecular oxygen (O_2) (Gutteridge and Halliwell, 1990). The main form of defence which is conserved across all aerobic life forms involves antioxidant enzymes (Gutteridge, 1995). Of particular interest to this study are the peroxiredoxin (Prx) and glutathione peroxidase (GPx) families of antioxidant enzymes. Previous studies in mammals have revealed that members of both of these families, specifically Prx 4 and GPx 4, play important roles in male fertility (Imai et al., 2009; Iuchi et al., 2009; Schneider et al., 2009). However, there has been limited investigation of the roles of these proteins in fish. Therefore, the overall aim of this project was to investigate the tissue distribution of the expression of Prx 4 and GPx 4 proteins in yellowtail kingfish (YTK, *Seriola lalandi*). The ultimate purpose of this was to determine any possible roles these proteins may play in male fertility in this important aquaculture species. In particular, we hypothesised that both Prx 4 and GPx 4 protein expression would be higher in testes than in any other organs as had been observed in studies with mammals (Imai et al., 2001; Iuchi et al., 2009).

1.2 Oxidative stress and reactive oxygen species

Many cellular processes in eukaryotes require molecular oxygen (O_2) to function correctly (Gutteridge and Halliwell, 1990). Processes that use oxygen, such as respiration in the mitochondria involve the reduction of O_2 to water (Gutteridge, 1995). However, electron leakage can occur at various stages during this process and this can lead to the partial reduction of O_2 as follows:

$$O_2 + e^- \rightarrow O_2^{\cdot -}$$

$$O_2^{\cdot -} + e^- + 2H^+ \rightarrow H_2O_2$$

$$H_2O_2 + e^- + H^+ \rightarrow \cdot OH + H_2O$$

These three reactions produce the superoxide radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($\cdot OH$), which are all categorised under the broad term of reactive oxygen species (ROS) (Mourente et al., 2007). ROS can also have exogenous origins and can be introduced into the body from sources such as the environment or food (Janssens et al., 2000). The toxicity of ROS is well documented with high levels of ROS in the cell increasing oxidative stress, leading to DNA damage, protein damage and lipid peroxidation (reviewed in Tocher et al., 2002). Furthermore, ROS have been implicated in ageing and the preliminary stages of many diseases (Janssens et al., 2000). Therefore, as a result of the detrimental effects of ROS, anti-oxidant defences have evolved to remove them (Mourente et al., 2007). The balance of pro-oxidant and anti-oxidant processes determines the level of ROS and thus the level of oxidative stress (Mourente et al., 2007)

1.3 Lipid peroxidation

Lipids, especially polyunsaturated fatty acids (PUFA), are highly susceptible to oxidative attack by ROS, in particular $\cdot OH$, which abstracts a hydrogen atom from the lipid hydrocarbon chain, yielding a lipid radical (Halliwell and Chirico, 1993). This lipid radical undergoes molecular rearrangement before reacting with O_2 to form a lipid peroxyl radical, which can then abstract a hydrogen atom from an adjacent lipid to form a lipid hydroperoxide (ROOH) and propagate a lipid peroxidation chain reaction (Fig 1.1; Gutteridge and Halliwell, 1990). The peroxidation of lipids by ROS can lead to secondary products such as aldehydes,

which contribute to the rancid flavour in oxidatively deteriorating meat (Sevanian and Hochstein, 1985; Gutteridge and Halliwell, 1990). Furthermore, lipid peroxidation can result in damage to cell membranes, leading to reduced function of membrane proteins, increased membrane permeability and possibly apoptosis (Mourente et al., 2007).

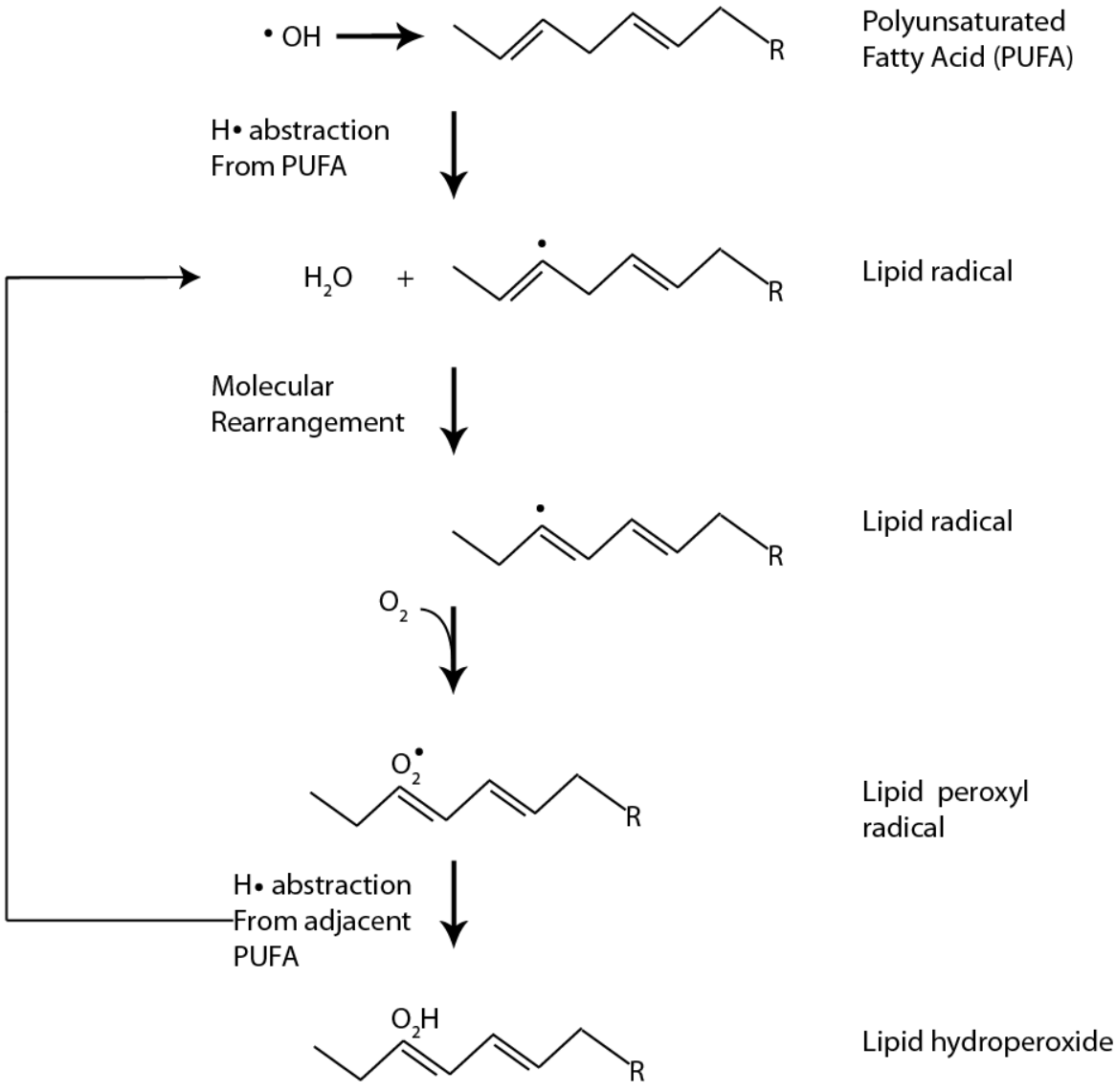


Figure 1.1: The mechanism of peroxidation of lipids, leading to a lipid peroxidation chain reaction. Figure adapted from Gutteridge and Halliwell (1990).

1.4 Antioxidant protection

The evolution of antioxidant defences against ROS is seen in all forms of life that have adapted to an oxygen-rich environment. Antioxidant defences include dietary antioxidants such as vitamin C and vitamin E, and endogenously produced antioxidant enzymes such as catalase, superoxide dismutase (SOD), peroxiredoxins (Prx) and glutathione peroxidases (GPx) (Tocher et al., 2002; Mourente et al., 2007). These antioxidant enzymes are expressed in nearly all forms of life and are highly conserved, suggesting that they have essential functions (Fujii and Ikeda, 2002; Toppo et al., 2008). SOD enzymes target superoxide radicals and catalases target hydrogen peroxide (Mourente et al., 2007), whereas Prx and GPx enzymes target hydrogen peroxide or lipid hydroperoxides, reducing them to water or the corresponding alcohol, respectively. This prevents hydrogen peroxide forming a hydroxyl radical or lipid hydroperoxides forming lipid peroxyl radicals which are highly reactive and damaging to cellular molecules, especially unsaturated fatty acids and lipids (Fujii and Ikeda, 2002; Yant et al., 2003). The Prx and GPx families have also been implicated in various developmental pathways in mammals and will be the focus of this study.

1.4.1 Peroxiredoxins

The defining feature of the peroxiredoxin (Prx) family is the presence of the peroxidatic cysteine residue (Cys-S_PH) in the active site which catalyses the reduction of H_2O_2 or complex hydroperoxides to water or the corresponding alcohols, respectively, and at the same time becomes oxidized to a Cys sulfenic acid residue (Cys-S_POH). (Wood et al., 2003). Peroxiredoxins are peroxidases, which are dependent on thioredoxin (Trx), glutathione (GSH) or other reducing agents to return the peroxidatic Cys residue to its reduced state at the end of the catalytic cycle (Fujii and Ikeda, 2002). Prx proteins can be divided broadly into

two classes: 1-Cys Prx, which contain only the peroxidatic Cys residue, and 2-Cys Prx which contain a resolving Cys residue (Cys-S_RH) as well as the peroxidatic Cys residue (Wood et al., 2003). This second conserved Cys residue is known as the resolving Cys because it is involved in the recycling of the peroxidatic Cys via a reduction reaction involving thioredoxin (or other reducing agents), hence the previous term for Prx proteins which was thioredoxin peroxidase (Wood et al., 2003). The majority of research interest has been targeted at the 2-Cys Prx proteins and they will be the focus of this study.

There are currently four subclasses of 2-Cys Prx proteins which have been defined and they are simply named Prx 1, Prx 2, Prx 3 and Prx 4 (Wood et al., 2003). Each of these subclasses has been well defined and characterised in humans and other mammals (Fujii and Ikeda, 2002; Wood et al., 2003). While limited studies have been conducted in fish, the nucleotide sequence and predicted amino acid sequence of each is known for zebrafish and various other fish species. Previous work in our group has resulted in successful cloning and functional characterisation by recombinant expression in *E. coli* of Prx 4 from YTK and Prx 2 from Southern Bluefin Tuna (SBT; Loo and Schuller, 2010; Sutton et al., 2010, respectively). Of particular interest to this study is the Prx 4 subclass.

Previous studies with mammals have shown that Prx 4 proteins are expressed at higher levels in the testes of rodents relative to other major organs (Iuchi et al., 2009), suggesting a role in male fertility. Prx 4 proteins are secreted via the Golgi apparatus as a 25 kDa protein in mice (Iuchi et al., 2009) and a 27 kDa protein in rats (Okado-Matsumoto et al., 2000; Sasagawa et al., 2001). In addition, there is a second, larger form of Prx 4, 27 kDa in mice (Iuchi et al., 2009) and 31 kDa in rats (Okado-Matsumoto et al., 2000; Sasagawa et al., 2001), found only

in testes. However, the most convincing evidence for a role for Prx 4 in male fertility comes from a recent gene knockout experiment in mice. In this experiment, a knockout of the Prx 4 gene increased oxidative stress and apoptosis in spermatogenic cells and decreased the number of sperm produced by half, however, the sperm that were produced were functionally normal (Iuchi et al., 2009). In addition, the testes of the Prx 4 knockout mice were significantly smaller, but the sizes of all other organs were unaffected (Iuchi et al., 2009). This indicates a role for Prx 4 in the differentiation of primordial male germ cells into mature spermatozoa.

1.4.2 Glutathione Peroxidases

The glutathione peroxidase (GPx) family has been widely studied and family members have been found in nearly all forms of life (Ursini et al., 1995; Toppo et al., 2008). In mammals, there are currently seven characterised proteins in the GPx family and the addition of an eighth member has been proposed (Toppo et al., 2008). Proteins from the GPx family reduce hydrogen peroxide or lipid hydroperoxides utilising reduced glutathione (GSH) as the electron donor (Takebe et al., 2002). The GPx family can be broadly divided into two groups. GPx 1, 2, 3 and 4 in mammals have a selenocysteine (Sec) residue in the active site, whereas GPx 5, GPx 7 and GPx 8 have a cysteine residue. GPx 6 is an exception, containing a Sec residue in humans but a Cys residue in rodents (Toppo et al., 2009).

We are particularly interested in the Sec-containing GPx proteins as these have been widely studied and, with the exception of GPx 2, have been extensively characterised in mammals (Toppo et al., 2009). Specifically, GPx 4 has been shown to be important in mammalian

spermatogenesis with roles in both structure (Ursini et al., 1999) and motility of sperm (Schneider et al., 2009). GPx 4, originally named phospholipid hydroperoxide glutathione peroxidase or PHGPx, is the only known antioxidant enzyme that can accept phospholipid hydroperoxides as substrates (Ursini et al., 1985; Toppo et al., 2008). Phospholipids are abundant in cell membranes and therefore GPx 4 (PHGPx) may have a special role to play in protecting cell membranes. In mice and other mammals, GPx 4 is present as 3 different isoforms, nuclear GPx 4 (nGPX 4) cytosolic GPx4 (cGPx 4) and mitochondrial GPx 4 (mGPx 4), which are all coded for by the same gene (Pushpa-Rekha et al., 1995; Maiorino et al., 2003). The GPx 4 gene contains two alternatives for the first exon with separate promoter sites for each (Maiorino et al., 2003). If exon 1A is translated then cytosolic or mitochondrial GPx 4 is produced but if exon 1B is translated then nuclear GPx 4 is produced. The first 84 N-terminal amino acids of nuclear GPx 4 are different from mitochondrial GPx 4 but the remainder of the proteins is identical (Maiorino et al., 2003). The cytosolic and mitochondrial forms are translated from the same mRNA but they are differentiated by alternative start codons (Pushpa-Rekha et al., 1995). A GPx 4 gene knockout mutant in mice, which removes all three isoforms, has been shown to be embryo lethal (Yant et al., 2003).

GPx 4 has also been shown to be essential for correct male reproductive development, playing a role in the function and motility of spermatozoa (Imai et al., 2009; Schneider et al., 2009). GPx 4 is highly expressed in the mitochondria at the mid-piece of spermatozoa and Imai et al. (2009) found that a spermatocyte-specific GPx 4 gene knockout resulted in increased oxidative stress, decreased testicular size and a 76% decrease in sperm motility. Fertilization success from these sperm in an *in vitro* fertilisation assay was zero. Schneider et al. (2009), conducted a similar study but with an mGPx4 knockout and found very similar results. These gene knockout studies confirmed the earlier work of Ursini et al. (1999) who

had found that GPx 4 had a role as a structural protein in the mitochondrial capsule of sperm. The mitochondrial capsule is a complex of keratinous fibres which embeds the mitochondria in the mid-piece of spermatozoa and the presence of GPx 4 in the capsule suggested a specific role for mGPx4 in sperm development. Schneider et al. (2009) took the analysis of fertilisation success one step further by discovering that *in vitro* fertilisation by direct injection of mGPx4 knockout sperm into an unfertilised egg could produce viable embryos. This further supported the notion that reduced sperm motility was the determining factor in the reduced fertility of mGPx 4 knockout mice.

1.5 Prx 4 and GPx 4 have similar roles but contrasting mechanisms

The results from the studies discussed in Section 1.4 show that Prx 4 and GPx 4 both play important roles in male fertility in mammals but their sites of action are different. On the one hand, Prx 4 is expressed at high levels in spermatogenic cells and protects them against oxidative stress allowing the production of viable sperm (Iuchi et al., 2009). On the other hand, GPx 4 is a structural protein of sperm which is essential for their normal development after they have been produced by the spermatogenic cells (Imai et al., 2009; Schneider et al., 2009).

1.6 Antioxidant enzymes in fish

In contrast to the wealth of information available regarding the structure and function of Prx and GPx proteins in mammals, very little is known about these proteins in fish. Fish flesh is especially rich in the omega-3 (n-3) long chain polyunsaturated fatty acids (LC-PUFA), docosahexanoic acid (DHA) and eicosapentanoic acid (EPA) that are highly susceptible to

lipid peroxidation because they are highly unsaturated with a large number of double bonds (Mourente et al., 2007). The n-3 LC-PUFA are produced by phytoplankton and accumulate in the food web (Moreno et al., 1979). The result of this is that higher order predators such as marine finfish contain very high levels of n-3 LC-PUFA, especially in their cell membranes (Sargent et al., 1999). It is important to maintain the high levels of these fatty acids in cultured fish as a human diet rich in these fatty acids has well-documented health benefits (Calder and Yaqoob, 2009). A review conducted by Connor (2000) summarised that a diet high in n-3 PUFA is required for correct brain development in childhood as well as being associated with a reduction in the risk of cardiovascular disease (CVD), breast, colon and prostate cancers and autoimmune disorders. The most researched benefit is reduction in CVD and some studies have found that populations with high fish intake have lower rates of CVD despite higher rates of smoking and obesity (Dewailly et al., 2003). This makes the consumption of foods that are high in n-3 LC-PUFA, such as fish, all the more important (Sargent et al., 1999).

Since antioxidant enzymes may play a role in protecting n-3 LC-PUFA against oxidative attack by ROS, they may also have an important role to play in maintaining the human health benefits gained from consuming fish. Furthermore, due to the higher levels of n-3 LC-PUFA found in fish than in terrestrial animals, the importance of antioxidant enzymes is potentially greater, particularly in marine fish species which generally have higher levels of n-3 LC-PUFA than fresh water fish species (Tocher et al., 1989). Previous work in our group has resulted in the successful cloning and, in some cases, functional characterisation of GPx 1, GPx 4, Prx 1, Prx 2 and Prx 4 cDNAs from the liver or muscle of either southern bluefin tuna (*Thunnus maccoyii*) (GPx 1, GPx 4, Prx 2) or yellowtail kingfish (*Seriola lalandi*) (Prx 1, Prx 4) (Thompson et al., 2006; Loo and Schuller, 2010; Sutton et al., 2010; Thompson et al.,

2010). These studies have been helpful in understanding the similarities of fish antioxidant enzymes to those of mammals. However, to our knowledge, no studies have yet been conducted to investigate the possible roles of these antioxidant enzymes in fertility in fish. Understanding the possible roles of Prx and GPx proteins in fish fertility may benefit the growing fish aquaculture industry in that it may provide information to improve the management and productivity of broodstock.

1.7 Aquaculture

It has been estimated that 68% of marine capture fisheries are fully or over-exploited (Garcia and Grainger, 2005). Aquaculture is an alternative to capture fisheries, and while capture fisheries production is remaining static, aquaculture production is consistently exhibiting growth (FAO, 2010). However, the majority of this growth is in lower trophic level marine species or freshwater fish, with marine finfish only contributing 1-2% of total finfish production (FAO, 2010). Furthermore, combined capture fisheries and aquaculture production of marine finfish has not significantly increased since 1986 (FAO, 2010) and capture fisheries are believed to already be at capacity (Garcia and Grainger, 2005). As a result, aquaculture has the potential for substantial growth and will contribute strongly towards meeting increased demand for marine fish in the future (Turchini et al., 2009).

True aquaculture involves captive-breeding in contrast to "ranching" which is the practice of taking fish from the wild and 'on-growing' or 'fattening' them in captivity. A major challenge to producing captive-bred finfish is maintaining healthy broodstock, both male and female, to maximise the number of offspring produced. To maintain healthy broodstock, it is

necessary to optimize nutrition (Watanabe and Vassallo-Agius, 2003) and prevent diseases (Lee and Ostrowski, 2001), thereby maximising reproductive output. Therefore, understanding the factors that affect nutrition, disease and reproductive output is vital for the growth of the marine finfish aquaculture industry.

1.8 Effects of oxidative stress on spermatogenesis and fertility in fish

The interactions between oxidative stress, spermatogenesis and fertility in fish have been extensively studied, mostly to assess the responses of fish to environmental contamination with heavy metals or other pollutants. For example, Al-Salahy (2011) found that catfish exposed to mercuric chloride at a level of 150 μg per litre of water showed 3 times higher levels of peroxides in ovaries than control fish, indicating high susceptibility of this tissue to oxidative stress and a link with female fertility. In another study, Celino et al. (2009) investigated the effects of arsenic on cultured cells from Japanese eel testicular tissue and found that arsenic levels as low as 0.1 μM inhibited germ line proliferation. Furthermore, arsenic toxicity resulted in increased concentrations of ROS which were associated with increased apoptosis in spermatogenic cells (Celino et al., 2009).

There is evidence that the genes/proteins and hormones involved in various reproductive processes are highly conserved between fish and mammals. For example, Celino et al. (2009) showed that human chorionic gonadotropin could stimulate proliferation of Japanese eel testicular cells. Similarly, Keen et al. (2008) showed that the KiSS1/GPR54 system plays a role in puberty in Rhesus monkeys and Elizur et al. (2009) found that the KiSS1/GPR54 system regulates fish reproduction in a manner very similar to that seen in mammals. These

studies provide evidence that the regulation of reproduction is highly conserved between mammals and fish and this provides confidence that antioxidant enzymes may play similar roles in fish spermatogenesis and sperm viability as they do in these processes in mammals.

1.9 Conclusions and aims of the study

The literature presented here indicates that antioxidant enzymes play important roles in protecting cells against the deleterious effects of oxidative stress caused by ROS. Furthermore, due to the high levels of n-3 LC-PUFA in fish, the need for efficient functioning of antioxidant enzymes in fish, especially marine fish, may be greater than in terrestrial animals. There is a wealth of information available regarding the roles of the GPx and Prx antioxidant enzyme families in mammals and, in particular, Prx 4 and GPx 4 have been shown to positively affect fertility, by protecting spermatogenic cells and spermatozoa, respectively. The long term success of the fish aquaculture industry is dependent on the fertility of broodstock. Therefore understanding the factors that affect fish fertility is important. There is evidence that at least some of the mechanisms of regulation of reproduction are conserved between fish and mammals. Therefore, here it is proposed to characterise the roles that GPx and Prx family members, particularly GPx 4 and Prx 4, may play in the fertility of male fish. The results of this study will provide information that may lead to strategies to better manage and improve the health and reproductive success of aquaculture broodstock, thus increasing the productivity of the industry.

The specific aims of this study were to:

1) use bioinformatics to investigate the conservation of Prx and GPx proteins between mammals and fish with a particular focus on the commercially important aquaculture species yellowtail kingfish (YTK, *Seriola lalandi*)

2) use immunoblotting to determine whether the antibodies we had at our disposal which had been raised against human Prx 4 and GPx 4 proteins would cross-react with related proteins in YTK and southern bluefin tuna (SBT, *Thunnus maccoyii*), another commercially important aquaculture species.
3) investigate the tissue distribution of expression of Prx and GPx proteins (especially Prx 4 and GPx 4) in YTK in order to test the hypothesis that these proteins are expressed at higher levels in the testes of fish as has been found in mammals.
4) investigate whether a second isoform of the Prx 4 protein which is present in the testes of sexually mature mammals is also present in the testes of sexually mature YTK in order to provide preliminary evidence that Prx 4 has a role to play in male fish fertility as it does in male mammal fertility.

Chapter 2 – General methods

2.1 General chemicals

All general chemicals used in this project and suppliers are listed below:

Chemical	Supplier
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Sigma
Acrylamide-Bis (40%) 37.5:1 solution	Fluka Analytical
Ammonium persulphate	Sigma
β-mercaptoethanol	Sigma
Bromophenol blue	Sigma
Coomassie Blue R-250	Bio-Rad
Dithiothreitol (DTT)	Astral Scientific
Ethanol (99-100%)	Sigma
Ethylenediaminetetraacetic acid (EDTA)	Sigma
Glacial Acetic Acid	Chem Supply
Glycerol	Sigma
Glycine	Chem supply
Hydrochloric acid (HCl)	Ajax Finechem
Dipotassium phosphate (K_2HPO_4)	Sigma
Monopotassium phosphate (KH_2PO_4)	Sigma
L-glutathione, reduced (GSH)	Sigma
Methanol	Merck Scientific
Polymethylsulfinyl fluoride (PMSF)	Sigma
RNA*later*®	Ambion Inc.
Sodium Chloride (NaCl)	Chem supply
Sodium Dodecyl Sulphate (SDS)	Amresco
Sodium hydroxide (NaOH)	Ajax Finechem
Tetramethylethylenediamine (TEMED)	Sigma
Tris	Sigma
Tris-HCl	Sigma
Tween® 20	Sigma
Urea	Sigma

2.2 Tissue sampling

2.2.1 Yellowtail kingfish obtained from SARDI Aquatic Sciences

Four-month old Yellowtail Kingfish (YTK, *Seriola lalandi*), weighing approximately 400 g each, were provided by Dr David Stone (SARDI Aquatic Sciences, West Beach, Adelaide). The fish were euthanized with a lethal dose of Aqui-S® and transported to Flinders University on ice. The fish were dissected and samples were taken of brain, liver, muscle, heart, intestine and gill tissue and placed in RNA*later*®. The tissues were left for >30 min to absorb the RNA*later*® and then stored at -20°C.

2.2.2 YTK obtained from Clean Seas Tuna Ltd.

Twelve- and eighteen-month old YTK weighing approximately 1.5 kg and 3 kg each, respectively, were provided by Clean Seas Tuna Ltd. from the YTK aquaculture facility at Arno Bay, Eyre Peninsula, South Australia. The size of these fish ranged from 47 cm to 49 cm for the 1.5 kg fish and 59 cm to 65 cm for the 3 kg fish. The fish were measured, sexed and gutted on site and samples were immediately taken from brain, liver, muscle, heart, intestine, spleen, kidney, gonad and gill tissue. These samples were cut into pieces of approximately 0.3 cm^3 and placed in RNA*later*® or cut into pieces of approximately 0.5 cm^3 and frozen in dry ice. The tissue samples were transported back to Flinders University and the samples stored in RNA*later*® were placed at -20°C and the frozen samples were placed at -80°C.

2.3 Protein extraction

2.3.1. Extraction of the YTK samples from SARDI Aquatic Sciences and other samples

A number of 10 mL tubes were prepared and labelled. Tissue samples were removed from RNA*later*® and dabbed dry on a Kimtech® absorbant tissue. The sample was then weighed and placed immediately into the appropriate 10 mL tube. A 2× to 10× volume of extraction buffer was then added to the tube. Various extraction buffers were used for these samples and are described in detail in Chapter 4. The tissues were then disrupted using a Polytron® tissue homogeniser and then the homogenate was placed in a Sigma 3-16 PK centrifuge at maximum speed (1,385 *g*) for 20 min. The supernatant was collected and frozen at -20°C.

2.3.2 Extraction of YTK samples from Clean Seas Tuna Ltd.

A number of 10 mL tubes were prepared and labelled and 300-1000 µl of extraction buffer was added to each tube (extraction buffers described in detail in Chapters 4 and 5). The weight of the tube was recorded before tissue samples were removed from RNA*later*® and dabbed dry on a Kimtech® absorbant tissue or removed from the -80°C freezer and added immediately to the extraction buffer in the appropriate tube. The tube was then weighed again to determine tissue wet weight and extraction buffer up to a 5× volume was added to the tube. The tissues were then disrupted using a Polytron® tissue homogeniser before being placed in a Sigma 3-16 PK centrifuge at maximum speed (1,385 *g*) for 20 min. The supernatant was collected and placed into 1.5 mL tubes and centrifuged for a further 10 min at 15,000 *g* in a Beckman-Coulter Microfuge® 16 microcentrifuge at 4°C. The volume of supernatant was measured and then it was stored at -20°C in 100 µl aliquots. Any undissolved material was dabbed dry using a Kimtech® absorbant tissue and weighed. The volume of supernatant per

mg of tissue wet weight was calculated using the following equation: $= \frac{V_S}{(W-M)}$ where Vs = volume of supernatant, W = tissue wet weight and M = weight of undissolved material.

2.4 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

A sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) apparatus was set up and for each gel; two glass plates were separated with 1 mm spacers. A plastic comb for 10 wells was inserted and the depth of the comb was marked on the glass plates. The comb was then removed and two parallel SDS-PAGE resolving gels (Table 1.1) were prepared and poured in between the glass plates to approximately 0.5-1 cm below the bottom of the comb. The resolving gels were covered with iso-propanol and allowed to set for 10-15 minutes. After the resolving gel was set, the iso-propanol was poured off and excess was removed by dabbing with filter paper. Two stacking gels (Table 1.2) were then prepared and poured on top of the set resolving gels and the plastic comb was inserted for each and the gels were left to set for at least 15 minutes. The gels were then transferred to the SDS-PAGE electrophoresis tank and the tank was filled with SDS-PAGE running buffer (Table 1.3). Extracted protein solutions were mixed with an equal volume of 2× SDS-PAGE loading buffer (Table 1.4) and heated at 95°C for 5 minutes. The heated extracts were clarified by centrifugation in a benchtop centrifuge for 5 minutes at 14,000 *g* and the appropriate volume of supernatant was loaded onto the gel (loading volumes described for each protocol in Chapters 4 and 5). The SDS-PAGE gels were then run at 45-50 mA for 45-55 minutes or until the bromophenol blue tracking dye reached the bottom of the gel.

One of the gels was taken and stained for 2 h in Coomassie Brilliant Blue R-250 stain (Table 1.5) and then destained in SDS-PAGE gel destain (staining solution minus Coomassie Brilliant Blue R-250). The destain solution was poured off after approximately 1 hour and

fresh destain was added and the gel was left on an orbital shaker to destain overnight. The following day, the stain was changed at least twice more before the gel was removed from the destain solution, sealed in a plastic sleeve with ultra-pure water and photographed.

2.5 Immunoblot Analysis

The second gel from Section 2.4 was arranged into an immunoblotting apparatus and immersed in immunoblotting transfer buffer (Table 1.6). The configuration of the immunoblotting apparatus was as described in Maniatis et al. (2001). The components were sandwiched together and placed into a Bio-Rad Mini Trans-Blot® cell immunoblotting electrophoresis tank and the proteins were transferred from the gel to the nitrocellulose membrane at 200 mA for 60 min. The nitrocellulose membrane was then placed in a small container and incubated in 15-25 mL of blocking buffer (Table 1.7) at room temperature for 1 hour on an orbital shaker. The blocking buffer was poured off and the membrane was transferred to a plastic sleeve and 10 mL of a 1:1000 dilution of primary antibodies in blocking buffer was added. The plastic sleeve was then sealed and placed at 4°C on a rocking shaker overnight. The membrane was then placed back into the plastic container, washed 5 × 5 minutes with washing buffer (blocking buffer minus skim milk powder) and incubated with a 1:1000 dilution of goat-anti-(rabbit-IgG) secondary antibody conjugated to horseradish peroxidase (Rockland Immunochemicals for Research) for 1 h on an orbital shaker at room temperature. The membrane was then washed 3 × 5 min in washing buffer on an orbital shaker at room temperature.

2.6 Chemiluminescent detection

The washing buffer from Section 2.5 was poured off the membrane and excess was dabbed off using a Kimtech® absorbent tissue. Development was conducted using the SuperSignal® West Pico Chemiluminescent Substrate kit (Pierce). Briefly, 1 mL of peroxide solution was mixed with 1 mL of luminol/enhancer solution and pipetted directly onto the membrane which was subsequently incubated in the dark for 3 minutes. The immunoblots were then photographed using a VersaDoc™ 4000 molecular imaging system (Bio-Rad).

2.7 Primary antibodies

2.7.1 Anti-(SBT Prx 2) antibodies

Antibodies raised in a rabbit against a recombinant peroxiredoxin 2 (Prx 2) protein from southern bluefin tuna (*Thunnus maccoyii*) expressed in *E. coli* were kindly provided by Mr Drew Sutton (Sutton et al., 2010; Sutton, D., Unpublished data).

2.7.2 Anti-(human Prx 4) antibodies

Antibodies raised in a rabbit against a human peroxiredoxin 4 (Prx 4) peptide with the sequence SRVSVADHSLHLSKAKISK were kindly provided by Associate Professor John Power, Flinders University.

2.7.3 Anti-(human GPx 4) antibodies

Antibodies raised in a rabbit against a human glutathione peroxidase 4 (GPx 4) peptide with the sequence VNYTQLVDLHARYAEC were kindly provided by Associate Professor John Power, Flinders University.

Table2.1 SDS-PAGE resolving gel reagents and final concentrations

Reagent	**Concentration**
Tris-HCl (pH 8.8)	375 mM
SDS	0.1% (w/v)
Acrylamide-Bis (37.5:1 solution)	10% (w/v)
TEMED	0.4% (v/v)
Ammonium persulphate	0.5% (w/v)

Table 2.2: SDS-PAGE stacking gel reagents and final concentrations

Reagent	**Concentration**
Tris-HCl (pH 6.8)	125 mM
SDS	0.1% (w/v)
Acrylamide-Bis (37.5:1 solution)	4% (w/v)
TEMED	0.2% (v/v)
Ammonium persulphate	0.5% (w/v)

Table 2.3: SDS-PAGE running buffer reagents and final concentrations

Reagent	**Concentration**
Tris base	25 mM
Glycine	200 mM
SDS	1% (w/v)

Table 2.4: SDS-PAGE loading buffer reagents and final concentrations

Reagent	**Concentration**
Tris-HCl (pH 6.8)	50 mM
DTT	50 mM
EDTA	2 mM
Glycerol	10% (v/v)
SDS	2% (w/v)
Urea	12 M
Bromophenol Blue	A few grains

Table 2.5: SDS-PAGE Coomassie Brilliant Blue R-250 stain reagents and final concentrations

Reagent	**Concentration**
Methanol	45% (v/v)
Glacial acetic acid	10% (v/v)
Coomassie Brilliant Blue R-250	0.25% (w/v)

Table 2.6: Immunoblotting transfer buffer reagents and final concentrations

Reagent	**Concentration**
Tris-Base	50 mM
Glycine	380 mM
SDS	0.1% (w/v)
Methanol	20% (v/v)

Table 2.7: Blocking buffer reagents and final concentrations

Reagent	**Concentration**
Tris-HCl (pH 7.5)	50 mM
NaCl	150 mM
Tween-20	0.2% (v/v)
Skim milk powder	5% (w/v)

Chapter 3 – Sequence analyses

3.1 Aims and introduction

The aims of this chapter were to use bioinformatics to:

1. investigate the relatedness of 2-Cys peroxiredoxin (Prx) proteins from fish and a selection of mammals
2. investigate the relatedness of selenium-containing glutathione peroxidase (GPx) proteins from fish and a selection of mammals
3. predict whether or not the antibodies we had at our disposal were likely to cross-react with Prx and GPx proteins from yellowtail kingfish (YTK, *Seriola lalandi*)
4. predict the subunit molecular masses of Prx and GPx proteins from YTK.

The antibodies we had at our disposal were raised in rabbits against:

1. a recombinant Prx 2 protein from southern bluefin tuna (SBT; *Thunnus maccoyii*)
2. a peptide with the sequence SRVSVADHSLHLSKAKISK from a human Prx 4 protein
3. a peptide with the sequence VNYTQLVDLHARYAEC from a human GPx 4 protein.

3.2 Methods

3.2.1 Phylogenetic analysis of 2-Cys Prx proteins from fish and selected mammals

An amino acid sequence alignment was conducted for the four subclasses of 2-Cys Prx proteins from human (*Homo sapiens*), rat (*Rattus norvegicus*), mouse (*Mus musculus*), zebrafish (*Danio rerio*), Atlantic salmon (*Salmo salar*), SBT and YTK in order to determine

their relatedness. The full list of sequences is given in the legend to Fig. 3.1. The sequences were imported into the multiple sequence alignment program Clustal X2 version 2.0.11 (Larkin et al., 2007) and an alignment was performed which was then used to generate a phylogenetic tree using the molecular evolutionary genetics analysis program version 5 (MEGA5; Tamura et al., 2011) with the maximum parsimony method and 1000 replicates for bootstrap analysis.

3.2.2 Amino acid sequence alignment of Prx 4 proteins from fish and selected mammals

An amino acid sequence alignment was conducted as described in Section 3.2.1 using Prx 4 amino acid sequences for human, rat, mouse, Atlantic salmon, zebrafish and YTK. The full list of sequences is given in the legend to Fig. 3.2. The aligned sequences were imported into the alignment editing software GeneDoc (Nicholas et al., 1997) and conserved sequences were highlighted and sequence similarity was recorded. The alignments were then annotated using Adobe® Illustrator® to highlight the conserved VCP motifs characteristic of all 2-Cys Prx proteins (Wood et al., 2003), the putative signal peptide cleavage site for the human Prx 4 (GenBank), the signal peptide cleavage site for YTK Prx 4 predicted by Loo and Schuller (2010) and the location of the peptide used to raise the anti-(human Prx 4) antibodies.

3.2.3 Amino acid sequence alignment of 2-Cys Prx proteins from fish

An amino acid sequence alignment was conducted as described in Section 3.2.1 using Prx 1, 2, 3 and 4 sequences for zebrafish, Atlantic salmon, SBT and YTK. The full list of sequences is given in the legend to Fig. 3.3. The aims were to highlight the N-terminal extensions present in the Prx 3 and 4 proteins which have been shown to be targeting signals in mammals (Wood et al., 2003) and to predict whether the anti-(human Prx 4) antibodies would cross react with other 2-Cys Prx proteins. In mammals, signal peptide cleavage occurs

between residues 63 and 64 in Prx 3 and between residues 37 and 38 in Prx 4 (Wood et al., 2003). However, in YTK, cleavage has been predicted to occur between residues 33 and 34 in Prx 4 (Loo and Schuller, 2010). Prx 3 has not been studied in fish. The aligned sequences were imported into the alignment editing software GeneDoc (Nicholas et al., 1997) and conserved sequences were highlighted and sequence similarity was recorded. The alignments were then annotated using Adobe® Illustrator® to highlight the conserved VCP motifs characteristic of all 2-Cys Prx proteins (Wood et al., 2003) and the location of the peptide used to raise the anti-(human Prx 4) antibodies. The subunit molecular masses of the YTK Prx 3 and 4 proteins with and without their N-terminal extensions were calculated using the protein size calculator from www.sciencegateway.org.

3.2.4 Phylogenetic analysis of selenium-containing GPx proteins from fish and selected mammals

An amino acid sequence alignment was conducted for the four subclasses of selenium-containing GPx proteins from human, mouse, zebrafish, SBT and YTK in order to determine their relatedness. The full list of sequences is given in the legend to Fig. 3.4. The sequences were imported into the multiple sequence alignment program Clustal X2 version 2.0.11 (Larkin et al., 2007) and an alignment was performed which was then used to generate a phylogenetic tree using the molecular evolutionary genetics analysis program version 5 (MEGA5; Tamura et al., 2011) with the maximum parsimony method and 1000 replicates for bootstrap analysis.

3.2.5 Amino acid sequence alignment of GPx 4 proteins from fish and selected mammals

An amino acid sequence alignment was performed as described in Section 3.2.3 using GPx 4 sequences for human, mouse variant A and B, zebrafish variant A and B, SBT and YTK variant A and B. The full list of sequences is given in the legend to Fig. 3.5. The aligned

sequences were imported into the alignment editing software GeneDoc (Nicholas et al., 1997) and conserved sequences were highlighted and sequence similarity was recorded. The alignments were then annotated using Adobe® Illustrator® to highlight the catalytic selenocysteine (Sec) residue characteristic of these proteins (Toppo et al., 2009) and the location of the peptide used to raise the anti-(human GPx 4) antibodies.

3.2.6 Amino acid sequence alignment of selenium-containing GPx proteins from fish

An amino acid sequence alignment was performed as described in Section 3.2.3 using GPx 1, 2, 3 and 4 sequences for zebrafish, SBT and YTK. The full list of sequences is given in the legend to Fig. 3.6. The aims were to highlight the divergence between the selenium-containing GPx proteins and to predict whether the anti-(human GPx 4) antibodies would cross react with the other selenium-containing GPx proteins. The aligned sequences were imported into the alignment editing software GeneDoc (Nicholas et al., 1997) and conserved sequences were highlighted and sequence similarity was recorded. The alignments were then annotated using Adobe® Illustrator® to highlight the catalytic selenocysteine (Sec) residue characteristic of these proteins (Toppo et al., 2009) and the location of the peptide used to raise the anti-(human GPx 4) antibodies. The subunit molecular mass of the YTK GPx 4 protein was calculated using the protein size calculator from www.sciencegateway.org.

3.3 Results and discussion

3.3.1 Phylogenetic analysis of 2-Cys Prx proteins from fish and selected mammals

The phylogenetic tree was separated into four major clades (Fig. 3.1). The fish and mammalian Prx 1 and 2 sequences were contained together within one of these four major clades. However, within this clade, the Prx 1 sequences from mammals all clustered together separate from the Prx 1 and 2 sequences from fish. Interestingly, the Atlantic salmon Prx 1

clustered together with the SBT Prx 2 and zebrafish Prx 2 sequences rather than with the zebrafish Prx 1 sequence suggesting that the Atlantic salmon Prx 1 sequence may have been misclassified. However more fish sequences are required to support this conclusion.

A separate clade contained all of the mammalian Prx 2 sequences (Fig. 3.1). A third clade contained all of the Prx 3 sequences with separation between fish and mammals within this clade (Fig. 3.1). Similarly, the fourth clade contained all of the Prx 4 sequences with fish and mammals forming separate clusters within this clade (Fig. 3.1). The antibodies raised against the recombinant SBT Prx 2 protein would therefore be most likely to cross-react with fish Prx 1 and 2 and human Prx 1 proteins. It is also possible that these antibodies may cross-react with other Prx subclasses in fish and mammals; however, this would depend on the specific epitopes recognised by the antibodies. The epitopes are not known because the anti-(SBT Prx 2) antibodies were raised against whole protein rather than small peptides.

3.3.2 Amino acid sequence alignment of Prx 4 proteins from fish and selected mammals

A high level of conservation was observed both within the mammalian Prx 4 proteins and also between the mammalian and fish proteins with ≥ 72% identity across all sequences (Fig. 3.2). The majority of the variation in the sequences was at the N-terminal end with 51 of the 70 divergent amino acids contained within the first 60 N-terminal amino acids. The remainder of the protein was extremely well conserved. In particular, the two VCP motifs containing the two catalytic cysteine (Cys) residues characteristic of 2-Cys Prx proteins (Wood et al., 2003) were both in highly conserved regions with ≥ 5 amino acids either side of the VCP motif 100% conserved across all compared species.

The sequence of the peptide used to raise the anti-(human Prx 4) antibodies was SRVSVADHSLHLSKAKISK and the alignment showed that 16 out of the 19 amino acids were 100% conserved between the sequences in the region corresponding to this peptide. The three amino acids which were not conserved were located near the N-terminal end of the peptide at amino acids 1, 4 and 6. Interestingly, the divergent amino acids were conserved amongst fish or amongst mammals but not between fish and mammals. The residues at positions 1 and 4 from the N-terminal end of the peptide sequence were both serine in mammals but they were phenylalanine and proline, respectively, in fish and the residue at position 6 from the N-terminal end of the peptide was alanine in mammals but serine in fish. The antibodies raised against the human Prx 4 peptide are therefore likely to cross-react with fish Prx 4 proteins. However this reaction would depend on the importance of the divergent amino acids at the N-terminal end of the peptide and the specific epitopes recognised by the anti-(human Prx 4) antibodies.

3.3.3 Amino acid sequence alignment of 2-Cys Prx proteins from fish

The alignment showed a large amount of variation at the N-terminal end, particularly in the region of the putative targeting sequences for Prx 3 and 4 (Fig. 3.3). In mammals, Prx 3 has been shown to be targeted to the mitochondria and Prx 4 has been shown to be targeted for secretion via the endoplasmic reticulum (Fujii and Ikeda, 2002; Wood et al., 2003). The sequence alignment in Fig. 3.3 suggests a possible targeting sequence cleavage site between residues 67 and 68 for the Prx 3 and Prx 4 sequences. If this is correct then, the mature Prx 3 protein (without the signal peptide) would have a size of 199 amino acids and a molecular mass of 22 kDa. Similarly, the mature Prx 4 protein would have a size of 197 amino acids and a molecular mass of 22 kDa too. This predicted size is the same as that predicted for the

199 amino acid Prx 1 protein from zebrafish and the 196 amino acid Prx 2 protein from SBT which both also had a predicted molecular mass of 22 kDa.

Using Signal P 3.0 software, Loo and Schuller (2010) predicted that the signal peptide cleavage site for YTK Prx 4 was between residues 33 and 34. This would give a mature protein of 231 amino acids with a predicted molecular mass of 25 kDa. Thus, there are three possible YTK Prx 4 proteins and they are a 264 amino acid unprocessed form with a predicted molecular mass of 29 kDa, a 231 amino acid truncated form with a predicted molecular mass of 25 kDa and a 197 amino acid truncated form with a predicted molecular mass of 22 kDa.

Interestingly, the peptide used to raise the anti-(human Prx 4) antibodies overlapped the putative cleavage site for the N-terminal extension of the Prx 3 and Prx 4 proteins with the first 9 of the 19 amino acids of the peptide overlapping the C-terminal end of the N-terminal extension of Prx 3 and 4 (Fig. 3.3). Therefore, it is unlikely that the anti-(human Prx 4) antibodies would recognize Prx 1 or 2 and it is also unlikely that they would recognize processed forms of Prx 3 or Prx 4 with their N-terminal extensions removed. Furthermore, extremely low sequence conservation was observed between the Prx 3 sequence and the sequence of the antigenic peptide derived from human Prx 4. Therefore it is unlikely that any form of Prx 3 would be recognised by the anti-(human Prx 4) antibodies. The amino acids following the putative N-terminal extension cleavage site for Prx 4 were 100% identical to the second half of the antigenic peptide but there was considerable dissimilarity at the C-terminal end of the N-terminal extension. Thus, whether or not the anti-(human Prx 4) antibodies recognize fish Prx 4 will depend on the actual epitopes recognized by the antibodies.

3.3.4 Phylogenetic analysis of selenium-containing GPx proteins from fish and selected mammals

Two major clades were observed on the phylogenetic tree (Fig. 3.4). The first clade contained all GPx 1, 2 and 3 sequences and the second clade contained all GPx 4 sequences (Fig. 3.4). Within the first clade all GPx 1 and 2 sequences formed one subgroup and the GPx 3 sequences formed a separate subgroup. The mammalian GPx 1 sequences all clustered together in a distinct clade separate from the fish GPx 1 sequences which also clustered together (Fig. 3.4). The second large group which contained all of the GPx 4 sequences was separated into two subgroups comprised of mammalian GPx 4 and fish GPx 4 sequences (Fig. 3.4). The grouping of the GPx 4 sequences separately from the GPx 1, 2 and 3 sequences is consistent with evolutionary data which has been previously described for GPx proteins from various vertebrate species (see Toppo et al., 2008). The clear phylogenetic divergence between GPx 4 and the other selenium-containing GPx proteins suggests strongly that the antibodies raised against the human GPx 4 peptide will react only with GPx 4 proteins and not with GPx 1, 2 or 3 proteins.

3.3.5 Amino acid sequence alignment of GPx 4 proteins from fish and selected mammals

The alignment, including the location of the catalytic selenocysteine (Sec) residue characteristic of these proteins (Toppo et al., 2009), and the peptide sequence against which the anti-(human GPx 4) antibodies were raised is shown in Fig. 3.5. A moderate level of conservation was observed across all sequences with ≥57% sequence identity (Fig. 3.5). This level of conservation was somewhat lower than that observed for Prx 4 (Section 3.3.2). The majority of the variation was due to the N-terminal extension present in the mouse GPx 4A sequence. GPx 4A has been shown to localise to the nucleus (Maiorino et al., 2003), whereas

the shorter GPx 4B is targeted to the mitochondria (Toppo et al., 2008). The GPx 4 gene contains two alternatives for the first exon with separate promoter sites for each (Maiorino et al., 2003). If exon 1A is translated then mitochondrial GPx 4 is produced but if exon 1B is translated then the nuclear GPx 4 is produced with the first 84 N-terminal amino acids different from mitochondrial GPx 4 but the remainder of the protein being identical (Maiorino et al., 2003). This finding is in agreement with the data seen in Fig. 3.4; with the first 84 amino acids of the mouse GPx 4A sequence differing from the mouse GPx 4B sequence, but the remainder of the sequence being identical.

The sequence of the peptide against which the anti-(human GPx 4) antibodies were raised showed some conservation across the species with 9 out of the 16 amino acids 100% conserved and a further 3 amino acids 80% conserved, and one amino acid 60% conserved (Fig. 3.5). All variation between the sequences was seen at the centre and the C-terminal end of the peptide. All mammalian sequences in the region of the peptide were identical. However, the fish sequences show marked variation between the species and even differed between the different GPx 4 forms from zebrafish. Thus, it will be surprising if the anti-(human GPx 4) antibodies recognize fish GPx 4 proteins but this will depend on the particular epitopes the anti-(human GPx 4) antibodies recognize.

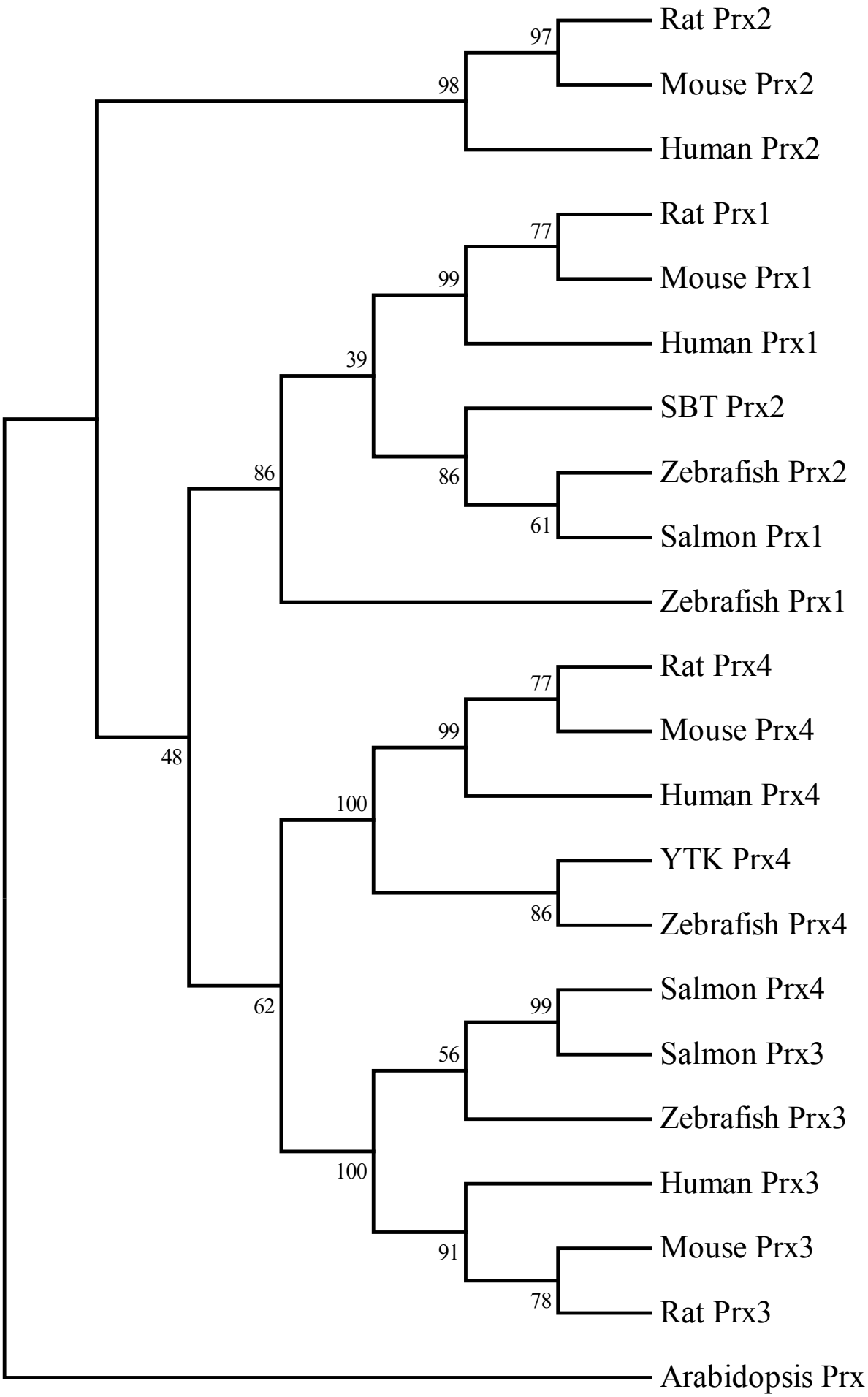
3.3.6 Amino acid sequence alignment of selenium-containing GPx proteins from fish

The alignment showed a very large amount of variation between the different GPx isoforms, especially in the region corresponding to the peptide used to raise the anti-(human GPx 4) antibodies (Fig. 3.6). N-terminal extensions were also observed in GPx 3 and GPx 4. The predicted molecular masses of the YTK GPx 4A and GPx 4B proteins were both approximately 21 kDa. A noteworthy difference between the GPx 4 proteins and the other

GPx proteins was the presence of two ‘gaps’ in the GPx 4 sequence. The first gap was 6 amino acids long from residue 113 to 118 and the second gap was 17 amino acids long from residue 157 to 173 (Fig. 3.6). These gaps may be due to deletions in the GPx 4 gene over evolutionary time. In a review of more than 700 GPx sequences, Toppo et al (2008) also discovered a similar gap in the region corresponding to resides 157-173 in the alignment (Fig. 3.5) and proposed that the amino acids in this region could be responsible for what was termed the ‘oligomerisation loop’ and contributed to the oligomerisation of GPx 1, 2 and 3 proteins to form tetramers (Toppo et al., 2008). Interestingly GPx 4 is the only selenium-containing GPx protein that does not form tetramers; it is functional as a monomer (Toppo et al., 2009). The gaps seen for GPx 4 in the sequence alignment (Fig. 3.6) could explain the difference in oligomerisation between GPx 4 and other selenocysteine GPx proteins. Furthermore, the gaps, along with other sequence divergence, could contribute to the clear separation of GPx 4 from the other selenocysteine GPx proteins in the phylogenetic analysis (Fig. 3.4).

Given the great sequence divergence, it is highly unlikely that the anti-(human GPx 4) antibodies will recognize GPx 1, 2 or 3 from either mammals or fish. However, this again will depend on the particular epitopes recognised by the anti-(human GPx 4) antibodies.

Figure 3.1: A phylogenetic tree comparing fish and mammalian amino acid sequences for 2-Cys Prx proteins. All sequences were sourced from the NCBI RefSeq database (indicated by the NP prefix) unless otherwise stated. The sequences were human Prx 1-4 (NP859048, and SwissProt accession numbers P32119, P30048, Q13162, respectively), rat Prx 1-4 (NP_476445, NP_058865, GenBank accession number EDL94585, NP_445964, respectively), mouse Prx 1-4 (NP_035164, NP_035693, GenBank accession number EDL01849, NP_058044, respectively), zebrafish Prx 1-4 (NP_001013489, NP_001002468, GenBank accession number AAH92846, NP_001082894, respectively), Atlantic salmon Prx 1, 3 and 4 (NP_001134295, and GenBank accession numbers ACH85347 and ACI69656, respectively), SBT Prx 2 (GenBank accession number ABW88997) and YTK Prx 4 (GenBank accession number ACM47312). A 2-Cys Prx from the plant *Arabidopsis thaliana* (NP_187769) was used to root the tree. The sequences were aligned using Clustal X2 and the tree was created using MEGA5 software with the maximum parsimony method. Numbers on nodes indicate bootstrap consensus values from 1000 replicates.


Rat Prx2
Mouse Prx2
Human Prx2
Rat Prx1
Mouse Prx1
Human Prx1
SBT Prx2
Zebrafish Prx2
Salmon Prx1
Zebrafish Prx1
Rat Prx4
Mouse Prx4
Human Prx4
YTK Prx4
Zebrafish Prx4
Salmon Prx4
Salmon Prx3
Zebrafish Prx3
Human Prx3
Mouse Prx3
Rat Prx3
Arabidopsis Prx
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99
56
100
91
78

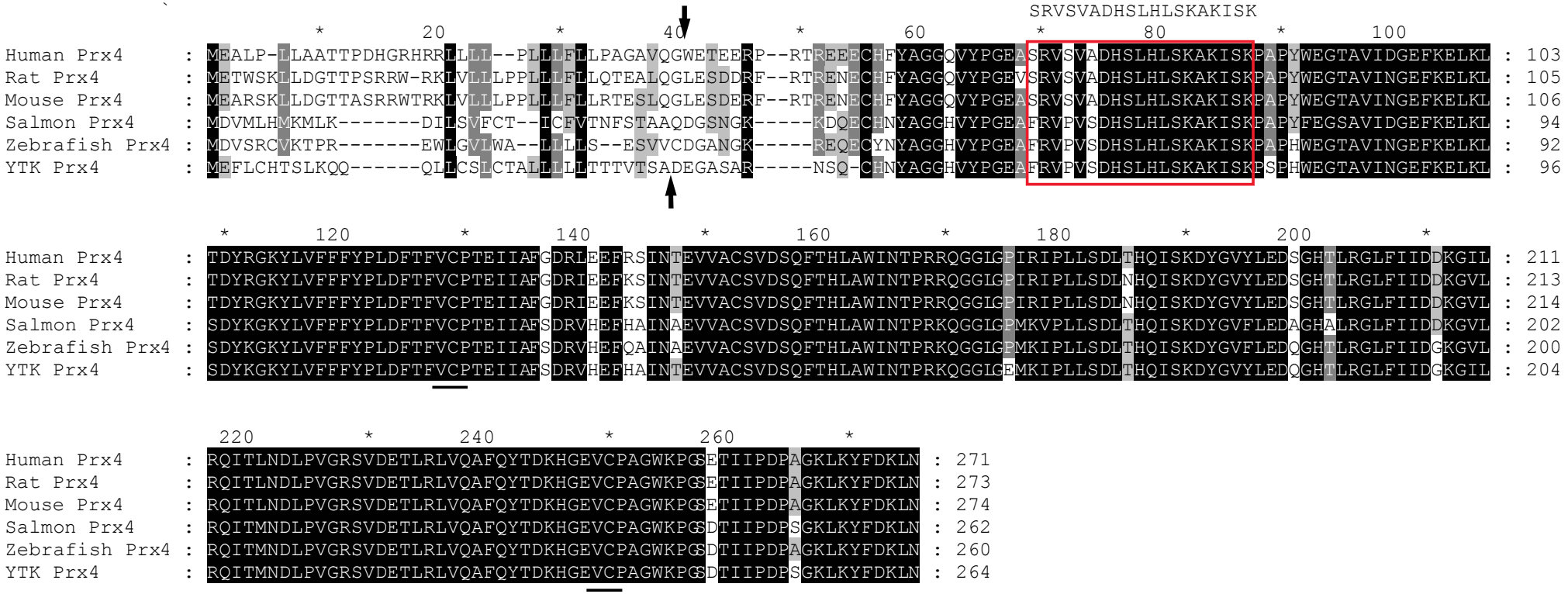


Figure 3.2: A multiple sequence alignment of Prx 4 amino acid sequences from fish and selected mammals. All sequences were sourced from the NCBI RefSeq database unless otherwise stated. Black shading indicates 100% identity between sequences, dark grey shading indicates 80% identity and light grey shading indicates 60% identity. The red box indicates the region corresponding to the peptide used to raise the anti-(human Prx 4) antibodies and the annotation above this region indicates the peptide sequence. The underlined sequences at residues 128-130 and 249-251 indicate the two catalytic cysteine residues within the conserved VCP motifs. The top arrow at residues 40-41 indicates the putative cleavage site for the putative targeting sequence for human Prx 4 and the bottom arrow at residues 39-40 indicates the predicted cleavage site for the putative targeting sequence for YTK Prx 4 (Loo and Schuller, 2010). The species and accession numbers were human (SwissProt accession no. Q13162; this is the sequence used to design the antigenic peptide), rat (NP_445964), mouse (NP_058044), Atlantic salmon (GenBank accession number ACI69656), zebrafish (NP_001082894), and YTK (GenBank accession number ACM47312).

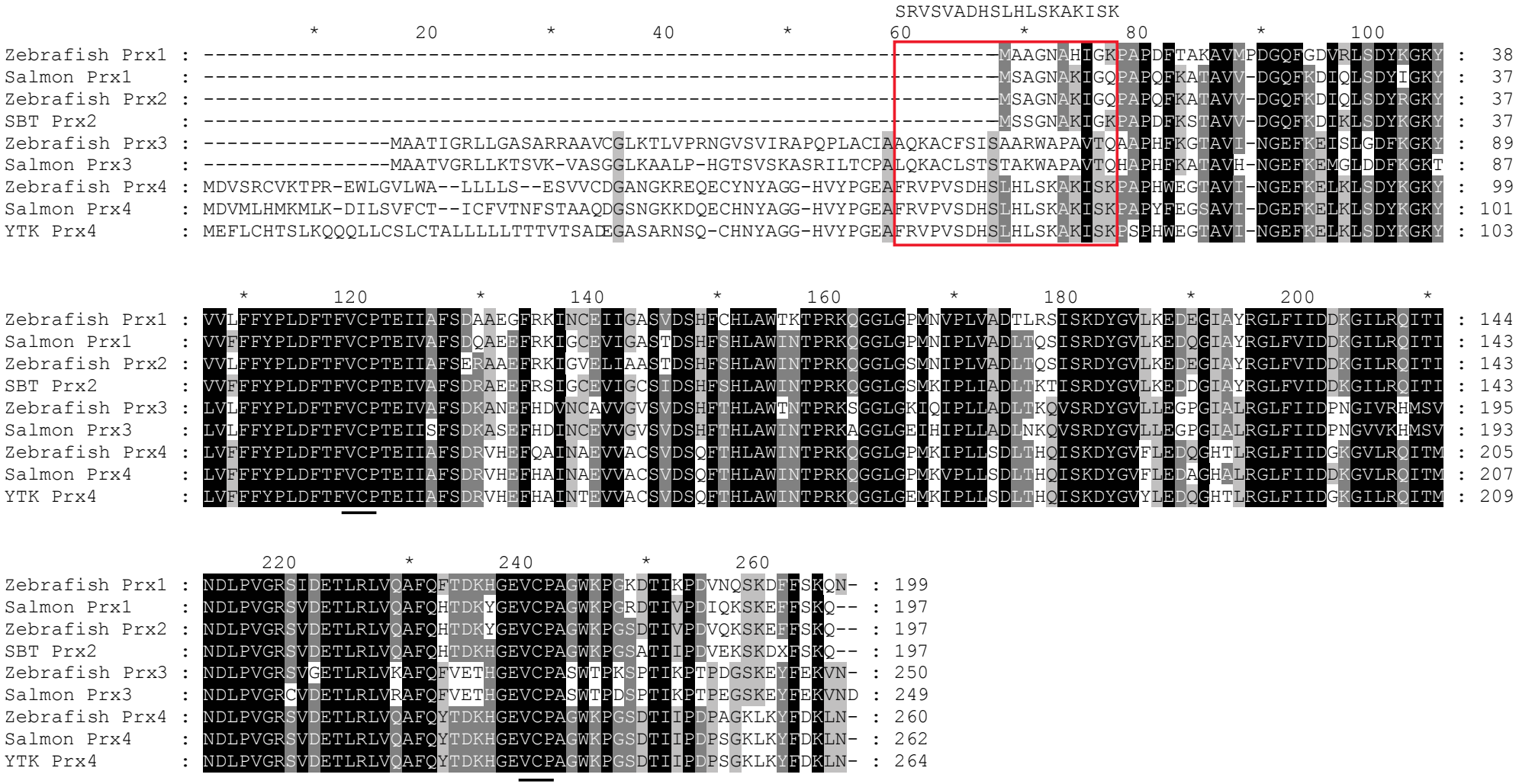
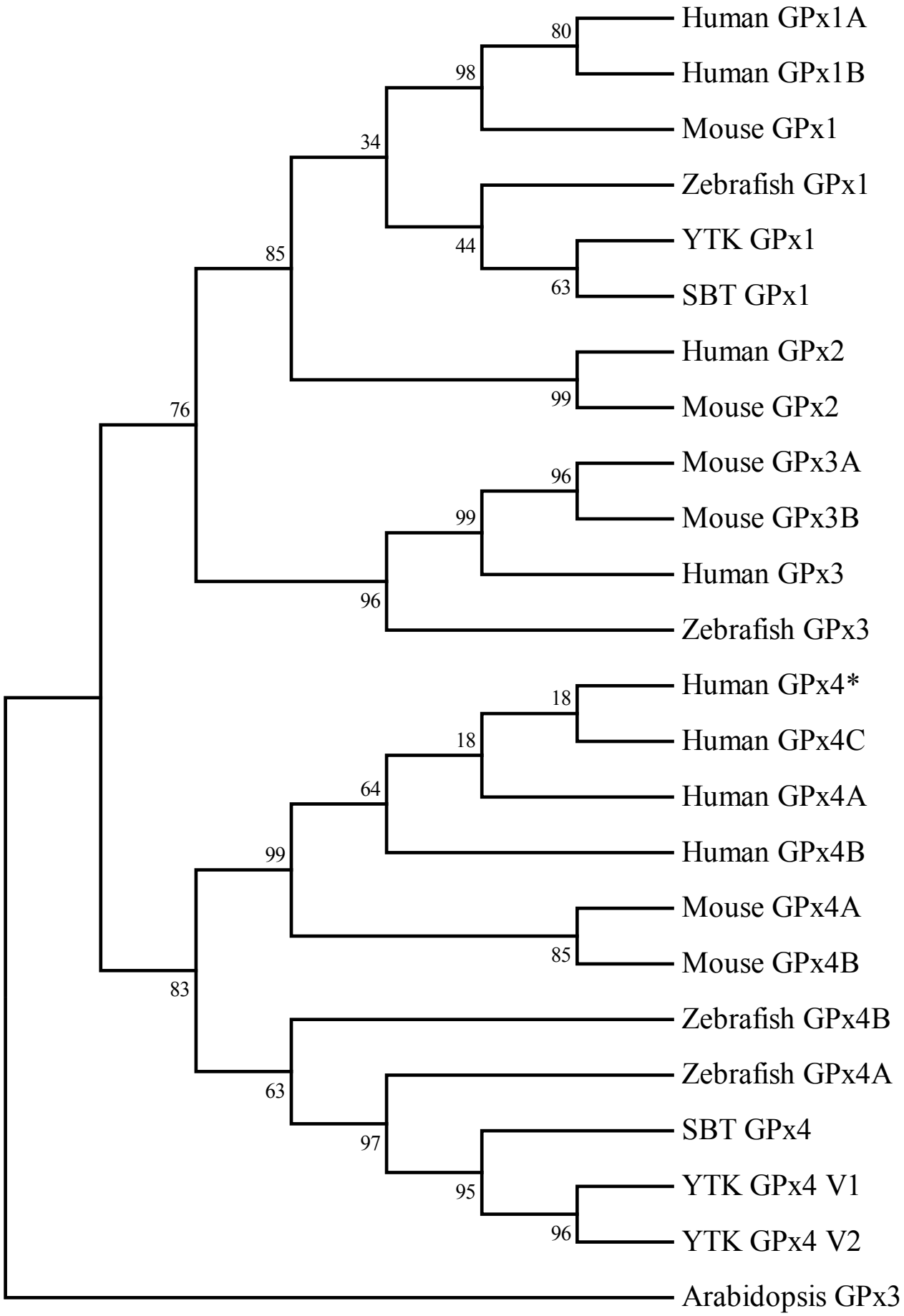


Figure 3.3: A multiple sequence alignment of 2-Cys Prx amino acid sequences from fish. All sequences were sourced from the NCBI RefSeq database unless otherwise stated. Black shading indicates 100% identity, dark grey 80% identity and light grey 60% identity. The region corresponding to the peptide used to raise the anti-(human Prx 4) antibodies is boxed in red and the annotation above this region indicates the peptide sequence. The underlined sequences at residues 118-120 and 239-241 indicate the two catalytic Cys residues within the conserved VCP motifs. The species and accession numbers were zebrafish Prx 1-4 (NP_001013489, NP_001002468, GenBank accession number AAH92846, NP_001082894, respectively), Atlantic salmon Prx 1, 3 and 4 (NP_001134295, and GenBank accession numbers ACH85347 and ACI69656, respectively), SBT Prx 2 (GenBank accession number ABW88997) and YTK Prx 4 (GenBank accession number ACM47312).

Figure 3.4: A phylogenetic tree comparing fish and mammalian amino acid sequences for selenium-containing GPx proteins. All sequences were sourced from the NCBI RefSeq database (indicated by the NP prefix) unless otherwise stated. The species and accession numbers were human GPx 1 variants A and B (NP_000572, NP_958799, respectively), GPx 2 (NP_002074), GPx3 (NP_002075), GPx 4 variants A, B and C (NP_002076, NP_001034936, NP_001034937, respectively) and the GPx 4 sequence that the anti-(human GPx 4) antibodies was raised against (SwissProt accession no. P36969; this is the sequence used to design the antigenic peptide denoted human GPx 4* on tree), mouse GPx 1 (NP_032186), GPx 2 (NP_109602), GPx 3 variants A and B (NP_001077398, NP_032187, respectively) and GPx 4 variants A and B (NP_001032830, NP_032188, respectively). Fish sequences were from zebrafish GPx 1 (NP_001007282), GPx 3 (NP_001131027), GPx 4 variants A and B (NP_001007283, NP_001025241, respectively), SBT GPx 1(GenBank accession number ABO38817) and GPx 4 (GenBank accession number ABO38818), YTK GPx 1 (GenBank accession number AEI91048) and GPx 4 variants A and B (GenBank accession numbers AEI91049, AEI91050, respectively). A GPx 3 sequence from the plant, *Arabidopsis thaliana* (NP_001189742) was used as an outgroup. The sequences were aligned using Clustal X2 and the tree was created using MEGA5 software with the maximum parsimony method. The MEGA5 software could not accommodate the selenocysteine (U) amino acid so it was replaced with a cysteine (C) for the construction of the tree. Numbers on nodes indicate bootstrap consensus values from 1000 replicates.


Human GPx1A
Human GPx1B
Mouse GPx1
Zebrafish GPx1
YTK GPx1
SBT GPx1
Human GPx2
Mouse GPx2
Mouse GPx3A
Mouse GPx3B
Human GPx3
Zebrafish GPx3
Human GPx4*
Human GPx4C
Human GPx4A
Human GPx4B
Mouse GPx4A
Mouse GPx4B
Zebrafish GPx4B
Zebrafish GPx4A
SBT GPx4
YTK GPx4 V1
YTK GPx4 V2
Arabidopsis GPx3
80
98
34
85
44
63
99
76
96
99
96
18
18
64
99
85
83
63
97
95
96

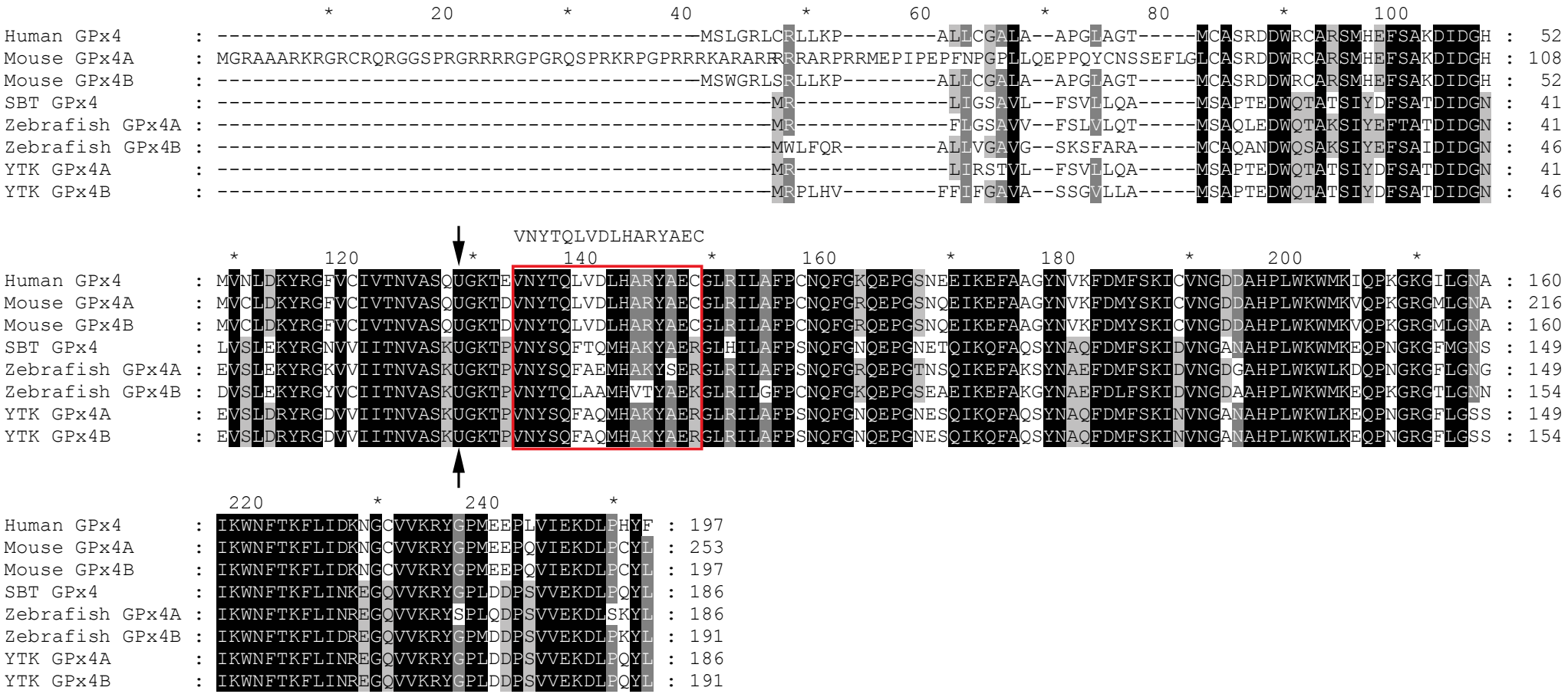


Figure 3.5: A multiple sequence alignment of GPx 4 proteins from mammals and fish. All sequences were sourced from the NCBI RefSeq database unless otherwise stated. Black shading indicates 100% identity, dark grey 80% identity and light grey 60% identity. The red box indicates the region containing the peptide sequence that the anti-(human GPx 4) antibodies were raised against and the annotation above this region indicates the peptide sequence. The arrow at residue 129 indicates the catalytic selenocysteine residue. The species and accession numbers were human GPx 4 (SwissProt accession number P36969), mouse GPx 4 variants A and B (NP_001032830, NP_032188, respectively), SBT GPx 4 (GenBank accession number ABO38818), zebrafish GPx 4 variants A and B (NP_001007283, NP_001025241, respectively) and YTK GPx 4 variants A and B (GenBank accession numbers AEI91049, AEI91050, respectively).

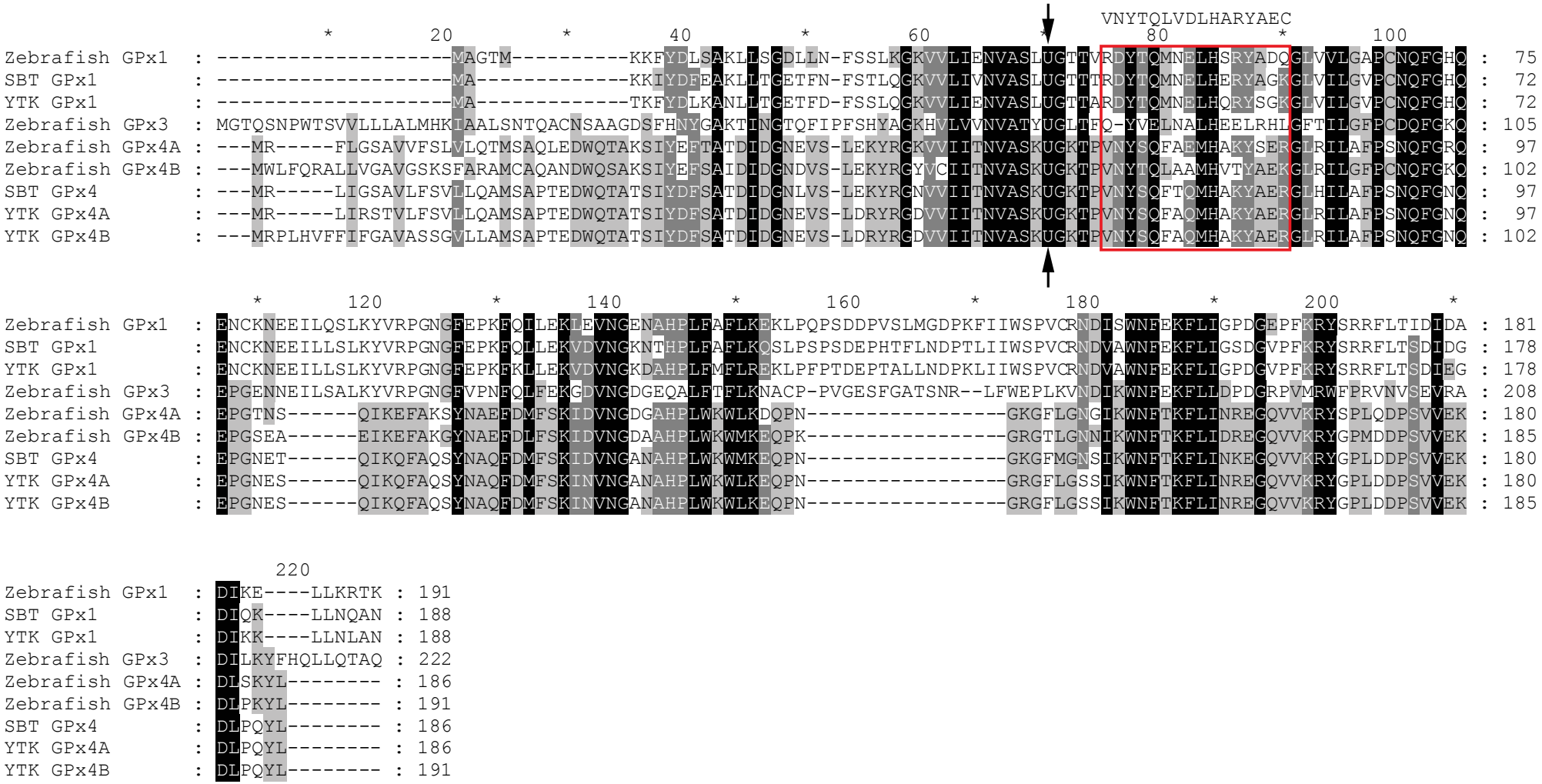


Figure 3.6: An amino acid sequence alignment of selenium-containing GPx proteins from fish. All sequences were sourced from the NCBI RefSeq database unless otherwise stated. Black shading indicates 100% identity, dark grey 80% identity and light grey 60% identity. The red box indicates the region containing the peptide sequence that the anti-(human GPx 4) antibodies were raised against and the annotation above this region indicates the peptide sequence. The arrow at residue 70 indicates the catalytic selenocysteine residue. The species and accession numbers were zebrafish GPx 1 (NP_001007282), GPx 3 (NP_001131027) and GPx 4 variant A and B (NP_001007283, NP_001025241, respectively), SBT GPx 1(GenBank accession number ABO38817) and GPx 4 (GenBank accession number ABO38818), YTK GPx 1 (GenBank accession number AEI91048) and GPx 4 variant A and B (GenBank accession numbers AEI91049, AEI91050, respectively).

Chapter 4 – Preliminary investigations

4.1 Background and aims

In Chapter 3, bioinformatics was used to predict that the anti-(SBT Prx 2) antibodies were likely to detect all 2-Cys Prx proteins in fish tissues. Furthermore, it was predicted that the anti-(human Prx 4) antibodies were likely to be highly specific for Prx 4 and may detect Prx 4 from fish. The anti-(human GPx 4) antibodies were predicted to be highly specific for GPx 4 from mammals but unlikely to detect fish GPx 4 due to divergence from the human sequence. Therefore, in Chapter 4, the aim was to experimentally verify these predictions using total protein extracts from SBT and YTK.

In previous work from our laboratory a GPx 1 protein with a subunit molecular mass of 24 kDa was purified from SBT liver (Thompson et al., 2006). Furthermore, we have recombinantly expressed a 22 kDa SBT Prx 2 protein in *E. coli* (Sutton et al., 2010) and two different forms of a YTK Prx 4 protein with subunit molecular masses of 22 kDa and 26 kDa were also recombinantly expressed in *E. coli* (Loo and Schuller, 2010).

Therefore, in Chapter 4, the aims were to:

1) determine experimentally whether the anti-(SBT Prx 2), anti-(human Prx 4) and anti-(human GPx 4) antibodies we had at our disposal would cross-react with related proteins from YTK
2) determine whether the subunit molecular masses of any cross-reacting proteins matched those expected based on the current literature, previous work in our laboratory and the bioinformatics analyses in Chapter 3

3) optimize the tissue extraction protocol through trialling different published buffers and methods in order to maximize the amounts of Prx 4 and GPx 4 proteins extracted from various YTK tissues
4) undertake a preliminary investigation of the tissue distribution of expression of Prx and GPx proteins in various YTK tissues (provided that Aims 1, 2 and 3 were met)

4.2 Methods

4.2.1 Fish tissues

The tissues used in Protocols 1, 2 and 3, their storage methods and their sources are shown in Table 4.1. The fish used in Protocol 4 were YTK of approximately 400 g obtained from SARDI Aquatic Sciences and they were euthanized on site with a lethal dose of Aqui-S® (Section 2.2.1). The fish were then transported on ice to laboratory facilities at Flinders University. The tissues harvested and their storage methods are summarised in Table 4.2. The fish used in Protocols 5 and 6 were obtained from Clean Seas Tuna Ltd and were approximately 3 kg in size (Table 4.3, Section 2.2.2). These fish were provided freshly killed and tissues were harvested by the author of this thesis and were stored initially in dry ice and then at -80°C until needed.

4.2.2 Protocol 1

The tissues used, their storage methods and their sources are summarized in Table 4.1. The SBT muscle and liver samples stored frozen at -80^{o}C since 2003 were placed in a 10 mL tube with a 2× volume of Extraction Buffer A (Table 4.4) whereas the SBT liver, YTK liver and YTK muscle samples stored in RNA*later*® since 2010 were placed in 10 mL tubes with 5×,

10× and 4× volumes, respectively, of Extraction Buffer A. This buffer, plus 5 mM imidazole, had previously been used in our group to extract recombinant YTK Prx 4 and SBT Prx 2 proteins from *E. coli* (Loo and Schuller, 2010; Sutton et al., 2010, respectively). All tissues were extracted using the method described in Section 2.3.1 and total protein was determined using the method of Bradford (1976). Following extraction, 100 µg of protein from each sample (with the exception of the YTK liver sample for which the amount of protein was 10 µg) was run on three replicate 10% SDS-PAGE gels using the protocol described in Section 2.4. One gel was stained for protein (Section 2.4) while the other 2 gels were used for immunoblot analysis (Section 2.5) using a 1:1000 dilution of anti-(SBT Prx 2) or anti-(human Prx 4) primary antibodies. Development and detection were conducted as described in Sections 2.5 and 2.6 with a 1:1000 dilution of the secondary antibodies in blocking buffer.

4.2.3 Protocol 2

The SBT muscle and liver samples stored frozen at minus -80^{o}C since 2003 (Table 4.1) were extracted as per Protocol 1 (Section 4.2.2) using Extraction Buffer B (Table 4.5) instead of Extraction Buffer A. Extraction Buffer B was selected as it had previously been shown to be an effective buffer for extracting GPx proteins from SBT liver and muscle (Thompson et al., 2006). One hundred µg of total protein from each sample was run on three replicate 10% SDS-PAGE gels using the protocol described in Section 2.4. One gel was stained with Coomassie Blue for 1 hour and destained over the weekend (Section 2.4) and the other two gels were used for immunoblot analysis (Section 2.5). The two immunoblots were blocked over the weekend in blocking buffer containing 0.04% (w/v) sodium azide and then incubated in a 1:1000 dilution of anti-(human GPx 4) antibodies in blocking buffer. Development and detection were conducted as described in Sections 2.5 and 2.6 with a 1:1000 dilution of secondary antibodies.

4.2.4 Protocol 3

The SBT liver samples stored frozen at -80^{o}C since 2003 (Table 4.1) were extracted into a 2× volume of four different extraction buffers: Buffer B (Table 4.5), Buffer B plus 1% (w/v) SDS, Buffer C (Table 4.6) or Buffer C plus 1% (w/v) SDS as per Section 2.3.1. Buffer C had previously been used in our group to extract total protein from liver and muscle tissues from Atlantic salmon (*Salmo salar*), barramundi (*Lates calcarifer*), SBT and YTK (Mr Drew Sutton, unpublished data). The method of Bradford (1976), could not be used to determine total protein in these buffers due to the presence of SDS which interferes with the Bradford assay. At a concentration of 1% (w/v), SDS induces dye binding equivalent to 44 µg of bovine serum albumin (BSA) markedly affecting the accuracy and sensitivity of this assay (Bradford, 1976; Friedenauer and Berlet, 1989). Initially, comparable extraction efficiency was assumed for all samples. Therefore, an equal volume of each extract was assumed to contain an approximately equal mass of protein. As a result, 5 µl of each sample was mixed with 5 µl of SDS-PAGE loading buffer and the resulting 10 µl was loaded onto each of four replicate 10% SDS-PAGE gels and the gels were run according to the protocol described in Section 2.4. One gel was stained for protein (Section 2.4) and the other three were used for immunoblot analysis as per the protocol described in Section 2.5 using the anti-(SBT Prx 2), anti-(human Prx 4) and anti-(human GPx 4) primary antibodies. Development and detection were conducted as described in Sections 2.5 and 2.6 with a 1:1000 dilution of secondary antibodies. The aim of this protocol was to test whether the addition of SDS could improve the extraction efficiency of any of the GPx or Prx proteins, especially GPx 4 and Prx 4 which have been shown to be either membrane-bound or at least membrane-associated in mammals (Wood et al., 2003; Herbette et al., 2007).

4.2.5 Protocol 4

Skeletal muscle, liver, brain and heart samples stored in RNA*later*® from a 400 g YTK of unknown gender (Table 4.2) along with an SBT liver sample stored frozen at -80°C and a Guinea pig liver sample stored in RNA*later*® (Table 4.1) were extracted into a 5× volume of Buffer D (Table 4.7) according to the method described in Section 2.3.1. The method of Bradford (1976), could not be used to determine the total protein concentration in this buffer because of the presence of SDS (see Section 4.2.4). As a result, an equal volume of extract was used and 5 µl of each sample was mixed with 5 µl of SDS-PAGE loading buffer and loaded onto each of two 10% SDS-PAGE gels and the gels were run according to the protocol described in Section 2.4. One gel was stained for protein for 1 hour and then destained overnight (Section 2.4) and the protein from the second gel was transferred to a nitrocellulose membrane and used for immunoblotting analysis as per the protocol described in Section 2.5 using a 1:1000 dilution of anti-(SBT Prx 2) primary antibodies. Development and detection were conducted as described in Sections 2.5 and 2.6 with a 1:1000 dilution of secondary antibodies. The aim of this protocol was to investigate protein expression in a broader range of tissues than had been tested so far using the buffer that had been shown to be most effective in the previous protocol (Protocol 3).

4.2.6 Protocol 5

Brain, liver, muscle, heart, intestine, spleen, kidney, gonad and gill samples stored in RNA*later*® from a 3.0 kg YTK female (YTK 1, Table 4.3) were taken and protein was extracted into a 5× volume of Buffer D (Table 4.7) according to the method described in Section 2.3.2. The method of Bradford (1976) could not be used to determine total protein in this buffer because of the presence of SDS (see Section 4.2.4). Therefore, a method was

developed to normalise the amount of extract loaded onto the gel to the tissue weight. Briefly, the tissue wet weight, supernatant volume and amount of undissolved material were measured and a volume of supernatant per mg of tissue wet weight was calculated using the following equation: $= \frac{V_S}{(W-M)}$ where Vs = volume of supernatant, W = tissue wet weight (mg) and M = weight of undissolved material (mg). One hundred µl of supernatant from each extract was mixed with 100 µl of SDS-PAGE loading buffer and a volume of the mixture equivalent to 2.5 mg of tissue wet weight was loaded onto each of four replicate 10% SDS-PAGE gels for each fish and run according to the protocol described in Section 2.4. One gel for each fish was stained for protein with Coomassie blue for 1 hour and destained overnight (Section 2.4) and the proteins from the other gels were transferred to nitrocellulose membranes and used for immunoblotting analysis as per the protocol described in Section 2.5 using a 1:1000 dilution of anti-(SBT Prx 2), anti-(human Prx 4) or anti-(human GPx 4) primary antibodies. Development and detection were conducted as described in Sections 2.5 and 2.6 with a 1:1000 dilution of secondary antibodies.

The aims of this protocol were (1) to determine if gender (male or female) had any effect on the expression of Prx or GPx proteins, since this was the first time that the sex of the fish was known and (2) to investigate the expression of Prx and GPx proteins in YTK, whilst determining the loading of each extract by the equation described above, as this corrected for possible differences in protein extraction efficiency between the different samples.

4.2.7 Protocol 6

Further trials involved modifications to Buffer D. The first modification involved the addition of more DTT, to a final concentration of 50 mM. This modified buffer was named Buffer E

(Table 4.8). The second modification involved the addition of DTT to a final concentration of 50 mM as well as the addition of urea to a final concentration of 5 M. This modified buffer was named Buffer F (Table 4.9). The purpose of the addition of DTT was to provide stronger reducing conditions. It was anticipated that this would improve disulphide bridge disruption thereby counteracting the apparent protein aggregation seen in some of the previous protocols. Urea is a chaotrophic agent which assists in the denaturation (unfolding) of proteins (McCarthy et al., 2003; Auton et al., 2007). Therefore, we anticipated that urea would promote the unfolding of the proteins thereby making any buried disulphide bonds more accessible for disruption (reduction) by DTT. Therefore, we hypothesized that the combination of DTT with urea would be more effective than DTT alone at counteracting the apparent protein aggregation seen in some of the previous protocols.

To test this hypothesis, brain, liver, intestine and kidney samples stored in RNA*later*® (Table 4.3) from a 3 kg YTK female (YTK 1, Table 4.3) were taken and protein was extracted into a 5× volume of Buffer E (containing extra DTT, Table 4.8) or Buffer F (containing urea as well as extra DTT, Table 4.9) according to the method described in Section 2.3.2. One hundred µl of each sample was mixed with 100 µl of SDS-PAGE loading buffer and a volume of the mixture equivalent to 2.5 mg of tissue wet weight was loaded onto each of three 10% SDS-PAGE gels and the gels were run according to the protocol described in Section 2.4. One gel was stained for protein with Coomassie blue for 1 hour and then destained overnight (Section 2.4) and the proteins from the other two gels were transferred to nitrocellulose membranes and used for immunoblotting analysis as per the protocol described in Section 2.5 using a 1:1000 dilution of the anti-(SBT Prx 2) or anti-(human Prx 4) primary antibodies. Development and detection were conducted as described in Sections 2.5 and 2.6 with a 1:1000 dilution of secondary antibodies.

Table 4.1: Tissues, tissue storage methods and sources of tissues used in Protocols 1, 2 and 3.

Species	Tissue	Storage	Source
SBT	Lean muscle (Akami)	Frozen (-80°C) since 2003	A commercial fish farm in Boston Bay, South Australia (Thompson et al., 2006)
SBT	Medium fat muscle (Chutoro)	Frozen (-80°C) since 2003	A commercial fish farm in Boston Bay, South Australia (Thompson et al., 2006)
SBT	High fat muscle (Otoro)	Frozen (-80°C) since 2003	A commercial fish farm in Boston Bay, South Australia (Thompson et al., 2006)
SBT	Liver	Frozen (-80°C) since 2003	A commercial fish farm in Boston Bay, South Australia (Thompson et al., 2006)
SBT	Liver	Stored in RNA*later*® since 2010	Blaslov fishing company and Clean Seas Tuna Ltd.
YTK	Liver	Stored in RNA*later*® since 2010	SARDI Aquatic Sciences
YTK	Muscle	Stored in RNA*later*® since 2010	SARDI Aquatic Sciences
Guinea pig (GP)	Liver	Stored in RNA*later*® since 2010	Ms. Bo Nan Chen, Flinders University, School of Medicine

Table 4.2: Tissues used in Protocol 4. All fish weighed approximately 400 g and were provided by SARDI Aquatic Sciences. They were freshly killed by a lethal dose of Aqui-S® and tissues were harvested by the author of this thesis in February 2011. All tissues were stored in RNA*later*® (Section 2.3.1).

Species	**Tissue**	**Storage**
YTK	Brain	RNA*later*®
YTK	Liver	RNA*later*®
YTK	Muscle	RNA*later*®
YTK	Heart	RNA*later*®

Table 4.3: Tissues used in Protocols 5 and 6. All fish were YTK of approximately 3 kg body weight provided freshly killed by Clean Seas Tuna Ltd, Arno Bay, South Australia. The tissues were harvested by the author of this thesis in February 2011 and all tissues were stored in RNA*later*® (Section 2.3.2). Gender was determined by the author of this thesis by visually identifying gonad tissue as ovaries (large, semi-transparent with a reddish colour) or testes (thin, white and non-transparent).

Fish	**Sex**	**Tissue**
YTK 1	Female	Brain
YTK 1	Female	Liver
YTK 1	Female	Muscle
YTK 1	Female	Heart
YTK 1	Female	Spleen
YTK 1	Female	Ovaries
YTK 1	Female	Intestine
YTK 1	Female	Kidney
YTK 1	Female	Gill
YTK 2	Male	Brain
YTK 2	Male	Liver
YTK 2	Male	Muscle
YTK 2	Male	Heart
YTK 2	Male	Spleen
YTK 2	Male	Intestine
YTK 2	Male	Testes

Table 4.4: Reagents and final concentrations for Buffer A[1]

Chemical	**Concentration**
Tris-base (pH 7.5)	20 mM
NaCl	500 mM
Dithiothreitol (DTT)	5 mM

[1]Buffer composition adapted from Loo and Schuller (2010) and Sutton et al. (2010)

Table 4.5: Reagents and final concentrations for Buffer B[2]

Chemical	**Concentration**
Potassium phosphate (pH 7.6)	50 mM
Dithiothreitol (DTT)	5 mM
L-glutathione, reduced (GSH)	6 mM
Polymethylsulfinyl fluoride (PMSF)	2 mM
Ethylenediaminetetraacetic acid (EDTA)	1 mM

[2]Buffer composition adapted from Thompson et al. (2006).

Table 4.6: Reagents and final concentrations for Buffer C[3]

Chemical	Concentration
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH 7.5)	50 mM
NaCl	150 mM
Dithiotheritol (DTT)	10 mM
Polymethylsulfinyl fluoride (PMSF)	1 mM
Ethylenediaminetetraacetic acid (EDTA)	1 mM

[3] Buffer composition sourced from Mr Drew Sutton (unpublished data).

Table 4.7: Reagents and final concentrations for Buffer D[4]

Chemical	Concentration
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH 7.5)	50 mM
NaCl	150 mM
Dithiothreitol (DTT)	10 mM
Polymethylsulfinyl fluoride (PMSF)	1 mM
Ethylenediaminetetraacetic acid (EDTA)	1 mM
Sodium dodecyl sulphate (SDS)	1% (w/v)

[4]Buffer D was the same as Buffer C but with the addition of SDS

Table 4.8: Reagents and final concentrations for Buffer E[5]

Chemical	Concentration
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH 7.5)	50 mM
NaCl	150 mM
Dithiothreitol (DTT)	50 mM
Polymethylsulfinyl fluoride (PMSF)	1 mM
Ethylenediaminetetraacetic acid (EDTA)	1 mM
Sodium dodecyl sulphate (SDS)	1% (w/v)

[5]Buffer E was the same as Buffer D but with the addition of 50 mM instead of 10 mM DTT

Table 4.9: Reagents and final concentrations for Buffer F[6]

Chemical	Concentration
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH 7.5)	50 mM
NaCl	150 mM
Dithiothreitol (DTT)	50 mM
Polymethylsulfinyl fluoride (PMSF)	1 mM
Ethylenediaminetetraacetic acid (EDTA)	1 mM
Sodium dodecyl sulphate (SDS)	1% (w/v)
Urea	5 M

[6]Buffer F was the same as Buffer E but with the addition of urea

4.3 Results

4.3.1 Protocol 1

The aims of Protocol 1 were to test Buffer A (Table 4.4) for efficient extraction of Prx proteins and to investigate whether the anti-(SBT Prx 2) antibodies and anti-(human Prx 4) antibodies would cross-react with related proteins in YTK. Buffer A, with some modifications, had previously been used by Loo and Schuller (2010) and Sutton et al. (2010) to extract Prx proteins from *E.coli* expressing recombinant Prx 4 and Prx 2 proteins from YTK and SBT, respectively.

The Coomassie Blue stained gel (Fig. 4.1A) showed a similar pattern of protein bands for all three SBT muscle samples (lanes 1-3). The main difference observed between the different tissues (muscle and liver) was that the muscle samples contained a small number of highly abundant proteins, compared to the liver samples which contained a larger number of proteins with equal abundance (Fig. 4.1A). In Fig. 4.1B, the anti-(SBT Prx 2) antibodies detected an intense protein band at approximately 20 kDa and a weaker protein band at approximately 45 kDa in the SBT liver sample stored in RNA*later*® (lane 5). However, no bands were detected in any of the other samples except for some very faint bands at approximately 80 kDa in all three SBT muscle tissue samples (lanes 1-3). The 20 kDa and 45 kDa sizes corresponded approximately to the expected monomer and dimer sizes of 2-Cys Prx proteins as calculated in Section 3.3.3 and also as seen in previous work from our group with a recombinant SBT Prx 2 protein expressed in *E. coli* (Sutton et al., 2010). Interestingly, the SBT liver sample stored at -80^{o}C (lane 4) and the YTK liver and muscle samples stored in RNA*later*® (lanes 5, 6 and 7, respectively) did not exhibit the same bands at 20 kDa and 45 kDa that were seen for the SBT liver stored in RNA*later*® (lane 5). In fact no protein bands were seen on the

immunoblot for the SBT liver sample stored at -80°C, or for any of the YTK samples. This result suggested that the Prx proteins in the SBT tissue samples stored at -80°C may have suffered proteolytic degradation over the 8 years of storage. The lack of reaction in any of the YTK tissues is harder to explain. It could be that the anti-(SBT Prx 2) antibodies could not detect the YTK Prx proteins.

The reactions at molecular masses higher than the calculated monomeric size (20 kDa) may be due to oligomerisation of the proteins. Prx proteins have been shown to be difficult to reduce to their monomeric form and even trace amounts of H_2O_2 in extraction buffers have been shown to cause dimerisation of human Prx 2 (Peskin et al., 2007). Furthermore, previous work in our group has found that Prx proteins from fish are very difficult to reduce to monomeric size (Mr Drew Sutton, unpublished data). This may explain the presence of a dimer-sized protein in the SBT liver sample (Fig. 4.1B, lane 5), and a tetramer-sized protein in the SBT muscle samples (Fig. 4.1B, lanes 1-3) despite the presence of the reducing agent DTT in the extraction buffer and the gel loading buffer.

The Coomassie Blue stained gel in Fig. 4.2A was the same as in Fig. 4.1A. It is included again here for comparison. In Fig. 4.2B, the anti-(human Prx 4) antibodies detected a protein band at approximately 20 kDa in SBT Otoro muscle (lane 3) but not in SBT Akami muscle (lane 1), SBT Chutoro muscle (lane 2) or SBT liver (lane 4), all stored at -80°C. In contrast, these same antibodies detected proteins at approximately 25 kDa and/or 45 kDa in SBT liver (lane 5), YTK liver (lane 6), YTK muscle (lane 7) and Guinea pig liver (lane 8) all stored in RNA*later*®. These results suggested Prx 4 may have been preserved better when tissues were stored in RNA*later*® than when they were stored at -80°C. Alternatively, the length of time

that these tissues were stored for was also different as the frozen tissues had been stored since 2003 and the tissues in RNA*later*® only stored since 2010. The long storage time for the frozen tissues may have resulted in degradation of Prx 4 protein and this could explain the lack of reaction seen on the immunoblot for these samples.

Bands at approximately the size of a Prx dimer (45 kDa) were seen in the SBT liver, YTK liver, YTK muscle and Guinea pig liver samples stored in RNA*later*® (Fig. 4.2B, lanes 5-8, respectively) and even larger oligomers (approximately the size of a Prx octomer) were seen in the SBT liver and YTK liver samples stored in RNA*later*® (Fig. 4.2B, lanes 5 and 6, respectively). These results suggested that storage in RNA*later*® may promote oligomerisation of Prx proteins from fish. The SBT and YTK liver samples in lanes 5 and 6 showed similar banding patterns with bands at similar sizes; however, the band at the monomeric size (25 kDa) in the YTK liver sample was much fainter than the band at the same size in the SBT liver sample suggesting that the dimer form may be more prevalent in YTK.

In summary, the results of Protocol 1 suggested that the anti-(human Prx 4) antibodies could indeed recognise related proteins from fish but the results were inconsistent and more research was needed to understand the factors determining the oligomeric states of the fish Prx proteins.

4.3.2 Protocol 2

The aim of Protocol 2 was to test if the anti-(human GPx 4) antibodies could detect GPx 4 and/or any other GPx proteins from fish tissues. To test this, Buffer B (Table 4.5), which had previously been used by Thompson et al. (2006) to extract GPx and other proteins from muscle and liver of SBT was used.

The Coomassie Blue stained gel (Fig. 4.3A) showed a similar pattern for all three SBT muscle samples with a relatively small number of highly abundant proteins observed between 20 and 70 kDa (lanes 1-3). The SBT and Guinea pig liver samples, however, appeared to have a large number of equally abundant proteins (lanes 4 and 5, respectively). In Fig. 4.3B, the anti-(human GPx 4) antibodies detected strong expression in the Guinea pig liver sample for proteins with sizes of approximately 45 kDa and 90 kDa (lane 5). However, no expression was detected in any of the fish samples (lanes 1-4). These results suggested that the anti-(human GPx 4) antibodies could cross-react with GPx 4 from a mammal (Guinea pig) but not from a fish (SBT). Furthermore, they suggested that Guinea pig GPx 4 was present mostly as a dimer and also as a tetramer but not as a monomer. This result is curious as GPx 4 is functional as a monomer in mammals and has only been reported to oligomerise in the mitochondrial capsule of sperm (Ursini et al., 1999; Toppo et al., 2008) and could indicate a problem with the storage of the Guinea pig samples or with the immunoblotting protocol.

4.3.3 Protocol 3

Protocol 1 showed that the anti-(SBT Prx 2) antibodies could detect Prx proteins in SBT but not in YTK (Fig. 4.1B) and that the anti-(human Prx 4) antibodies could detect Prx proteins

in both SBT and YTK in both muscle and liver, but the results were inconsistent (Fig. 4.2B). Protocol 2 showed that the anti-(human GPx 4) antibodies could detect GPx 4 proteins in Guinea pig, as a representative mammal, but not in SBT, as a representative fish (Fig. 4.3). Therefore, the aims of Protocol 3 were (1) to test a third buffer (Buffer C, Table 4.6) that had been used in our group to successfully extract Prx proteins from muscle and liver tissues from Atlantic salmon, YTK, SBT and barramundi (Mr Drew Sutton, unpublished data), (2) to determine whether Buffer C was more effective than Buffer B which had been used in Protocol 2 and (3) to investigate the effects of adding a detergent (SDS) to both Buffer B and Buffer C. One of the forms of GPx 4 in mammals has been shown to be targeted to the mitochondria (Maiorino et al., 2003) and Prx 4 is believed to lodge in the endoplasmic reticulum (ER) via an N-terminal hydrophobic region prior to cleavage of this region and secretion of the mature protein (Iuchi et al., 2009). Therefore we hypothesised that the addition of SDS would solubilise the membranes of these subcellular organelles, releasing GPx 4 and/or Prx 4 into solution, overcoming, in the case of GPx 4, the lack of detection seen in Protocol 2.

In Fig. 4.4A, the Coomassie Blue stained gel showed a similar pattern of protein bands for the SBT liver samples in each extraction buffer. Therefore, there were no obvious differences between the buffers with respect to protein extraction efficiency. In Fig. 4.4B, the anti-(SBT Prx 2) antibodies detected intense protein bands in all four buffers at approximately monomer (20 kDa) and dimer (40 kDa) size with the monomer size being somewhat more abundant than the dimer size. Other than that, there were no noteworthy differences in size or intensity of the bands between the different buffers. Therefore, the four different buffers appeared to be equally effective in extracting Prx proteins that could be detected by the anti-(SBT Prx 2) antibodies.

The Coomassie Blue stained gel in Fig. 4.5A was the same as in Fig. 4.4A. It is included again here for comparison. In Fig. 4.5B, the anti-(human Prx 4) antibodies detected proteins in all four buffers at approximately Prx monomer (20 kDa) and Prx dimer (40 kDa) size but the amount of protein as dimer was much greater than the amount as monomer. This suggested that Prx 4 had not been completely reduced to monomeric size by any of the four different extraction buffers. The samples extracted into the buffers containing SDS showed additional protein bands slightly larger than the presumed Prx dimer and also in the range 60-200 kDa (lanes 2 and 4).

The Coomassie Blue stained gel in Fig. 4.6A was the same as in Figs. 4.4A and 4.5A. It is included again here for comparison. In Fig. 4.6B, the anti-(human GPx 4) antibodies detected proteins in all four different extraction buffers at a molecular mass of approximately 40 kDa which is the expected dimer size for GPx proteins. Otherwise, there were no noteworthy differences between the different extraction buffers.

In summary, the most important results from this protocol were (1) that the anti-(human GPx 4) antibodies detected a cross-reacting protein (possibly a GPx 4 protein) in SBT liver extracted in Buffer B and C both with or without added SDS (Fig. 4.6) and (2) that the addition of SDS to either Buffer B or C resulted in extraction of additional proteins recognized by the anti-(human Prx 4) antibodies (Fig. 4.5). This finding supported our hypothesis that the addition of SDS would solubulise the Prx 4 protein that was associated

with intracellular membranes such as the ER. Buffer C plus SDS was selected for further trials and will be referred to as Buffer D in subsequent protocols.

4.3.4 Protocol 4

Protocol 3 showed that Buffer C plus SDS (from now on referred to as Buffer D) was effective at extracting Prx proteins from SBT liver. Therefore, the aims of Protocol 4 were (1) to determine whether Buffer D was also effective at extracting Prx proteins from a range of YTK tissues as well and (2) to conduct a preliminary investigation of the tissue distribution of expression of Prx proteins YTK.

In Fig. 4.7A, the Coomassie Blue stained gel showed that the amounts of the proteins extracted from the YTK tissues appeared to be low (lanes 1-4) compared with the amounts extracted from Guinea pig liver (lane 5) and SBT liver (lane 6). However there was good resolution of the proteins in the YTK samples with distinct bands seen in each lane (lanes 1-4). In Fig. 4.7B, no reaction was seen with the anti-(SBT Prx 2) antibodies in YTK muscle and only a weak reaction was seen in YTK heart. In contrast, a strong reaction was seen, at Prx monomer size (approximately 20 kDa), in YTK liver and brain (lanes 2 and 3) and also in SBT liver (lane 6). In Guinea pig liver (lane 5), there was a strong reaction at Prx dimer size (approximately 40 kDa) but no reaction at Prx monomer size. In SBT liver (lane 6) there were also strong reactions at several higher molecular masses. These results suggest (1) that Prx protein expression is higher in YTK liver and brain than in YTK muscle and heart, (2) that Prx protein expression is very high in SBT liver and (3) that in Guinea pig liver, Prx dimers are more abundant than Prx monomers.

4.3.5 Protocol 5

In Protocol 4, Prx protein expression was investigated in YTK muscle, liver, brain and heart from a fish of unknown gender. Therefore, the aims of Protocol 5 were (1) to investigate Prx protein expression in other tissues as well, (2) to investigate the effect of gender (male or female) on the tissue distribution of Prx protein expression and (3) to investigate the tissue distribution of GPx 4 expression. Previous studies with mammals had shown that Prx 4 protein expression was higher in testes than in other tissues (Iuchi et al. 2009) and that GPx 4 was expressed to a high level in spermatocytes and spermatids (Imai et al., 2001; Imai et al., 2009). Therefore, we hypothesized that Prx 4 protein expression would also be higher in the testes of fish and that GPx would show abundant expression in the testes but would not necessarily be more abundant than in other organs.

4.3.5.1 Expression of Prx and GPx proteins in a broad range of tissues from a 3.0 kg YTK female (YTK 1)

The Coomassie Blue stained gel (Fig. 4.8A) showed relatively similar amounts of protein for most tissues except the spleen and gill tissues (lanes 5 and 8) which showed slightly less protein. As previously observed, skeletal muscle showed a small number of highly abundant proteins (lane 3) whereas the other tissues showed a large number of proteins of equal abundance. The only other noteworthy difference was the presence of a single highly abundant protein in the intestine (lane 7) at approximately 45 kDa. In Fig. 4.8B, expression was detected using the anti-(SBT Prx 2) antibodies at Prx monomer size (approximately 20 kDa) in YTK brain, liver, heart, spleen and ovaries (lanes 1, 2, 4, 5 and 6, respectively). The strongest expression was observed in brain, liver and ovaries (lanes 1, 2 and 6). In muscle (lane 3), a strong band was seen at approximately 35 kDa and this band was much stronger

than the faint band seen at 20 kDa. Similarly, in Guinea pig liver (lane 9, used as a positive control), a strong band was observed at 45 kDa but no band was observed at 20 kDa. In intestine (lane 7), a strong band was observed between 116 and 200 kDa but no bands were observed at any other sizes. The 20 kDa band seen in the majority of the samples was at the expected size for a Prx monomer, however, the 35 kDa band in muscle did not correspond to any predicted molecular mass for Prx proteins.

Broadly speaking, the results shown in Fig. 4.8 confirmed those shown in Fig. 4.7 in that Prx expression at the monomer size was high in YTK liver and brain but low in YTK muscle and heart. In addition, Fig. 4.8 showed that Prx expression at the monomer size was high in YTK ovaries (as high as in YTK liver and brain), somewhat lower in YTK spleen and absent from YTK intestine or gill. Interestingly, the approximately 31 kDa cross-reacting protein in muscle which was faint in Fig. 4.7 was strong in Fig. 4.8 indicating that this may be a different form of Prx recognized by the anti-(SBT Prx 2) antibodies or a different protein altogether. The very large cross-reacting protein in intestine is also interesting. This could be a highly aggregated form of a Prx protein or a different protein that cross-reacts with the anti-(SBT Prx 2) antibodies.

The Coomassie Blue stained gel in Fig. 4.9A had the same contents as the gel in Fig. 4.8A except that the gill sample was excluded due to low protein abundance and the Guinea pig liver sample was replaced with a human brain extract (Fig. 4.9A, lane 8) as a more reliable positive control. The human brain extract showed a large number of proteins of approximately equal abundance (lane 8). In Fig. 4.9B, the anti-(human Prx 4) antibodies detected strong protein bands at approximately 45 kDa in YTK liver (lane 2) and intestine

(lane 7) and a weak protein band at this same molecular mass in human brain (lane 8). There was also a band at 80-100 kDa in YTK liver (lane 2) and YTK heart (lane 4). Interestingly, the anti-(human Prx 4) antibodies did not detect any proteins at 20 kDa, the approximate monomer size for Prx proteins. This suggests either that Prx 4 had formed oligomers during extraction or that the anti-(human Prx 4) antibodies were recognizing a different protein or possibly that Prx 4 was associating with another protein increasing its molecular mass. Comparing Figs. 4.2B, 4.5B and 4.9B, all of which were produced using the anti-(human Prx 4) antibodies, it can be seen that very different banding patterns were produced. Thus, it is difficult to draw any firm conclusions from these results.

The Coomassie Blue stained gel in Fig. 4.10A contained the same samples as the gel in Fig. 4.9A. It is included here for comparison. In Fig. 4.10B, the anti-(human GPx 4) antibodies detected multiple protein bands in YTK brain between 45 and 116 kDa (lane 1). They also detected three bands in the ovaries with the strongest at 45 kDa and two fainter bands at slightly higher molecular masses (lane 6). The same pattern was seen in the human brain sample (lane 8) and a single band the same size as the largest band in the ovaries and human brain was observed in the intestine (lane 7). Some reaction at a higher molecular mass (greater than 116 kDa) was also observed in the ovaries and intestine (Fig. 4.11). None of these sizes, except perhaps the 45 kDa size, corresponded to any known sizes for GPx proteins. Thus, it is unclear whether the anti-(human GPx 4) antibodies were recognizing genuine GPx proteins in YTK. If we compare Fig. 4.6B with Fig. 4.10B, both of which were produced from experiments with the anti-(human GPx 4) antibodies, it is clear that the molecular masses of the proteins recognized by these antibodies are different and therefore it is difficult to draw any conclusions.

4.3.5.2 Expression of Prx and GPx proteins in a broad range of tissues from a 3.0 kg YTK male (YTK 2)

Previous studies with mammals have found significantly higher expression of Prx 4 protein (Iuchi et al., 2009) in testes than in any other tissues and GPx 4 protein has been shown to be highly abundant in late spermatocytes and spermatids (Imai et al., 2001; Imai et al., 2009). Therefore, the aim of this Protocol was to determine if the expression of Prx and GPx proteins was higher in testes than in other organs of YTK. A secondary aim was to determine if protein detected by the anti-(SBT Prx 2), anti-(human Prx 4) and anti-(human GPx 4) antibodies was any different in male fish compared with female fish (see Section 4.3.5.1). We hypothesised that expression would be higher in testes than in any other organs investigated so far, and that expression in female fish would be similar to male fish, with the exception of the gonad tissues (testes and ovaries).

In Fig. 4.11A, the Coomassie Blue stained gel showed a similar pattern of protein banding as was seen in previous gels. Most samples had an even distribution of proteins of approximately equal abundance. The exception to this was skeletal muscle (lane 3) which had a small number of highly abundant proteins and heart (lane 4) showed two bands at approximately 35 and 45 kDa that were more abundant than other proteins in the sample. The intestine (lane 5) and spleen (lane 6) both exhibited a band at approximately 7 kDa that was stronger than any other bands in the sample. In Fig. 4.11B, the anti-(SBT Prx 2) antibodies detected strong expression at the Prx monomer size (approximately 20 kDa) in spleen (lane 6) but only weak expression at this size or any other sizes in any of the other tissues. The reactions in the brain, liver, skeletal muscle and heart tissues were much lower in the male YTK (Fig. 4.11B) than were observed in the female YTK (Fig. 4.8B), and this suggests either (a) that the extraction efficiency was lower for the males than for the females, (b) that the

expression of Prx proteins in male YTK is significantly lower than in female YTK or (c) that there was a mistake in the immunoblotting protocol which decreased the sensitivity of the antibody reaction with the samples from the males. The most likely explanation is (c), an error in the immunoblotting protocol.

The Coomassie Blue stained gel in Fig. 4.12A was the same as in Fig. 4.11A. It is included again here for comparison. In Fig. 4.12B, the anti-(human Prx 4) antibodies detected prominent proteins at approximately 50 and 80 kDa in YTK liver (lane 2) and at approximately 80 kDa in YTK heart (lane 4). No bands were detected for any of the other tissues.

These results were consistent with the results seen for the 3.0 kg YTK female (Fig. 4.9B) and suggest that the reducing conditions in the extraction buffer were not sufficient to reduce the Prx 4 proteins to monomeric size. Importantly, the testes tissue (Fig. 4.12B, lane 7) did not exhibit the expected high level of expression and this could be because the fish were not old enough to express Prx 4 in the testes. Alternatively, the Prx 4 protein expression profile in fish tissues may be different to what has been observed for mammals (Iuchi et al., 2009) and Prx 4 may not be expressed at a high level in fish testes.

The Coomassie Blue stained gel in Fig. 4.13A was the same as in Fig. 4.11A. It is included again here for comparison. In Fig. 4.13B, the anti-(human GPx 4) antibodies detected multiple protein bands between 50 and 80 kDa in the 3.0 kg YTK male brain (lane 1). This was similar to what had been observed for the 3.0 kg YTK female (Fig. 4.10B). Reaction was

also seen at a higher molecular mass (>100 kDa) in intestine (lane 6) and spleen (lane 7). This reaction at high molecular mass was also similar to what was seen for the 3.0 kg YTK female (Fig. 4.10B).

The reaction at higher molecular masses using the anti-(human Prx 4) and anti-(human GPx 4) antibodies was consistent for both the 3 kg YTK female and the 3 kg YTK male (YTK 1 and YTK 2). Therefore, in Protocol 6 the concentration of DTT was raised to promote stronger reducing conditions and urea was added to promote unfolding of the protein, in an effort to reduce a greater amount of protein to the monomeric form.

4.3.6 Protocol 6

The results obtained using the previous protocols suggested that Extraction Buffer D may not have provided the appropriate conditions to completely reduce the Prx and GPx proteins to their monomeric states. Therefore, the aims of Protocol 6 were to investigate the effect of increasing the DTT concentration from 10 mM (Buffer D) to 50 mM (Buffer E) and (2) to investigate the combined effects of increasing the DTT concentration from 10 mM (Buffer D) to 50 mM and adding a 5 M concentration of urea (Buffer F). The purpose of addition of extra DTT was to provide stronger reducing conditions in the buffer. It was hypothesised that this would improve disulphide bridge disruption and reduce the apparent protein aggregation seen in some of the previous protocols. Urea is a chaotrophic agent which assists in the denaturation (unfolding) of proteins (McCarthy et al., 2003; Auton et al., 2007). Therefore, we anticipated that urea would promote the unfolding of the proteins thereby making any buried disulphide bonds more accessible for disruption (reduction) by DTT. We hypothesized

that the combination of DTT with urea would be more effective than DTT alone at counteracting the apparent protein aggregation seen in some of the previous protocols.

The Coomassie Blue stained gel in Fig. 4.14A showed an approximately equal concentration of protein for all samples with the exclusion of the YTK brain sample in Buffer F (lane 5), which exhibited a larger amount of protein than all of the other samples. All of the samples had a large number of equally abundant proteins and no highly prominent bands were observed in any of the extracts. In Fig. 4.14B, the anti-(SBT Prx 2) antibodies gave a strong reaction at the predicted Prx monomer size (approximately 20 kDa) in YTK brain and liver in Buffer E (lanes 1 and 2, respectively) and in YTK brain and liver in Buffer F (lanes 5 and 6, respectively. Only a weak reaction was seen at this size in any of the other samples in either Buffer E or Buffer F. Therefore, it can be concluded that both Buffer E and Buffer F were equally effective at extracting the Prx proteins detected by the anti-(SBT Prx 2) antibodies from YTK and reducing these proteins to monomeric form.

The Coomassie Blue stained gel in Fig. 4.15A was the same as in Fig. 4.14A. It is included again here for comparison. In Fig. 4.15B, the anti-(human Prx 4) antibodies detected prominent protein bands at approximately 25 and 50 kDa in YTK liver for both Extraction Buffer E (lane 2) and Extraction Buffer F (lane 6) but they did not detect any prominent bands for any of the other tissues regardless of the buffer used. Interestingly, the 50 kDa protein was more abundant than the 25 kDa protein. These results suggested that the additional DTT in Buffer E was sufficient to reduce some, but not all of the Prx 4 proteins in the liver sample to the Prx monomeric size. If we compare Fig. 4.15B with Fig. 4.9B (both YTK 1 with the anti-(human Prx 4) antibodies) it is clear that there is greater reduction of Prx

4 from YTK liver to monomeric size in both Buffer E (Fig. 4.15B, lane 2) and Buffer F (Fig. 4.15B, lane 6) than was observed for Buffer D (Fig. 4.9B, lane 2).

4.4 Discussion

In Protocols 1-3, three buffers were used that had previously been reported as effective at extraction Prx or GPx proteins from fish tissues. Out of these three buffers, Buffer C (Table 4.6) plus 1% SDS (renamed Buffer D, Table 4.7) was found to be most effective. Buffer D was used to successfully extract Prx and GPx proteins from SBT liver and showed two bands of Prx 4 at dimer size and just above dimer size where Buffer C only showed the dimer sized band (Section 4.3.3). The anti-(SBT Prx 2) antibodies were shown to cross-react with Prx proteins in all YTK tissues with the exception of gill (Fig. 4.8). The low reaction in gill may be explained by low extraction efficiency resulting in a limited amount of protein being loaded on the gel or just simply a low amount of protein per mg wet weight in gill (Fig. 4.8A). The strong cross-reaction in the majority of YTK tissues (Fig. 4.8) supports the hypothesis that these antibodies detect multiple sub-classes of Prx proteins in fish tissues and that those Prx proteins are expressed at high levels. Reaction was mostly at the expected monomer size of a 2-Cys Prx protein. The exception to this was muscle, which consistently showed a band at approximately 34 kDa.

The anti-(human Prx 4) antibodies showed consistent cross-reaction in YTK liver. This reaction was observed at dimer size for the extracts in Buffer D (Fig. 4.9) but the modifications made in Buffer F resulted in a large amount, but not all, of this protein in liver being reduced to the predicted size of a Prx 4 monomer (Fig. 4.15). Importantly, the predicted high abundance of Prx 4 in the testes of male YTK was not observed.

The anti-(human GPx 4) antibodies cross-reacted with a protein at approximately GPx 4 dimer size (40 kDa) in SBT liver (Fig. 4.6) but failed to detect any protein in YTK liver. The only reaction seen in YTK was with 3 proteins at approximately 50, 60 and 80 kDa in brain (Figs. 4.10 and 4.13). The 50 and 60 kDa sizes do not correspond to any predicted molecular mass of GPx 4 but the 80 kDa band was approximately the size of a GPx 4 tetramer.

In Section 4.3.5, the conditions provided by Buffer D were found to be insufficient to reduce many of the Prx and GPx proteins to their monomeric form. Therefore, in Section 4.3.6, further modifications were made to Buffer D and the resulting Buffer F was found to provide stronger reducing conditions and was selected for use in subsequent chapters.

The successful extraction of Prx and GPx proteins in Protocols 1-3 was as expected, as the buffers used had previously been successful at extracting these proteins from fish tissues (Thompson et al., 2006; Loo and Schuller, 2010; Sutton et al., 2010; Mr Drew Sutton, unpublished data). However, the detection of an additional band by the anti-(human Prx 4) antibodies around dimer size in extracts containing SDS (Fig. 4.6B) was unexpected as Prx 4 has traditionally been described as a cytosolic or secreted protein (Wood et al., 2003). The presence of this additional form may be explained by the hydrophobic sequence found at the N-terminal end of the unprocessed Prx 4 proteins in mammals (Jin et al., 1997). In mammals, this sequence anchors the unprocessed form of Prx 4 to the endoplasmic reticulum (ER) lumen, and is then cleaved off prior to secretion in all tissues except for sexually mature testes (Iuchi et al., 2009). It is possible, therefore, that in fish, the unprocessed form is

remaining anchored to the ER similar to what is seen in mammalian testes. The addition of SDS to the extraction buffer would have then allowed solubilisation of the endoplasmic reticulum, releasing unprocessed forms of Prx 4 which formed dimers, resulting in the additional bands seen on the immunoblot.

The almost ubiquitous reaction detected by the anti-(SBT Prx 2) antibodies was expected as it was believed that these antibodies were detecting all 2-Cys Prx proteins from fish as the antibodies were raised against a whole protein rather than a small peptide. Members of the 2-Cys Prx family are known to be almost ubiquitous in mammals (Leyens et al., 2003; Iuchi et al., 2009) and have been found in the liver and muscle of fish (Loo and Schuller, 2010; Sutton et al., 2010). The band at approximately 35 kDa that was consistently observed in YTK skeletal muscle, did not correspond to any predicted size of a Prx protein. Prx proteins use thioredoxin as an electron acceptor (Wood et al., 2003) and thioredoxin is approximately 12 kDa in size (calculated from the zebrafish sequence GenBank accession number NP_001002461). It is possible, therefore, that the 35 kDa band in skeletal muscle was a result of the 22 kDa Prx protein associating with the 12 kDa thioredoxin protein.

In reactions with the anti-(human Prx 4) antibodies, the ubiquitous expression of Prx 4 that has been observed in mammalian tissues (Iuchi et al., 2009), was not observed in many of the YTK tissues (Figs. 4.9, 4.12 and 4.16). The only consistent expression was observed in liver and in the majority of cases, this reaction was intense indicating high expression. Importantly, the testes tissue (Fig. 4.13B, lane 7) did not exhibit the expected high level of expression based on the mammalian literature. The literature for mammals indicates that Prx 4 is most abundant in the testes of sexually mature animals (Sasagawa et al., 2001). This result could

indicate that the 3.0 kg fish were not sexually mature. However, a recent development of a YTK pedigree by Associate Professor Wayne Knibb and colleagues indicates that the 3.0 kg fish used in this study were likely to be sexually mature (personal communications). Alternatively, the expression profile of Prx 4 in fish tissues may be different to what has been observed for mammals (Iuchi et al., 2009) and Prx 4 may not be expressed at a high level in fish testes.

The reaction with the anti-(human GPx 4) antibodies at sizes larger than the predicted monomer size (21 kDa) was unexpected as GPx 4 is known, in mammals, to be functional as a monomer (Toppo et al., 2008) and has not been reported to oligomerise. This suggests that either (a) GPx 4 from fish forms oligomers that are not observed for mammalian GPx 4, (b) the anti-(human GPx 4) antibodies are not specific for fish GPx 4 and are binding with some other protein or (c) GPx 4 is associating with another protein of a similar molecular mass and producing the reactions at dimer size seen on the immunoblot.

The experiments in Section 4.3.6 indicated that the addition of more DTT to the extraction buffer (Buffer E) resulted in a greater reduction of Prx 4 proteins to monomeric size (Fig. 4.15) than was seen for Buffer D (Section 4.3.5). Furthermore, since the reaction at monomeric size was more intense in YTK liver in the buffer containing urea (Buffer F), we concluded the addition of urea to the extraction buffer allowed greater unfolding of Prx 4 which in turn allowed greater accessibility of the disulphide bonds to be reduced by DTT. There were, however, still bands in the liver samples at dimer size in both Buffer E and Buffer F (Fig. 4.15) and this may be due to the presence of trace amounts of hydrogen peroxide in the extraction buffer solution, which have been shown to cause dimerisation in

Prx proteins (Peskin et al., 2007). Previous work in our group with Prx 4 has also found this protein to be very difficult to reduce to monomeric form (Mr D. Sutton, unpublished data) and it is possible that some of the oligomerisation of these proteins is not reversible by traditional reducing agents. Therefore, despite the presence of some dimer of Prx 4, Buffer F was chosen to further investigate Prx and GPx expression in various tissues of YTK in Chapter 5.

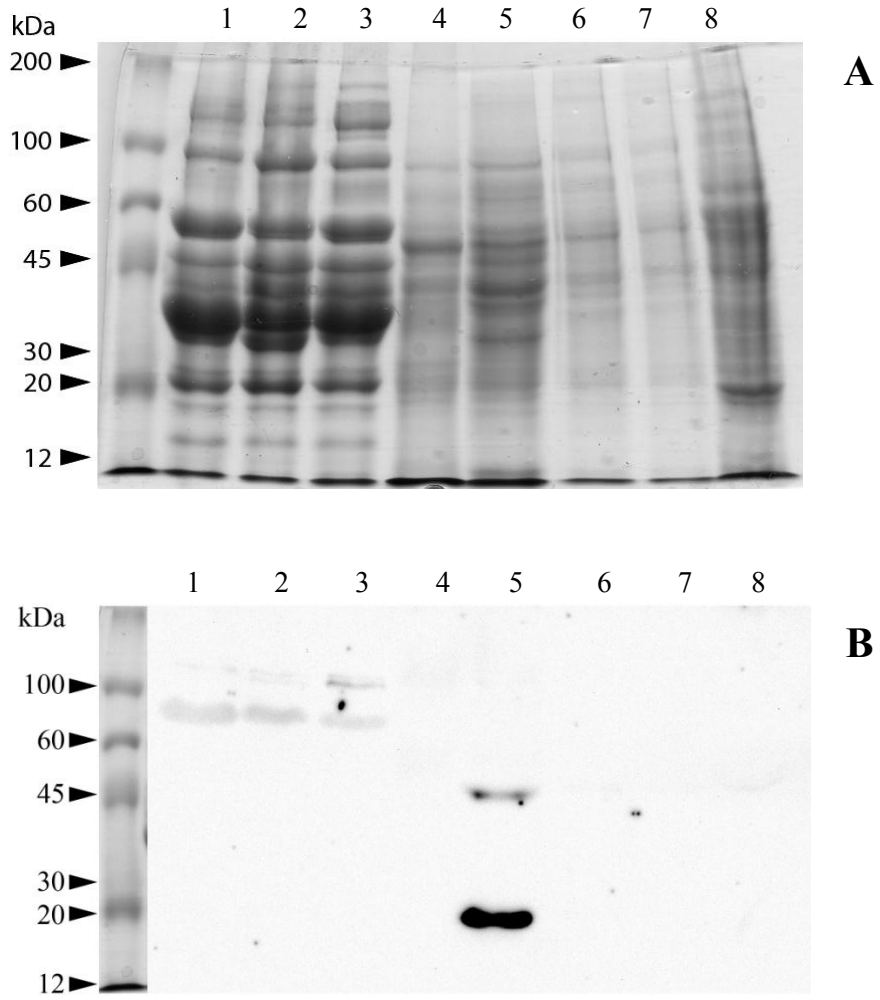


Figure 4.1: Expression of Prx proteins in various tissues of SBT and YTK. The lanes contained 1 – SBT Akami muscle (stored at -80^{o}C); 2 – SBT Chutoro muscle (stored at -80^{o}C); 3 – SBT Otoro muscle (stored at -80°C); 4 SBT liver (stored at -80°C); 5 – SBT liver (stored in RNA*later*®); 6 – YTK Liver (stored in RNA*later*®); 7 – YTK muscle (stored in RNA*later*®); 8 – Guinea Pig liver (stored in RNA*later*®). The sources of the tissue samples are shown in Table 1.

(A) SDS-PAGE conducted on a 10% (w/v) acrylamide gel with the indicated tissues of SBT and YTK extracted into extraction Buffer A (20 mM Tris-base, 500 mM NaCl and 5 mM DTT). Total protein was estimated using the method of Bradford (1976) and 100 µg protein was loaded into each lane of the gel. .

(B) Immunoblot analysis of a parallel gel to panel A using a 1:1000 dilution of anti-(SBT-Prx 2) antibodies.

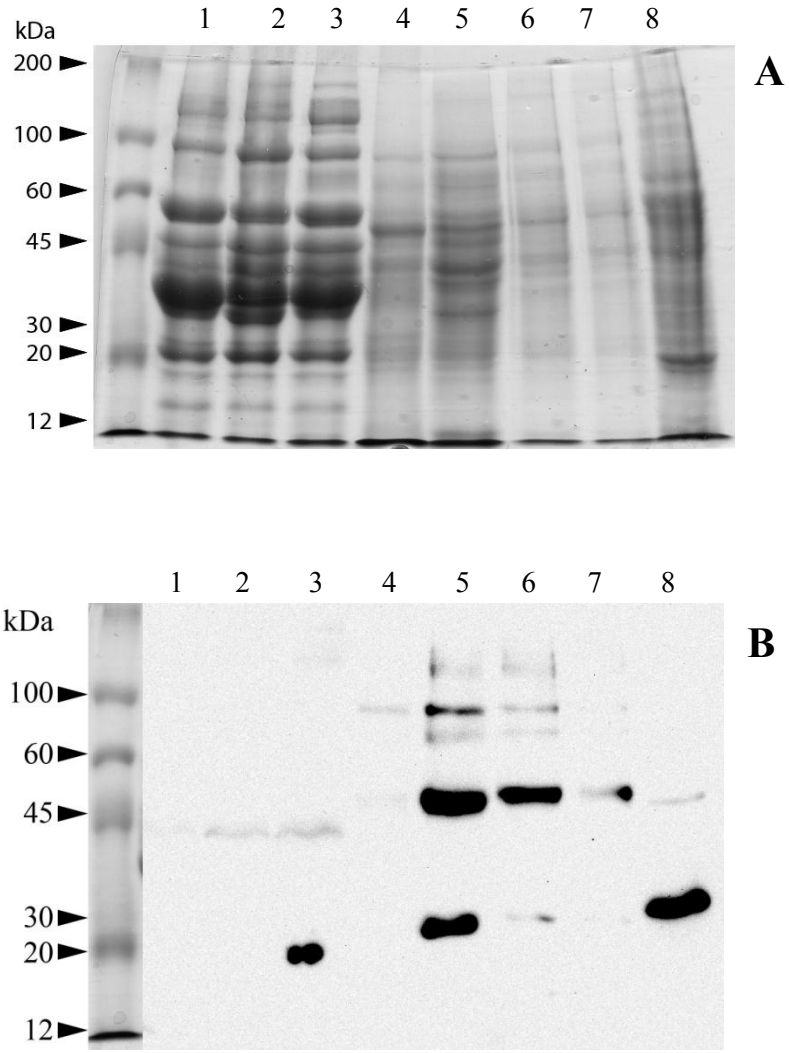


Figure 4.2: Expression of Prx 4 in various tissues of SBT and YTK. The lanes contained 1 – SBT Akami muscle (stored at -80°C); 2 – SBT Chutoro muscle (stored at -80°C); 3 – SBT Otoro muscle (stored at -80°C); 4 – SBT liver (stored at -80°C); 5 – SBT liver (stored in RNA*later*®); 6 – YTK liver (stored in RNA*later*®); 7 – YTK muscle (stored in RNA*later*®); 8 – Guinea Pig liver (stored in RNA*later*®). The sources of the tissue samples are shown in Table 1.

(A) SDS-PAGE conducted on a 10% (w/v) acrylamide gel with the indicated tissues of SBT and YTK extracted into extraction Buffer A (20 mM Tris-base, 500 mM NaCl and 5 mM DTT). Total protein was estimated using the method of Bradford (1976) and 100 µg protein was loaded into each lane of the gel. .

(B) Immunoblot analysis of a parallel gel to panel A using a 1:1000 dilution of anti-(human Prx 4) antibodies.

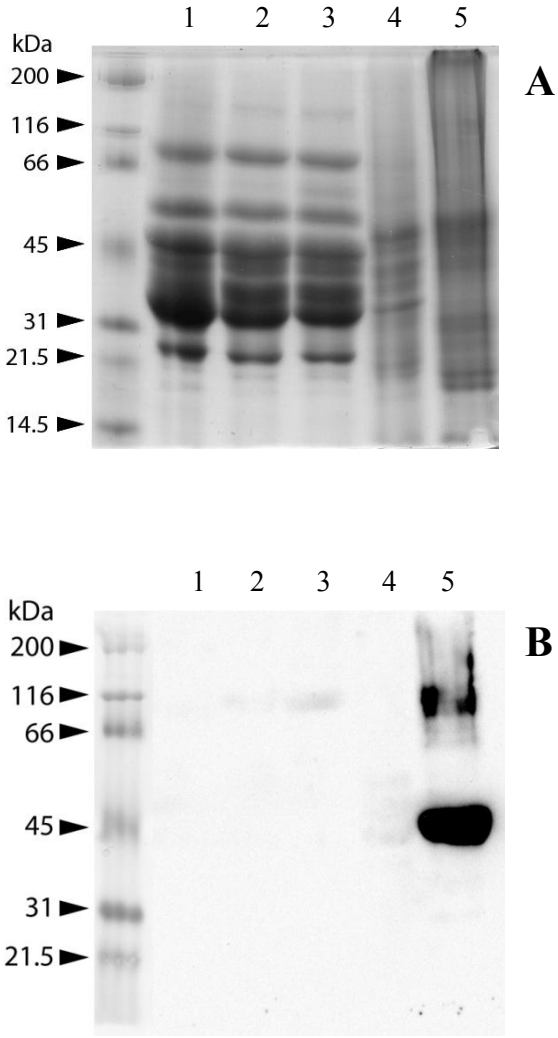


Figure 4.3: Expression of GPx 4 in various tissues of SBT. The lanes contained 1 – SBT Akami muscle (stored at -80°C); 2 – SBT Chutoro muscle (stored at -80°C); 3 – SBT Otoro muscle (stored at -80°C); 4 – SBT liver (stored in RNA*later*®); 5 – Guinea Pig liver (stored in RNA*later*®). The sources of the tissue samples are shown in Table 1.

(A) SDS-PAGE conducted on a 10% (w/v) acrylamide gel with the indicated tissues of SBT extracted into extraction Buffer B (50 mM K-phosphate, 5 mM DTT, 6 mM GSH, 2 mM PMSF and 1 mM EDTA). Total protein was estimated using the method of Bradford (1976) and 100 µg protein was loaded in each lane of the gel. .

(B) Immunoblot analysis of a parallel gel to panel A using a 1:1000 dilution of anti-(human GPx 4) antibodies.

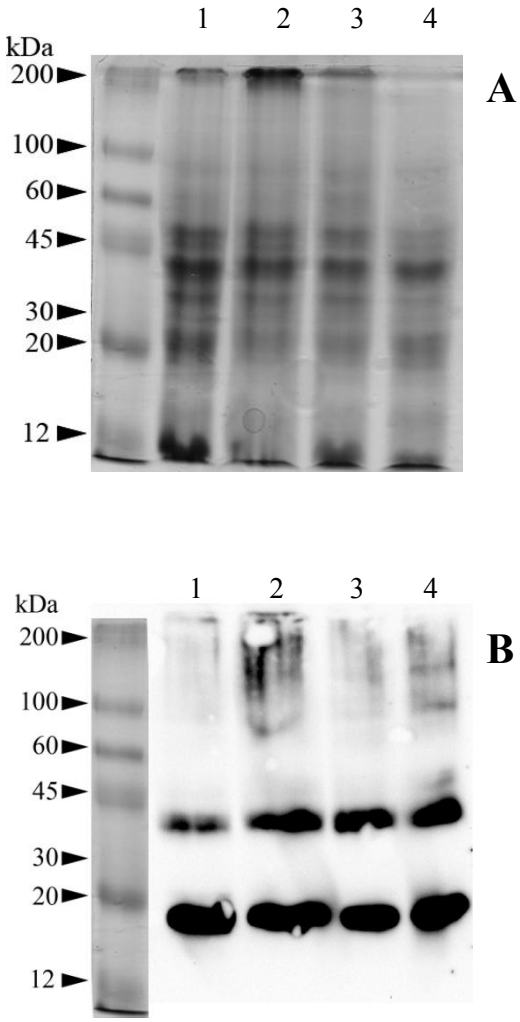


Figure 4.4: Expression of Prx proteins in SBT liver (stored at -80°C) using four different extraction buffers. The lanes contained 1 – SBT liver in Buffer C; 2 – SBT liver in Buffer C + SDS; 3 – SBT liver in Buffer B; 4 – SBT liver in Buffer B + SDS. The source of the tissue samples is shown in Table 1.

(A) SDS-PAGE conducted on a 10% (w/v) acrylamide gel with SBT liver extracts in four different extraction buffers. Buffer C contained 50 mM HEPES, 150 mM NaCl, 10 mM DTT, 1 mM PMSF and 1 mM EDTA. Buffer C + SDS was the same as Buffer C plus 1 % (w/v) SDS. Buffer B contained 50 mM K-phosphate, 5 mM DTT, 6 mM GSH, 2 mM PMSF and 1 mM EDTA. Buffer B + SDS was the same as Buffer B plus 1 % (w/v) SDS. An equal volume of extract from an equal mass of tissue was loaded into each lane of the gel because protein concentration could not be determined due to interference of SDS with the protein assay. .

(B) Immunoblot analysis of a parallel gel to panel A using a 1:1000 dilution of anti-(SBT-Prx 2) antibodies.

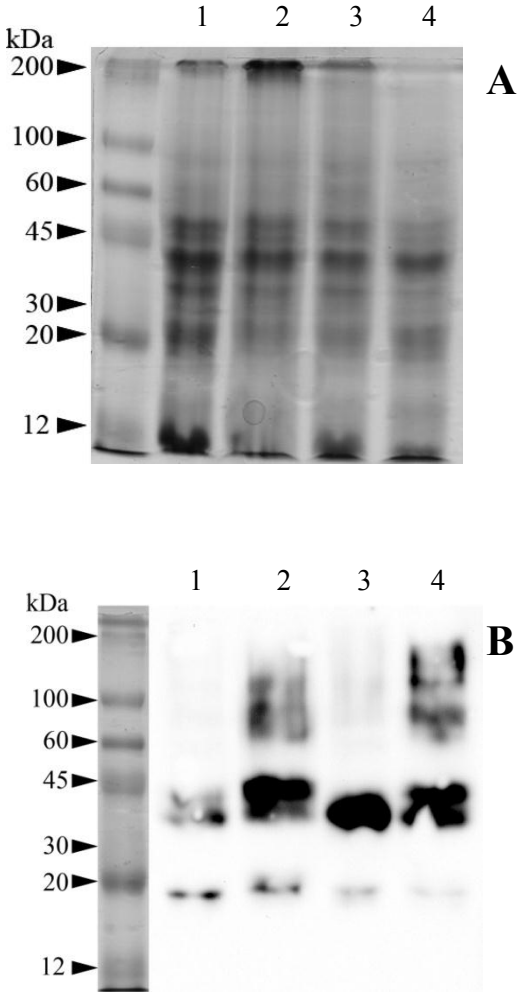


Figure 4.5: Expression of Prx 4 in SBT liver (stored at -80°C) using four different extraction buffers. The lanes contained 1 – SBT liver in Buffer C; 2 – SBT liver in Buffer C + SDS; 3 – SBT liver in Buffer B; 4 – SBT liver in Buffer B + SDS. The source of the tissue samples is shown in Table 1.

(A) SDS-PAGE conducted on a 10% (w/v) acrylamide gel with SBT liver extracts in four different extraction buffers. Buffer C contained 50 mM HEPES, 150 mM NaCl, 10 mM DTT, 1 mM PMSF and 1 mM EDTA. Buffer C + SDS was the same as Buffer C plus 1 % (w/v) SDS. Buffer B contained 50 mM K-phosphate, 5 mM DTT, 6 mM GSH, 2 mM PMSF and 1 mM EDTA. Buffer B + SDS was the same as Buffer B plus 1 % (w/v) SDS. An equal volume of extract from an equal mass of tissue was loaded into each lane of the gel because protein concentration could not be determined due to interference of SDS with the protein assay. .

(B) Immunoblot analysis of a parallel gel to panel A using a 1:1000 dilution of anti-(human Prx 4) antibodies.

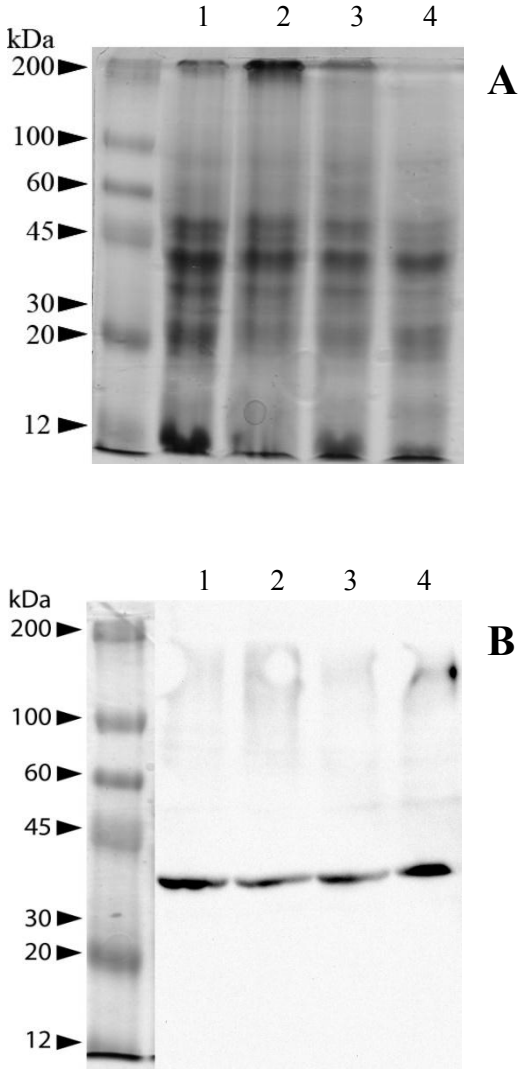


Figure 4.6: Expression of GPx 4 in SBT liver (stored at -80°C) using four different extraction buffers. The lanes contained 1 – SBT liver in Buffer C; 2 – SBT liver in Buffer C + SDS; 3 – SBT liver in Buffer B; 4 – SBT liver in Buffer B + SDS. The source of the tissue samples is shown in Table 1.

(A) SDS-PAGE conducted on a 10% (w/v) acrylamide gel with SBT liver extracts in four different extraction buffers. Buffer C contained 50 mM HEPES, 150 mM NaCl, 10 mM DTT, 1 mM PMSF and 1 mM EDTA. Buffer C + SDS was the same as Buffer C plus 1 % (w/v) SDS. Buffer B contained 50 mM K-phosphate, 5 mM DTT, 6 mM GSH, 2 mM PMSF and 1 mM EDTA. Buffer B + SDS was the same as Buffer B plus 1 % (w/v) SDS. An equal volume of extract from an equal mass of tissue was loaded into each lane of the gel because protein concentration could not be determined due to interference of SDS with the protein assay. .

(B) Immunoblot analysis of a parallel gel to panel A using a 1:1000 dilution of anti-(human GPx 4) antibodies.

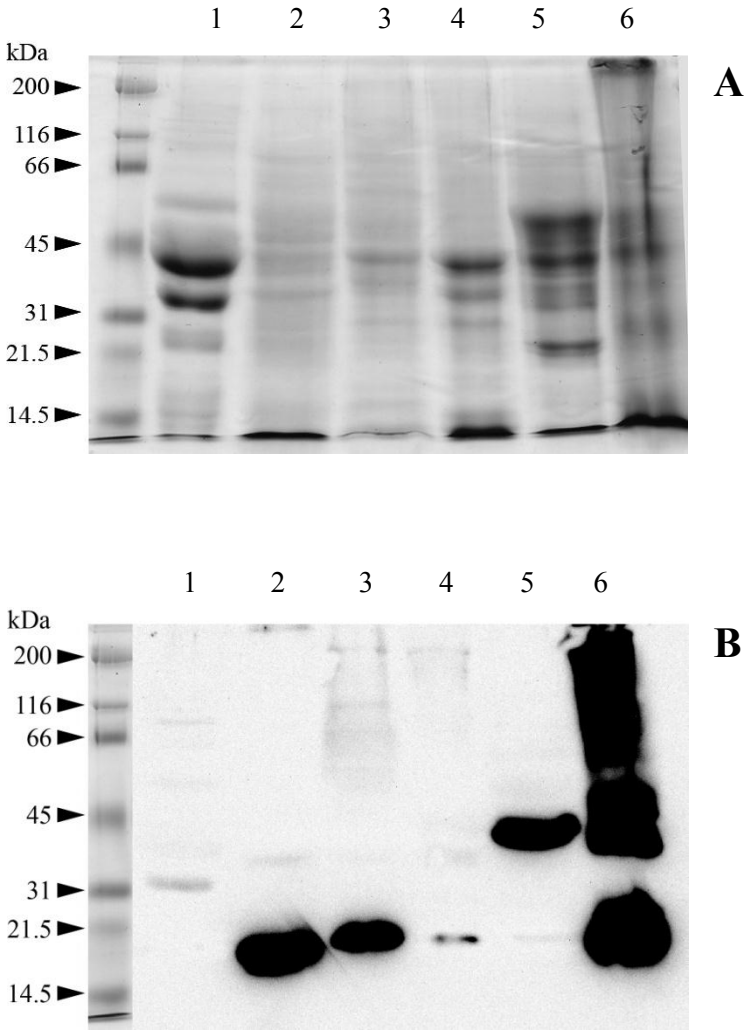


Figure 4.7: Expression of Prx proteins in various tissues of YTK (stored in RNA*later*®). The lanes contained 1 – Muscle; 2 – Liver; 3 – Brain; 4 – Heart; 5 – Guinea pig liver; 6 – SBT liver (stored at -80°C). The sources of the SBT and Guinea pig tissue samples are shown in Table 1 and the source of the YTK samples is shown in Table 2.

(A) SDS-PAGE conducted on a 10% (w/v) acrylamide gel with the indicated tissues of YTK extracted into extraction Buffer D (50 mM HEPES, 150 mM NaCl, 10 mM DTT, 1 mM PMSF, 1 mM EDTA and 1 % (w/v) SDS). An equal volume of extract from an equal mass of tissue was loaded into each lane of the gel because protein concentration could not be determined due to interference of SDS with the protein assay. .

(B) Immunoblot analysis of a parallel gel to panel A using a 1:1000 dilution of anti-(SBT-Prx 2) antibodies.

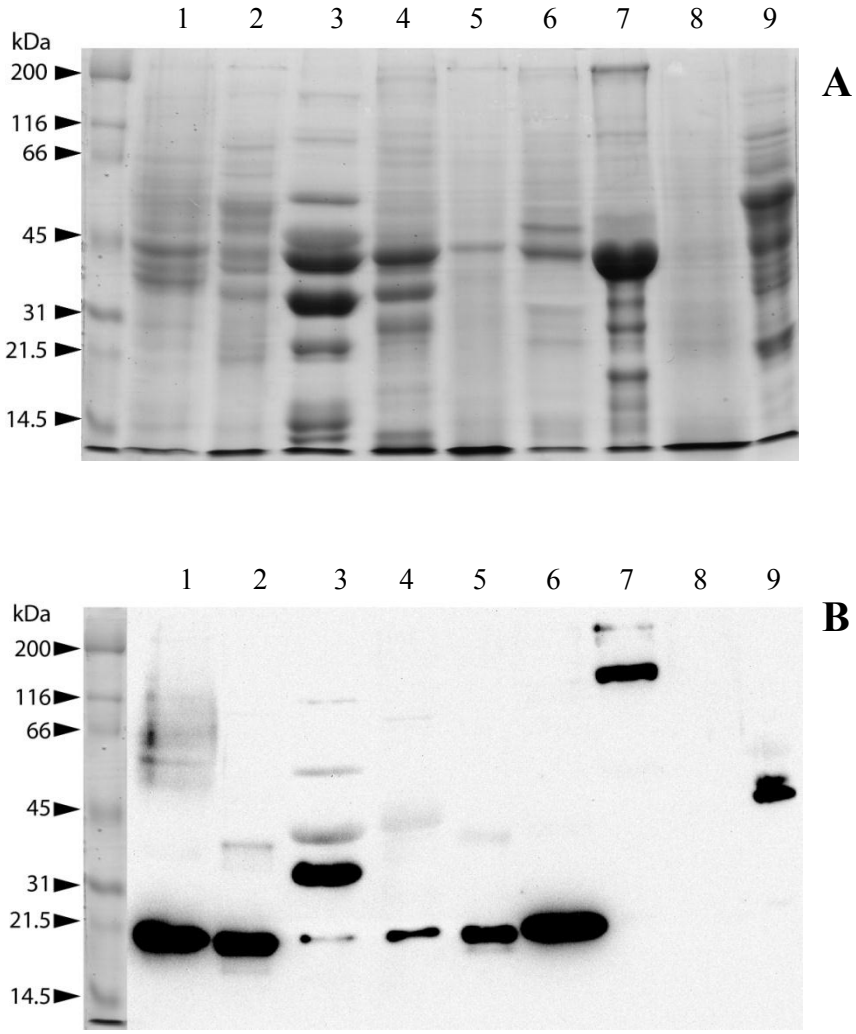


Figure 4.8: Expression of Prx proteins in various tissues of a 3 kg female YTK (YTK 1; stored in RNA*later*®). The lanes contained 1 – Brain; 2 – Liver; 3 – Muscle; 4 – Heart; 5 – Spleen; 6 – Ovaries; 7 – Intestine; 8 – Gill; 9 – Guinea pig liver (Stored in RNA*later*®). The source of the Guinea pig samples is shown in Table 1 and the sources of the YTK samples are shown in Table 3.

(A) SDS-PAGE conducted on a 10% (w/v) acrylamide gel with the indicated tissues of YTK 1 extracted into extraction Buffer D (50 mM HEPES, 150 mM NaCl, 10 mM DTT, 1 mM PMSF, 1 mM EDTA and 1 % (w/v) SDS). A volume of extract equivalent to that which would have been obtained from 2.5 mg of tissue wet weight was loaded into each lane of the gel. .

(B) Immunoblot analysis of a parallel gel to panel A using a 1:1000 dilution of anti-(SBT-Prx 2) antibodies.

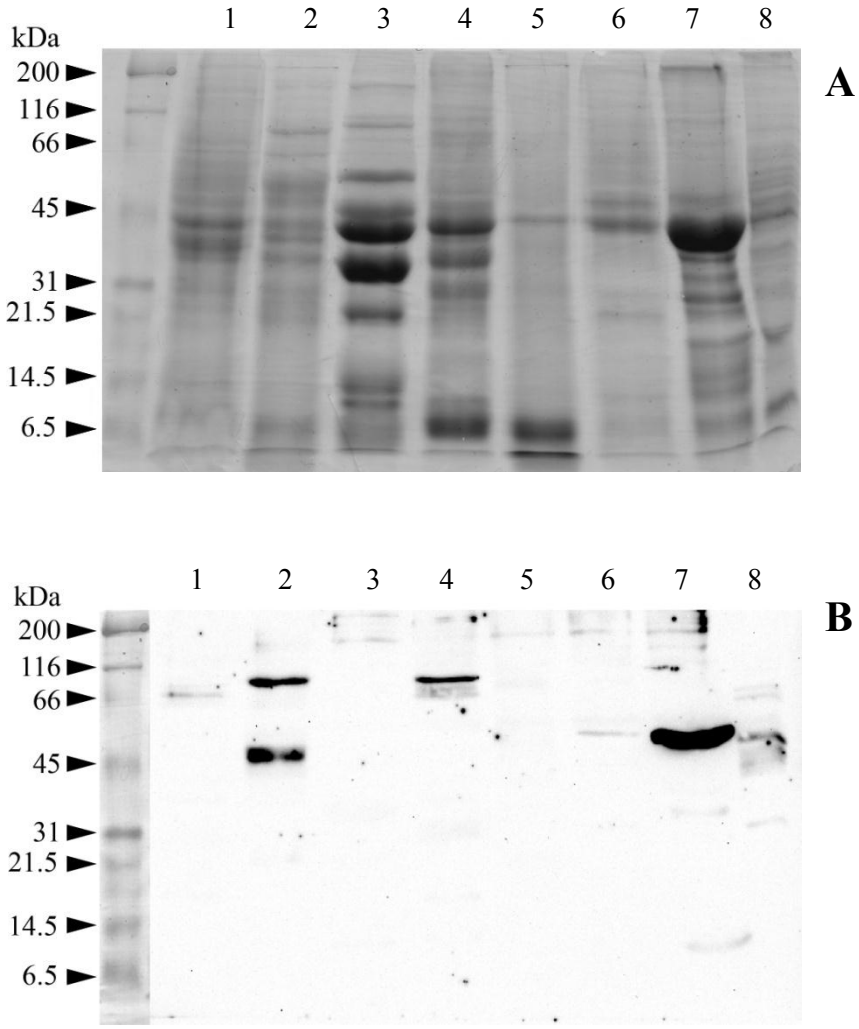


Figure 4.9: Expression of Prx 4 in various tissues of a 3 kg female YTK (YTK 1; stored in RNA*later*®). The lanes contained 1 – Brain; 2 – Liver; 3 – Muscle; 4 – Heart; 5 – Spleen; 6 – Ovaries; 7 – Intestine; 8 – Human brain. The human brain tissue was sourced from Dr John Power, Flinders University, School of Medicine and the source of the YTK tissues is shown in Table 3.

(A) SDS-PAGE conducted on a 10% (w/v) acrylamide gel with the indicated tissues of YTK 1 extracted into extraction Buffer D (50 mM HEPES, 150 mM NaCl, 10 mM DTT, 1 mM PMSF, 1 mM EDTA and 1 % (w/v) SDS). A volume of extract equivalent to that which would have been obtained from 2.5 mg of tissue wet weight was loaded into each lane of the gel. .

(B) Immunoblot analysis of a parallel gel to panel A using a 1:1000 dilution of anti-(human Prx 4) antibodies.

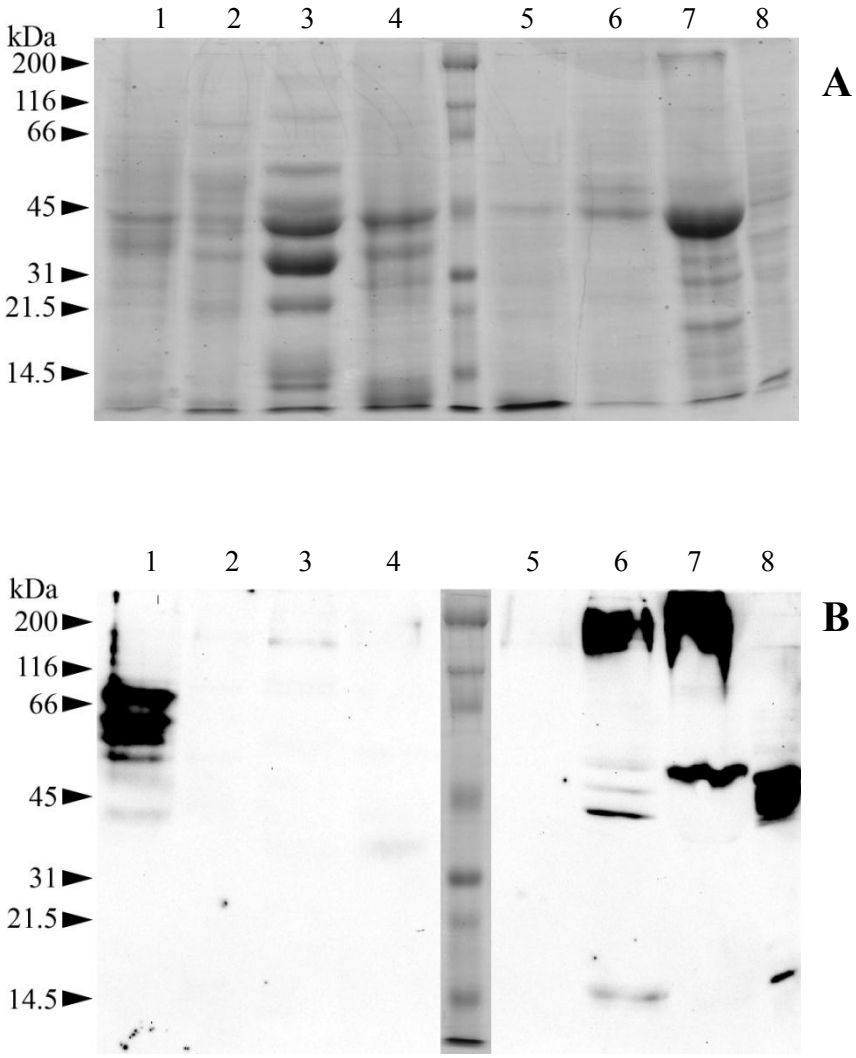


Figure 4.10: Expression of GPx 4 in various tissues of a 3 kg female YTK (YTK 1; stored in RNA*later*®). The lanes contained 1 – Brain; 2 – Liver; 3 – Muscle; 4 – Heart; 5 – Spleen; 6 – Ovaries; 7 – Intestine; 8 – Human brain. The human brain tissue was sourced from Dr John Power, Flinders University, School of Medicine and the source of the YTK tissues is shown in Table 3.

(A) SDS-PAGE conducted on a 10% (w/v) acrylamide gel with the indicated tissues of YTK 1 extracted into extraction Buffer D (50 mM HEPES, 150 mM NaCl, 10 mM DTT, 1 mM PMSF, 1 mM EDTA and 1 % (w/v) SDS). A volume of extract equivalent to that which would have been obtained from 2.5 mg of tissue wet weight was loaded into each lane of the gel. .

(B) Immunoblot analysis of a parallel gel to panel A using a 1:1000 dilution of anti-(human GPx 4) antibodies.

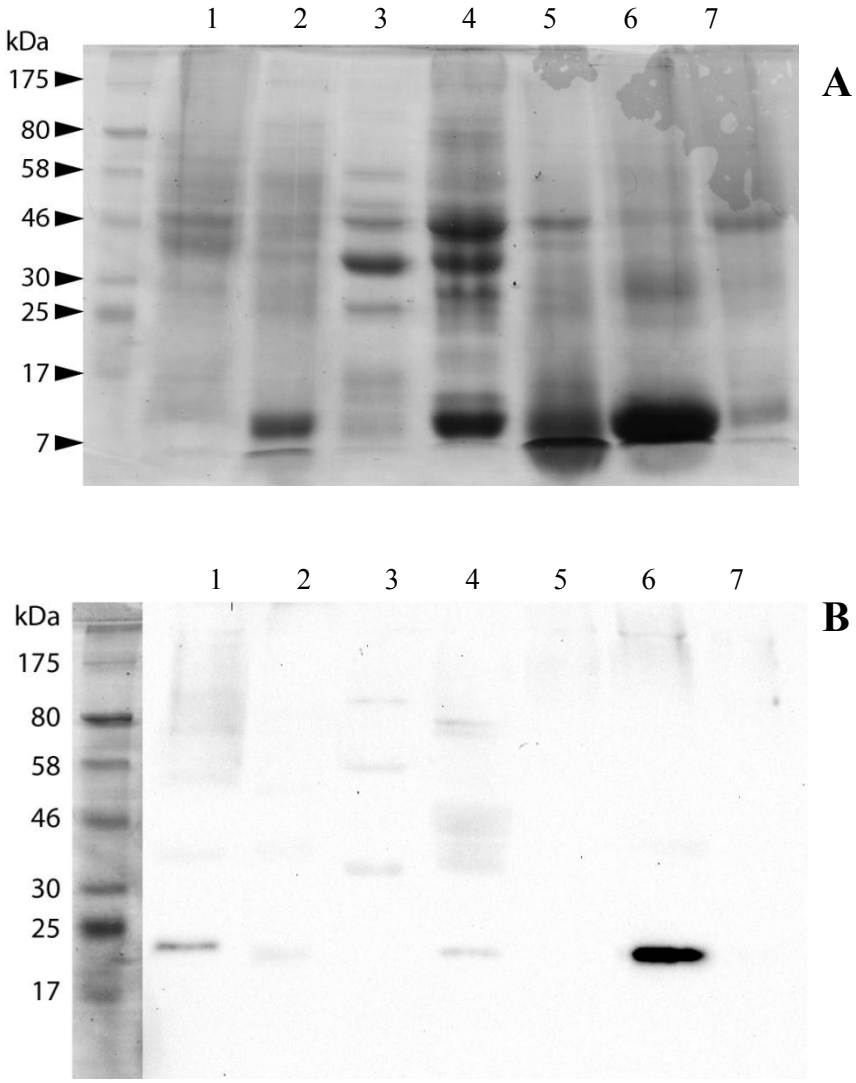


Figure 4.11: Expression of Prx proteins in various tissues of a 3 kg male YTK (YTK 2; stored in RNA*later*®). 1 – Brain; 2 – Liver; 3 – Muscle; 4 – Heart; 5 – Intestine; 6 – Spleen; 7 – Testes. The source of the tissue samples is shown in Table 3.

(A) SDS-PAGE conducted on a 10% (w/v) acrylamide gel with the indicated tissues of YTK 2 extracted into extraction Buffer D (50 mM HEPES, 150 mM NaCl, 10 mM DTT, 1 mM PMSF, 1 mM EDTA and 1 % (w/v) SDS). A volume of extract equivalent to that which would have been obtained from 2.5 mg of tissue wet weight was loaded into each lane of the gel. .

(B) Immunoblot analysis of a parallel gel to panel A using a 1:1000 dilution of anti-(SBT-Prx 2) antibodies.

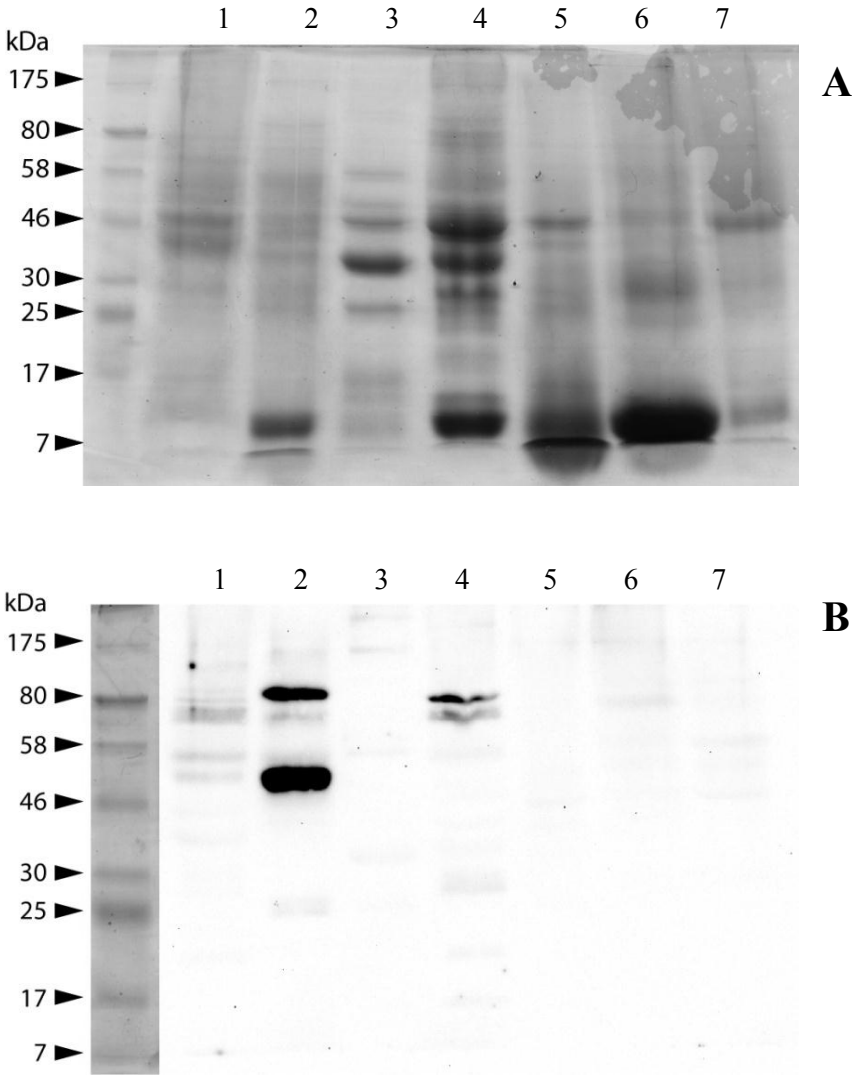


Figure 4.12: Expression of Prx 4 in various tissues of a 3 kg male YTK (YTK 2; stored in RNA*later*®). The lanes contained 1 – Brain; 2 – Liver; 3 – Muscle; 4 – Heart; 5 – Intestine; 6 – Spleen; 7 – Testes. The source of the tissue samples is shown in Table 3.

(A) SDS-PAGE conducted on a 10% (w/v) acrylamide gel with the indicated tissues of YTK 2 extracted into extraction Buffer D (50 mM HEPES, 150 mM NaCl, 10 mM DTT, 1 mM PMSF, 1 mM EDTA and 1 % (w/v) SDS). A volume of extract equivalent to that which would have been obtained from 2.5 mg of tissue wet weight was loaded into each lane of the gel. .

(B) Immunoblot analysis of a parallel gel to panel A using a 1:1000 dilution of anti-(human Prx 4) antibodies.

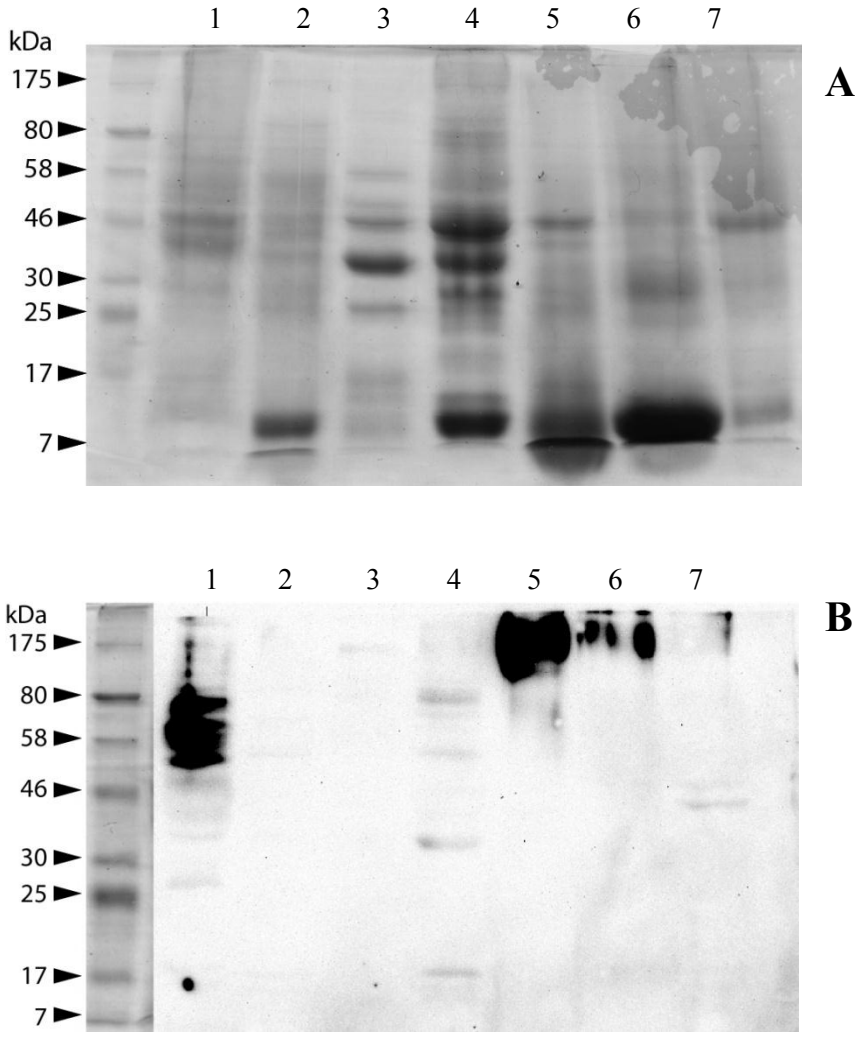


Figure 4.13: Expression of GPx 4 in various tissues of a 3 kg male YTK (YTK 2; stored in RNA*later*®). The lanes contained 1 – Brain; 2 – Liver; 3 – Muscle; 4 – Heart; 5 – Intestine; 6 – Spleen; 7 – Testes. The source of the tissue samples is shown in Table 3.

(A) SDS-PAGE conducted on a 10% (w/v) acrylamide gel with the indicated tissues of YTK 2 extracted into extraction Buffer D (50 mM HEPES, 150 mM NaCl, 10 mM DTT, 1 mM PMSF, 1 mM EDTA and 1 % (w/v) SDS). A volume of extract equivalent to that which would have been obtained from 2.5 mg of tissue wet weight was loaded into each lane of the gel. .

(B) Immunoblot analysis of a parallel gel to panel A using a 1:1000 dilution of anti-(human GPx 4) antibodies.

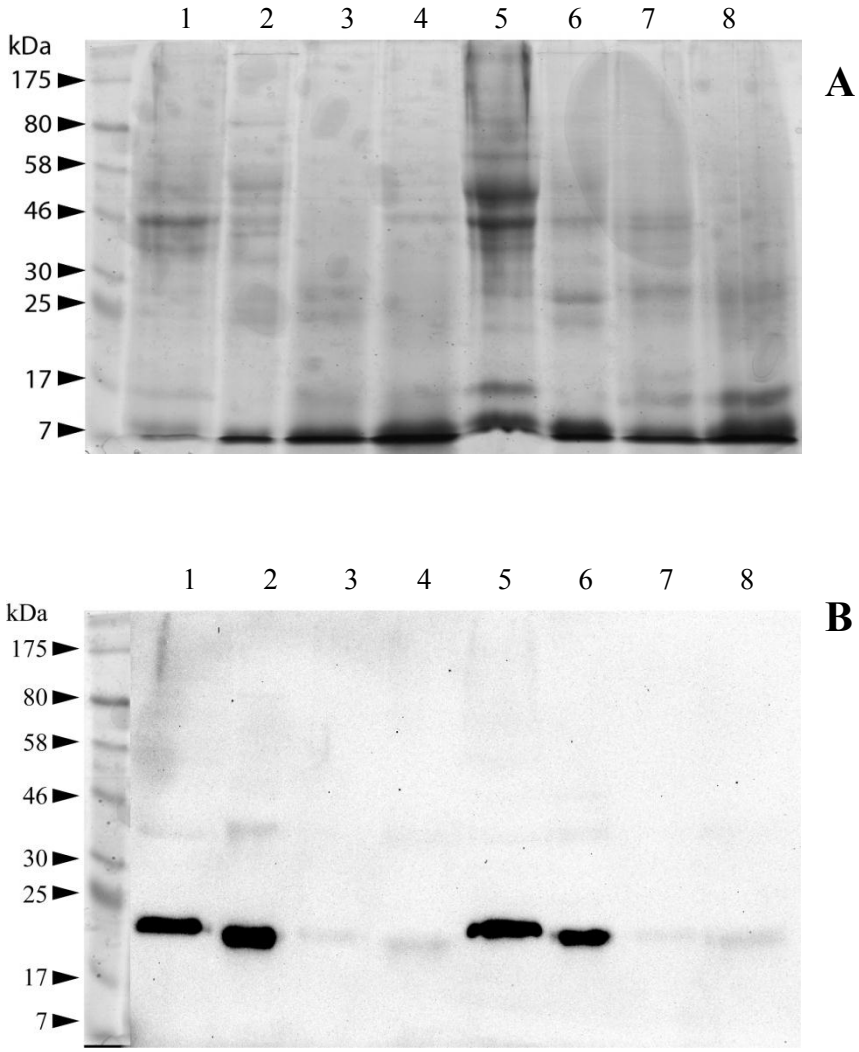


Figure 4.14: Expression of Prx proteins in various tissues of a 3 kg female YTK (YTK 1; stored in RNA*later*®) with extra DTT in the extraction buffer and with and without urea in the extraction buffer. The lanes contained 1 – Brain (Buffer E); 2 – Liver (Buffer E); 3 – Intestine (Buffer E); 4 – Kidney (Buffer E); 5 – Brain (Buffer F); 6 – Liver (Buffer F); 7 – Intestine (Buffer F); 8 – Kidney (Buffer F). The source of the tissue samples is shown in Table 3.

(A) SDS-PAGE conducted on a 10% (w/v) acrylamide gel with the indicated tissues of YTK 1 extracted into Extraction Buffer E (50 mM HEPES, 150 mM NaCl, 50 mM DTT, 1 mM PMSF, 1 mM EDTA and 1 % (w/v) SDS) in lanes 1-4 and Extraction Buffer E + urea (Buffer F; 50 mM HEPES, 150 mM NaCl, 50 mM DTT, 1 mM PMSF, 1 mM EDTA, 1 % (w/v) SDS and 5 M urea) in lanes 5-8. A volume of extract equivalent to that which would have been obtained from 2.5 mg of tissue wet weight was loaded into each lane of the gel. .

(B) Immunoblot analysis of a parallel gel to panel A using a 1:1000 dilution of anti-(SBT-Prx 2) antibodies.

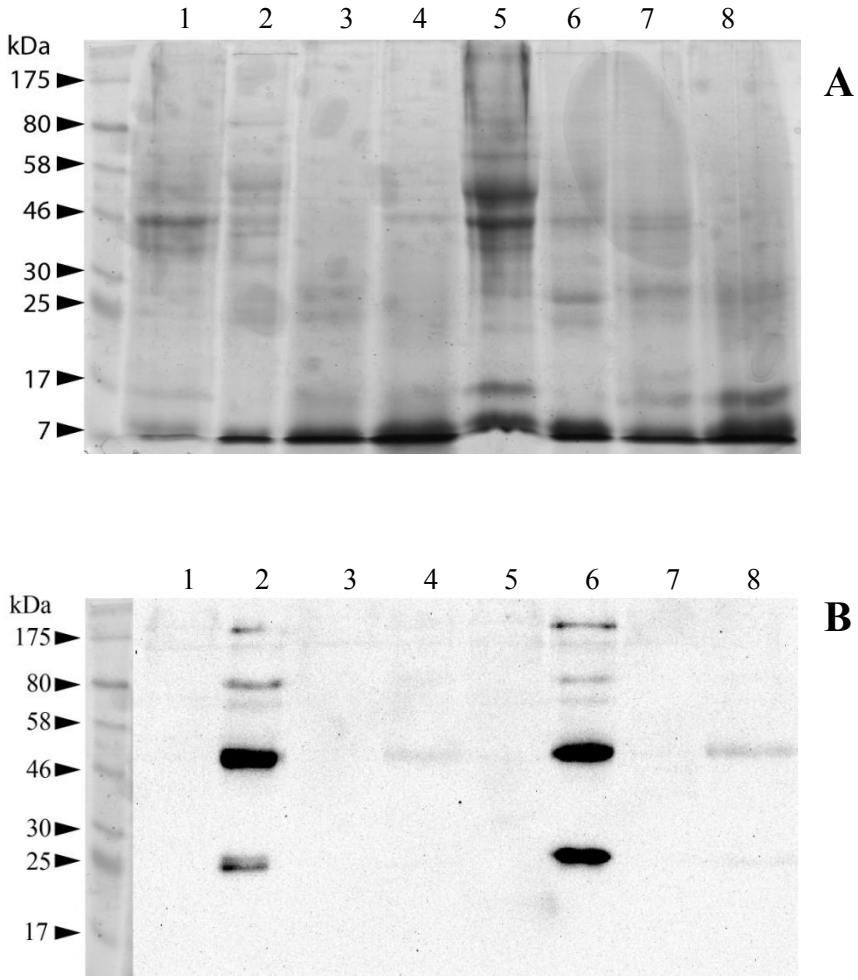


Figure 4.15: Expression of Prx 4 in various tissues of a 3 kg YTK (YTK 1; stored in RNAlater®) with extra DTT in the extraction buffer and with and without urea in the extraction buffer. The lanes contained 1 – Brain (Buffer E); 2 – Liver (Buffer E); 3 – Intestine (Buffer E); 4 – Kidney (Buffer E); 5 – Brain (Buffer F); 6 – Liver (Buffer F); 7 – Intestine (Buffer F); 8 – Kidney (Buffer F). The source of the tissue samples is shown in Table 3.

(A) SDS-PAGE conducted on a 10% (w/v) acrylamide gel with the indicated tissues of YTK 1 extracted into extraction Buffer E (50 mM HEPES, 150 mM NaCl, 50 mM DTT, 1 mM PMSF, 1 mM EDTA and 1 % (w/v) SDS) in lanes 1-4 and extraction Buffer E + urea (Buffer F; 50 mM HEPES, 150 mM NaCl, 50 mM DTT, 1 mM PMSF, 1 mM EDTA, 1 % (w/v) SDS and 5 M urea) in lanes 4-8. A volume of extract equivalent to that which would have been obtained from 2.5 mg of tissue wet weight was loaded into each lane of the gel. .

(B) Immunoblot analysis of a parallel gel to panel A using a 1:1000 dilution of anti-(human Prx 4) antibodies.

Chapter 5 – Tissue distribution and age dependence of the expression of Prx and GPx proteins in YTK

5.1 Aims and Background

The aims of this chapter were:

1. to investigate Prx and GPx protein expression in YTK in a broad range of tissues using the methods established in Chapter 4
2. to investigate the effects of fish size, as a proxy for stage of development, on Prx and GPx protein expression in YTK
3. to investigate the effects of fish gender (male or female) on Prx and GPx protein expression in YTK

Previous studies in mammals have found high Prx 4 expression in testes compared to other tissues (Iuchi et al., 2009). Furthermore, Prx 4 knockout mice exhibited reduced testicular size and weight and increased spermatogenic cell death, even though the small number of sperm produced were normal (Iuchi et al., 2009). Mammalian GPx 4 has been found to be highly expressed in spermatocytes (Imai et al., 2001; Imai et al., 2009) and a spermatocyte-specific GPx 4 knockout decreased sperm motility by 76 % and reduced fertilisation success to zero (Imai et al., 2009). A similar result was seen with mitochondrial GPx 4 knockout mice. Sperm from these mice exhibited lower motility, progressivity and zero reproductive success (Schneider et al., 2009).

In summary, the literature indicates that Prx 4 and GPx 4 proteins are important in male fertility in mammals. Therefore, we hypothesized that the same would be the case in fish. Specifically, we hypothesized that Prx 4 protein expression would be higher in fish testes

than in other organs or tissues and that the expression of these proteins would increase as the fish reached sexual maturity. We also hypothesised that GPx 4 protein would be abundant in spermatocytes and therefore GPx 4 would also show a high level of expression in the testes of sexually mature fish.

5.2 Methods

5.2.1 Fish tissue sampling

The fish used in this chapter were 1.5 – 3.0 kg in weight and samples were taken of various tissues from both male and female fish (summarised in Table 1). The 3.0 kg fish were selected as they were the largest YTK that we had access to. Fish of similar size had been shown to be sexually mature (Assoc. Prof. W. Knibb, personal communications). The 1.5 kg fish provided a comparison to younger fish raised in the same conditions. It is likely that the 1.5 kg fish were sexually immature. The tissues were harvested as described in Section 2.2.2, frozen in dry ice, transported to Flinders University and then frozen at -80°C until needed.

5.2.2 Prx and GPx protein expression in various tissues from a 1.5 kg YTK female

Total protein was extracted from brain, liver, skeletal muscle, heart, intestine, spleen, ovary and gill of a 1.5 kg YTK female (YTK 13, Table 1) into buffer F (Table 2) according to the protocol for tissues frozen at -80°C described in Section 2.3.2. An amount of protein equal to that which would have been obtained from 2.5 mg of tissue wet weight (calculation described in Section 4.2.5) was loaded into each lane of four replicate 10% SDS-PAGE gels and the gels were run according to the protocol described in Section 2.4. One gel was stained for total protein, while the other three were used for immunoblot analysis with a 1:1000 dilution of the

anti-(SBT Prx 2), anti-(human Prx 4) or anti-(human GPx 4) primary antibodies in blocking buffer (Section 2.5). Development and detection were conducted as described in Sections 2.5 and 2.6.

5.2.3 Prx and GPx protein expression in various tissues from two 1.5 kg YTK males

Total protein was extracted from brain, liver, skeletal muscle, heart, intestine, spleen, testes and gill of two 1.5 kg YTK males (YTK 8 and YTK 12, Table 1) into buffer F (Table 2) according to the protocol for tissues frozen at -80°C described in Section 2.3.2. The extracts were then analysed as described in Section 5.2.2.

5.2.4 Prx and GPx expression in various tissues from a 3.0 kg YTK female

Total protein was extracted from the brain, liver, skeletal muscle, heart, intestine, spleen, kidney, ovaries and gill tissue of a 3 kg YTK female (YTK 6, Table 1) into Buffer F (Table 2) according to the protocol for tissues frozen at -80°C described in Section 2.3.2. The extracts were then analysed as described in Section 5.2.2.

5.2.5 Prx and GPx expression in various tissues of two 3.0 kg YTK males

Total protein was extracted from brain, liver, skeletal muscle, heart, intestine, spleen, kidney, testes and gill of two 3 kg YTK males (YTK 7 and YTK 9, Table 1) into buffer F (Table 2) according to the protocol for tissues frozen at -80°C described in Section 2.3.2. The extracts were then analysed as described in Section 5.2.2.

5.3 Results

5.3.1 Protein expression analysis using anti-(SBT Prx 2) antibodies

Previous studies with mice have shown that Prx 4 protein expression is higher in testes than in any other organs/tissues and that there is a second form of the protein with a molecular mass of 27 kDa in addition to the usual form with a molecular mass of 25 kDa (Iuchi et al., 2009). Similar results have also been obtained for rats and, in addition, it has been shown that the larger form of the Prx 4 protein (31 kDa in rats) does not appear until the animals are sexually mature (Sasagawa et al., 2001). Therefore, the aims of this section were (a) to investigate the tissue distribution of Prx protein expression in YTK and (b) to determine whether expression varied with gender or size of the fish. In particular, we hypothesised that expression would be higher in the testes of the large 3.0 kg male fish which we believed to be old enough to be sexually mature.

5.3.1.1 Prx expression in various tissues from a 1.5 kg YTK female

The Coomassie Blue stained gel showed that a large amount of protein had been extracted from all of the tissues (Fig. 5.1A). In skeletal muscle (lane 3) and ovaries (lane 7), there was a particularly abundant protein at approximately 46 kDa. Otherwise, there were no noteworthy differences between the tissues.

In Fig. 5.1B, the anti-(SBT Prx 2) antibodies detected intense protein bands at the expected size for a Prx monomer (approximately 22 kDa) in all tissues except for skeletal muscle (lane 3), which showed a weaker reaction at this molecular mass indicating that expression was low in skeletal muscle. The strongest expression was observed in brain (lane 1), liver (lane 2)

and ovaries (lane 7). There also appeared to be a strong reaction in skeletal muscle at approximately 80 kDa (lane 3) and in intestine at an even higher molecular mass (lane 5).

5.3.1.2 Prx expression in various tissues from two 1.5 kg YTK males

In the Coomassie Blue stained gels (Figs. 5.2A and 5.3A), a large amount of protein was observed for all tissues except for intestine for YTK 12 (Fig. 5.3A, lane 5). Particularly high protein abundance was seen for liver, skeletal muscle and heart. The abundant protein at approximately 46 kDa that was observed for YTK 13 (Fig. 5.1A), was also observed for YTK 8 (Fig. 5.2A) and YTK 12 (Fig. 5.3A).

In Figs. 5.2B and 5.3B, strong expression was detected using the anti-(SBT Prx 2) antibodies at approximately the Prx monomer size of 22 kDa. As seen for the YTK female (Fig. 5.1B), expression was seen for the YTK males (Figs. 5.2B and 5.3B) at the Prx monomer size in all the tissues. Expression was weaker in the skeletal muscle and heart of YTK 8 (Fig. 5.2B, lanes 3 and 4) and was also weaker in the skeletal muscle and intestine of YTK 12 (Fig. 5.3B, lanes 3 and 5). Consistent with what was seen for the 1.5 kg female (Section 5.3.1.1), the strongest reaction for both 1.5 kg males was seen in brain and liver. Liver showed a band at approximately dimer size (44 kDa) as well in YTK 8 (Fig. 5.2B, lane 2), but not in YTK 12 (Fig. 5.3B, lane 2). Likewise, strong bands were observed at approximately 34 kDa, 45 kDa, 60 kDa and 80 kDa in skeletal muscle for YTK 8 (Fig. 5.2B, lane 3), but only at 45 kDa and 80 kDa in skeletal muscle for YTK 12 (Fig. 5.3B, lane 3). The 80 kDa band was also seen in heart for YTK 12 (Fig. 5.3B, lane 4), but not for YTK 8 (Fig. 5.2B, lane 4). There was also a large amount of cross-reaction at higher molecular masses for all tissues for YTK 8 (Fig. 5.2B).

In summary Prx proteins were detected at monomer size in all tissues of the 1.5 kg fish, both male and female. This reaction was intense in the majority of tissues but was weaker in skeletal muscle. There was also consistent reaction at approximately 80 kDa in muscle, which could indicate the formation of Prx oligomers in this tissue.

5.3.1.3 Prx expression in various tissues from a 3.0 kg YTK female

The Coomassie Blue stained gel for YTK 6 (Fig. 5.4A) showed that a large amount of protein had been extracted from all tissues. An especially abundant protein was observed at approximately 46 kDa in skeletal muscle (lane 3) and ovaries (lane 8). The spleen sample (lane 6) had a low abundance of protein above approximately 50 kDa; the majority of the protein in this sample was at low molecular mass.

In Fig. 5.4B, the anti-(SBT Prx 2) antibodies detected expression in all tissues of YTK 6 at approximately the size of a Prx monomer (22 kDa). Consistent with what was observed for the 1.5 kg fish in Sections 5.3.1.1 and 5.3.1.2, the strongest expression at the monomeric size was observed in brain (lane 1), liver (lane 2) and ovaries (lane 8). The weakest expression at monomeric size was again observed in skeletal muscle (lane 3), with a strong reaction at approximately tetramer size (80 kDa) in skeletal muscle. Weak reaction was also observed in kidney (lane 7) at approximately 46 kDa which corresponds to the predicted Prx dimer size.

5.3.1.4 Prx expression in various tissues from two 3.0 kg YTK males

In the Coomassie Blue stained gel for YTK 7 (Fig. 5.5A) a large amount of protein was seen for all samples indicating that protein extraction had been successful. Consistent with what was seen in previous sections, a prominent band was observed in the skeletal muscle sample at approximately 45 kDa (lane 3). Interestingly, the intestine also had a prominent protein band at approximately 45 kDa (lane 5). The spleen sample (lane 6) showed a similar pattern of protein banding as seen in previous sections with a large quantity of low molecular mass proteins and a very low concentration of proteins above approximately 50 kDa.

In Fig. 5.5B, the anti-(SBT Prx 2) antibodies detected a strong protein band at the size expected for a Prx monomer (22 kDa) in all tissues of YTK 7 except for the skeletal muscle, which only showed a faint band at this size (lane 3). There was also strong reaction at approximately 46 kDa in the kidney sample (lane 7). Consistent with what was observed in Sections 5.3.1.1 – 5.3.1.3, a band at approximately 80 kDa was observed for skeletal muscle (lane 3) and there was also a strong reaction at a high molecular mass in intestine (lane 5).

The Coomassie Blue stained gel for YTK 9 (Fig. 5.6A) again showed a high abundance of protein indicating successful protein extraction. The prominent band in the skeletal muscle sample at approximately 46 kDa (lane 3) was observed once again. Extraction efficiency for the spleen sample (lane 6) appeared to be higher than had been observed previously with some protein detected above 50 kDa.

In Fig. 5.6B, a similar pattern of reaction was seen in YTK 9 as was observed for YTK 7 (Fig. 5.5B) with strong reaction at Prx monomer size for all tissues except skeletal muscle (lane 3). Skeletal muscle (lane 3) showed a band at approximately 33 kDa, which was not observed in any other samples. Also, weak reaction was seen at approximately 46 kDa in kidney (lane 7), testes (lane 8) and gill (lane 9). A band at approximately 80 kDa was observed in skeletal muscle (lane 3) and there was some binding at high molecular masses in brain (lane 1), skeletal muscle (lane 3) and heart (lane 4).

In summary, the anti-(SBT Prx 2) antibodies detected a band at the size of a 2-Cys Prx monomer (22 kDa) in all tissues of every fish analysed. The most intense bands at monomer size were in brain and liver and the weakest reaction at this size was in skeletal muscle. The skeletal muscle consistently exhibited an additional band at approximately 80 kDa which does not correspond to any reported size of a Prx protein.

5.3.2 Protein expression analysis using anti-(human Prx 4) antibodies

In the previous section (Section 5.3.1), it was shown that Prx protein expression, detected by the anti-(SBT Prx 2) antibodies at the expected size for the Prx monomer, was high in all organs, except skeletal muscle, in both male and female YTK, regardless of size. Therefore, there did not appear to be any effect of gender or stage of development on Prx protein expression. In particular, the 3.0 kg male fish, which were believed to be sexually mature, did not show higher expression in their testes than the 1.5 kg male fish which were believed to be sexually immature. However, it should be noted that the antibodies used in the previous section were raised against whole Prx 2 protein from SBT. Therefore, these antibodies are likely to have recognized many different epitopes and therefore many different Prx protein

subclasses, not only Prx 4. Therefore, they were not specific to Prx 4. As a result, the aim of the present section was to investigate, specifically, the expression of Prx 4 using the anti-(human Prx 4) antibodies we had at our disposal.

5.3.2.1 Prx 4 expression in various tissues of a 1.5 kg YTK female

The Coomassie Blue stained gel (Fig. 5.7A) was the same gel as shown in Fig. 5.1A and described in Section 5.3.1.1. It is included again here for comparison.

In Fig. 5.7B, the anti-(human Prx 4) antibodies detected intense protein bands at approximately 25 and 50 kDa in liver (lane 2) but the smaller sized protein was not observed in any other tissues. These sizes corresponded to the expected sizes for a Prx 4 monomer and dimer, respectively (Section 3.3.3). There was a strong reaction at approximately 50 kDa in the intestine sample (lane 5) and at approximately 46 kDa in the ovaries sample (lane 7). There also appeared to be a large amount of cross-reaction above about 58 kDa in the majority of the tissues (Fig. 5.7B).

5.3.2.2 Prx 4 expression in various tissues of two 1.5 kg YTK males

The Coomassie Blue stained gels (Figs. 5.8A and 5.9A) were the same gels as shown in Figs. 5.2A and 5.3A and described in Section 5.3.1.2. They are included again here for comparison.

In Fig. 5.8B, the anti-(human Prx 4) antibodies detected an intense protein band at the predicted Prx 4 monomer size (approximately 25 kDa) in the liver of YTK 8 (lane 2) and

lower intensity reactions were seen at the 25 kDa size in brain (lane 1), intestine (lane 5), testes (lane 7) and gill (lane 8). Strong bands were also observed at the predicted Prx 4 dimer size (approximately 50 kDa) in liver (lane 2), intestine (lane 5), spleen (lane 6), testes (lane 7) and gill (lane 8). There was also a large amount of non-specific reaction extending from about 50 kDa up to the origin of the immunoblot for all samples (Fig. 5.8B).

Similarly, in the tissues of YTK 12 (Fig. 5.9B), the anti-(human Prx 4) antibodies detected an intense band at approximately 25 kDa in the liver of YTK 12 (lane 2) and lower intensity reactions were observed at 25 kDa in intestine (lane 5) and testes (lane 7). A strong band was also observed at approximately 50 kDa in liver (lane 2). Interestingly, YTK 12 (Fig. 5.9B) did not exhibit the same intensity of binding at high molecular masses as seen in YTK 8 (Fig. 5.8B). However, there was a strong reaction in the heart sample at approximately 175 kDa (Fig. 5.9B, lane 4). Overall, the results presented in Figs. 5.8B and 5.9B are consistent in that they show strong expression of Prx 4 in liver at both the monomer size and the dimer size. They are also consistent in that they show moderate expression in intestine at the monomer size. Therefore, we can be reasonably sure that Prx 4 is expressed at high levels in liver.

In summary, for the 1.5 kg YTK females and males, Prx 4 expression was highest in the liver with intense bands observed at both monomer and dimer size. Intestine showed consistent reaction at dimer size in both males and females, and at monomer size in males only.

5.3.2.3 Prx 4 expression in various tissues of a 3.0 kg YTK female

The Coomassie Blue stained gel (Fig. 5.10A) was the same gel as shown in Fig. 5.4A and described in Section 5.3.1.3. It is included again here for comparison.

In Fig. 5.10B, the anti-(human Prx 4) antibodies detected an intense protein band at approximately the Prx 4 monomer size of 25 kDa in the liver of YTK 6 (lane 2). Lower intensity reactions were seen at monomeric size in intestine (lane 5), kidney (lane 7) and gill (lane 9). Bands were also observed at the predicted Prx 4 dimer size of 50 kDa in liver (lane 2), spleen (lane 6), kidney (lane 7) and gill (lane 9). Ovaries showed reactions at approximately 30 and 40 kDa (lane 8), which were not observed in any of the other tissues. Interestingly, the 40 kDa band in the ovaries was similar to what was observed in the ovaries of the 1.5 kg YTK female (Fig. 5.7B, lane 7). Therefore, this result appears to be consistent. There was also a large amount of non-specific reaction extending from about 45 kDa up to the origin of the immunoblot for all samples (Fig. 5.10B).

5.3.2.4 Prx 4 expression in various tissues of two 3.0 kg YTK males

The Coomassie Blue stained gels (Figs. 5.11A and 5.12A) were the same gels as shown in Figs. 5.5A and 5.6A and described in Section 5.3.1.4. They are included again here for comparison.

In Fig. 5.11B, the anti-(human Prx4) antibodies detected an intense protein band at approximately Prx 4 monomer size (25 kDa) in the liver of YTK 7 (lane 2). Lower intensity reaction was seen at 25 kDa in intestine (lane 5). Very faint bands were also seen at 25 kDa in brain (lane 1), kidney (lane 7) and gill (lane 9). A band at approximately Prx 4 dimer size (46 kDa) was observed at high intensity in both liver (lane 2) and intestine (lane 5). A band of this same size was observed at lower intensity in spleen (lane 6), kidney (lane 7) and gill

(lane 9). A faint band at approximately 30 kDa was observed in intestine (lane 5) and bands were observed at just above 80 kDa in both liver (lane 2) and intestine (lane 5).

The immunoblot of the YTK 9 tissues (Fig. 5.12B) showed a high intensity reaction at the 25 kDa Prx 4 monomer size in liver (lane 2). A less intense reaction was observed at 25 kDa in intestine (lane 5) along with a faint band in gill (lane 9). The liver sample also had a band at approximately the 50 kDa dimer size (lane 2). Faint reactions were also seen at dimer size in intestine (lane 5), spleen (land 6), kidney (lane 7) and gill (lane 9).

Interestingly, the immunoblots for both YTK 7 (Fig. 5.11B) and YTK 9 (Fig. 5.12B) did not show the same intensity of non-specific reaction at high molecular masses as was seen for YTK 8 (Fig. 5.8B) and YTK 6 (Fig. 5.10B) even though the same antibodies were used.

In summary, a high level of expression was seen in liver at both monomer and dimer size in every fish, regardless of size or gender. Intestine also showed bands of varying intensity at both monomer and dimer size in every fish regardless of size or gender. In most cases, a band at monomer size was observed in gill and the female YTK of both sizes showed two bands at 35 kDa and 45 kDa in the ovaries that were not present in any other organ.

5.3.3 Protein expression analysis using anti-(human GPx4) antibodies

Previous studies in mammals have shown that GPx 4 is expressed in most organs (Yant et al., 2003) and is found at high levels in spermatocytes and spermatids in the testicular tissues of male mice (Imai et al., 2001). Knockouts of the entire GPx 4 gene are embryo-lethal and a

knockout of only mitochondrial GPx 4 has detrimental effects on male fertility but mice are otherwise normal (Schneider et al., 2009). Therefore, the aims of this section were (a) to investigate the tissue distribution of GPx 4 protein expression in YTK to determine whether it followed the same pattern as seen in mammals (Yant et al., 2003) and (b) to determine whether GPx 4 expression was higher in the testes of large 3.0 kg YTK males that were considered to be sexually mature than it was in the testes of small 1.5 kg YTK males that were considered to be sexually immature.

5.3.3.1 GPx 4 expression in various tissues of a 1.5 kg YTK female

The Coomassie Blue stained gel (Fig. 5.13A) was the same gel as shown in Fig. 5.1A and described in Section 5.3.1.1. It is included again here for comparison.

In Fig. 5.13B, the anti-(human GPx 4) antibodies did not detect any protein in YTK 13 at the predicted GPx 4 monomer size of 21 kDa (Section 3.3.6). However, distinct bands were seen close to the predicted GPx 4 dimer size (42 kDa) in liver (lane 2), muscle (lane 3) and ovaries (lane 7). This 42 kDa band was also observed, albeit at lower intensity, in brain (lane 1) and heart (lane 4). Strong cross-reaction was seen in all lanes extending from approximately 45 kDa up to the origin of the immunoblot and it was difficult to distinguish any individual bands in this region (Fig. 5.13B). Therefore we assumed this to be non-specific binding of the primary antibodies or background signal from the secondary antibodies.

5.3.3.2 GPx 4 expression in various tissues of two 1.5 kg YTK males

The Coomassie Blue stained gels (Figs. 5.14A and 5.15A) were the same gels as shown in Figs. 5.2A and 5.3A and described in Section 5.3.1.2. They are included again here for comparison.

In Fig. 5.14B, a very faint band was observed at approximately the size of a GPx 4 monomer (21 kDa) in YTK 8 liver (lane 2). However, much stronger bands were observed at approximately 42 kDa, the size of a GPx 4 dimer, in brain (lane 1), liver (lane 2) and muscle (lane 3). A band was also seen at approximately 27 kDa in brain (lane 1). This did not correspond to any predicted size for GPx 4. Again, strong cross-reaction was seen in all lanes above approximately 45 kDa up to the origin of the immunoblot (Fig. 5.14B).

The samples from YTK 12 (Fig. 5.15B) showed a reaction at approximately GPx monomer size (21 kDa) in liver (lane 2) and heart (lane 4). Bands were also observed at approximately GPx dimer size (42 kDa) in brain (lane 1), liver (lane 2), muscle (lane 3) and heart (lane 4). There were also faint bands at approximately 25 kDa and 50 kDa in brain (lane 1) and at 45 and 50 kDa in intestine (lane 5). A band was observed at approximately 60 kDa in brain (lane 1), liver (lane 2), heart (lane 4), intestine (lane 5) and testes (lane 7). Again, strong cross-reaction was seen in all lanes above approximately 60 kDa up to the origin of the immunoblot, making it difficult to distinguish between individual bands in this region (Fig. 5.15B).

5.3.3.3 GPx 4 expression in various tissues of a 3.0 kg YTK female

The Coomassie Blue stained gel (Fig. 5.16A) was the same gel as shown in Fig. 5.4A and described in Section 5.3.1.3. It is included again here for comparison.

In Fig. 5.16B, the anti-(human GPx 4) antibodies detected faint bands at approximately GPx 4 monomer size (21 kDa) in YTK 6 liver (lane 2), heart (lane 4), intestine (lane 5), kidney (lane 7) and ovaries (lane 8). A strong reaction was also observed at the predicted GPx 4 dimer size (42 kDa) in ovaries (lane 8). Weaker reactions were observed at dimer size in liver (lane 2), skeletal muscle (lane 3), heart (lane 4) and intestine (lanes 5). A band was also observed at approximately 50 kDa in brain (lane 1). As with the smaller fish, strong cross-reaction was seen in all lanes above approximately 45-60 kDa up to the origin of the immunoblot, making it difficult to distinguish between individual bands in this region (Fig. 5.16B).

5.3.3.4 GPx 4 expression in various tissues of two 3.0 kg YTK males

The Coomassie Blue stained gels (Figs. 5.17A and 5.18A) were the same gels as shown in Figs. 5.5A and 5.6A and described in Section 5.3.1.4. They are included again here for comparison.

In Fig. 5.17B, the anti-(human GPx 4) antibodies did not detect any bands at the size of a GPx 4 monomer in the tissues of YTK 7. Extremely faint bands were observed at the size of a GPx 4 dimer (42 kDa) in liver (lane 2), intestine (lane 5), and gill (lane 9). There was also some reaction in brain at approximately 27 kDa and 50 kDa (lane 1). A strong protein band was observed at approximately 50 kDa in the intestine sample (lane 5) and gill also exhibited

bands at approximately 27 and 30 kDa (lane 9). There was some cross-reaction at high molecular masses for all samples except for liver (lane 2) and spleen (lane 6). This reaction was most intense in kidney (lane 7) and gill (lane 9) and made it difficult to distinguish individual bands above approximately 50 kDa (Fig. 5.17B).

In Fig. 5.18B, the anti-(human GPx 4) antibodies reacted with a protein at approximately GPx 4 monomer size (21 kDa) in the YTK 9 liver (lane 2) and intestine (lane 5). Reaction at dimer size (42 kDa) was also observed in brain (lane 1), liver (lane 2), skeletal muscle (lane 3), heart (lane 4) and intestine (lanes 5). Intestine showed a prominent band at approximately 30 kDa, in between the monomer and dimer sized bands (lane 5). Bands were observed at approximately 50 kDa in intestine (lane 5), kidney (lane 7), testes (lane 8) and gill (lane 9) at approximately 50 kDa. However, it was difficult to distinguish these bands from the large amount of binding at high molecular mass that was seen in all tissues extending from 50-60 kDa up to the origin of the immunoblot (Fig. 5.18B).

In summary, bands the size of a GPx 4 monomer (21 kDa) and dimer (42 kDa) were consistently observed in liver and heart in the majority of the fish analysed regardless of size or gender. A dimer sized band was also observed in skeletal muscle for most fish with the exception of one of the 3.0 kg males (YTK 7). Strong reaction was seen at high molecular masses in the majority of the fish tissues regardless of age or gender.

5.4 Discussion

5.4.1 Protein expression analysis using anti-(SBT Prx 2) antibodies

A band at the size of a 2-Cys Prx monomer (22 kDa) was detected in all tissues of every fish analysed and these reactions were intense in the majority of tissues, but strongest in brain and liver. Skeletal muscle usually showed the weakest reaction at monomer size but consistently exhibited a band at approximately 80 kDa. Interestingly, the ovaries of both the 1.5 kg and 3.0 kg female fish showed a higher abundance of Prx protein than in other organs, whereas Prx abundance in testes was approximately equal to what was seen in other organs.

The high level of Prx protein expression is consistent with the mammalian literature for multiple Prx proteins (Leyens et al., 2003; Iuchi et al., 2009). However, the width of some bands at monomer size extended from approximately 22 kDa up to just above 25 kDa. As a result, it was not possible to determine which sub-classes of 2-Cys Prx proteins the antibodies were detecting. However, it was unlikely that they were detecting the unprocessed form of Prx 3 (27 kDa) or the 264 amino acid full-length form of Prx 4 (29 kDa). It is, however, possible that the antibodies were detecting the 231 amino acid truncated form of Prx 4 (25 kDa). It is not clear why the anti-(SBT Prx 2) antibodies could not detect the 27 kDa unprocessed form of Prx 3 or the 264 amino acid full-length form of Prx 4 because all four 2-Cys Prx proteins (Prx 1, Prx 2, Prx 3 and Prx 4) are highly conserved at the amino acid level and the anti-(SBT Prx 2) antibodies were raised against whole protein and should, therefore, have recognized many different epitopes (regions) of 2-Cys Prx proteins. Further experiments are required to resolve this apparent anomaly.

The high level of Prx protein expression observed in brain and liver showed no noticeable variation with the size or gender of the fish. This high level of expression could be due to (a) high cumulative abundance of 2-Cys Prx proteins in these tissues, (b) high abundance of the 231 amino acid truncated form of Prx 4 or (c) a combination of both (a) and (b).

The strong cross-reaction of the anti-(SBT Prx 2) antibodies at approximately 80 kDa in both skeletal muscle and intestine could be due to aggregation of the Prx proteins or their association with other proteins in these tissues. It is known that Prx proteins undergo oxidation by H_2O_2 to form dimers and decamers and it is proposed that this is involved in cell signalling (Wood et al., 2003; Rhee et al., 2005a; Rhee et al., 2005b). Since the fish were supplied dead, it is possible that they were stressed at the time of capture and that this caused a rise in the level of ROS such as H_2O_2 causing oligomerisation of the Prx proteins. This dimerization would normally have been disrupted by the addition of the reducing agent DTT which was present in the extraction buffer and the SDS-PAGE sample buffer, but Prx proteins from fish appear to show resistance to reduction by traditional reducing agents (Mr Drew Sutton, unpublished data).

It is not known why the Prx proteins in skeletal muscle and intestine showed a reaction with the anti-(SBT Prx 2) antibodies at dimer size and above, whereas other tissues showed expression at Prx monomer size. However it is possible, that this could be due to specific proteins the Prx proteins are associating with in these tissues, which could affect the

accessibility of the disulphide bonds between the Prx monomers thus limiting the disruption of these disulphide bonds by the reducing agent DTT.

The elevated level of Prx protein expression in the ovaries, but not in the testes of YTK is interesting since the literature available for Prx proteins in mammals suggests that Prx 1, 2 and 3 are expressed at approximately the same level in reproductive tissues compared to other organs whereas Prx 4 is express at higher levels in testes compared to other organs (e.g. Leyens et al., 2003). Furthermore, in the case of Prx 4 in mammals, expression is higher in testes than in any other tissue and a second form of the monomer is present that is not seen in any other tissues (Sasagawa et al., 2001; Iuchi et al., 2009). The results presented here suggest that expression of 2-Cys Prx proteins in YTK is higher in the ovaries but not higher in the testes when compared to other organs. Interestingly, only the 22 kDa forms of the 2-Cys Prx proteins appeared to be present in both the ovaries and the testes but it is possible that either of these bands were intense enough to contain two bands which were not able to be distinguished by the current resolution of the SDS-PAGE gel and immunoblot.

5.4.2 Protein expression analysis using anti-(human Prx 4) antibodies

A high level of expression was seen in liver at both Prx monomer and dimer size in every fish, regardless of fish size or gender. Intestine also showed bands of varying intensity at both monomer and dimer size in every fish regardless of size or gender. In the female YTK of both 1.5 kg and 3.0 kg, two bands at 35 kDa and 45 kDa were detected in the ovaries that were not present in any other organ.

The reaction at 25-30 kDa in the liver was always intense, yielding a large band. This intense reaction could be indicative of either an extremely high abundance of Prx 4 in the liver tissue, or the presence of two or more different sizes of Prx 4 which could not be distinguished from each other at the resolution of the gels that were used. Both these hypotheses are in contrast to the mammalian literature. Prx 4 has been reported to be no more abundant in liver than in many other organs (Iuchi et al., 2009) and multiple isoforms of Prx 4 at the monomer size have only been reported in testes (Sasagawa et al., 2001; Iuchi et al., 2009). The size of the band at the monomer size (25-30 kDa) indicated that the anti-(human Prx 4) antibodies were detecting either the full-length 264 amino acid form of Prx 4 (29 kDa), or the truncated 231 amino acid form of Prx 4 (25 kDa), or a combination of both forms. This hypothesis was supported by the analysis in Chapter 3, which showed that half of the region corresponding to the peptide used to raise the anti-(human Prx 4) antibodies is removed in the 197 amino acid (22 kDa) truncated form of Prx 4. The band at approximately 50 kDa in liver was at the estimated dimer size for the 231 amino acid form of Prx 4 (Section 3.3.2). Therefore, we hypothesised that the anti-(human Prx 4) antibodies were detecting both the 231 and 264 amino acid forms of Prx 4 at monomer size and that the 231 amino acid form was forming dimers resulting in the band seen at 50 kDa.

The reaction in the intestine followed the same protein size patterns as seen in liver. The reactions in intestine showed variation in intensity between the different fish, but no discernable pattern emerged in regards to fish size or gender. The variation in intensity of the antibody reaction suggests that the amount of Prx 4 was different in each sample. This could be explained by high levels of proteases in the intestine which may have degraded Prx 4 during extraction or storage, thus affecting the amount of Prx 4 that could be detected from intestinal tissue (Chong et al., 2002).

The presence of two bands in the ovaries of both the 1.5 kg female (Fig. 5.7B) and the 3.0 kg female (Fig. 5.10B) at approximately 40 and 45 kDa was unexpected. The 40 kDa size does not correspond to any estimated size of Prx 4 but the 45 kDa band could represent a dimer form of the 197 amino acid truncated form of Prx 4. However, this is unlikely because only half the region corresponding to the peptide used to raise the anti-(human Prx 4) antibodies is present in the 197 amino acid form of Prx 4 (Section 3.3.3). The two bands do, however, correspond to extremely abundant protein bands seen in the Coomassie Blue stained gels (Fig. 5.7A and Fig. 5.10A) and therefore, they could be due to non-specific binding of the anti-(human Prx 4) antibodies.

Interestingly, little to no reaction was observed at monomer size in the testes tissues of the male fish with the anti-(human Prx 4) antibodies. This finding is in contrast to what has been observed in mammals where Prx 4 is expressed at much higher levels in the testes than in any other organs (Sasagawa et al., 2001; Iuchi et al., 2009).

Furthermore, the literature for mammals reported two forms of Prx 4 monomer in the testes (Sasagawa et al., 2001). These experiments, however, were conducted on sexually mature rats and the high level of expression, as well as the second isoform of the Prx 4 monomer, only appeared at sexual maturity (Sasagawa et al., 2001). It is not currently known with certainty what the exact age of sexual maturity is for YTK as there is conflict in the literature on this subject. For example, Poortenaar et al. (2001) found that sexual maturity was not reached in wild-caught male YTK until they were at least 750 mm in length (The mean length

of the 3.0 kg fish in this study was 600 mm). However, this appears to be dependent on environmental factors. For example, Gillanders et al. (1999) reported that YTK from warm waters in New South Wales could be sexually mature, in exceptional cases, at sizes as small as 360 mm, with 50% of males maturing by 490 mm. Furthermore, a recent development of a YTK pedigree by Assoc. Prof. W. Knibb and colleagues (personal communication), has found that when raised in aquaculture conditions, male YTK of 18 months (approximately 600 mm) contributed genetically to progeny. To test whether the expression of Prx proteins is indeed similar throughout reproductive development in males, sampling of larger YTK that are certainly sexually mature will be needed. However, due to the high commercial value of large YTK and because the fish need to be euthanized to sample the tissues, this investigation could not be covered in the scope of the current study.

5.4.3 Protein expression analysis using anti-(human GPx 4) antibodies

GPx 4 expression was seen in the liver at dimer (42 kDa) size in every fish. The most reproducible detection at monomer size was also in liver but the monomer was not found in the 1.5 kg YTK female (Fig. 5.13B, Section 5.3.3.1) and was only found in one of the 3.0 kg YTK males (Section 5.3.3.4).

The only other reaction that was observed in all the fish was the binding at high molecular mass extending from approximately 60 kDa up to the origin of the immunoblots. This could possibly indicate that the anti-(human GPx 4) antibodies were reacting non-specifically with large proteins that were not necessarily GPx 4, causing the strong reaction at high molecular masses.

The expression of GPx 4 at dimer size is curious because in mammals, GPx 4 is the only selenium-containing GPx protein that is functional as a monomer (Toppo et al., 2008). Relatively few studies have been conducted on GPx proteins in fish and it is possible that GPx 4 may not be functional as a monomer in fish and this could explain the dimer sized bands. However, it is also possible that the anti-(human GPx 4) antibodies were not detecting GPx 4 in fish and the reaction that was seen was non-specific binding of the anti-(human GPx 4) antibodies to some other protein. The large amount of binding at higher molecular masses and the curiosity of the unexpected dimer sized bands, even in the presence of reducing agent, indicates that the latter hypothesis (non-specific binding) is the more likely.

5.4.4 Summary and conclusions

In this chapter the expression of 2-Cys Prx proteins, in particular Prx 4, and selenium-containing GPx proteins, in particular GPx 4, was investigated in the tissues of male and female YTK at different stages of development This investigation showed that 2-Cys Prx proteins are expressed at similar levels in virtually all YTK tissues except for muscle where expression is lower but still present. Prx 4 expression was consistently detected at very high levels in liver and at lower levels in intestine. There was also some evidence for detection of Prx 4 expression in kidney, testes and gill but the results were inconsistent. There was some evidence for detection of GPx 4 expression in brain, liver, muscle and heart but again the results were inconsistent. There appeared to be high levels of non-specific binding in the blots with the anti-(human GPx 4) antibodies suggesting that these antibodies may not be detecting GPx 4 in fish.

The size of the fish, as a proxy for the stage of development, did not appear to have any noticeable effects on the expression of Prx or GPx proteins in YTK. This suggests either (a) that the differences in stage of development were not great enough to show changes in expression or (b) that the large YTK were not, in fact, sexually mature or (c) that the expression of Prx and GPx proteins does not change as YTK mature.

Similarly, the gender of the fish did not appear to have any effect on the level Prx or GPx protein expression in YTK. There was, however, detection of two bands in the ovaries at 35 kDa and 45 kDa with the anti-(human GPx 4) antibodies. These bands may correspond to hitherto unknown forms of GPx 4 but it is more likely that the anti-(human GPx 4) antibodies were not specific for GPx proteins

Table 5.1: Summary of fish and tissues used for the analyses in this chapter. All tissues were harvested from freshly killed YTK obtained by the author of this thesis from Clean Seas Tuna Ltd in June 2011. The tissues were frozen in dry ice on site at the Arno Bay wharf and transported in dry ice to Flinders University where they were frozen at -80°C until they were analysed.

Fish	Size	Sex	Tissues harvested
YTK 6	3 kg	Female	Brain, liver, skeletal muscle, heart, intestine, spleen, kidney, ovaries, gill
YTK 7	3 kg	Male	Brain, liver, skeletal muscle, heart, intestine, spleen, kidney, testes, gill
YTK 8	1.5 kg	Male	Brain, liver, skeletal muscle, heart, intestine, spleen, testes, gill
YTK 9	3 kg	Male	Brain, liver, skeletal muscle, heart, intestine, spleen, kidney, testes, gill
YTK 12	1.5 kg	Male	Brain, liver, skeletal muscle, heart, intestine, spleen, testes
YTK 13	1.5 kg	Female	Brain, liver, skeletal muscle, heart, intestine, spleen, ovaries, gill

Table 5.2: Reagents and final concentrations for Buffer F[1]

Chemical	Concentration
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.5	50 mM
NaCl	150 mM
Dithiothreitol (DTT)	50 mM
Polymethylsulfinyl fluoride (PMSF)	1 mM
Ethylenediaminetetraacetic acid (EDTA)	1 mM
Sodium dodecyl sulphate (SDS)	1% (w/v)
Urea	5 M

[1] Buffer composition adapted from a recipe from Mr Drew Sutton (unpublished data)

Table 5.3: Reagents and final concentrations for Buffer B[2]

Chemical	Concentration
Potassium phosphate (pH 7.6)	50 mM
Dithiothreitol (DTT)	5 mM
L-Glutathione, reduced (GSH)	6 mM
Polymethylsulfinyl fluoride (PMSF)	2 mM
Ethylenediaminetetraacetic acid (EDTA)	1 mM

[2] Buffer composition sourced from Thompson et al. (2006)

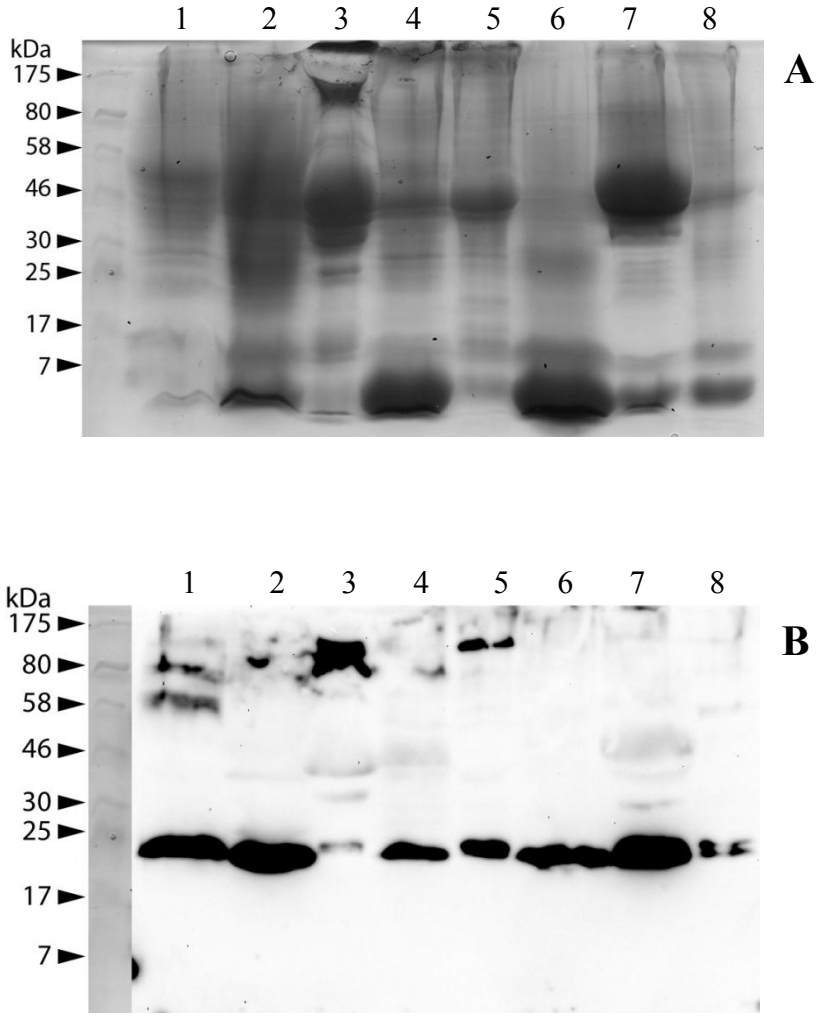


Figure 5.1: Expression of Prx proteins in various tissues of a 1.5 kg YTK female (YTK 13). The lanes contained 1 – Brain; 2 – Liver; 3 – Skeletal muscle; 4 – Heart; 5 – Intestine; 6 – Spleen; 7 – Ovaries; 8 – Gill

(A) SDS-PAGE conducted on a 10% (w/v) acrylamide gel with the indicated tissues from YTK 13 extracted into extraction Buffer F. Each lane was loaded with the amount of protein extracted from 2.5 mg of tissue. .

(B) Immunoblot analysis of a parallel gel to panel A using a 1:1000 dilution of anti-(SBT-Prx 2) antibodies.

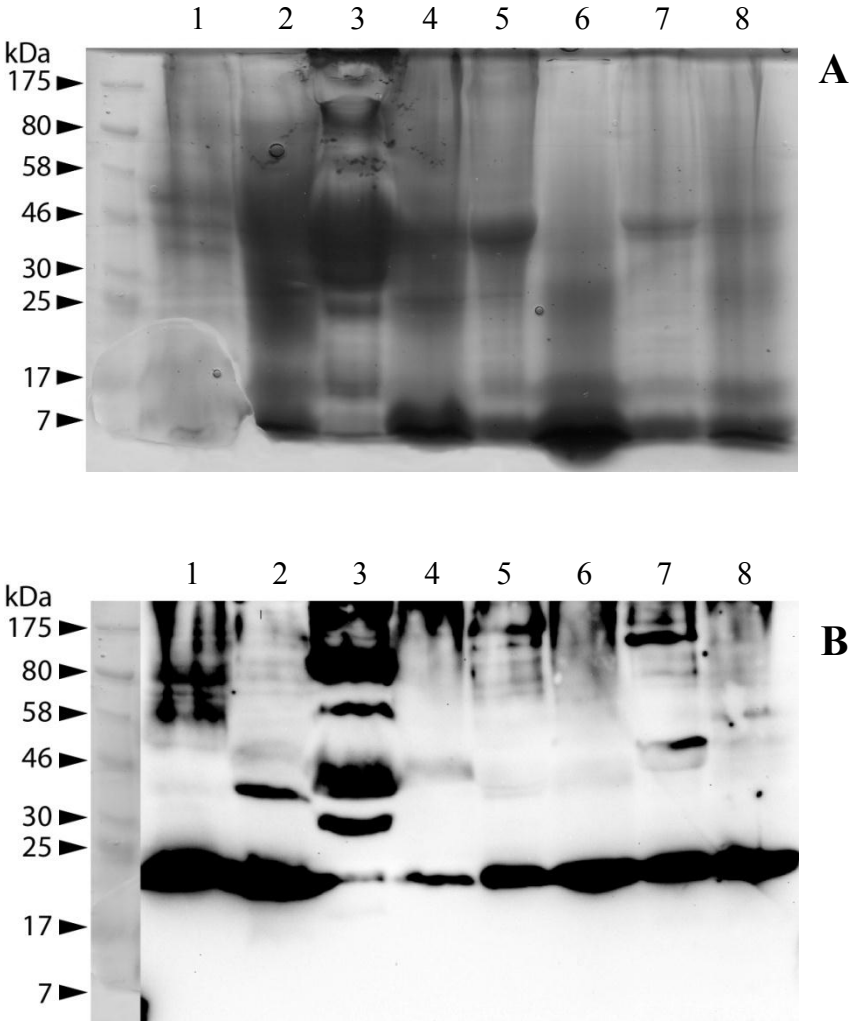


Figure 5.2: Expression of Prx proteins in various tissues of a 1.5 kg YTK male (YTK 8). The lanes contained 1 – Brain; 2 – Liver; 3 – Skeletal muscle; 4 – Heart; 5 – Intestine; 6 – Spleen; 7 – Testes; 8 – Gill.

(A) SDS-PAGE conducted on a 10% (w/v) acrylamide gel with the indicated tissues from YTK 8 extracted into Extraction buffer F. Each lane was loaded with the amount of protein extracted from 2.5 mg of tissue. .

(B) Immunoblot analysis of a parallel gel to panel A using a 1:1000 dilution of anti-(SBT-Prx 2) antibodies.

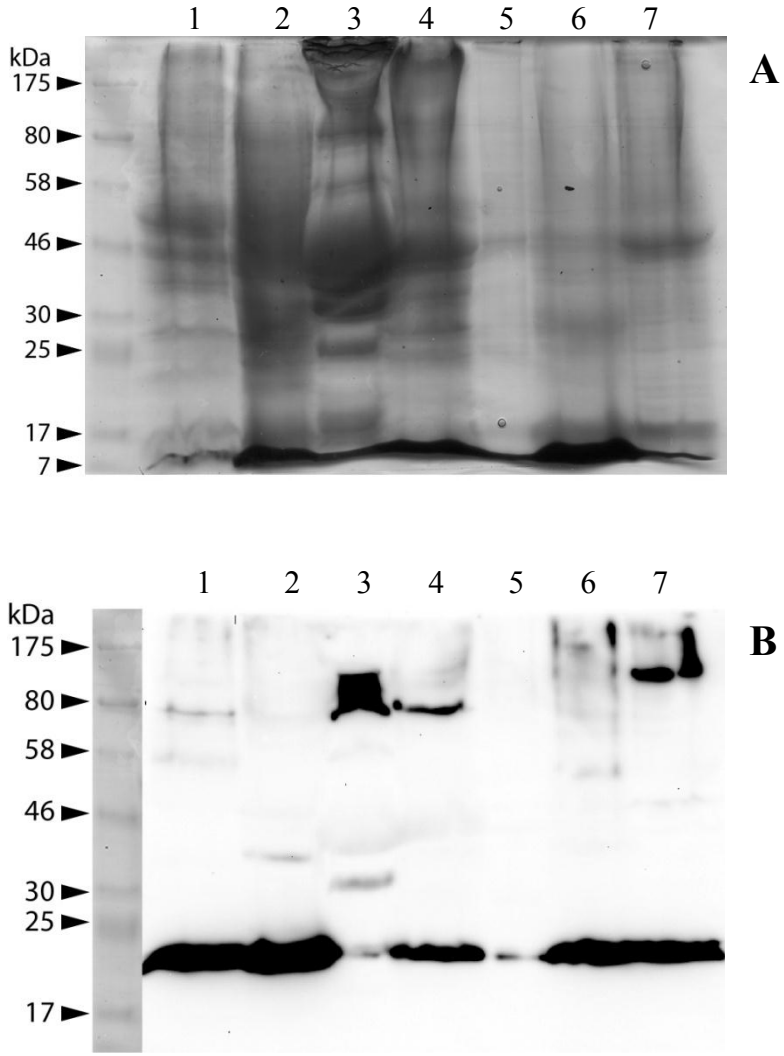


Figure 5.3: Expression of Prx proteins in various tissues of a 1.5 kg YTK male (YTK 12). The lanes contained 1 – Brain; 2 – Liver; 3 – Skeletal muscle; 4 – Heart; 5 – Intestine; 6 – Spleen; 7 – Testes

(A) SDS-PAGE conducted on a 10% (w/v) acrylamide gel with the indicated tissues of YTK 12 extracted into extraction buffer F. Each lane was loaded with the amount of protein extracted from 2.5 mg of tissue.

(B) Immunoblot analysis of a parallel gel to panel A using a 1:1000 dilution of anti-(SBT-Prx 2) antibodies.

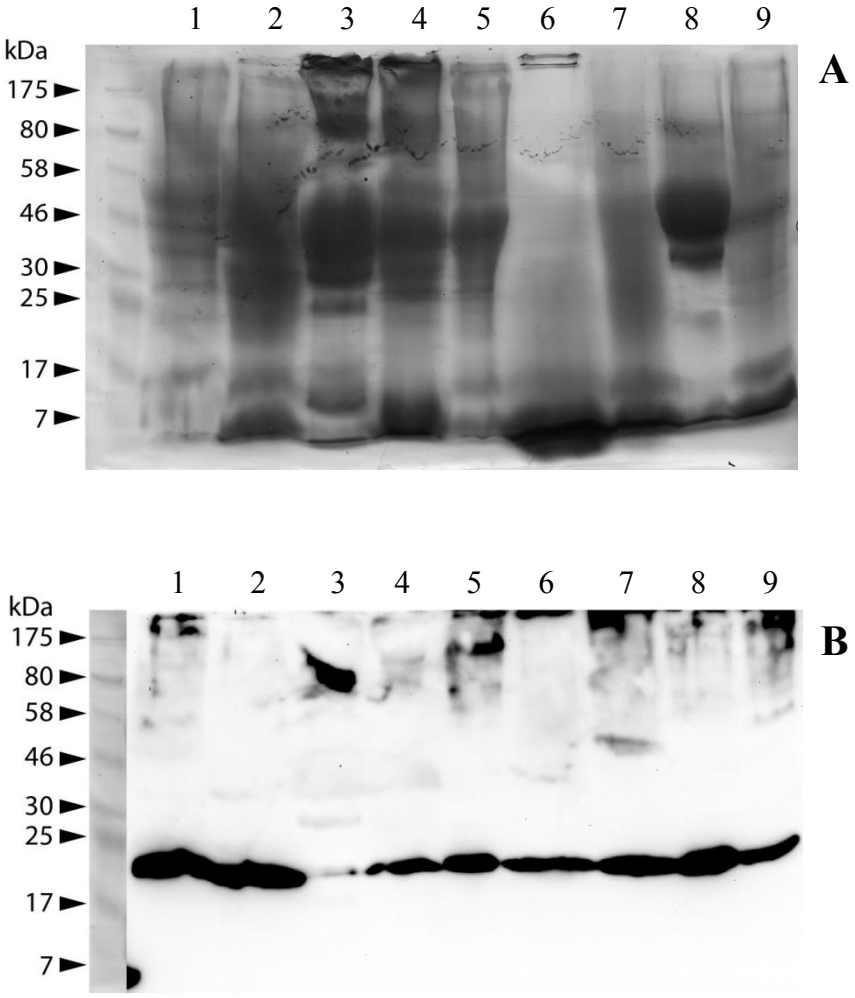


Figure 5.4: Expression of Prx proteins in various tissues of a 3 kg YTK female (YTK 6). The lanes contained 1 – Brain; 2 – Liver; 3 – Skeletal muscle; 4 – Heart; 5 – Intestine; 6 – Spleen; 7 – Kidney; 8 – Ovaries; 9 – Gill.

(C) SDS-PAGE conducted on a 10% (w/v) acrylamide gel with the indicated tissues of YTK 6 extracted into Extraction Buffer F. Each lane was loaded with the amount of protein extracted from 2.5 mg of tissue. .

(A) Immunoblot analysis of a parallel gel to panel A using a 1:1000 dilution of anti-(SBT-Prx 2) antibodies.

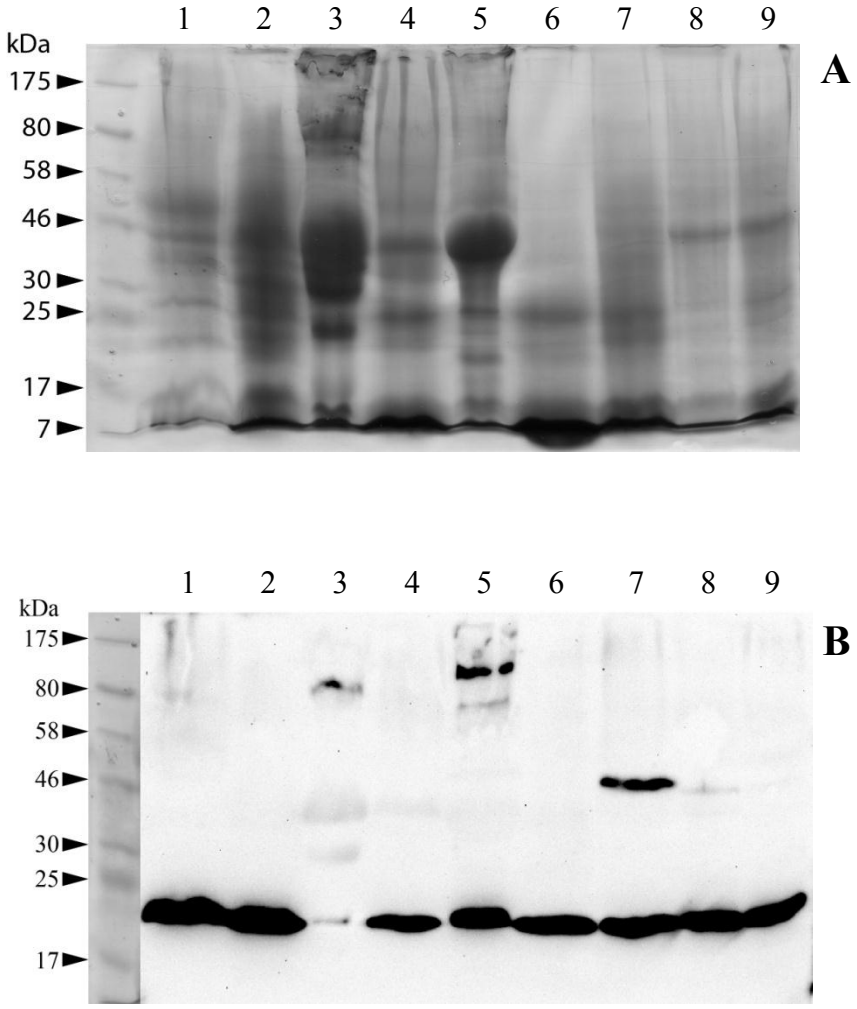


Figure 5.5: Expression of Prx proteins in various tissues of a 3 kg YTK male (YTK 7). The lanes contained 1 – Brain; 2 – Liver; 3 – Skeletal muscle; 4 – Heart; 5 – Intestine; 6 – Spleen; 7 – Kidney; 8 – Testes; 9 – Gill.

(D) SDS-PAGE conducted on a 10% (w/v) acrylamide gel with the indicated tissues of YTK 7 extracted into extraction buffer F. Each lane was loaded with the amount of protein extracted from 2.5 mg of tissue. .

(A) Immunoblot analysis of a parallel gel to panel A using a 1:1000 dilution of anti-(SBT-Prx 2) antibodies.

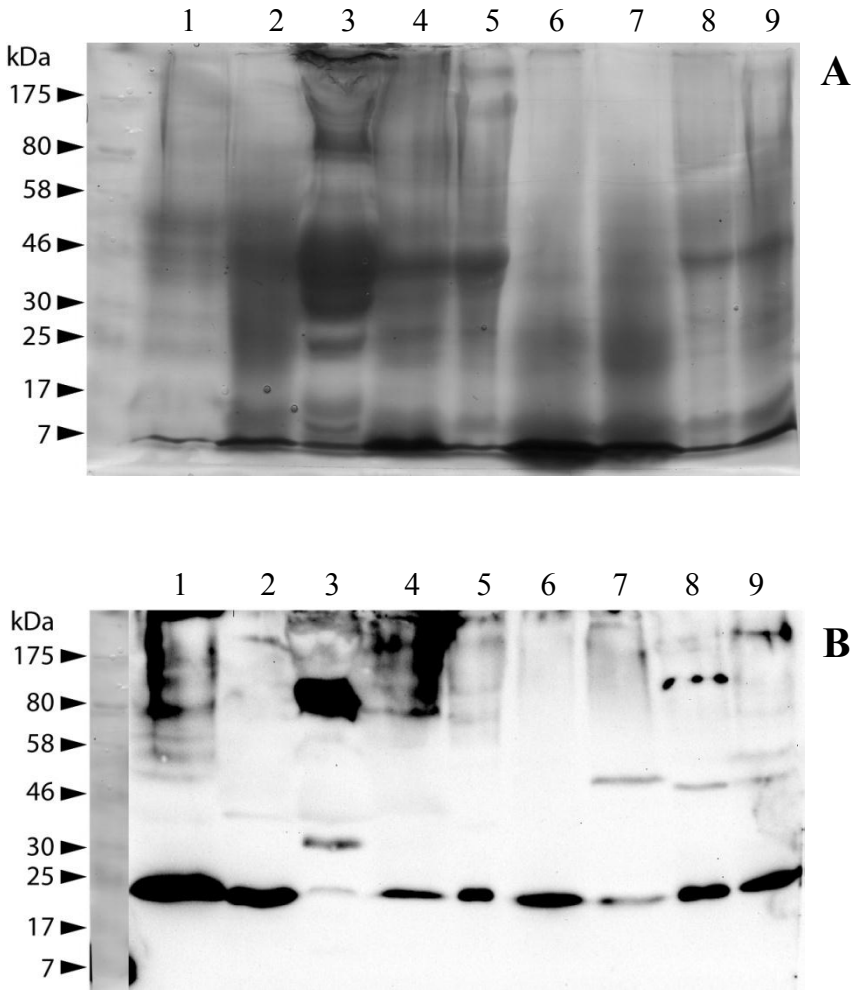


Figure 5.6: Expression of Prx proteins in various tissues of a 3 kg YTK male (YTK 9). The lanes contained 1 – Brain; 2 – Liver; 3 – Skeletal muscle; 4 – Heart; 5 – Intestine; 6 – Spleen; 7 – Kidney; 8 – Testes; 9 – Gill

(A) SDS-PAGE conducted on a 10% (w/v) acrylamide gel with the indicated tissues of YTK 9 extracted into Extraction Buffer F. Each lane was loaded with the amount of protein extracted from 2.5 mg of tissue. .

(B) Immunoblot analysis of a parallel gel to panel A using a 1:1000 dilution of anti-(SBT-Prx2) antibodies.

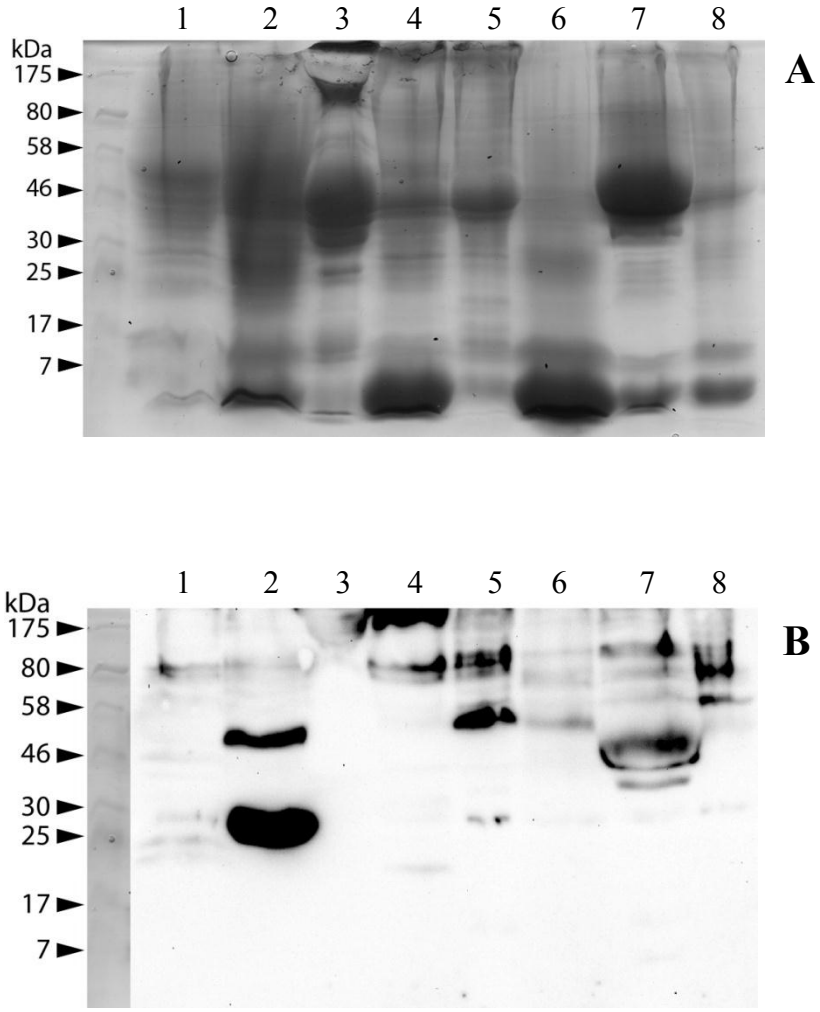


Figure 5.7: Expression of Prx 4 protein in various tissues of a 1.5 kg YTK female (YTK 13). The lanes contained 1 – Brain; 2 – Liver; 3 – Skeletal muscle; 4 – Heart; 5 – Intestine; 6 – Spleen; 7 – Ovaries; 8 – Gill.

(A) SDS-PAGE conducted on a 10% (w/v) acrylamide gel with the indicated tissues of YTK 13 extracted into Extraction Buffer F. Each lane was loaded with the amount of protein extracted from 2.5 mg of tissue. .

(B) Immunoblot analysis of a parallel gel to panel A using a 1:1000 dilution of anti-(human Prx 4) antibodies.

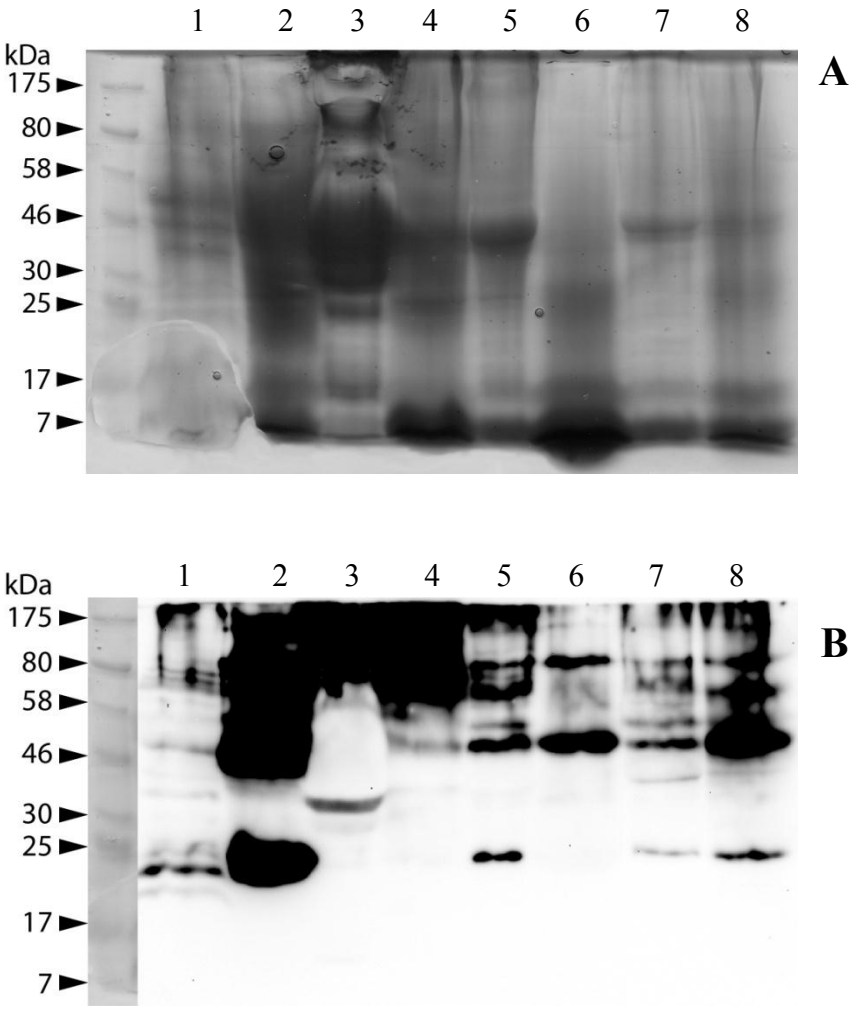


Figure 5.8: Expression of Prx 4 proteins in various tissues of a 1.5 kg YTK male (YTK 8). The lanes contained 1 – Brain; 2 – Liver; 3 – Skeletal muscle; 4 – Heart; 5 – Intestine; 6 – Spleen; 7 - Testes; 8 - Gill

(A) SDS-PAGE conducted on a 10% (w/v) acrylamide gel with the indicated tissues of YTK 8 extracted into Extraction Buffer F. Each lane was loaded with the amount of protein extracted from 2.5 mg of tissue. .

(B) Immunoblot analysis of a parallel gel to panel A using a 1:1000 dilution of anti-(human-Prx 4) antibodies.

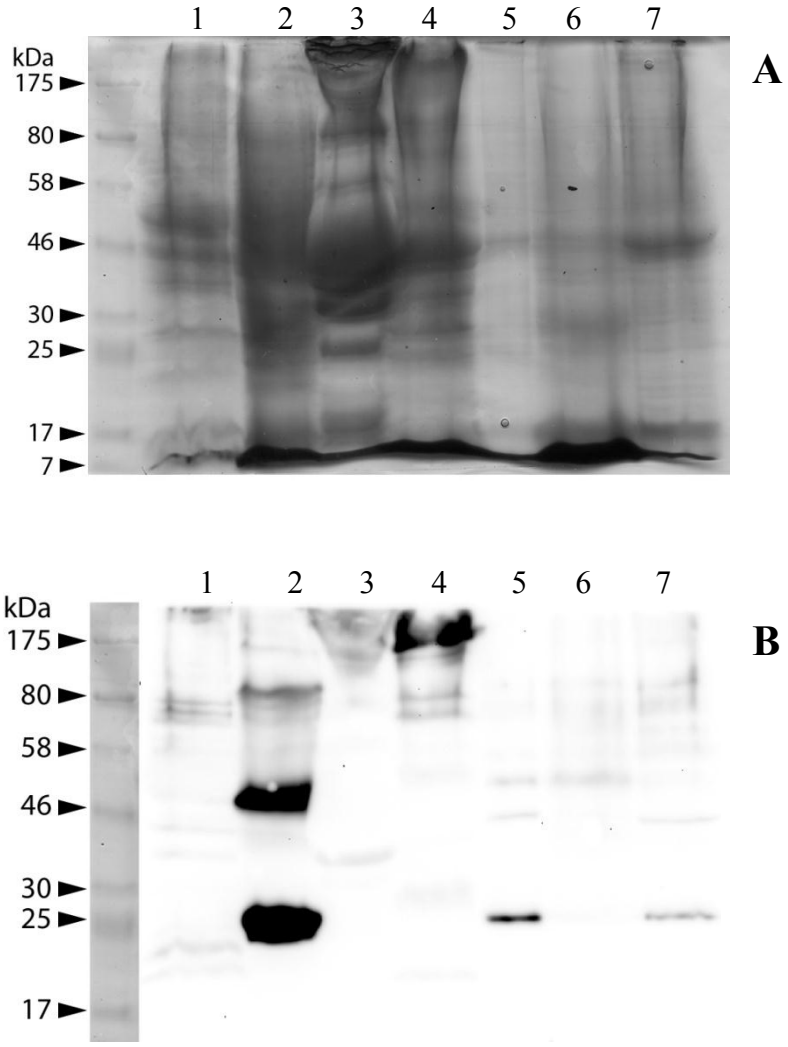


Figure 5.9: Expression of Prx 4 proteins in various tissues of a 1.5 kg YTK male (YTK 12). The lanes contained 1 – Brain; 2 – Liver; 3 – Skeletal muscle; 4 – Heart; 5 – Intestine; 6 – Spleen; 7 – Testes

(A) SDS-PAGE conducted on a 10% (w/v) acrylamide gel with the indicated tissues of YTK 12 extracted into Extraction Buffer F. Each lane was loaded with the amount of protein extracted from 2.5 mg of tissue.

(B) Immunoblot analysis of a parallel gel to panel A using a 1:1000 dilution of anti-(human-Prx 4) antibodies.

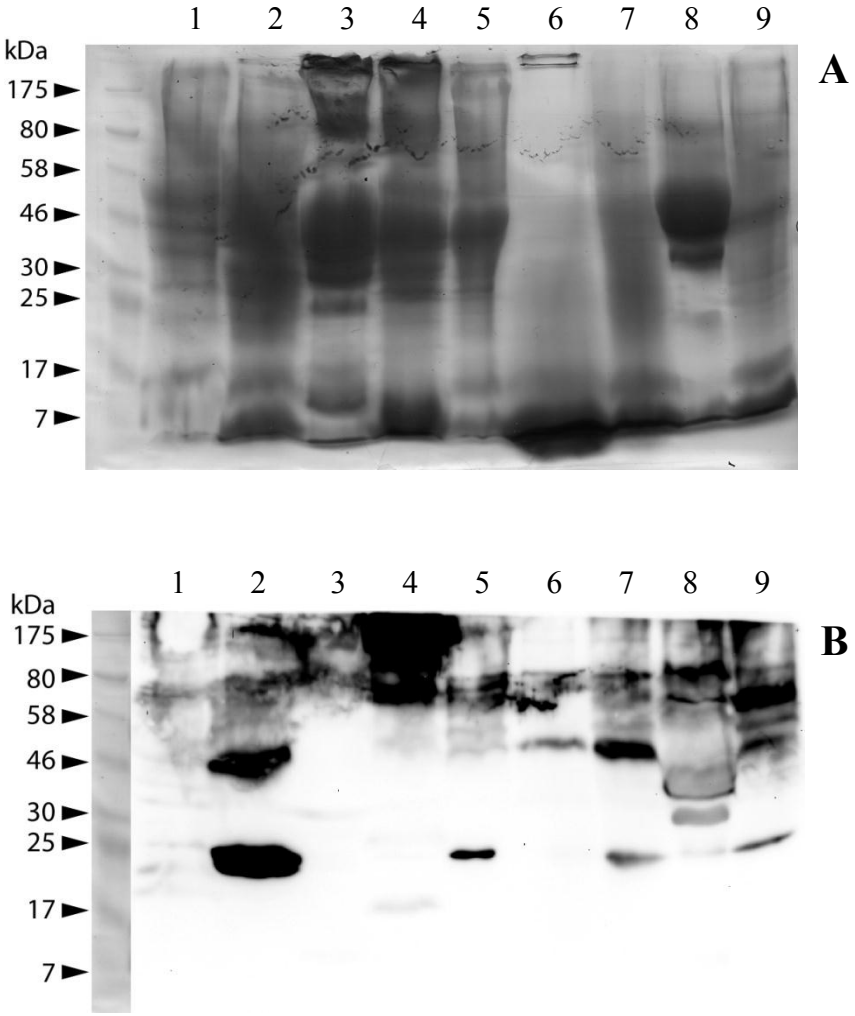


Figure 5.10: Expression of Prx 4 proteins in various tissues of a 3 kg YTK female (YTK 6). The lanes contained 1 – Brain; 2 – Liver; 3 – Skeletal muscle; 4 – Heart; 5 – Intestine; 6 – Spleen; 7 – Kidney; 8 – Ovaries; 9 - Gill

(A) SDS-PAGE conducted on a 10% (w/v) acrylamide gel with the indicated tissues of YTK 6 extracted into extraction buffer F. Each lane was loaded with the amount of protein extracted from 2.5 mg of tissue. .

(B) Immunoblot analysis of a parallel gel to panel A using a 1:1000 dilution of anti-(human Prx 4) antibodies.

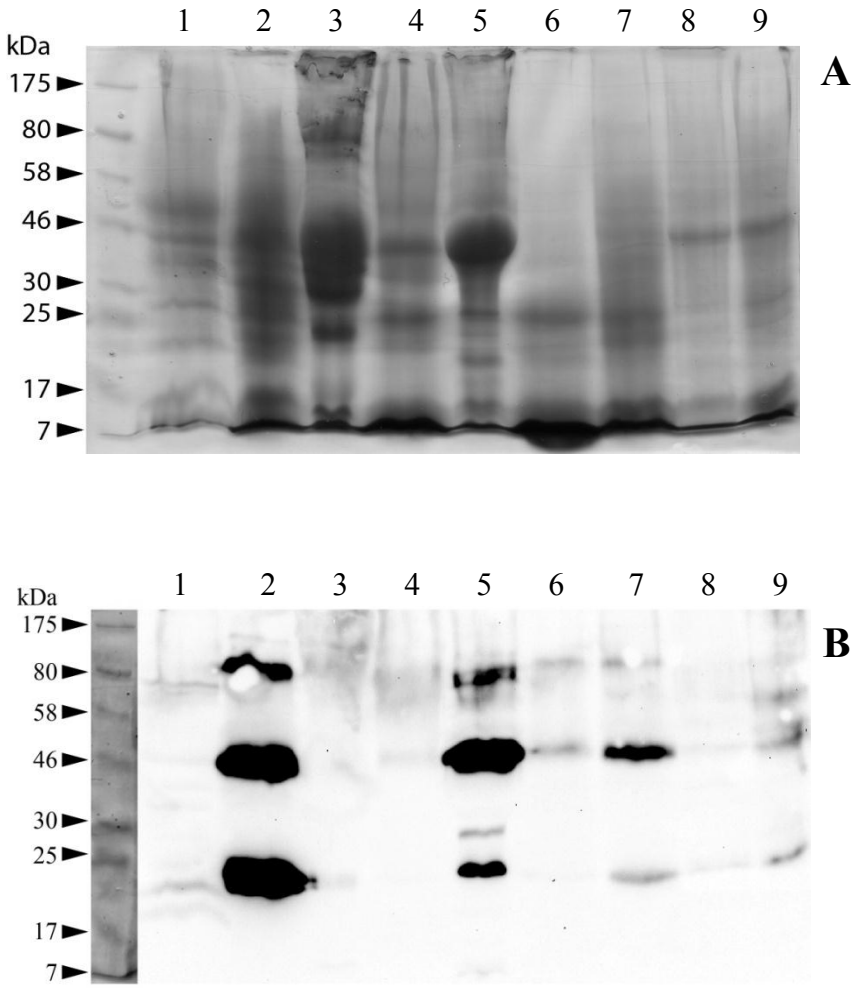


Figure 5.11: Expression of Prx 4 proteins in various tissues of a 3 kg YTK male (YTK 7). The lanes contained 1 – Brain; 2 – Liver; 3 – Skeletal muscle; 4 – Heart; 5 – Intestine; 6 – Spleen; 7 – Kidney; 8 - Testes; 9 - Gill

(A) SDS-PAGE conducted on a 10% (w/v) acrylamide gel with the indicated tissues of YTK 5 extracted into extraction buffer F. Each lane was loaded with the amount of protein extracted from 2.5 mg of tissue. .

(B) Immunoblot analysis of a parallel gel to panel A using a 1:1000 dilution of anti-(human Prx 4) antibodies.

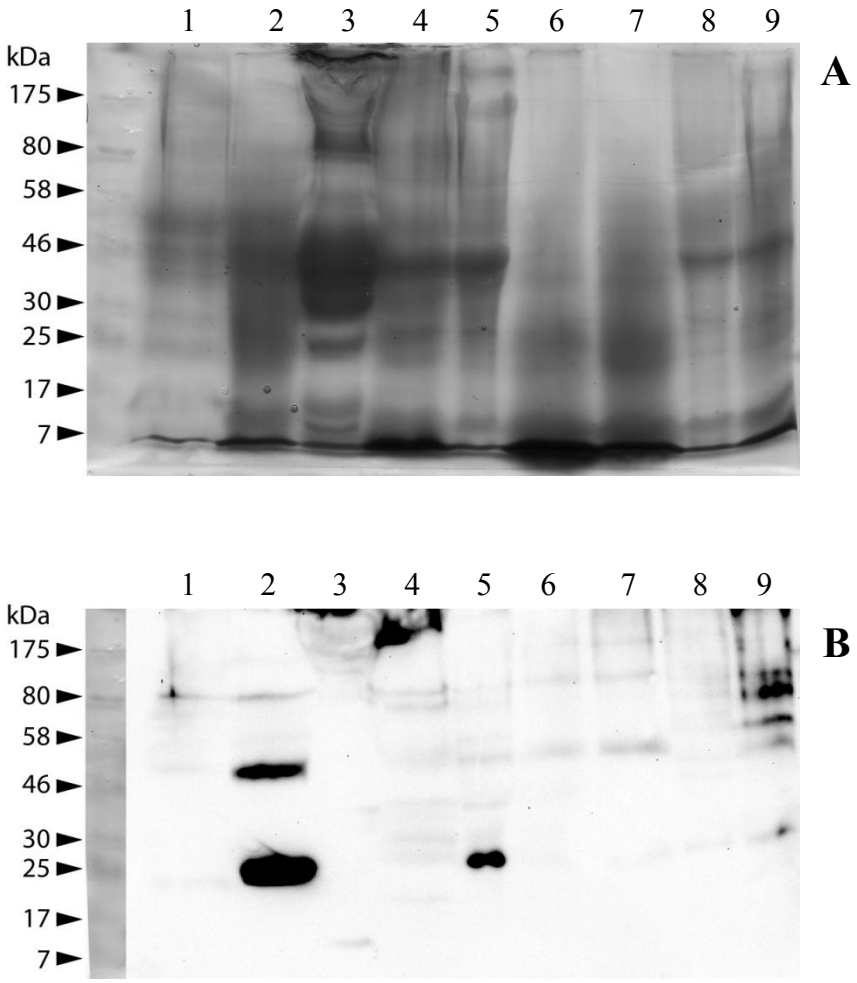


Figure 5.12: Expression of Prx 4 proteins in various tissues of a 3 kg YTK male (YTK 9). The lanes contained 1 – Brain; 2 – Liver; 3 – Skeletal muscle; 4 – Heart; 5 – Intestine; 6 – Spleen; 7 – Kidney; 8 - Testes; 9 - Gill

(A) SDS-PAGE conducted on a 10% (w/v) acrylamide gel with the indicated tissues of YTK 9 extracted into extraction buffer F. Each lane was loaded with the amount of protein extracted from 2.5 mg of tissue. .

(B) Immunoblot analysis of a parallel gel to panel A using a 1:1000 dilution of anti-(human Prx 4) antibodies.

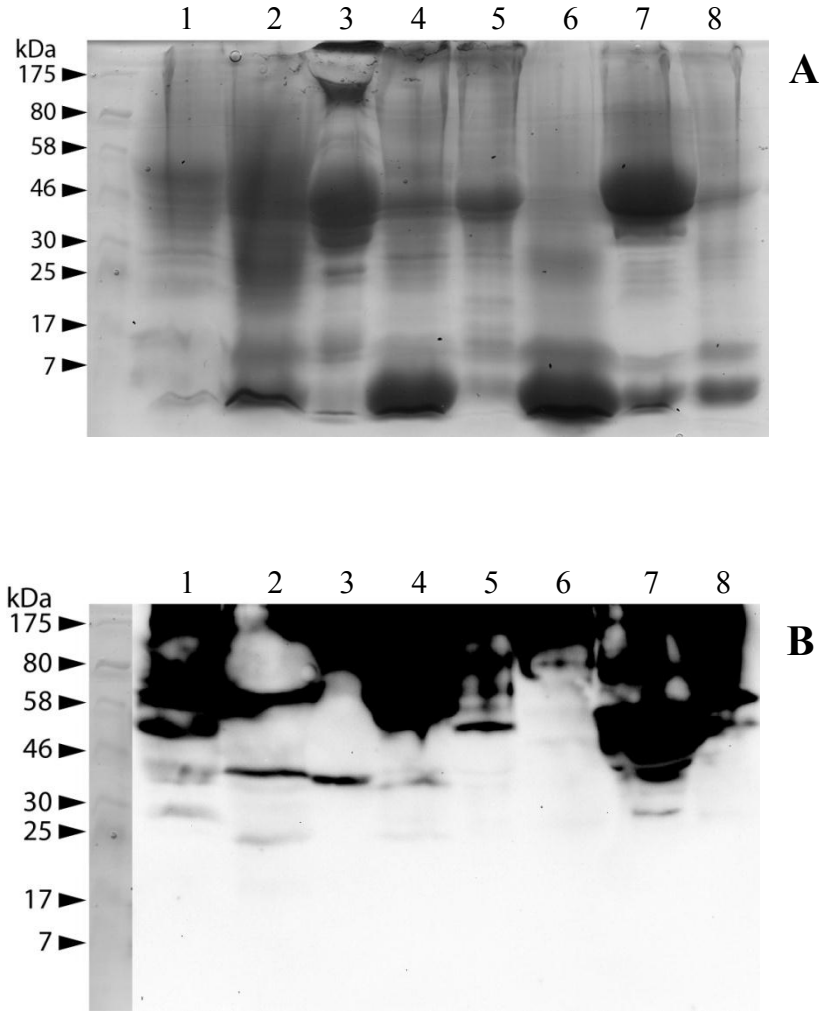


Figure 5.13: Expression of GPx 4 proteins in various tissues of a 1.5 kg YTK female (YTK 13). The lanes contained 1 – Brain; 2 – Liver; 3 – Skeletal muscle; 4 – Heart; 5 – Intestine; 6 – Spleen; 7 – Ovaries; 8 - Gill

(A) SDS-PAGE conducted on a 10% (w/v) acrylamide gel with the indicated tissues of YTK 13 extracted into extraction buffer F. Each lane was loaded with the amount of protein extracted from 2.5 mg of tissue. .

(B) Immunoblot analysis of a parallel gel to panel A using a 1:1000 dilution of anti-(human GPx 4) antibodies.

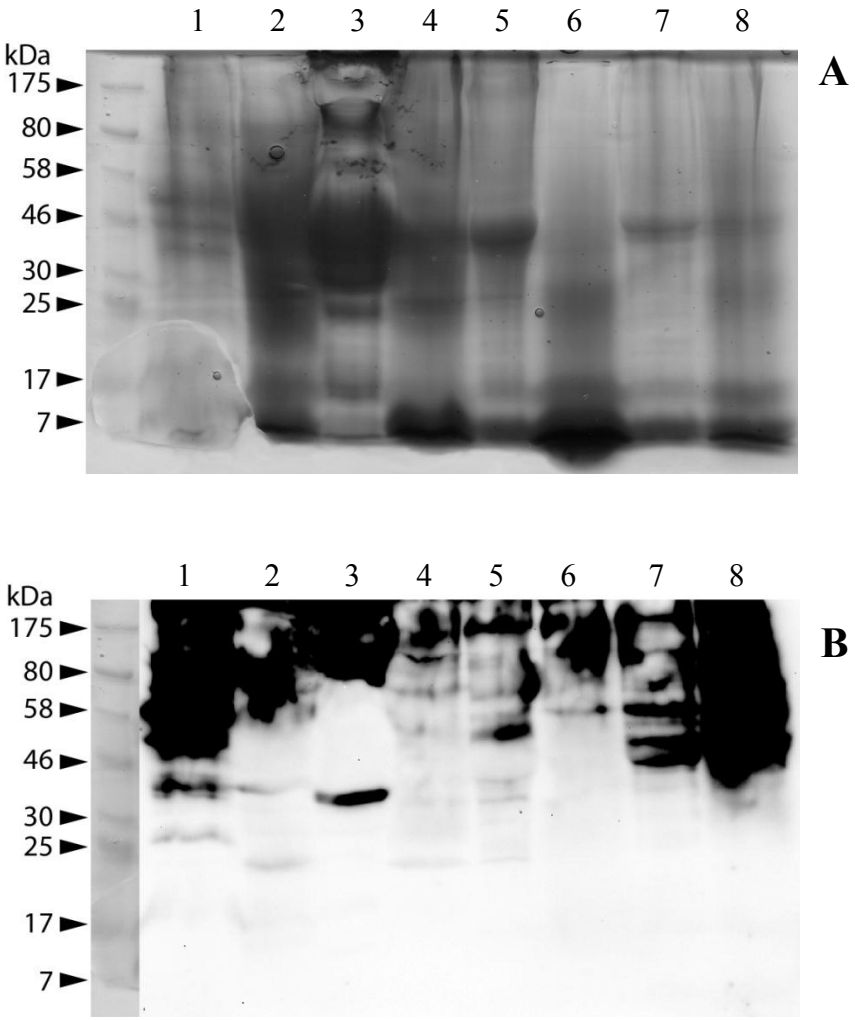


Figure 5.14: Expression of GPx 4 proteins in various tissues of a 1.5 kg YTK male (YTK 8). The lanes contained 1 – Brain; 2 – Liver; 3 – Skeletal muscle; 4 – Heart; 5 – Intestine; 6 – Spleen; 7 – Testes; 8 – Gill

(A) SDS-PAGE conducted on a 10% (w/v) acrylamide gel with the indicated tissues of YTK 8 extracted into extraction buffer F. Each lane was loaded with the amount of protein extracted from 2.5 mg of tissue. .

(B) Immunoblot analysis of a parallel gel to panel A using a 1:1000 dilution of anti-(human GPx 4) antibodies.

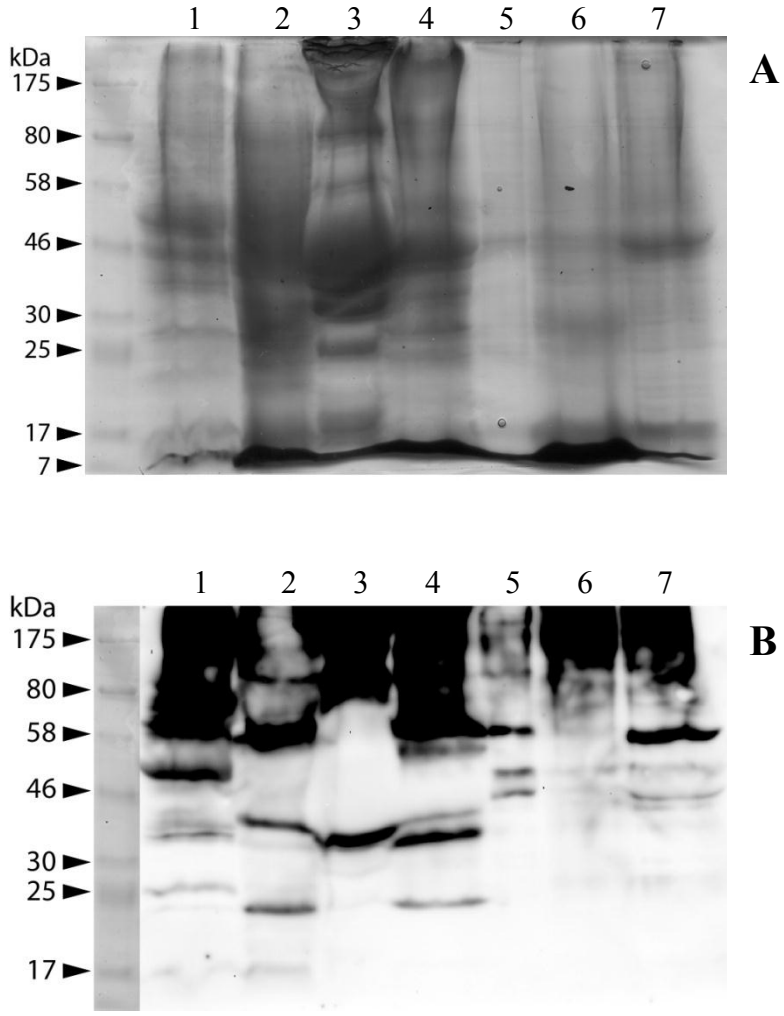


Figure 5.15: Expression of GPx 4 proteins in various tissues of a 1.5 kg YTK male (YTK 12). The lanes contained 1 – Brain; 2 – Liver; 3 – Skeletal muscle; 4 – Heart; 5 – Intestine; 6 – Spleen; 7 – Testes

(A) SDS-PAGE conducted on a 10% (w/v) acrylamide gel with the indicated tissues of YTK 12 extracted into extraction buffer F. Each lane was loaded with the amount of protein extracted from 2.5 mg of tissue. .

(B) Immunoblot analysis of a parallel gel to panel A using a 1:1000 dilution of anti-(human GPx 4) antibodies.

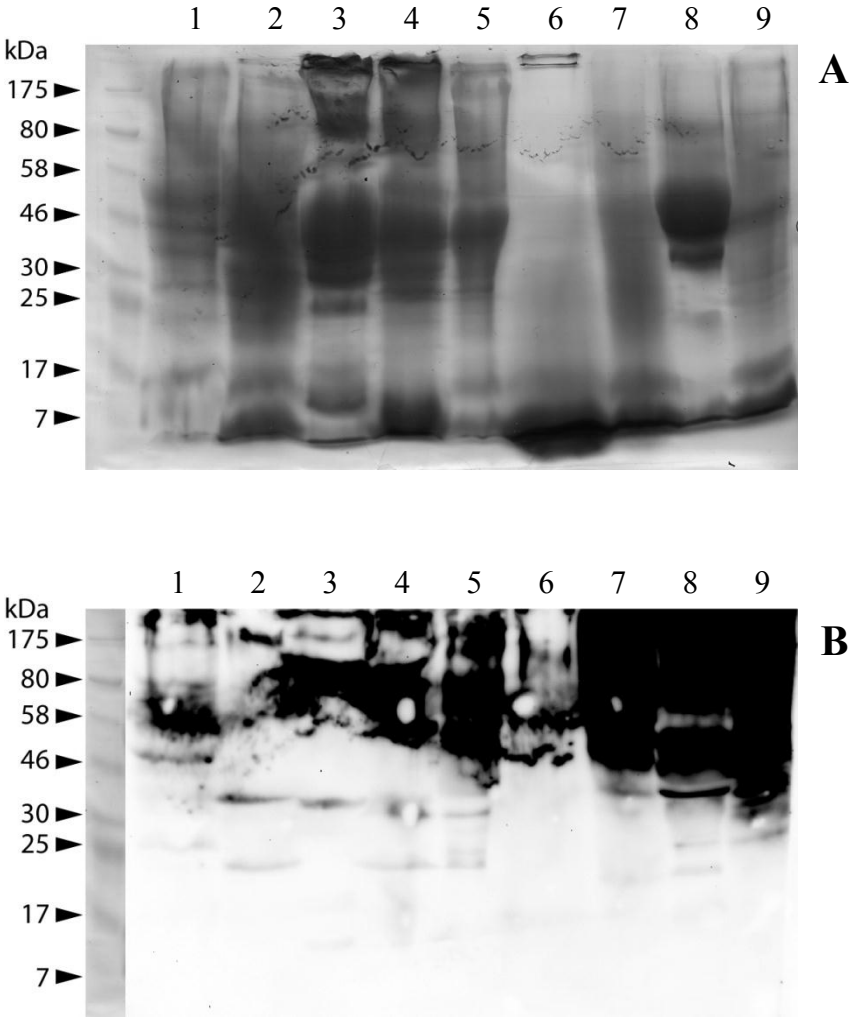


Figure 5.16: Expression of GPx 4 proteins in various tissues of a 3 kg YTK female (YTK 6). The lanes contained 1 – Brain; 2 – Liver; 3 – Skeletal muscle; 4 – Heart; 5 – Intestine; 6 – Spleen; 7 – Kidney; 8 – Ovaries; 9 - Gill

(A) SDS-PAGE conducted on a 10% (w/v) acrylamide gel with the indicated tissues of YTK 6 extracted into extraction buffer F. Each lane was loaded with the amount of protein extracted from 2.5 mg of tissue. .

(B) Immunoblot analysis of a parallel gel to panel A using a 1:1000 dilution of anti-(human GPx 4) antibodies.

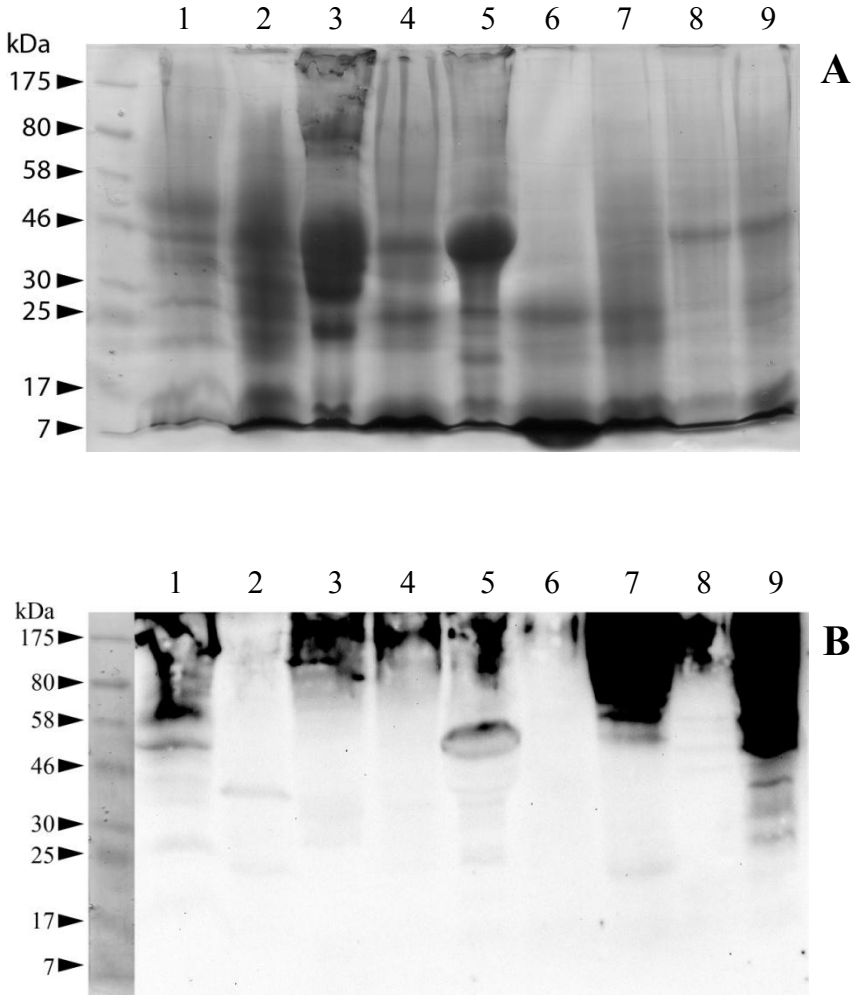


Figure 5.17: Expression of GPx 4 proteins in various tissues of a 3 kg YTK male (YTK 7). The lanes contained 1 – Brain; 2 – Liver; 3 – Skeletal muscle; 4 – Heart; 5 – Intestine; 6 – Spleen; 7 – Kidney; 8 - Testes; 9 - Gill

(A) SDS-PAGE conducted on a 10% (w/v) acrylamide gel with the indicated tissues of YTK 7 extracted into extraction buffer F. Each lane was loaded with the amount of protein extracted from 2.5 mg of tissue. .

(B) Immunoblot analysis of a parallel gel to panel A using a 1:1000 dilution of anti-(human GPx 4) antibodies.

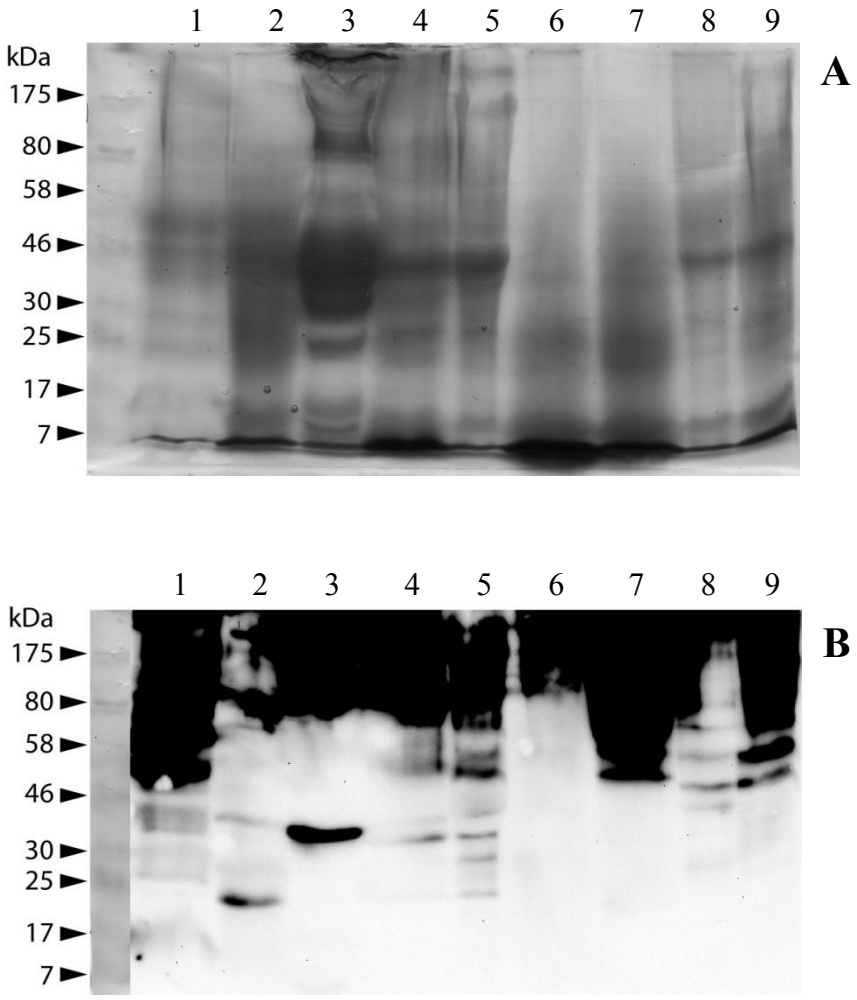


Figure 5.18: Expression of GPx 4 proteins in various tissues of a 3 kg YTK male (YTK 9). The lanes contained 1 – Brain; 2 – Liver; 3 – Skeletal muscle; 4 – Heart; 5 – Intestine; 6 – Spleen; 7 – Kidney; 8 - Testes; 9 - Gill

(A) SDS-PAGE conducted on a 10% (w/v) acrylamide gel with the indicated tissues of YTK 9 extracted into extraction buffer F. Each lane was loaded with the amount of protein extracted from 2.5 mg of tissue. .

(B) Immunoblot analysis of a parallel gel to panel A using a 1:1000 dilution of anti-(human GPx 4) antibodies.

Chapter 6 – Further analysis of Prx expression in YTK to determine whether there is evidence for post-translational processing of Prx 4

6.1 Background and Aims

Previous studies with mammals have shown that Prx 4 is expressed at a higher level in the testes of rodents relative to other major organs (Okado-Matsumoto et al., 2000; Iuchi et al., 2009). Furthermore, a second, larger isoform of the Prx 4 monomer is found only in the testes of sexually mature animals (Okado-Matsumoto et al., 2000; Sasagawa et al., 2001; Iuchi et al., 2009). The results from Chapter 5 showed that Prx expression in YTK was not, in fact, higher in the testes of large, male fish. Interestingly, high expression was seen in the female gonad tissue and two bands were present at 35 and 45 kDa which were not present in other tissues. However, in the majority of cases, the resolution of the SDS-PAGE gels and the immunoblots was not sufficient to distinguish between different isoforms of the Prx 4 monomer.

Therefore, the aims of this chapter were to:

1. further investigate the expression of Prx 4 in the ovaries of a sexually immature 1.5 kg YTK female (YTK 13) and a large 3.0 kg YTK female (YTK 6) that was thought to be sexually mature (Dr Wayne Knibb, personal communication). In particular, the aim was to confirm the reaction of the anti-(human Prx 4) antibodies with the proteins at 35 and 45 kDa that was seen in these fish in Chapter 5 (Sections 5.3.2.1 and 5.3.2.3)
2. further investigate the expression of Prx proteins in the testes and various other tissues of large 3.0 kg YTK males that were thought to be sexually mature (Dr Wayne Knibb, personal communication). In particular, the aim was to determine whether different isoforms of the Prx 4 monomer were to be found in YTK testes indicating possible

post-translational removal of an endoplasmic reticulum targeting sequence similar to what has been observed in studies with mammals.

Based on the bioinformatics analyses in Chapter 3, we expected to see reaction with the anti-(human Prx 4) antibodies at the sizes of the 264 amino acid unprocessed form of Prx 4 (29 kDa) and the 231 amino acid truncated form of Prx 4 (25 kDa) but not at the size of the 197 amino acid truncated form of Prx 4 (22 kDa). The reason for this was that only half the region corresponding to the peptide used to raise the anti-(human Prx 4) antibodies was present in the 197 amino acid truncated form of Prx 4 (22 kDa; Section 3.3.3). Therefore, this form was not expected to be detected by the anti-(human Prx 4) antibodies but the full length 264 amino acid and the truncated 231 amino acid forms were. We also expected to see reaction with the anti-(SBT Prx 2) antibodies at the sizes of the 231 amino acid truncated form of Prx 4 (25 kDa) and the mature 2-Cys Prx monomers (22 kDa). However, based on the results seen in Chapter 5, we did not expect to see a band at the size of the 264 amino acid unprocessed form of Prx 4 (29 kDa).

6.2 Methods

6.2.1 Expression of Prx 4 in the ovaries of a 1.5 kg YTK female and a 3.0 kg YTK female

Protein extracts from the ovaries of a 1.5 kg YTK female (YTK 13, Section 5.2.2) and a 3.0 kg YTK female (YTK 6, Section 5.2.4) were taken and serial dilutions from 1:10 to 1:10,000 were prepared in Buffer F (Section 4.2.6) with the aim of decreasing the apparent non-specific binding on the immunoblots due to the high concentrations of some proteins. The various dilutions were loaded on two replicate 10% SDS-PAGE gels for each fish and the

gels were run according to the protocol described in Section 2.4. One gel for each fish was stained for protein (Section 2.4), while the other was used for immunoblot analysis (Section 2.5) using a 1:1000 dilution of the anti-(human Prx 4) primary antibodies. Development and detection were conducted as described in Sections 2.5 and 2.6 using a 1:2000 dilution of secondary antibodies.

6.2.2 Expression of 2-Cys Prx proteins in the testes of two 3.0 kg YTK males

The protein extracts from the testes of YTK 7 and YTK 9 described in Section 5.2.5 were taken and serial dilutions from 1:2 to 1:8 were prepared in Buffer F (Section 4.2.6) with the aim of decreasing the intensity of the antibody reaction and resolving any possible different isoforms of Prx 4 at the monomer size. The various dilutions were loaded on two replicate 10% SDS-PAGE gels and the gels were run according to the protocol described in Section 2.4. One gel was stained for protein (Section 2.4), while the other was used for immunoblot analysis as per Section 2.5 using a 1:1000 dilution of anti-(SBT Prx 2) primary antibodies. Development and detection were conducted as described in Sections 2.5 and 2.6 using a 1:2000 dilution of secondary antibodies.

6.2.3 High resolution SDS-PAGE of proteins from a 3.0 kg YTK male

Total protein was extracted from brain, liver, skeletal muscle, heart, intestine, spleen, kidney, testes and gill of a 3.0 kg YTK male (YTK 7) into Buffer B (Section 4.2.2) according to the protocol for tissues frozen at -80°C described in Section 2.3.2. Total protein was calculated using the method of Bradford (1976) and 250 µg of protein was loaded into each lane of three replicate 12% acrylamide SDS-PAGE gels and the gels were run at 50 mA for approximately 60-70 minutes or until the 7 kDa marker protein had just run off the bottom of the gel. The

aim of using a 12% rather than a 10% acrylamide gel was to provide better separation of proteins at low molecular masses with the hope of resolving any different isoforms of Prx 4 at the monomeric size. One gel was stained for protein (Section 2.4), while the other two were used for immunoblot analysis (Section 2.5) using a 1:1000 dilution of the anti-(SBT Prx 2) or anti-(human Prx 4) primary antibodies. Development and detection were conducted as described in Sections 2.5 and 2.6 using a 1:2000 dilution of secondary antibodies.

6.3 Results

6.3.1 Expression of Prx 4 in the ovaries of a 1.5 kg YTK female and a 3.0 kg YTK female

The aim of this section was to investigate whether the reactions seen at the unexpected molecular masses of 35 and 45 kDa in Sections 5.3.2.1 and 5.3.2.3 were reactions with previously unknown forms of Prx 4 or just simply non-specific binding of the primary antibodies to abundant proteins that were not necessarily Prx proteins. It was hypothesized that dilution of the ovaries extracts would reduce non-specific binding of the primary antibodies.

The Coomassie Blue stained gel for the 1.5 kg YTK female (YTK 13, Fig. 6.1A) showed proteins in the undiluted ovaries extract in distinct bands from approximately 30 to 50 kDa (lane 2). The same pattern was observed at reduced intensity in the 1:10 dilution (lane 4). The intensity was further reduced in the 1:1000 dilution (lane 5). Protein was undetectable by Coomassie Blue staining in the 1:1,000 and 1:10,000 dilutions (lanes 6 and 7).

In Fig. 6.1B, the anti-(human Prx 4) antibodies reacted with all of the proteins that were visible in the Coomassie Blue stained gel for YTK 13. The undiluted ovaries extract (lane 2) showed a large amount of reaction from approximately 40 kDa up to the origin of the immunoblot. This reaction resolved into 3 distinct bands at approximately 40, 45 and 50 kDa in the 1:10 dilution (lane 4). The reaction in the 1:100 dilution (lane 5) was the same as seen in the 1:10 dilution, but at reduced intensity. Therefore, it can be concluded that dilution of the extract reduced the intensity of reaction but the antibodies appeared to be reacting in a non-specific manner with all abundant proteins in the immunoblot. Therefore, it is most likely that the anti-(human Prx 4) antibodies were reacting non-specifically with YTK ovary proteins.

The Coomassie Blue stained gel for the 3.0 kg YTK female (YTK 6, Fig. 6.2A) showed a large amount of protein with two highly abundant proteins at approximately 40 kDa and 45 kDa in the undiluted extract (lane 2). The 1:10 dilution showed a similar pattern but with lower intensity due to the lower abundance of protein (lane 4). The protein abundance in the 1:100 (lane 5), 1:1,000 (lane 6) and 1:10,000 (lane 7) dilutions was too low to be detected by the Coomassie Blue stain.

Fig. 6.2B showed that the anti-(human Prx 4) antibodies reacted strongly with proteins in the undiluted ovaries extract from approximately 25 kDa up to the origin of the immunoblot (lane 2). In the 1:10 dilution (lane 4), three prominent protein bands were observed at approximately 58, 45 and 40 kDa with the 45 kDa protein band being the most abundant of the three. The size of the 45 kDa band corresponded with the predicted dimer size of the 197 amino acid truncated form of Prx 4. The same reactions were also observed in the 1:100

dilution (lane 5) albeit at lower intensity. No reaction was observed in the 1:1,000 or 1:10,000 dilutions (lanes 6 and 7, respectively). Therefore, similarly to what had been seen in Fig. 6.1B, dilution of the ovaries extract reduced the intensity of the reaction and therefore the antibodies appeared to be reacting in a non-specific manner with any abundant proteins in the immunoblot. Again, this suggests non-specific binding to various proteins rather than specific binding to Prx 4.

6.3.2 Expression of 2-Cys Prx proteins in the testes of two 3.0 kg YTK males

The aim of this section was to determine whether dilution of the testes extracts from two of the large YTK males would allow resolution of two different molecular mass forms of Prx 4 at the monomer size. The large 3.0 kg YTK males were considered to be sexually mature. This was important because, the mammalian literature had shown that sexually mature rats expressed two different isoforms of Prx 4 at the monomer size, whereas immature rats did not (Sasagawa et al., 2001). Specifically, it was hypothesised that dilution of the YTK testes extracts would decrease the intensity of the antibody reaction and allow the resolution of two protein bands at approximately 25 kDa (the 231 amino acid truncated form of the Prx 4 monomer) and 22 kDa (the 197 amino acid truncated form of the Prx 4 monomer) using the anti-(SBT Prx 2) antibodies which had been predicted to react with all forms of 2-Cys Prx proteins in fish (Chapter 3).

The Coomassie Blue stained gel (Fig. 6.3A) showed the highest abundance of protein in the undiluted extract of YTK 9 testes (lane 5) and this concentration noticeably decreased over the dilution series (lanes 6-8). A lower abundance of protein was observed in the testes of

YTK 7 (lane 1) and a similar decrease in concentration was observed over the dilution series (lanes 2-4).

In Fig. 6.3B, the anti-(SBT Prx 2) antibodies detected protein at approximately the size of a 2-Cys Prx monomer (22 kDa) in all dilutions of the testes extracts from both YTK 7 (lanes 1-4) and YTK 9 (lanes 5-8). The reaction in the YTK 9 testes (lanes 5-8) was stronger than the reaction in the YTK 7 testes (lanes 1-4) which was consistent with the observation that the amount of protein loaded appeared to be more for YTK 9 than YTK 7 (Fig. 6.3A). The intensity of the 22 kDa protein band decreased in proportion to the decrease in total protein. Interestingly, a less intense, but distinct protein band was seen at approximately 50 kDa in the crude extract and the 1:2 dilution for the YTK 9 testes (Fig. 6.3B, lanes 6 and 7, respectively). This size corresponds to the predicted dimer size for Prx proteins. Importantly, there was no evidence for two different sizes for the monomeric form of Prx 4. However, it is still possible that the resolution of the SDS-PAGE gel was not sufficient to distinguish between two protein forms of very similar size. This hypothesis was tested in Section 6.3.3 below by increasing the acrylamide concentration in the gel to 12% with the aim of better resolving small proteins.

6.3.3 High resolution SDS-PAGE of proteins from a 3.0 kg YTK male

The aim of this section was to determine whether SDS-PAGE at a higher acrylamide concentration would be able to resolve different isoforms of Prx 4 at monomer size in the tissues of a large 3.0 kg YTK male thought to be sexually mature. This was important as the mammalian literature had suggested that sexually mature rats expressed 2 different isoforms of Prx 4 at the monomer size, whereas immature rats did not (Sasagawa et al., 2001) and it

was hypothesized that a similar age-dependent expression might be present in fish. Based on the results presented in the previous chapters, we hypothesised that the anti-(SBT Prx 2) antibodies would be able to detect two protein bands at approximately 25 kDa (the 231 amino acid truncated form of Prx 4) and 22 kDa (the 197 amino acid truncated form of Prx 4) in the liver and testes of YTK 7. We also hypothesised that the anti-(human Prx 4) antibodies would also be able to detect two protein bands in liver at 29 kDa (the 264 amino acid unprocessed form of Prx 4) and 25 kDa (the 231 amino acid truncated form of Prx 4).

The Coomassie Blue stained gel for the 3.0 kg YTK male (YTK 7, Fig. 6.4A) showed a high abundance of protein for all tissues except spleen (lane 6). A high abundance of proteins was observed above approximately 35 kDa in brain (lane 1) along with a low abundance of proteins at 10-35 kDa. The other tissues all showed a large number of equally abundant proteins (Fig. 6.4A) with the exception of skeletal muscle (lane 3), which showed only 7-8 bands of highly abundant proteins.

In Fig. 6.4B, the anti-(SBT Prx 2) antibodies detected two prominent protein bands, one at approximately 22 kDa and the other at approximately 25 kDa. The 22 kDa size corresponded to the predicted size of all mature 2-Cys Prx monomers (Section 3.3.3) and the 25 kDa size corresponded to the predicted size of the 231 amino acid Prx 4 monomer (Section 3.3.3). These strong bands were observed in brain (lane 1), liver (lane 2) and testes (lane 8) and weaker bands were observed in gill (lane 9). A single band was observed at the 22 kDa size in skeletal muscle (lane 3) and heart (lane 4) and a single band at the 25 kDa size was observed in intestine (lane 5). There was reaction in spleen (lane 7) and kidney (lane 8) at

approximately 22 and 25 kDa but the reaction was faint and it was difficult to determine which size band(s) were present in these tissues (lanes 6 and 7, respectively).

The Coomassie Blue stained gel in Fig. 6.5A was the same gel as in Fig. 6.4A. It is included again for comparison.

In Fig. 6.5B, the anti-(human Prx 4) antibodies detected two bands in the liver tissue of YTK 7 at approximately 25 kDa and 29 kDa (lane 2). The 25 kDa band corresponded to the predicted size of the 231 amino acid form of Prx 4 (Section 3.3.3) and the 29 kDa band corresponded to the predicted size of the 264 amino acid form of Prx 4 (Section 3.3.3). The 25 kDa size was also observed in kidney (lane 7) and the 29 kDa band was also observed in intestine (lane 5), testes (lane 8) and gill (lane 9). There was also reaction at approximately Prx 4 dimer size (50 kDa) in liver (lane 2), intestine (lane 5), spleen (lane 6), kidney (lane 7), testes (lane 8) and gill (lane 9). However, most importantly, there was no reaction at 22 kDa (the size of the 197 amino acid truncated form of Prx 4).

6.4 Discussion

6.4.1 Expression of Prx 4 in the ovaries of a 1.5 kg YTK female and a 3.0 kg YTK female

The anti-(human Prx 4) antibodies detected proteins in the ovaries of the 1.5 kg YTK female (Fig. 6.1B) and the 3.0 kg YTK female (Fig. 6.2B). However, when this reaction was compared to their respective Coomassie Blue stained gels (Figs. 6.1A and 6.2A, respectively), the bands that exhibited reaction corresponded in size to the most abundant proteins present on the gels. This result showed a high probability that the reactions observed

were due to non-specific binding of the antibodies to any abundant proteins on the blot and not specifically to Prx proteins. It appeared that the anti-(human Prx 4) antibodies reacted with all proteins on the immunoblot above approximately 25 kDa for both YTK 13 (Fig. 6.1B) and YTK 6 (Fig. 6.2B). The prominent bands at approximately 40-45 kDa seen in Section 5.3.3.1 and 5.3.3.3 were seen in these results but the antibodies again seemed to be reacting with all of the most abundant proteins present on the immunoblot.

6.4.2 Expression of 2-Cys Prx proteins in the testes of two 3.0 kg YTK males

The anti-(SBT Prx 2) antibodies detected clear bands at approximately the expected size of a 22 kDa 2-Cys Prx monomer in both YTK 7 and YTK 9 (Fig. 6.3B). This reaction decreased proportionally to the serial dilutions for both YTK 7 (Fig. 6.3B, lanes 1-4) and YTK 9 (Fig. 6.3B, lanes 5-8), however, the lower concentration of the protein extracts failed to resolve any additional bands at the Prx monomer size which was contrary to what had been predicted based on the mammalian literature. Interestingly, the band at approximately 50 kDa in the YTK 9 undiluted testes extract and the 1:2 dilution of this extract (Fig. 6.3B, lanes 5 and 6, respectively) corresponded to the predicted size of a dimer of the 231 amino acid form of Prx 4.

This suggested that the anti-(SBT Prx 2) antibodies were detecting the 231 amino acid form of Prx 4 in these extracts but this reaction was not observed at the monomer size. Since, to the best of our knowledge, expression or characterisation of Prx proteins in fish testes has not been conducted in the past, the best literature to compare this result to would be similar studies in mammals. Sasagawa et al. (2001) and Iuchi et al. (2009) found that in mice, Prx 4 expression was higher in testes than other tissues and the testes also contained a second,

larger form of Prx 4 at the monomer size, which was not present in other tissues. The results presented here indicate that only one form of Prx 4 was present at monomeric size in YTK. However, Sasagawa et al. (2001) also found that the second form of Prx 4 could not be detected in testes until the mice reached sexual maturity. Thus, our inability to detect two different sizes of the monomer form of Prx 4 could be due to either (a) the fish were not old enough to be expressing the 25 kDa form of Prx 4 in the testes, or (b) the resolution of the gel was not sufficient to distinguish between these two bands. Due to the high value of YTK and difficulties accessing individuals larger than 3.0 kg, hypothesis (a) could not be tested. The experiments described in Section 6.3.3 tested hypothesis (b).

6.4.3 High resolution SDS-PAGE of proteins from a 3.0 kg YTK male

The anti-(SBT Prx 2) antibodies detected two bands on the immunoblot (Fig. 6.4B) at 22 kDa and 25 kDa in the brain (lane 1), liver (lane 2), testes (lane 8) and gill (lane 9). Due to the high level of amino acid sequence conservation between SBT Prx 2 and other fish Prx proteins (Section 3.3.3), it is likely that these antibodies are detecting multiple Prx protein subclasses in the YTK tissues including the Prx 4 subclass. The estimated sizes for Prx 1, Prx 2, the truncated form of Prx 3 missing its putative mitochondrial targeting sequence and the 197 amino acid form of Prx 4 are all approximately 22 kDa (Section 3.3.3). Interestingly only Prx 3 and Prx 4 have forms larger than 22 kDa, and the predicted size of the 231 amino acid truncated form of Prx 4 corresponds closely to the 25 kDa bands seen on the immunoblot (Fig. 6.5B). Skeletal muscle and heart both showed a single band at approximately 22 kDa and, interestingly, intestine showed a band at approximately 25 kDa, but not at 22 kDa.

The detection of a 25 kDa form of a 2-Cys Prx protein was confirmed by the reaction with the anti-(human Prx 4) antibodies which detected two bands at approximately 29 kDa and 25 kDa in YTK 7 liver (Fig. 6.5B, lane 2). These sizes correspond approximately to the predicted sizes of the 264 and 231 amino acid forms of YTK Prx 4, respectively (Section 3.3.3). There was also reaction at approximately 25 kDa in kidney (Fig. 6.5B lane 7) and at 29 kDa in intestine (lane 5), testes (lane 8) and gill (lane 9). There was no reaction at the predicted size for the 197 amino acid form (22 kDa) of Prx 4 and this may be because the region corresponding to the peptide used to raise the anti-(human Prx 4) antibodies overlaps the putative cleavage site for the 197 amino acid truncated form of Prx 4 (Section 3.3.3). This suggests that, as expected, the sequence conservation at the C-terminal end of the region corresponding to the antigenic peptide used to generate the anti-(human Prx 4) antibodies was not sufficient for the anti-(human Prx 4) antibodies to detect the 197 amino acid form of YTK Prx 4.

This is the first time that a larger form of the Prx 4 monomer has been reported in the testes of YTK or any fish. Thus, it appears as if fish have similarities with mammals in this regard (Sasagawa et al., 2001; Iuchi et al., 2009). Furthermore, the expression of three distinct forms of Prx 4 described here in the brain, intestine and gill has not been previously described for YTK. This novel discovery of multiple forms of Prx 4 in various tissues of YTK clearly warrants further investigation.

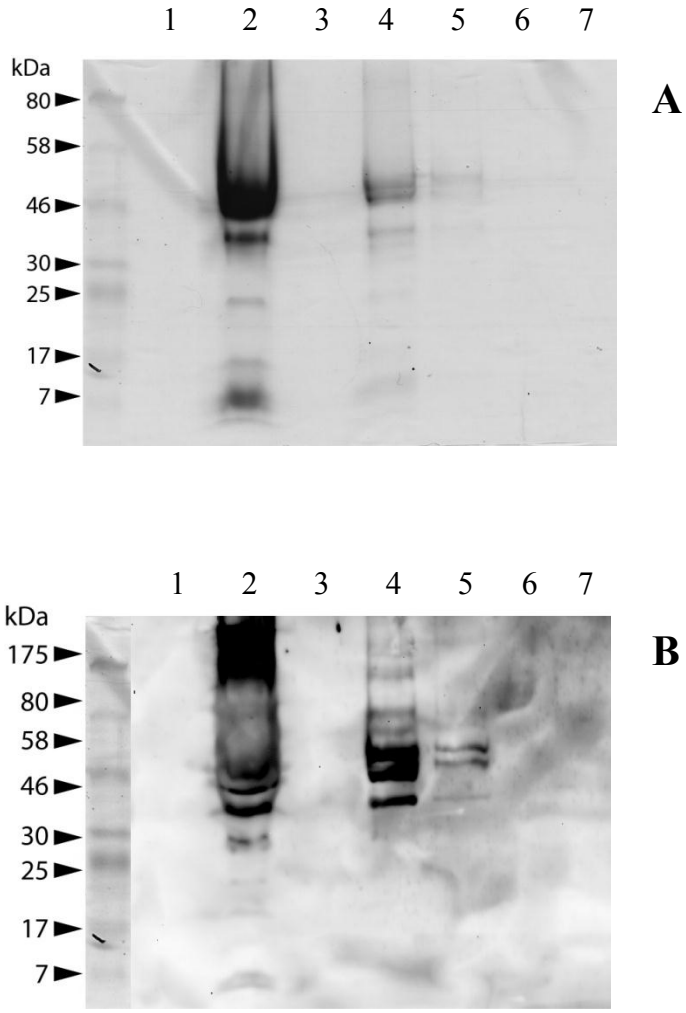


Figure 6.1: Expression of Prx 4 in the ovaries of a 1.5 kg YTK female (YTK 13). The lanes contained 1 – Blank; 2 – undiluted extract; 3 – Blank; 4 – 1:10 dilution); 5 – 1:100 dilution; 6 – 1:1,000 dilution; 7 – 1:10,000 dilution.

(A) SDS-PAGE conducted on a 10% (w/v) acrylamide gel. The ovaries were extracted in Extraction Buffer F (50 mM HEPES (pH 7.5), 150 mM NaCl, 50 mM DTT, 1 mM PMSF, 1 mM EDTA, 1% (w/v) SDS and 5 M urea) as described in Section 2.3.2 The gel was loaded based on protein obtained from a given amount of tissue (Section 4.2.6). Lane 2 – 2.5 mg tissue; lane 4 – 2.5 x 10^{-1} mg tissue; lane 5 – 2.5 x 10^{-2} mg tissue; lane 6 – 2.5 x 10^{-3} mg tissue; lane 7 – 2.5 x 10^{-4} mg tissue. .

(B) Immunoblot analysis of a parallel gel to panel A using a 1:1000 dilution of anti-(human Prx 4) antibodies.

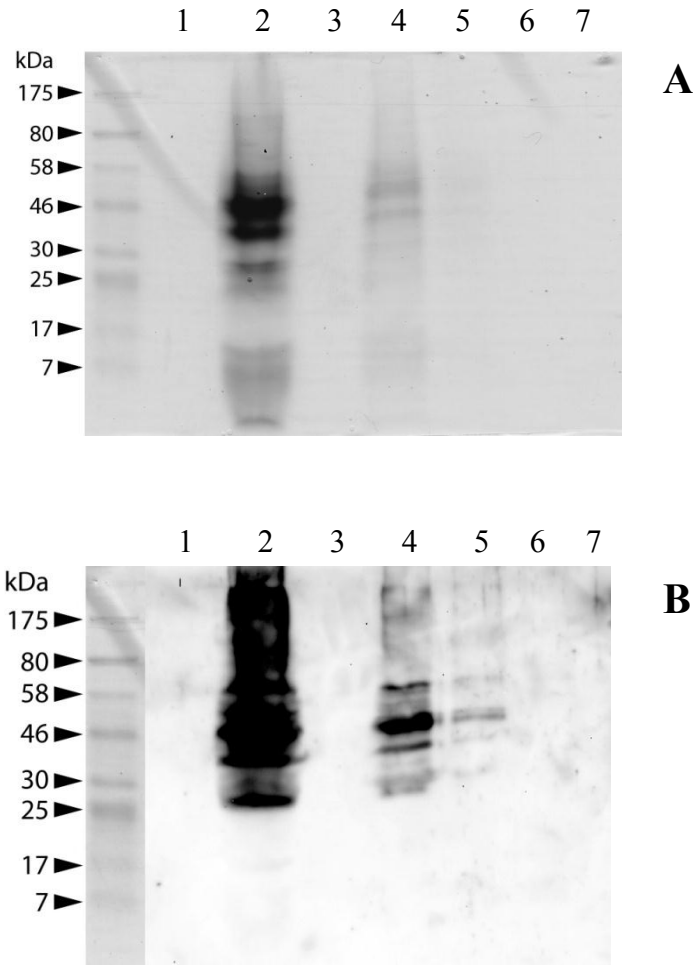


Figure 6.2: Expression of Prx 4 in the ovaries of a 3 kg YTK female (YTK 6). The lanes contained 1 – Blank; 2 – undiluted extract; 3 – Blank; 4 – 1:10 dilution; 5 – 1:100 dilution; 6 – 1:1,000 dilution; 7 – 1:10,000 dilution.

(A) SDS-PAGE conducted on a 10% (w/v) acrylamide gel. The ovaries were extracted in Extraction Buffer F (50 mM HEPES (pH 7.5), 150 mM NaCl, 50 mM DTT, 1 mM PMSF, 1 mM EDTA, 1% (w/v) SDS and 5 M urea) as described in Section 2.3.2. The gel was loaded based on protein obtained from a given amount of tissue (Section 4.2.6). Lane 2 – 2.5 mg tissue; lane 4 – 2.5 x 10^{-1} mg tissue; lane 5 – 2.5 x 10^{-2} mg tissue; lane 6 – 2.5 x 10^{-3} mg tissue; lane 7 – 2.5 x 10^{-4} mg tissue. .

(B) Immunoblot analysis of a parallel gel to panel A using a 1:1000 dilution of anti-(human Prx 4) antibodies.

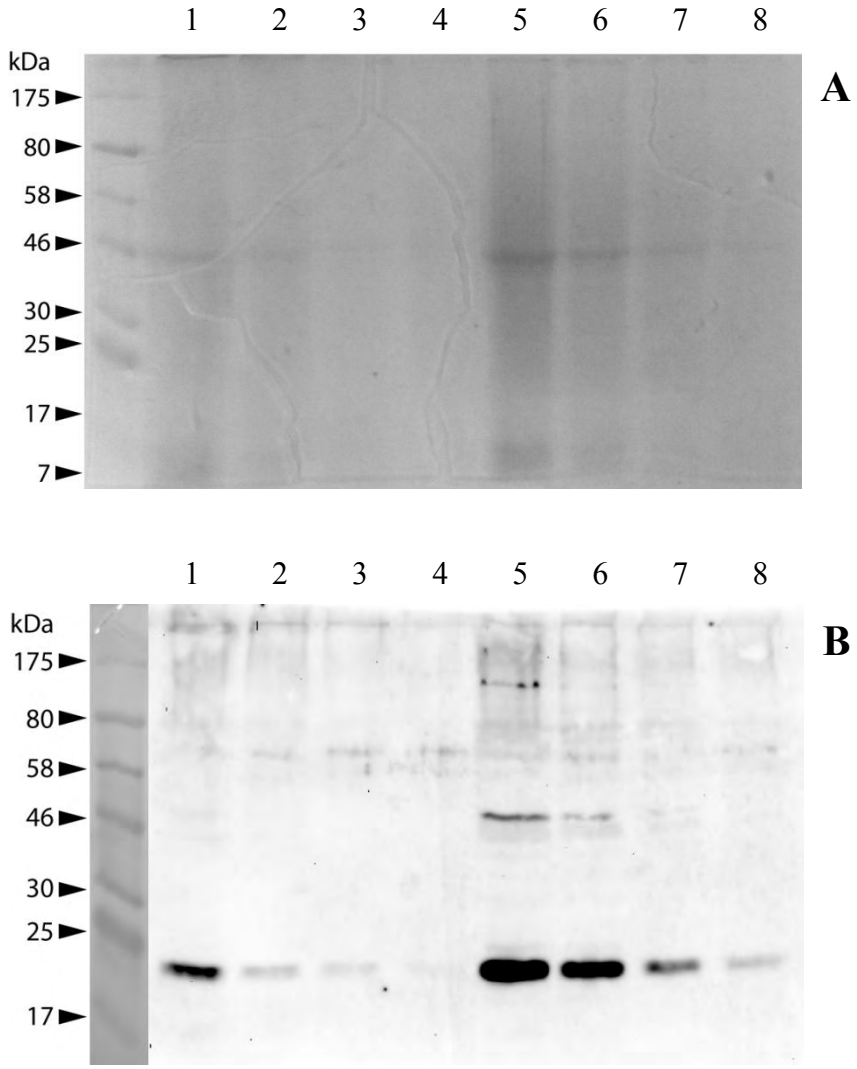


Figure 6.3: Expression of Prx proteins in various dilutions of testes extracts from two 3 kg YTK males (YTK 7 and YTK 9). The lanes contained 1 – YTK 7 undiluted extract; 2 – YTK 7 1:2 dilution; 3 – YTK 7 1:4 dilution; 4 – YTK 7 1:8 dilution; 5 – YTK 9 undiluted; 6 – YTK 9 1:2 dilution; 7 – YTK 9 1:4 dilution; 8 – YTK 9 1:8 dilution.

(C) SDS-PAGE conducted on a 10% (w/v) acrylamide gel. The tissue was extracted in Extraction Buffer F (50 mM HEPES (pH 7.5), 150 mM NaCl, 50 mM DTT, 1 mM PMSF, 1 mM EDTA, 1% (w/v) SDS and 5 M urea) as described in Section 2.3.2. The gel was loaded based on protein obtained from a given amount of tissue (Section 4.2.6). Lane 1 – 2 mg tissue; lane 2 – 1 mg tissue; lane 3 – 0.5 mg tissue; lane 4 – 0.25 mg tissue; lane 5 – 2 mg tissue; lane 6 – 1 mg tissue; lane 7 – 0.5 mg tissue; lane 8 – 0.25 mg tissue. .

(D) Immunoblot analysis of a parallel gel to panel A using a 1:1000 dilution of anti-(SBT-Prx 2) antibodies.

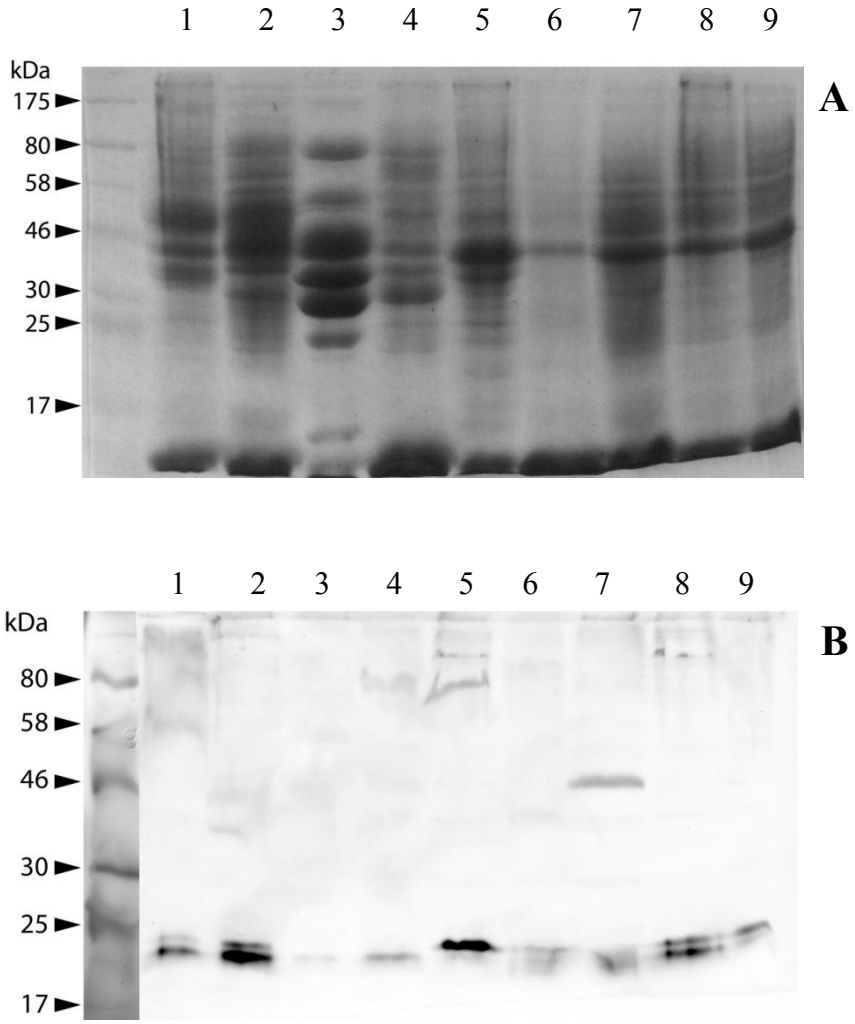


Figure 6.4: Expression of Prx proteins in the tissues of a 3 kg YTK male (YTK 7). The lanes contained 1 – Brain; 2 – Liver; 3 – Muscle; 4 – Heart; 5 – Intestine; 6 – Spleen; 7 – Kidney; 8 – Testes; 9 – Gill

(A) SDS-PAGE conducted on a 12% (w/v) acrylamide gel with the indicated tissues of YTK 7 extracted into Extraction Buffer B (50 mM K-phosphate, 5 mM DTT, 6 mM GSH, 2 mM PMSF and 1 mM EDTA) as described in Section 2.3.2. Total protein was estimated using the method of Bradford (1976) and 250 µg protein was loaded in each lane of the gel. .

(B) Immunoblot analysis of a parallel gel to panel A using a 1:1000 dilution of anti-(SBT-Prx 2) antibodies.

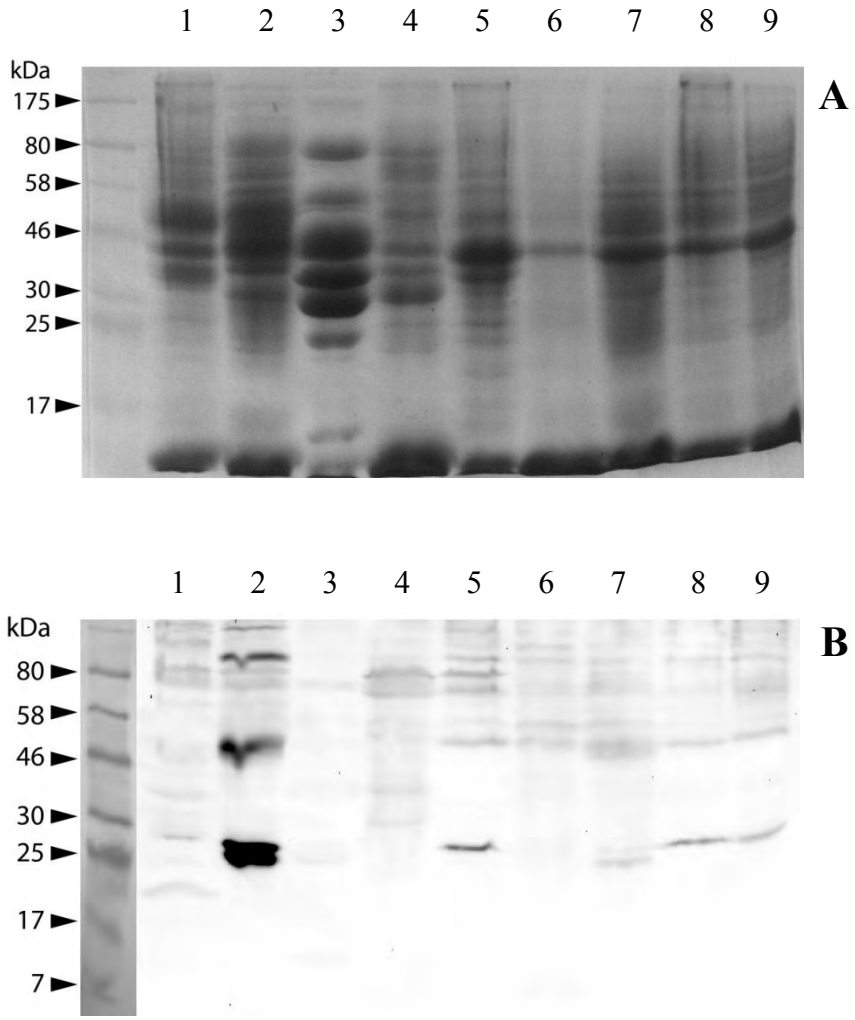


Figure 6.5: Expression of Prx 4 in various tissues of a 3 kg YTK male (YTK 7). The lanes contained 1 – Brain; 2 – Liver; 3 – Muscle; 4 – Heart; 5 – Intestine; 6 – Spleen; 7 – Kidney; 8 – Testes; 9 – Gill

(A) SDS-PAGE conducted on a 12% (w/v) acrylamide gel with the indicated tissues of YTK 7 extracted into Extraction Buffer B (50 mM K-phosphate, 5 mM DTT, 6 mM GSH, 2 mM PMSF and 1 mM EDTA) as described in Section 2.3.2. Total protein was estimated using the method of Bradford (1976) and 250 µg protein was loaded in each lane of the gel. .

(B) Immunoblot analysis of a parallel gel to panel A using a 1:1000 dilution of anti-(human-Prx 4) antibodies.

Chapter 7 – Conclusions and Future Work

7.1 Conclusions

This study investigated the tissue distribution of the expression of selected Prx and GPx protein family members in YTK, an important fish aquaculture species in South Australia. In Chapter 3 it was shown that the individual members of these protein families are highly conserved between mammals and fish. In Chapter 4 it was shown that (a) anti-(SBT Prx 2) antibodies detected multiple Prx proteins in brain, liver, muscle, heart, intestine, spleen and ovaries of YTK (b) anti-(human Prx 4) antibodies detected a protein with a size equal to that expected for Prx 4 in YTK liver and intestine and (c) anti-(human GPx 4) antibodies cross-reacted with a protein with a size equal to that expected for GPx 4 in YTK liver but showed a large amount of non-specific reaction as well. In Chapter 5, 2-Cys Prx proteins were found to be almost ubiquitously expressed in YTK. Prx 4 was found to be highly abundant in liver, and was also detected in intestine. GPx 4 expression was only found consistently in liver. In Chapter 6 it was shown that (a) anti-(SBT Prx 2) antibodies detected two monomeric Prx proteins at 22 kDa and 25 kDa and (b) anti-(human Prx 4) antibodies detected two monomeric Prx 4 proteins at 25 kDa and 29 kDa in liver and the latter size was also detected in intestine, testes and gill. The analyses in Chapter 3 predicted that the anti-(SBT Prx 2) antibodies would detect all four sub-classes of 2-Cys Prx proteins from fish due to the high degree of conservation between all 2-Cys Prx proteins. However, the results in Chapter 5 suggested that they were not detecting the 264 amino acid unprocessed form of Prx 4 in YTK. This lack of reaction could not be explained and further experiments are needed to resolve this issue in the future.

7.1 Future directions

In Chapter 5, almost ubiquitous expression of Prx proteins was found in the organs of YTK and Prx 4 and GPx 4 were found to be most abundant in liver. The detection of three isoforms of monomeric Prx 4 in Chapter 6 was a very significant result of this study and the presence of these forms has not been previously described in fish. This finding warrants further investigation into the function of the different forms of Prx 4 in YTK and other fish. For example, immunoblotting experiments could be performed using a mixture of anti-(SBT Prx 2) and anti-(human Prx 4) primary antibodies on the same immunoblot to verify that three forms of Prx 4 are indeed expressed in YTK tissues. In addition, since we are not sure whether or not the YTK used were sexually mature, the expression analyses could be conducted again using samples from very large YTK that are certainly sexually mature. This would allow us to determine with certainty whether expression of Prx or GPx proteins increases as the fish mature sexually.

Another interesting direction would be to determine the sub-cellular localisation of the different forms of Prx 4 in fish. This could be done using immunohistochemistry with the various tissues of YTK that were collected as part of this study. Determining the sub-cellular localisation would provide additional information regarding the possible roles of Prx and GPx proteins in YTK and could assist in formulating hypotheses about the functions of the different isoforms of YTK Prx 4 that have been discovered in this study.

In conclusion, this study has made a significant contribution to our understanding of the expression of Prx and GPx antioxidant proteins in a commercially important aquaculture

species and it will provide a strong basis for future research into the roles of these important proteins in fertility in fish and other vertebrates.

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