The Chemical Characterization and Biological Evaluation of Selenium-enriched
*Agaricus bisporus* Mushroom Organic Selenium Species

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Abstract

The research study was divided into two main objectives, the chemical characterization and speciation of organic selenium species in cultivated selenium-enriched *Agaricus bisporus* mushroom and the biological evaluation of the organic Se species using a rat model. The biological evaluation of the mushroom organic Se species determined the effect of dietary supplementation with mushroom Se on the colonic antioxidant selenoprotein status markers cytosolic glutathione peroxidase-1 (GPx-1), gastrointestinal tract glutathione peroxidase-2 (GPx-2), thioredoxin reductase-1 (TrxR-1) and selenoprotein P (SeP). Furthermore, dietary effects of the mushroom organic Se supplementation on gut physiological function, (ileal) mucosal permeability and antioxidant selenoenzymes status during heat induced oxidative stress in rats were evaluated. We also evaluated the effects of dietary Se supplementation with the mushroom Se and with or without additional α-tocopherol on the regulation of the expression of colonic selenoproteins GPx-1, GPx-2, TrxR-1 and SeP in hyperthermally induced oxidative stress in rat. Selenocystine (SeCys), selenomethionine (SeMet), and methyl-selenocysteine (MeSeCys) were separated, identified and quantified by liquid chromatography–electrospray ionisation-mass spectrometry from water solubilised and acetone precipitated proteins. Although all three selenoamino acids were detected for both the control and selenium-enriched mushrooms, significant increases of the selenoamino acids were observed for the selenium-enriched mushrooms. The most notable result was the much higher levels of SeCys accumulated by *A. bisporus* compared to SeMet and MeSeCys, for both control and selenium-enriched *A. bisporus*. The maximum selenoamino acids concentration recorded in caps and stalks of control/selenised mushrooms was 4.16/9.65µg/g dried weight (DW) for SeCys, 0.08/0.58µg/g DW for SeMet, and 0.031/0.10µg/g DW for MeSeCys, respectively. The protein bound selenocysteine rich mushroom Se fed to the rats was observed to up-regulate all colonic selenoproteins under study. Rats maintained under thermoneutral conditions and fed the mushroom Se in dietary supplementation at a 1µg Se/g feed showed a significant (P<0.05) fold increase in mRNA expression relative to the basal treatment (rats fed the control diet) of GPx-1 (2.98±0.82), GPx-2 (1.8±1.4), TrxR-1 (1.83±0.64) and SeP (2.77±0.92). However, supplementation with α-tocopherol alone under thermoneutral conditions only affected the expression of GPx-1 (1.83±0.82) and SeP (2.21±0.92) and no effect was observed with GPx-2 and TrxR-1. Rats exposed to heat stress and placed on the low Se control diet also showed a significant (P<0.05) fold increase in mRNA expression relative to the basal treatment for all colonic selenoproteins at GPx-1 (3.15±0.82), GPx-2 (1.6±1.4), TrxR-1 (1.55±0.64) and SeP (1.93±0.92). However, heat stressed rats on the mushroom Se diet displayed a much higher mRNA expression of GPx-2 (3.2±1.4) and SeP (4.23±0.92). Heat stressed rats placed on the α-tocopherol diet alone displayed the highest
expression level of selenoproteins relative to all diets with TrxR-1 at 4.10±0.64 and SeP at 7.44±0.92 fold increase. In correlation with mRNA expression, GPx-1 activity was observed to increase in rats placed on the mushroom Se diet and maintained under thermoneutral conditions, however in contrast with mRNA expression, GPx-1 activity was not up-regulated by α-tocopherol alone. Heat stress was observed to have a negative impact on GPx-1 activity which was recovered by mushroom Se, but not by α-tocopherol, in the diet. Although supplementation with α-tocopherol to the mushroom Se diet also displayed an increase in GPx-1 activity under thermoneutral conditions and also restored GPx-1 activity during heat stress, α-tocopherol did not enhance the Se effect. Furthermore, dietary supplementation with Se-enriched A. bisporus mushroom (1 µg Se/g feed) reduced (P < 0.05) gut (ileum) permeability markers baseline short-circuit current (Isc) and epithelium conductance (Ge) during heat stress to 1.74 and 1.91 fold, respectively, indicating protection from heat stress-induced mucosal permeability increase and restoration of gut barrier function. Thus, Se-enriched A. bisporus mushroom presented an efficient source of functional organic Se with demonstrated beneficial biological effects of up-regulating gut antioxidant selenoproteins linked to anti-cancer and anti-inflammatory mechanisms and disease prevention and protection against heat induced oxidative stress and gut dysfunction and injury.
This is to certify that:

i. the thesis comprises only my original work towards the PhD except where indicated in the Preface,

ii. due acknowledgement has been made in the text to all other material used,

iii. the thesis is fewer than 100 000 words in length, exclusive of tables, maps, bibliographies and appendices OR the thesis is [number of words] as approved by the Research Higher Degrees Committee.
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The thesis includes three multi-author published papers and one manuscript under review, where as the PhD candidate I am first author for all papers with a contribution of 60% towards the published work for each paper. Co-author forms indicating the multi-authors and contributions are attached. The multi-author published papers and the manuscript under review are presented in the thesis and the author contributions are as follows;

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The chemical structures of the seleno amino acids were drawn using the ChemDraw Pro 13 software.
CHAPTER 1

Literature Review

1.0 Introduction

1.1.0 What is Selenium (Se)?

Chemical and physical properties

Selenium is a chemical element that belongs to Group 16 of the periodic table of elements and is represented on the table by the chemical symbol Se. It has an atomic number of 34 and an atomic mass of 78.96g.mol\(^{-1}\). Selenium is a chalcogen and a non-metal that lies beneath sulphur in group 16 of the periodic table, and has its chemical properties and reactivity similar to those of sulphur. It exists in many allotropes able to interconvert when heated and cooled at different temperatures and rates (House 2008). Selenium usually occurs as amorphous brick red powder. It converts to a black vitreous form usually sold industrially as beads, when rapidly melted (House 2008). Black selenium is irregular and complex consisting of polymeric rings with up to 1000 atoms per ring and is a brittle, lustrous solid slightly soluble in carbon disulphide. When heated, black Se softens at 50°C and converts to gray Se at 180°C (Greenwood & Earnshaw 1997). Selenium has six naturally occurring isotopes, five of which are stable in states \(^{74}\text{Se},~^{76}\text{Se},~^{77}\text{Se},~^{78}\text{Se}\) and \(^{80}\text{Se}\) and relatively unstable \(^{79}\text{Se}\) with a half-life of 327 000 years (Jörg et al. 2010).

Selenium rarely occurs in its elemental state in nature or as a pure ore compounds (Kabata-Pendias 1998). The element naturally occurs in the environment, especially soil and water as selenium compounds commonly existing in oxidations states -2, +2, +4 and +6 (Fordyce 2007). It is a relatively least-reactive element, stable in air, water and most acids but reactive to alkaline compounds. However, elemental Se is able to react with oxygen in some cases to form different oxides, selenium dioxide (SeO\(_2\)) and selenium trioxide (SeO\(_3\)) with selenium dioxide being the most stable (House 2008). As pointed out earlier in this section, native elemental Se is a rare mineral and its isolation is often complicated by the presence of other elements and compounds. In nature, selenium occurs in a number of inorganic forms including the major ones selenide (Se\(^2^-\)), selenate (SeO\(_4^{2-}\)) and selenite (SeO\(_3^{2-}\)) containing minerals, however the minerals are rare. Selenium in nature mostly occurs in its water soluble form selenate, found in soils allowing the bio-
concentration of selenium by certain plants (Fordyce 2007). The selenate form analogous to sulphate (SO\textsubscript{4}\textsuperscript{2-}) is readily leached into rivers and lakes by runoff water (Kabata-Pendias 1998, Fordyce 2007). Consequently, sea water contains significant amounts of selenium (Amouroux et al. 2001, Haug et al. 2007). Selenium in nature mostly occurs in an impure form, replacing a small part of the sulphur and sulphide ores of several metals (Kabata-Pendias 1998). Further, in living systems, selenium is found in organic seleno-amino acids selenomethionine (SeMet), selenocysteine (Sec) and methylselenocysteine (MSeCys) (a metabolite of selenocysteine) where Se replaces sulphur in side chains of amino acids methionine and cysteine. Selenium plays a role analogous to that of sulphur in the selenoamino acids (Wessjohann et al. 2007). Dimethyl selenide is another organic selenium compound (Wessjohann et al. 2007).

1.1.1 The Essential Trace Element and Micronutrient: Selenium

Selenium is an essential trace element and a micronutrient required at minute amounts for various metabolic selenium dependent enzymes and physiological functions in human and animal systems (Rayman 2000). Selenium is also known to be of fundamental importance to human health and prevention of disease (Clark et al. 1996, Rayman 2000, Flores-Mateo et al. 2006). Selenium can be acquired from the diet as it occurs naturally in foods such as nuts, eggs, fish, liver, chicken, red meat, garlic and some species of mushrooms. In Australia and New Zealand, the major dietary sources of selenium are poultry, eggs, seafood and, to a lesser extent, other muscle meats (NHMRC 2006). Cereal products constitute another Se contributor, although levels depend on the source (NHMRC 2006). Although Se can occur naturally in certain foods, especially vegetables, there are regions in the world with a low Se content in the soil. The Se content of foods therefore varies with geographic sources of the food. Reports reveal that soil Se concentrations can range from <0.01 µg/g to >1,000 µg/g with plant food sources content reflecting the range (NHMRC 2006). However, variability of selenium content is not so marked in animal food products (NHMRC 2006). Low levels of Se in New Zealand soils mean that selenium dietary intakes and status in the population are lower than several other countries (Thomson 2004). In Australia, there are regions with Se deficient soils, such as those along the coasts of Queensland, New South Wales, South Australia, Victoria and Western Australia (Whanger 2004) and several primary products originating from the regions have relatively minute levels of Se (Reilly 1992, Combs 2001). For regions with low Se levels in soils, it is recommended to monitor Se levels in animals and plants and where necessary supplementation must be considered to improve the low Se status (Misurova et al. 2009). Selenium deficiencies in Australia have been largely dealt with by intervention programs such as supplementation to farm animals (Tinggi 2003, Heard et al. 2007).
Selenium supplementation can be administered in organic forms and inorganic forms (e.g., sodium selenite and sodium selenate) used in human and animal nutrition (Schrauzer 2001, Bosse et al. 2010). They serve as precursors for the biosynthesis of the Se dependent enzymes and bioactive Se compounds (discussed later in text in section 1.2.1) needed to drive essential biological processes in the body (Bosse et al. 2010). Sodium selenite and sodium selenate are predominantly used as Se sources in multivitamin preparations, infant formulas, weight-loss products, protein mixes and animal feed (Schrauzer 2001). However in recent years, the use of organic Se forms such as selenomethionine and selenised yeast (contains Se predominantly as SeMet), Se chelates (with Se as part of a chelate complex with proteins or amino acids) and methylselenocysteine have been adopted as supplements (Misurova et al. 2009). Although Se supplementation has been administered in inorganic forms selenite and selenate in earlier years, organic forms are preferable as they possess a higher bioavailability, are easily absorbed and effective at raising blood Se levels and easily incorporate Se into tissues (Mahan & Kim 1996, Schrauzer 2000, Ravn-Haren et al. 2008). Organic forms of Se also have a lower toxicity effect than inorganic species (Mahan & Kim 1996). The different chemical forms of Se are discussed in more detail in the later text in section 1.2.1.

Selenium is a micronutrient that is an essential component of the diet as already pointed out. It is imperative that it is consumed at adequate levels to drive biological processes and at correct levels to avoid adverse effects as there is a narrow range between its toxicity level and beneficial level (WHO 1996). Levels falling below the daily recommended intake can lead to accelerated aging, a less robust immune system, increased risk of cardiovascular ailments and other degenerative diseases (WHO 1996, Rayman 2000). Furthermore, Se deficiency has been linked with reduced response to influenza infections (Jaspers et al. 2007), osteoporosis (Ebert & Jakob 2007), epilepsy (Ashrafi et al. 2007), leukemia and other cancers (Ip 1998, Peters et al. 2007) and progression of HIV to AIDS (Campa et al. 2000). Combs (2001) and Whanger (2004) report deficient selenium intake as falling below 40µg Se per person per day, whereas Ebert and Jakob (2007) state symptoms of Se deficiency occur at intakes below 20µg per day. Adult recommended daily intake of Se is 60 µg and 70 µg per day for females and males respectively as reported by the Australian National Health and Medical Research Council (NHMRC 2006). At the nutritional recommended levels, Se is required for healthy brain and bone development, thermoregulation, normal growth, production of thyroid hormone triiodothyronine (Ip 1998) and proper immune system functioning (WHO 1996, Rayman 2000). Intakes of Se above recommended nutritional intake, supra-nutritional, are linked to reduced risk for various types of cancers (Clark et al. 1996, Rayman 2000, Naithani 2008).
However intake of Se above the tolerable upper intake level (UL) of 400 µg per day (USFDA 2002, IOM 2009) leads to gastrointestinal disturbances, skin rashes, fatigue, nervous system abnormalities, hair and nail brittleness and loss, garlic breath and irritability (DRI 2000). Ebert and Jakob (2007) report that symptoms of selenium toxicity show at levels more than 800 µg per day in adults. Chronic Se toxicity occurs at levels of 3200 to 5000 µg per day triggering selenosis, a state of chronic poisoning caused by acutely high levels of Se and in some cases leading to liver failure and having lethal consequences (Combs 2001, Whanger 2004, Kaur et al. 2005,).

1.1.2 The Discovery of Selenium as a ‘Super’ Element

For many years, selenium in trace amounts has been known to be an undesirable and a toxic element to animals. Selenium first attracted attention in the early 1930s as a toxic element that caused ‘alkali disease’ at minute levels in livestock that consumed high-selenium plants (Thomson 2003). The element was known for its poisoning and adverse effects on animals and the ecosystem as a whole (Ge et al. 1983). Environments near metal industries, waste treatment and selenium recovery plants were more prone to Se contamination as high levels of the element ended up in soil, water sources and assimilated by vegetation (Lemly 1997). The element got introduced into the animal food chain and its accumulation caused various ailments in animals (Lemly 1997).

Lemly (1997) reports that wastewater discharged from a coal-fired electric generating facility contaminated a neighbouring water source (Belews Lake) with selenium having catastrophic effects on the ecosystem. The study observed significant selenium bio-accumulations in aquatic food chains causing severe reproductive failure and teratogenic deformities in aquatic animals, mostly fish and aquatic birds (Lemly 1997). However, when the coal-fired electric facility changed its discharge practices of the selenium contaminated wastewater such that it no longer entered the water source, findings after 10 years revealed waterborne selenium levels dropped from a peak of 20 µg/L to 1 µg/L and Se concentrations in biota showed a 85-95% decrease (Lemly 1997). Field selenium hazard assessments were done to determine whether ecosystem-level hazards to fish and aquatic birds had changed as well and it was observed that hazard ratings dropped from high hazard to moderate hazard primarily due to selenium in the sediment-detrital food pathway (Lemly 1997). Selenium levels in sediments dropped by 65-75% but remained elevated enough (1-4 µg/g) to contaminate benthic food organisms of fish and aquatic birds (Lemly 1997). Field findings confirmed the validity of the hazard ratings as developmental anomalies in young fish indicated that selenium induced teratogenesis and reproductive impairment were still occurring. Selenium levels in benthic food organisms were sufficient to cause mortality in young blue-gill and other
centrarchid fish due to winter stress syndrome (Lemly 1997). Recovery was slow at ecosystem level and toxic effects of Se were still evident 10 years after Se contaminated wastewater discharges into water sources had stopped (Lemly 1997).

**Keshan disease and selenium**

In early 1980 Se was discovered as an essential component to life at micro levels as observed by the correlation of its deficiency with some diseases (Chen et al. 1980, Ge et al. 1983). More than 100 years ago, a condition known as Keshan disease was first discovered and described in Chinese medical literature (Ge & Yang 1993). However, it was not until 1935, that it was discovered that the health condition was associated with Se status, Se deficiency (Ge et al. 1983, Ge & Yang 1993). Keshan disease was named after a county in China correlating to the low selenium levels characterizing the soils found in the region (Chen et al. 1980). Keshan disease, an endemic cardiomyopathy, with the main pathological sign being multiple focal myocardial necrosis distributed throughout the heart muscle and with varying levels of cell infiltration and different stages of fibrosis (Chen et al. 1980). The condition was common in rural areas of China found in a long belt region where selenium levels in soils and foods were low (Ge et al. 1983). The levels of Se in the food produce of the disease belt were observed to be 0.025 µg/g whereas the disease free region recorded Se levels of 0.040 µg/g (Chen et al. 1980).

The cause of Keshan disease following its discovery was initially not known, with the only observation that the condition was prevalent in children under the age of 15 years and women of child bearing age (Chen et al. 1980). Studies of young children aged 1 to 9 years old revealed that Keshan disease was a selenium responsive condition (Chen et al. 1980). It was observed that incidence rates of 9.5 to 13.5/1000 in 1974 to 1975 were reduced to 1 to 2/1000 in children treated with weekly doses of 0.5-1mg sodium selenite (Chen et al. 1980). Reports revealed that only 21 cases of Keshan disease occurred in 36 603 treated children compared with 106 cases in 9430 untreated children of whom 53 died and 5 had insufficient heart function (Chen et al. 1980). The occurrence and prevalence of the condition was invariably linked with lower Se contents of cereals and of hair, less than 0.12 µg/g in residents from the affected in comparison with non-affected regions (Chen et al. 1980). The studies made conclusions that the dose relationship between Se and regional characteristics of Keshan condition suggested that the ailment was a biogeochemical condition (Chen et al. 1980).
In the last 20 years, animal models and human clinical trials have illustrated that trace amounts of Se above the recommended nutritional intake in the diet can reduce incidence and mortality of several types of cancers, including lung, colorectal and prostate (Clark et al. 1996, Finley et al. 2000, Naithani 2008). However, much is still unknown about the precise anti-cancer mechanism of Se although animals serve as good models in understanding the human selenium metabolism and physiology. Selenium has however been reported to exert its bioactive and anti-carcinogenic effects through the action of selenoenzymes and selenoproteins in reducing oxidative stress in the body (Mork et al. 1998, Gromadzinska et al. 2008, Pagmantidis et al. 2008,). Organic Se is an essential structural component of the selenoenzymes and selenoproteins for their activity as antioxidant and detoxifying agents in the body (Ip 1998, Peters et al. 2007, Gromadzinska et al. 2008). The function of Se as part of the endogenous antioxidant system, apart from its anti-cancer action is also linked to many other cardiovascular ailments and conditions such as coronary heart disease, atherosclerosis, high blood pressure, and aging (Flores-Mateo et al. 2006, Nawrot et al. 2007).

1.1.3 Food Safety Organizations and Legislative Bodies: Selenium

Selenium has been controversial and a challenge in its use as a supplement because it has a narrow range between toxicity level and beneficial level thus requiring very close monitoring of the total concentration and chemical forms found in the source. The recommended daily allowance for Se differs between different food regulatory agencies.

Food and Drug Administration (FDA)

The US Food and Drug Administration (FDA) approved the recommended dietary allowance (RDA) for Se to be 55 µg per day for adults, level required for normal physiological functions in the body. Although reports reveal higher Se amounts beyond the nutritional requirement levels are required to reduce incidence of cancer (Clark et al. 1996, Finley et al. 2000, Naithani 2008), FDA cautions the daily intake of Se should not exceed 400 µg as the tolerable upper intake level.

Institute of Medicine (IOM)

The US Institute of Medicine (2009) has also recommended 55 µg as the highest amount of daily intake of selenium for adults. Furthermore, the IOM recognized 900 µg per day as the lowest-observed-adverse-effect level (LOAEL) of selenium intake and 800 µg per day as the no-observed-adverse-effect level (NOAEL) of selenium intake (IOM 2009). The Institute of Medicine (2009)
characterized the adverse health effects of Se observed at the LOAEL (900 µg per day) as non-severe but most likely not easily reversible and therefore justifying an uncertainty factor of 2. The NOAEL (800 µg per day) was then divided by the uncertainty factor of 2 and IOM (2009) made a conclusion that 400µg per day is the tolerable upper limit (UL) of selenium from food and supplements likely to pose no risk of adverse health effects such as selenosis (state of chronic poisoning from acutely high levels of Se), in almost all people of adult age.

National Health and Medical Research Council (NHMRC)

The Australian National Health and Medical Research Council (NHMRC) consider gender and life stage for defining the Se intake recommendations. It recommends selenium daily intake of 60 µg and 70 µg per day for adult females and males respectively in the 19 to 50 years age bracket (NHMRC 2006). The recommended Se level for pregnant women is slightly higher at 65 µg per day as estimates from New Zealand, Germany and Poland studies showed additional requirements for foetal needs and development to be 1-2 µg per day (Casey et al. 1982, FAO: WHO 2001, Zachara 2001). Lactating mothers require a much higher Se level compared to their non-lactating counterparts as NHMRC (2006) recommends their daily Se intake be 75 µg. A level including an allowance of 12 µg per day for Se secreted in the breast milk which adds to the mother’s Se requirement.

On the other hand, children have a much lower recommended daily intake of Se and adolescents’ intake being gender determined. Children in the age bracket of 1 to 3 years and 4 to 8 years have RDA levels of 25 µg and 30 µg respectively for both genders (NHMRC 2006). Adolescents aged 9 to 13 years have an RDA of 50 µg for both genders. Whereas adolescents aged 14 to 18 years have RDAs of 70 µg and 60 µg for boys and girls respectively (NHMRC 2006). New born babies to infants of 6 months have an allowed intake of 12 µg per day and 15 µg daily is recommended for infants 7 to 12 months (NHMRC 2006).

World Health Organization (WHO)

The World Health Organization (WHO) in contrast with FDA, NHMRC and IOM has recommended a much lower level of Se daily intake. The joint efforts between WHO and Food and Agriculture Organization of the United Nations (FAO) consultation on preparation and use of food based dietary guidelines (WHO/FAO 1998) recommended intakes of 35 µg and 26 µg Se per day for adult males and females respectively. Further, intake of 30 µg and 26 µg Se per day was
recommended for adolescent males and females respectively. The WHO/FAO (1998) report stated RDAs of 6-21 µg Se per day for infants and children, depending on age. However other reports revealed WHO recommended 30 µg Se per day (WHO 1996) and a range between 30 and 40 µg per day (WHO 2004) for men and women. However, the United States National Academy of Sciences Panel on Dietary Oxidants and Related Compounds (2000) reviewed and revised the RDA values of Se to 55 µg per day for adults of both genders and 70 µg per day for pregnant and lactating women.

1.2.0 Selenium role in human health

As mentioned in earlier text, selenium is an essential trace element of fundamental importance to human health and prevention of disease (WHO 1996, Rayman 2000,). Selenium exerts its biological effect as a structural constituent of a number of selenoproteins including seleno-enzymes whose primary structures contain the element in the form of the 21st amino acid selenocysteine (Rayman 2000, Kasaikina et al. 2012, Ogra & Anan 2012). Selenium constitutes the active centre (site) of the selenoproteins (and selenoenzymes) that serve structural and enzymatic roles, the latter function being the main one where Se serves as an antioxidant and catalyst for the production of active thyroid hormone (Rayman 2000, Ogra & Anan 2012,) and participating in DNA synthesis and fertilization (Suzuki & Ogra 2002, Burk & Hill 2005, Bock et al. 2007). Literature reports that the Se biological functions and prevention of diseases are facilitated by more than 30 selenoproteins types in mammalian systems (Holben & Smith 1999) with at least 25 selenoprotein types reported in human systems(Gromadzinska et al. 2008). The reported 25 human selenoproteins have some particular relevance to anti-carcinogenic effects and protection of the gastrointestinal tract from oxidative stress and its associated ailments (Mork et al. 1998, Pagmantidis et al. 2008,). The selenoproteins and selenoenzymes of great importance to health and prevention of disease belong to the selenoenzyme families glutathione peroxidase (GPx), thioredoxin reductase (TrxR), iodothyronine 50-deiodinase (IDI) and a major selenoprotein selenoprotein P (SeP) (Holben & Smith 1999).

The antioxidant selenoenzymes of the GPx family and some selenoproteins, namely selenoprotein P, shield cells against oxidative damage and adverse effects of free radicals largely produced during aerobic metabolism in biological systems (Brigelius-Fohle 1999, Burk & Hill 2005, Bartel et al. 2007). Oxidative damage to cells and important biomolecules is a prominent pathway to the development of inflammations, cancers, cardiovascular and neurodegenerative diseases and aging (Pokorny et al. 2001). In mammalian systems, significant cell oxidative damage poses a health threat when free radicals accumulate to high levels and react with cellular biomolecules such as
DNA, proteins, sugars, lipoproteins and cell membrane lipids and lipoproteins causing mutations (DNA) and altering their structure and biological functions (Bellomo 1991, Pokorny et al. 2001). The biological role of antioxidant selenoenzymes and selenoproteins is to counteract these adverse effects by inactivating the free radicals and other reactive aerobic metabolite species (Brigelius-Fohle 1999, Bartel et al. 2007).

Selenium (selenoproteins) and Disease Prevention

Muscle disorders

Severe deficiencies in Se give rise to muscle disorders in mammals including humans. Papp et al. (2007) reports white muscle disease as a condition associated with selenium deficiency, a disorder observed in livestock reared in regions whose soils are low in Se. The affected animals’ muscle appear paler than usual and in some cases display pronounced longitudinal striations or prominent chalky appearance as a result of an anomaly in calcium deposition observed with very low Se levels (Papp et al. 2007). White muscle disorder affects the skeletal and cardiac muscles where selenoprotein W (SelW) is prominently expressed (Beilstein et al. 1996). The selenoprotein, SelW named after the condition white muscle disorder, expression is up-regulated in response to exogenous oxidants in muscle cells (Vendeland et al. 1995, Beilstein et al. 1996). Muscle disorders are also observed in humans deficient in Se, usually populations inhabiting farming regions with poor selenium levels (Bellinger et al. 2010). Amongst the muscle disorders associated with Se deficiency in humans is myotonic dystrophy, a condition that causes muscle pain and weakness (Bellinger et al. 2010).

Multiminicore disease

A collection of genetic disorders involving slow degeneration of muscle tissue is known as muscular dystrophy (NG 2007), and multiminicore disease characterized by a distinct loss of muscle fibres organization is one type of congenital muscular dystrophy (Jungbluth 2007). It has been reported that mutations in ryanodine receptors and selenoprotein N (SelN) cause multiminicore disease (Zorzato et al. 2007). SelN mutations have been associated with severe multiminicore myopathy, rigid spine muscular dystrophy-1 and desmin related myopathy with Mallory bodies (Moghadaszadeh et al. 2001, Petit et al. 2003, Tajsharghi et al. 2005,). Ryanodine receptors are channels in sarcoplasmic reticulum in muscle tissue that play a role in calcium stimulated release of calcium from intracellular stores (Zalk et al. 2007). The receptors enhance
calcium signals that may be initiated from membrane calcium channels and receptors, and mutations in the receptors lead to their disruption of function causing muscle fibres disorganization (Treves et al. 2005). On the other hand, SelN’s role in multiminicore disease has been ill defined because the function of SelN remains unclear (Bellinger et al. 2010). However, studies have shown ‘SelN knockdown’ in fish caused the disorganization of muscle fibres that matched multiminicore disease in humans (Deniziak et al. 2007). A SelN mutation responsible for causing multiminicore condition involves a loss of a selenium-response element (SRE), a cis element occurring in some selenoproteins additional to the selenocysteine insertion sequence (SECIS) element required for the expression of all selenoproteins (Maiti et al. 2008). The SRE is located within the RNA-coding region after the UGA codon, and its (SRE) mutation disrupts sequence ‘read through’ causing premature termination of translation of SelN (Maiti et al. 2008). Studies have demonstrated that SelN links with ryanodine receptors and the association is needed for proper functioning of the receptors (Jury nec et al. 2008) thus demonstrating that SelN mutations result in multiminicore disease because they impair calcium signalling by disrupting proper functioning of ryanodine receptors (Jury nec et al. 2008).

**Cardiovascular diseases**

The role of selenoproteins in cardiovascular diseases has been extensively studied mainly by analysing oxidative stress under selenium supplementation and selenium deficiency scenarios. Oxidative stress as a result of accumulated free radical action damages vascular endothelial cells and presents a platform for the development of cardiovascular disorders such as hypertension, coronary heart disease, congestive heart failure and atherosclerosis (Lum & Roebuck 2001, Flores-Mateo 2006, Nawrot et al. 2007). Selenium use to prevent and treat cardiovascular diseases has been under investigation for many years because seleproteins play a crucial role in cellular antioxidant defence system (Lum & Roebuck 2001, Bartel et al. 2007). Reports reveal that the expression and activities of selenoenzymes cytosolic glutathione peroxidise-1 (GPx1), phopholipid hydroperoxidase (GPx4) and thioredoxin reductase-1 (TrxR-1 are up-regulated by selenium supplementation (Thomas et al. 1993, Miller et al. 2001, Tang et al. 2005, Steinbrenner et al. 2006). The increased expression and activity of these selenoenzymes in vascular endothelial and smooth muscle cells prevent oxidative stress, cell damage and apoptosis from oxidized low-density lipoprotein (LDL) or triol, a cytotoxic hydroxylated cholesterol derivative present in cells, tissues, blood and atherosclerotic plaques in humans (Thomas et al. 1993, Miller et al. 2001, Steinbrenner et al. 2006, Tang et al. 2005). Huang et al. (2002) and Wu and Huang (2004) reveal that long term Se deficiency in rats severely decreased GPx enzyme expression and activity and this elevated
physiological and cholesterol oxide induced damage to the heart and vasculature, conditions which were reversed by dietary Se fortification (Huang et al. 2002, Wu & Huang 2004).

**Cardiac function and selenoproteins**

Reports by Maulik et al (1999) state that GPx1 enzyme prevents ischaemia/reperfusion-induced apoptosis of cardiac myocytes in mice. Furthermore, studies by Forgione et al (2002) reveal that genetic deletion of GPx1 in mice, caused heart and vascular malfunction and tissue irregularities. Atherogenic effects of lysophosphatidylcholine and 7-oxocholesterol, necrosis and endothelial cell apoptosis is reduced by GPx4 over-expression in mice (Guo et al. 2001). Another GPx isoform, GPx3, highly occurring in plasma; decreased GPx3 activity as a result of excessive accumulation of reactive oxygen species (ROS) leads to inadequate nitric oxide levels which disrupts platelet inhibitory mechanisms and increases arterial thrombosis (Kenet et al. 1999).

The thioredoxin reductase/thioredox (TRxR/TRx) system has been linked with regulating several processes of the cardiovascular system and ensuring its proper functioning (Ago & Sadoshima 2006, Berndt et al. 2007). The inter and intra cellular signalling pathways and activation of hypertrophic and apoptotic pathways in cardiac myocytes are altered by changes in the intracellular redox environment (Arner & Holmgren 2000, Pimentel et al. 2006). The TRxR/TRx system plays a role in regulating myocardial remodelling through the reversible oxidation of signalling molecules (Ago & Sadoshima 2006, Berndt et al. 2007). Studies by Kuster et al (2005) reveal this role showing that adrenergic receptor activation-induced hypertrophy in adult rat cardiac myocytes is affected by oxidation of cysteine thiols of Ras that are reduced by TrxR-1. It must be pointed out that TrxRs are able to directly reduce other substrates apart from TRx, which may have relevant roles on vascular function and heart health (Andersson et al. 1996).

Non enzymatic selenoproteins also play key roles in the functioning of the cardiovascular system. Selenoprotein K an endoplasmic reticulum protein, has an antioxidant function in cardiomyocytes and high mRNA expression in the heart (Lu et al. 2006). Another important non enzymatic selenoprotein, selenoprotein P, SeP transports Se to cells (Burk & Hill 2009) and supports optimal expression of the GPxs, TrxRs, and other important selenoenzymes in various tissues (Esworthy et al. 1998, Hill et al. 2003, Hoffmann et al. 2007, Bellinger et al. 2010). In addition, SeP reduces lipid and LDL peroxidation and peroxynitrite-induced protein oxidation and nitration (Arteel et al. 1998), this occurs at the expense of oxidizing TRx (Takebe et al. 2002).
Neurological disorders

In periods of dietary selenium deficiency, selenium is stored in the brain, suggesting the potential significance of the trace mineral in neurological disorders (Behne et al. 1988; Nakayama et al. 2007). Neurodegenerative conditions such as Alzheimer’s disease (AD) and Parkinson’s disease (PD) involve damage from ROS and other reactive species (Chen & Berry 2003).

Alzheimer’s disease (AD)

The oxidative damage of macromolecules is reported to be an early onset of AD that can occur before clinical symptoms (Moreira et al. 2006). Patients of AD display symptoms of impaired cognitive function, changes in behaviour and personality and suffer memory loss (Reddy & Beal 2005). Extracellular plaques containing amyloid β protein and the presence of intracellular neurofibrillary tangles characterise brains of Alzheimer disease patients (Reddy & Beal 2005). A lot of the AD cases are described as ‘late onset’ and progressing with age, however many autosomal dominant mutations have been identified and linked with ‘early onset’ of AD (Kowalska et al. 2004). Studies by Kowalska et al (2004) state that a mutation in an enzyme presenilin-2, involved in the processing of amyloid precursor protein causes early onset of AD. Hwang et al (2005) observed that a mouse model over expressing the human mutation had low levels of brain selenoprotein M (SelM), an endoplasmic reticulum specific selenoprotein whose precise role is not clear (Hwang et al. 2005, Bellinger et al. 2010). Bellinger et al (2010) states that selenoprotein M, SelM, may play a protective role in AD.

Furthermore, neurons and ependymal cells in the brain have abundant SeP, a selenoprotein previously identified as a plasma protein (Scharpf et al. 2007). Reports reveal that SeP expression in the brain elevates with aging, an indication it may have a role in mitigating oxidative stress (Lu et al. 2004). Peters et al (2006) reveal that genetic deletion of SeP damages synaptic function in the hippo-campus, an area of the brain involved in memory. Brain SeP deletion also decreases spatial learning and long term potentiation, a cellular model for learning and memory (Peters et al. 2006). Further, studies by Miller et al (2008) reveal that expression of SeP was up-regulated in AD beyond expression levels found in aging. Bellinger et al (2008) investigated SeP expression in post-mortem human brain and observed a different expression pattern of the selenoprotein within the centre of neuritic plaques and co-localization of the selenoprotein with plaques and neurofibrillary tangles. Studies have not precisely identified the specific role of SeP in AD, however its (SeP) location suggests it could have a function in mitigating the plaques accompanying oxidation (Bellinger et al. 2008).
Dietary Se greatly influences serum SeP and therefore Se supplementation may play a direct neuroprotective role by elevating the expression of SeP (Burk & Hill 2005).

**Parkinson’s disease (PD)**

Dopamine, a neurotransmitter controls numerous important brain functions although released from only 2% of brain neurons (Chinta & Andersen 2005). The severe loss of neurons releasing dopamine in the brain substantia nigra, is pivotal to the development of neurodegenerative disorder parkinson’s disease (Iversen & Iversen 2007). Parkinson’s disease symptoms include tremor and loss of movement control, rigidity, mood changes and cognitive impairments characterising the later stages of the condition (Fahn 2003). The loss of dopamine terminals in caudate and putamen within the striatum from neurons projecting from the substantia nigra (the nigrostriatal pathway) characterize PD (Fahn 2003). Studies link selenoproteins with preserving the nigrostriatal pathway (substantia nigra) (Chen & Berry 2003).

The substantia nigra and putamen are reported to possess higher levels of Se than other parts of the brain (Chen & Berry 2003). Studies by Kim et al (1999), Iman et al (1999) and Virmani et al (2003) reveal that Se deficiency elevates development of PD in mouse models. Zeevak et al (2008) reports that PD sufferers have a 50% decrease in glutathione levels, indicating an impaired GPx function. Chemical lesions of dopamine releasing terminals and neurons are reported to be significantly enhanced in Se deficient animals (Kim et al. 1999, Kim et al. 2000) and in contrast, Se supplementation offered protection to dopamine releasing neurons and increased GPx activity (Islam et al. 2002, Zafar et al. 2003). More studies reveal the link between selenoproteins and PD, GPx1 knockout in mice greatly potentiated dopamine loss and PD pathology (Klivenyi et al. 2004) and in contrast, GPx1 over-expression displayed a protective role from PD (Bensadoun et al. 1998, Ridet et al. 2006). These findings show that GPxs and other selenoproteins likely play an important function in protecting dopamine release and thus PD prevention (Ridet et al. 2006, Bellinger et al. 2010).

**Epilepsy**

Selenium status has also been linked to epilepsy, a chronic neurological disorder characterized by seizures which disrupt normal functions of the brain (Fisher et al. 2005, Ashrafi et al. 2007). A clinical study revealed that low selenium levels in blood lead to infant seizures and neurological disorders (Ashrafi et al. 2007). Epilepsy and brain trauma cause a free radicals signal cascade and activation of pro-apoptotic transcription factors which lead to neural loss (Savaskan et al. 2003). Savaskan et al (2003) showed that rats on Se deficient diets had elevated susceptibility to kainite-
induced seizures and cell loss. Furthermore, Naziroglu et al (2008) showed a synergistic protective effect of combining Se and topiramate (anti-epileptic drug) following pentylentetrazol-induced seizures in rats. Glutathione peroxidise and plasma membrane calcium ATPase activities were up-regulated upon pentylentetrazol challenge in rats administered Se and topiramate, thus preventing free radical production and regulating calcium driven processes (Kutluhan et al. 2009). Other selenoproteins are reported to also play a role in the prevention of epilepsy, studies by Schomburg et al (2003) and Hill et al (2003) reveal that SeP knockout mice had neurological seizures and movement disorders when placed on low Se diets.

**Endocrine disorders**

**Diabetes**

Diabetes is a condition caused by an inability to control glucose levels in blood, either as a result of impaired release of insulin (Type 1) or impaired insulin function or insulin resistance (Type 2) (Avery 1998). The consequential elevated blood glucose levels, hyperglycaemia increases ROS production, which contributes to the progression of the condition (Roberts & Sindhu 2009). Studies using the rat model have suggested that selenium supplementation may be used to treat diabetes (Battell et al. 1998, Ozdemir et al. 2005, Aydemir-Koksoy & Turan 2008). However recent clinical studies suggest selenium supplementation raises a possible risk of developing Type 2 diabetes (Bleys et al. 2007, Stranges et al. 2007). Ghosh et al (1994) and McNeill et al (1991) state that Se possesses insulin-mimetic properties in vitro and in vivo appearing to be autonomous of insulin release and this has a potential to accelerate insulin resistance development. Furthermore, a rat model for diabetes showed that selenoprotein S, SelS is glucose regulated (Walder et al. 2002, Karlsson et al. 2004) and other studies showed that insulin resistance causing Type 2 diabetes in mice developed as a result of GPx1 over-expression (McClung et al. 2004). Selenium supplementation up-regulates GPx1 expression (Sunde et al. 2005, Hu et al. 2010) in rats and mice and therefore may play a role in increasing risk of type 2 diabetes as reported by recent selenium fortification studies (Stranges et al. 2007, Lippman et al. 2009). These findings call for further research on the precise selenoproteins role in diabetes.
1.2.1 Selenium chemical forms and biological activity

Organic form versus inorganic form

As revealed in the earlier text in section 1.1.0, there are two chemical forms of selenium, namely organic and inorganic forms. The chemical form of selenium is an important factor as it determines the amount that can be safely ingested, its toxicity, nutritional importance, bioavailability and absorption, metabolic processing and fate (Mahan & Kim 1996, Shiobara et al. 1998). The chemical form of selenium ingested also influences the element’s tissue distribution (Mahan & Kim 1996) and affects biological activity such as the expression of endogenous selenoproteins (Ravn-Haren et al. 2008) linked to mechanisms by which selenium contributes to human health and disease prevention (Rayman 2000, Kasaikina et al. 2012). Organic forms of selenium are known to exhibit greater bioavailability than inorganic forms and are thus more readily absorbed and more effective at raising selenium levels in the blood and influencing selenium biological effects (Mahan & Kim 1996, Ravn-Haren et al. 2008). Studies by Lee et al (2007) evaluated the effects of different selenium sources (chemical forms) on performance, carcass properties, whole blood selenium concentration, plasma glutathione peroxidase activity and tissue selenium concentrations in finishing Hanwoo steers. The steers were placed on four treatment diets for 16 weeks; control (CON), spent mushroom composts from Se-enriched *Flammulina velutipes* mushrooms (Se-SMC), selenized yeast (Se-Y), and sodium selenite (SS) and dietary selenium levels of all treatments except for the control were an equal dosage of 0.9 mg Se/kg on dry matter basis (Lee et al. 2007). The control diet contained selenium levels of 0.08mg Se/kg on dry matter basis (Lee et al. 2007).

The study observed no significant differences among treatments with body weight gain and carcass characteristics in terms of dry matter intake (Lee et al. 2007). However, selenium concentrations in whole blood were observed to be significantly higher (P<0.05) for Se-SMC and Se-Y treatments compared to the control throughout treatment period with no significant difference in blood selenium concentrations between inorganic SS and the control (Lee et al. 2007). At 4 weeks, only the organic Se sources Se-SMC and Se-Y and not inorganic SS displayed a significant increase (P<0.05) in GPx3 activity compared to the control (Lee et al. 2007). The study also observed significantly higher (P<0.05) tissue Se concentrations in hind leg and liver with the organic Se sources Se-Y and Se-SMC compared to inorganic SS and the control, with the Se-Y displaying the highest Se concentrations of all treatments in both tissues (Lee et al. 2007). It was however observed that tissue selenium concentration for dietary treatment with inorganic SS was not significantly different to the control for both the hind leg muscle and liver tissues (Lee et al. 2007).
The findings from the study by Lee et al (2007) correlated with those of Ravn-Haren et al (2008) and Mahan and Kim (1996), that organic Se is more readily absorbed, more effective at raising blood Se levels and influencing Se driven biological activities. Ingestion of organic Se sources such as Se-SMC and Se-Y enhanced tissue Se concentration and blood Se levels and up-regulated plasma glutathione peroxidase activity more efficiently compared to dietary supplementation with inorganic Se, sodium selenite (Lee et al. 2007). Selenized yeast is reported by various studies to primarily occur as 83% (Rayman 2004), 70-80% (Ip et al. 2003) free organic selenomethionine. Studies by Maseko et al (2013) demonstrated in the Se-enriched Agaricus bisporus mushroom that the organic Se content was primarily made up of protein bound selenocysteine (95.5%) and selenomethionine (3.6%) and free methylselenocysteine (0.9%) all organic forms of selenium. Studies by Lee et al (2005) also showed that Se-SMC, by-products from the production of Se-enriched mushrooms cultivated by adding inorganic sodium selenite to mushroom growth compost, from the cultivation of Se-enriched Flammulina velutipes mushroom comprised of significant amounts of organic Se which was produced from the inorganic Se by metabolic processing of mushroom and their mycelia. Lee et al (2005) observed that about 70% of Se contained in Se-SMC from the Se-enriched F. velutipes mushrooms was organic Se bound to protein. Studies by Stefanka et al (2001) also demonstrated that inorganic Se added to mushroom compost is converted to organic Se by mycelia and the predominant form of Se being mostly selenocystine.

In another study, Lee et al (2006) also reports a poor intestinal absorption and lower Se concentration in whole blood following sodium selenite dietary treatment in steers, as most of the inorganic selenite fed to the steers was excreted as faeces. It is revealed that in ruminants, organically bound Se in the diet is more effective in the intestinal absorption, raising whole blood Se levels and Se accumulation in tissues compared to inorganic Se sources (van Ryssen et al. 1989). Earlier studies have observed that considerable amounts of inorganic Se is formed as insoluble selenide in the rumen, and subsequently excreted into faeces (Peterson & Spedding 1963, Wright & Bell 1966).

Studies by Lane et al (1990) also supported evidence that dietary (chemical) forms of selenium are differentially available for Se driven biological activity. Lane et al (1990) evaluated the effect of various forms of Se on GPx activity in the liver, heart, kidney and eyes of developing rats. Female rats were throughout mating, pregnancy and lactation placed on different diets of basal (<0.05 µg Se/g), selenite (0.15 µg Se/g) or selenomethionine (0.15µg Se/g) supplemented diets (Lane et al 1990). The study also had some pups born to dams on the basal diet given intraperitoneal doses of saline, selenite or selenomethionine. The study analysed GPx activity in the tissues (liver, heart,
kidney and eyes) from foetuses, 7 days-old and 14 days-old nursing pups and the dams. It was observed that in all tissues investigated, the 14 days-old pups from mothers that were placed on the selenomethionine diet, displayed the highest GPx compared to pups from mothers placed on the basal and selenite diets (Lane et al. 1990). Furthermore, the study also observed that eye tissues from foetuses from the selenomethionine treatment group displayed significantly higher (P<0.05) GPx activity compared to eye tissues from other treatments groups (Lane et al. 1990). With regards to the dams, supplementation with selenite and selenomethionine showed significantly higher (P<0.05) hepatic GPx activity compared to the basal diet. However, it is the dams on the selenomethionine supplementation and killed 14 days after parturition that displayed the highest liver GPx activity (Lane et al. 1990). Regarding selenium treatment of pups (nursing from dams fed a selenium deficient diet) by intraperitoneal injection of 3 µg Se/kg body weight as either selenite, selenomethionine or no selenium (saline), it was observed that there was no significant differences between tissue GPx activity at days 7 and 14 studied (Lane et al. 1990). However, hepatic GPx activity was significantly lower for the saline treated pups compared to the selenite and selenomethionine treated pups and renal GPx activity was observed to be significantly (P<0.05) the highest for the pups treated with selenite (Lane et al. 1990). Furthermore, for the heart and eye tissues, the chemical form of selenium displayed no effect on GPx activity (Lane et al. 1990).

Furthermore, pups were weaned to diets containing 0.1 or 0.2 µg Se/g of one of three forms of selenium (selenite, selenomethionine or selenocystine) or no added selenium. There were no significant differences in GPx activity between the two selenium levels 0.1 and 0.2 µg Se/g of diet. It was observed that after 14 days of selenium repletion, the basal diet group had the lowest (P<0.05) GPx activity in the heart, kidney and liver, compared to all the selenium treatment groups with the highest hepatic GPx activity occurring in the selenite group (Lane et al. 1990). Liver and heart GPx activity was observed to be generally higher (P<0.05) in the selenite and selenomethionine treatment groups compared to the basal or selenocystine treatment groups (Lane et al. 1990). However, the form of selenium displayed no effect on GPx activity in the eye tissues. The study further observed that in contrast to GPx activity, pups fed the selenocystine diet, displayed the highest tissue selenium concentration with the highest selenium tissue concentration found in the kidney (Lane et al. 1990).

More studies demonstrated the difference in bioavailability between different selenium chemical forms. Uglietta et al (2008) observed higher (P<0.001) plasma and muscle tissue selenium concentrations in reared neonatal pigs fed 1070 µg Se/kg dry matter milk powder, selenium-enriched whole milk obtained from cows supplemented with selenium yeast (comprised of 83%
selenomethionine) (Rayman 2004) in their diet, compared to pigs fed milk powder from non-supplemented cows and the milk powder supplemented with selenate. The study further observed that colonic expression of selenoprotein P was higher (P=0.024) in pigs fed the selenium-enriched milk powder from selenium yeast supplemented cows, compared to pigs fed milk powder from non-supplemented cows and the milk powder supplemented with selenate (Uglietta et al. 2008).

Furthermore, inorganic and organic forms of selenium, sodium selenite and selenomethionine respectively, are known to be used in cancer chemoprevention studies in food supplements and fortified foods. Both chemical forms, commercially available, are known to suppress carcinogenesis in animal models (Spolar et al. 1999, Hu et al. 2008, Yang et al. 2009). According to studies by Ammar and Couri (1981), in mice, the median lethal dose (LD50) of selenomethionine administered intravenously was determined to be 8.8 mg/kg whereas sodium selenite was found to be fourfold more toxic. However, the rat model showed contrasting results where selenomethionine displayed less cancer inhibition compared to selenite (Ammar & Couri 1981). The findings demonstrated that measuring the dosage of selenium used in chemoprevention is not enough, but it is also critical to be aware of the bioavailability, toxicity and anti-carcinogenic activity of the seleno-compounds used (Ammar & Couri 1989).

Studies by Ip (1998) and Spallholz (1994) also report anticancer activity for both selenite and selenomethionine, but reveal selenomethionine is less effective compared to selenite, particularly in vitro. It was demonstrated that selenite induced apoptosis of human prostate cancer cells was superoxide mediated and p53 dependent via mitochondrial pathways (Zhong & Oberley 2002; Zhao et al. 2006) and other studies revealed selenite induced apoptosis was mediated by reactive oxygen species production (Spallholz 1994, Kim et al. 2003). Spallholz (1994) reports that in contrast, selenomethionine possesses weaker anticancer action than most selenium compounds and that the low anticancer activity of selenomethionine is most likely linked with its metabolism within cells. Studies by Wang et al (2002) and Yamamoto et al (2003) showed that non effective levels of selenomethionine in the presence of methioninase (METase) or methionine β-lyase induced apoptosis in human cancer cells, indicating that active anticancer metabolites are generated from the catalysis of selenomethionine by the enzymes (Yamamoto et al. 2003).
1.2.2 Selenium Antioxidant System: seleno-enzymes and seleno-proteins

1.2.2.1 Glutathione peroxidases, Thioredoxin reductases and Thyroid hormone deiodinases

As earlier mentioned in text, selenium renders its valuable biological functions and health benefits through the various types of selenoproteins including selenoenzymes. The common feature shared by all of the selenoproteins including the selenoenzymes, whose functions have been identified, is their oxidoreductase activities catalysing the transfer of electrons from one molecule (reductant, electron donor) to another molecule (oxidant, electron acceptor) in oxidoreduction (redox) reactions (Kasaikina et al. 2012, Meplan & Hesketh 2012). The redox reaction is important in intracellular redox homeostasis and antioxidant defence (Kasaikina et al. 2012). So the selenoproteins and selenoenzymes participate in maintaining cellular redox homeostasis and constitute part of the antioxidant defence system that protects cells from reactive oxygen species, reactive nitrogen species and oxidative damage (Kasaikina et al. 2012, Meplan & Hesketh 2012). The selenoproteins require organic Se in the form of selenocysteine in their active site, as an essential structural component for their activity (Ogra & Anan 2012) to reduce oxidative stress in the body (Meplan & Hesketh 2012).

The selenoproteins that participate in maintaining cellular redox homeostasis and antioxidant defence are the five glutathione peroxidases, GPx-1, GPx-2, GPx-3, GPx-4 and GPx-6, three thioredoxin reductases, TrxR-1, TrxR-2 and TrxR-3, and three thyroid hormone deiodinases, DI1, DI2, DI3 (Kasaikina et al. 2012, Meplan & Hesketh 2012). The GPxs occur in different isoforms and have unique roles depending on where they are expressed. The different GPx forms exist in the cytosol of almost all tissues, particularly enriched in the liver, kidney and erythrocytes (GPx-1) and enriched exclusively in the epithelium of gastrointestinal tract (GPx-2) (Esworthy et al. 1998, Chu et al. 2004, Kasaikina et al. 2012). The plasma GPx, GPx3 is synthesized predominantly by the kidneys and secreted to the plasma (Kasaikina et al. 2012). Another form of GPx, GPx4 is represented by cytosolic (cGPx4), nuclear (nGPx4, testis-specific), and mitochondrial (mGPx4) isoforms (Conrad et al 2007, Kasaikina et al. 2012). The fifth GPx, GPx6 also occurs in the cytosol and is enriched in the olfactory epithelium (Brigelius-Flohe 2006). The GPx forms are all glutathione dependent in their detoxification of hydrogen peroxide and are also capable of reducing various other peroxides (Takebe et al. 2002, Lubos, Loscalzo & Handy 2011) in protecting cells from oxidative damage. Brigelius-Flohe et al (2001) states that plasma GPx, GPx3, in addition to playing an antioxidant role in kidneys, it also acts as a secretory protein with antioxidant tasks in extra-cellular space.
The three isoforms identified of thioredoxin reductase (TrxR) also occur in different places, with TrxR-1 and TrxR-2 occurring in the cytosol and TrxR-1 occurring in the mitochondria and all perform different tasks (Kasaikina et al. 2012). Thioredoxin (Trx) is a multifunctional protein with a redox-active disulphide/dithiol in the active site, and possessing redox-regulating and reactive oxygen species scavenging properties, plays a significant role in intracellular and extracellular compartments protection from oxidative stress (Yalcin 2004). Thioredoxin reductases are the only enzymes known to reduce thioredoxin and together with the ubiquitously occurring protein (thioredoxin) catalyse disulphide/dithiol exchange reactions hence making up the thioredoxin system that constitutes an important part of the body’s antioxidant defence system (Arner & Holmgren 2000, Yalcin 2004). TrxR-1 has at least 6 isoforms differing in N-terminal sequences and it reduces the oxidized form of cytosolic thioredoxin, whereas TrxR-2 has a glutaredoxin domain is involved in catalysis of a variety of reactions specific for thioredoxin and glutaredoxin systems (Su et al. 2005, Holmgren & Lu 2010). Thioredoxin reductase-3 reduces the oxidized form of mitochondrial thioredoxin and glutaredoxin 2 (Turanov, Su & Gladyshev 2006). Thioredoxin reductases are members of the type-I pyridine nucleotide-disulfide oxidoreductase enzyme family (Holmgren & Bjornstedt 1995), and similar to other members of the enzyme family, catalytically transfer two electrons from NADPH, via FAD and an N-terminal disulphide active site, to the substrate (Zhong et al. 2000). Unique to TrxR members of the family, mammalian TrxRs contain a penultimate selenocysteine residue at the carboxyl terminus (Gasdaska et al. 1996). Studies by Zhong, Arner and Holmgren (2000) to determine the role of the penultimate selenocysteine residue in thioredoxin reductases during catalysis showed that the Se atom transfers electrons to oxidized thioredoxin. The reduced form of thioredoxin then subsequently serves as an important source of reducing equivalents for ribonucleotide reductase and other proteins and enzymes with redox-active disulphide groups (Sun et al. 1999). Thioredoxin reductases have also been recently reported to reduce dehydroascorbate and ascorbyl free radical to ascorbate giving a new antioxidant function for selenium (May et al. 1997, May et al. 1998).

Another family of selenoenzymes that functions on the basis of selenocysteine contributing to their oxidoreductase activity are the thyroid hormone deiodinases, catalysing the reductive removal of iodine (I) from the outer ring of the prohormone thyroxine (T4) producing different forms of thyroid hormones (Kohrle & Gartner 2009, Marsili et al. 2011) whose primary role is metabolism regulation. Thyroid hormone deiodinase-1 (DI1) occurring in the plasma membrane removes iodine from the outer ring of thyroxine (T4) to produce plasma (triiodothyronine) T3, the active thyroid hormone form that is more potent than T4 and DI1 also catalyses deiodination and thus inactivating T3 (Maia et al. 2011) as required in cells. Thyroid hormone deiodinase-2 (DI2) occurs in the
endoplasmic reticulum and converts T4 to T3 locally in tissues (Ng et al. 2011). The catalysis of deiodination of T4 to T3 in peripheral tissues is handled by thyroid hormone deiodinase-3 (D13) also plasma membrane localized like D11 (Schomburg & Khorle 2008).

1.2.2.2 Selenoprotein P and other selenoproteins

Selenoprotein P (SeP) an extracellular glycoprotein secreted by most cells, including colonic cells, is uniquely made up of 10 selenocysteine residues, 1 residue in the N-terminus and 9 residues in the C-terminal domain sequence in rodents and humans (Burk & Hill 2009). Accounting for more than 50% of the total plasma Se content, selenoprotein P occurs in the blood or plasma as two isoforms of 50 and 60 kDa molecular weight (Burk & Hill 2009, Meplan et al. 2009). Studies have demonstrated that SeP plays a key role in the delivery of Se from the liver to extra-hepatic tissues like the testis and brain (Hill et al. 2003, Schomburg et al. 2003). Although SeP is mainly expressed in the liver, it is also expressed in a variety of tissues including the colon (Burk & Hill 2009). According to reports by Hill et al (2003) the presence of SeP is vital to influencing the expression of other individual selenoproteins in different tissues. It is reported that due to SeP’s function as a transport protein for Se, it explains its effective influence on the expression of other individual selenoproteins in various tissues (Hill et al. 2003, Hoffman et al. 2007). Furthermore, in addition to its key transport role, SeP has been reported to function as an antioxidant defence (Diwadkar-Navsariwala et al. 2006, Meplan & Hesketh 2012) and play a role in cancer prevention (Diwadkar-Navsariwala et al. 2006). Studies in presenting evidence to support the role of SeP as an antioxidant and its involvement in general Se homeostasis reveal that SeP knockout mice have very low Se concentrations in the brain, testis and foetus with severe pathophysiological consequential conditions in the tissues related to oxidative stress (Burk & Hill 2005). Burk and Hill (2005) further report that SeP binds to endothelial cells in rats, and concentrations of SeP in plasma correlate with inhibition of aliquot induced lipid peroxidation and hepatic endothelial cell injury. Cellular component damage by oxidative stress is known to initiate tumorigenesis (Ames et al. 1993, Bernstein et al. 2001).

In addition, selenoprotein P knockout mice have been associated with increased cancer development (Diwadkar-Navsariwala et al. 2006) and colorectal cancer was linked with a significant reduction or loss in mRNA expression of SeP (Al-Taie et al. 2004). Furthermore, human advanced colorectal adenoma was linked to some genetic variants in selenoprotein P (Persson-Moschos et al. 2000).
There are various other selenoproteins, relatively less characterised than SeP, however, it is reported that most of them are also oxidoreductases with a selenocysteine in their active sites (Kasaikina et al. 2012). Studies by Bellinger et al (2009) and Kelly et al (2009) state that selenoproteins H, L, T, and W (SelH, SelL, SelT and SelW) and the 15kDa seleniprotein belong to the novel family of selenoproteins that consist of a thioredoxin-like redox fold. Selenoprotein S, selenoprotein N, selenoprotein M and 15kDa selenoprotein are proteins of the endoplasmic reticulum involved in redox balance and unfolded protein response (Bellinger et al. 2009). Selenoprotein S and the 15kDa selenoprotein have been observed to play roles in the endoplasmic reticulum stress response (Kelly et al. 2009, Korotkov et al. 2001). Oxidative stress reduces the protein folding capacity of the endoplasmic reticulum consequentially causing endoplasmic reticulum stress and leading to the accumulation and aggregation of misfolded proteins (Li, Zhang & Li 2011). Continued or severe stress can ultimately lead to apoptotic cell death (Verfaillie et al. 2010). Reports by Kelly et al (2009) and Petit et al (2003) reveal that several selenoproteins expressed in the endoplasmic reticulum, SePS, SelN, SelM, SelH and SeP15 play a role in the removal of misfolded proteins from the endoplasmic reticulum lumen and influence inflammatory signalling pathways thereby reducing stress in the endoplasmic reticulum. It is reported that the selenoproteins are sensitive to Se status and reduction in Se supply exacerbates stress in the endoplasmic reticulum (Curran et al. 2005).

1.3.0 Selenium and Cancer

1.3.1 Epidemiological and Intervention Trials

Intervention studies have linked increased Se consumption from Se supplementation in the diet and correlating blood concentrations with reduced incidence of certain types of cancers. Reports reveal that Se consumption of at least 200 µg per day for adults offers protection against and reduces incidence of lung, liver, colorectal, prostate and breast cancers (Clark et al. 1996, Diwadkar-Vavsariwala & Diamond 2004). Studies by Clark et al. (1996) reveal that dietary Se supplementation of 200 µg per day in the form of selenium-enriched yeast decreased the incidence and mortality rates of colorectal, lung and prostate by 50%. Although the study by Clark et al. (1996) showed that over a period of 10 years, consumption of Se at 200 µg per day reduced total cancer incidence and cancers of the lung, prostate and colorectum, it however showed no effect on squamous cell skin cancer in human subjects. Although Duffield-Lillico et al (2002) intervention study supported conclusions by Clark et al (1996) on the effect of Se supplementation on total cancer incidence, it did not show significant links with the reduction in lung and colorectal cancers incidence. Furthermore, Rayman (2012) also concurred with evidence presented by Clark et al.
(1996) and Duffield-Lillico et al. (2002) that higher Se status reduces the risk of the cancers types earlier mentioned including bladder cancer. However, Rayman (2012) points out that due to mixed findings from trials, it is deduced that selenium supplementation will confer benefit in cases where the nutrient intake is inadequate. Reports reveal that supplementation of people who already have an adequate selenium intake and status with additional selenium increases their risk of developing type-2 diabetes (Bleys et al. 2007, Stranges et al. 2007, Rayman 2012). That notwithstanding, relatively more human intervention studies continue to show the positive potential role of selenium in cancer prevention. However, it was observed that the use of purified selenomethionine in supplements (as opposed to selenized yeast) and the combination of Se and vitamin E did not increase the risk of type-2 diabetes (Lippman et al. 2009). A study of 34 000 men using a nested case control study design demonstrated that elevated intakes of Se protected against prostate cancer (Yoshizawa et al. 1998) and reduced mortality rates from oesophageal cancer with a dietary supplemental combination of Se, beta-carotene and vitamin E (Blot et al. 1993).

Moreover, animal intervention studies and animal models have demonstrated in-vivo the anticarcinogenic effects of selenium supplementation. A study by Hu et al. (2008) investigated the effects of organic Se from two sources, dairy selenium occurring as selenomethionine rich milk selenoproteins (Heard et al. 2007) and yeast selenium primarily 90% free selenomethionine (Ip et al. 2003, Rayman 2004) on oncogenesis inhibition in carcinogen-treated mice and whether Se regulated the homeostatic response to carcinogen-induced DNA damage. The study observed that dairy selenoproteins at 1ppm dosage in the diet significantly suppressed aberrant crypt foci (ACF), clusters of abnormal tubular glands in the lining of the colon and rectum forming before colorectal polyps and serving as early indicators of colorectal cancer. Dairy Se at 1ppm also inhibited cancer of the colon whereas yeast Se at an equal dosage had no effect in azoxymethane challenged mice subjects (Hu et al. 2008). Furthermore, dairy Se at 1ppm was observed to significantly elevate plasma Se levels and acute apoptotic response to azoxymethane and a decrease in proliferation of cells and frequency of K-ras mutations in ACF was observed whereas the same dose of yeast Se did not display the same effects (Hu et al. 2008).

Studies by Unni et al (2005) demonstrated that another naturally occurring organic selenium compound, methyl-selenocysteine (MeSeCys) exhibits chemopreventive properties against in vivo and in vitro models of carcinogen-induced mouse and rat mammary tumorigenesis. Methyl-selenocysteine was observed to block several signalling pathways in mouse mammary tumour cells in vitro by inhibiting the activity of phosphatidylinositol 3-kinase (PI3-K) and its downstream effector molecules stopping tumor cells growth (Unni et al. 2005). Spolar et al. (1999) further
showed anticarcinogenic effects of another organic Se source, where dietary supplementation of selenium-enriched *Agaricus bisporus* mushroom at 1ppm was observed to significantly reduce total and anti-3, 4-dihydrodiol-1, 2-epoxide-deoxyguanosine DNA adducts induced by 7, 12-dimethylbenz[a] anthracene compared to dietary fortification with Se-unenriched *A. bisporus* in rats. The findings provided further evidence that organic Se, occurring as rich selenocysteine selenoproteins in *A. bisporus* (Maseko et al. 2013) is effective at inhibiting proliferation of carcinogen induced tumour cells.

1.3.2. Selenoproteins and cancer; selenoproteins expression and anti-cancer mechanisms

As earlier mentioned, there is evidence from animal and epidemiological studies that selenium has cancer chemopreventive characteristics. However, the biological mechanisms through which selenium renders its chemopreventive action (s) are not clearly understood. It is not clearly revealed whether selenium offers its anti-cancer properties in its elemental state, through its incorporation into organic molecules, through its selenoproteins or as a combination of these (Hudson et al. 2012). However, several studies have linked the anti-cancer action of Se to the selenoproteins’ expression and activity (Mork et al. 1998, Early et al. 2002, Chu et al. 2004, Iron et al. 2006; Pagmantidis et al. 2008) that primarily involves the carrying out of antioxidant and detoxification functions in cells (Mork et al. 1998, Chu et al. 2004). Hudson et al (2012) evaluated whether the selenoproteins mitigated the risk of developing carcinogen induced mammary cancer in mice. In the study (Hudson et al. 2012), the expression of selenoproteins in mouse was ablated in mammary epithelial cells by genetic deletion of selenocysteine tRNA gene (Trsp) whose product selenocysteine tRNA is needed for the translation of selenoproteins. Trsp floxed and mouse mammary tumour virus (MMTV)-cre mice were crossed to give tissue-specific excision of Trsp in targeted mammary glands. Standard doses of carcinogenic 7,12- dimethylbenzylbenz[a]antracene was administered to eight to twelve week old second generation of Trspfl/+; wild type, Trspfl/+; MMTV-cre, Trspfl/fl; wild type and Trspfl/fl; MMTV-cre female mice. Findings from the study revealed no difference in tumour incidence, tumor rate and survival for the heterozygous Trspfl/+; MMTV-cre mice compared to the Trspfl/+; wild type mice (Hudson et al. 2012). However, a significant 54.8% of the homozygous Trspfl/fl; MMTV-cre mice was observed to have developed mammary tumors and displayed significantly shorter survival periods compared to the corresponding Trspfl/fl; wild type mice with only 36.4% developing tumors (Hudson et al. 2012). The loss of the homozygous Trsp alleles was linked with the reduction in expression of selenoproteins and the findings suggested that mice with a reduction in selenoprotein expression have increased susceptibility to developing mammary
tumors induced by the carcinogen (Hudson et al. 2012). The findings also illustrated that the major protective mechanism against carcinogenic induced mammary cancer required expression of the selenoproteins (Hudson et al. 2012).

Furthermore, more animal studies further supported the evidence of the involvement of selenoproteins in the mitigation against carcinogenesis. Irons et al (2006) also evaluated the role played by selenoproteins in cancer protective effects of selenium using transgenic mice that carried a mutant selenocysteine transfer RNA gene which caused reduced selenoprotein expression. The study characterized selenium homeostasis in the liver and colon tissues of wild type and transgenic mice placed on a selenium deficient diet supplemented with 0, 0.1 or 2.0 microgram Se (as selenite/g diet. Transgenic mice were observed to display reduced (P<0.05) glutathione peroxidase expression of the liver and colon but conserved thioredoxin reductase expression compared to wild type immaterial of the selenium status (Irons et al. 2006). It was further observed from the study that transgenic mice showed more (P<0.05) azoxymethane-induced aberrant crypt foci (preneoplastic lesion, early indicators of colon cancer) formation compared to the wild type mice (Irons et al 2006). However selenium supplementation was observed to reduce (P<0.05) the number of aberrant crypt foci for both transgenic and wild type mice (Irons et al. 2006). It is important to state that the transgenic mice comprised Se in non-protein fractions of the liver and the colon compared to the wild type mice showing presence of a higher amount of low molecular weight selenocompounds and not selenoproteins (Irons et al. 2006). It was deduced from the findings that lack of selenoprotein activity elevates susceptibility to colon cancer and it was further observed that low molecular weight selenocompounds reduce preneoplastic lesions autonomous from the selenoprotein genotype (Irons et al. 2006). It was evident from the study that both selenoproteins and low molecular weight selenocompounds play an important role in the cancer-protective effects of Se (Irons et al. 2006).

Glutathione peroxidases

Studies by Chu et al (2004) observed that the targeted inactivation of GPx1 and GPx2 genes jointly lead to high incidence of ileocolitis (inflammation and ulceration of the mucous membrane of the ileum and colon) in mice raised under conventional conditions which include the harbouring of Helicobacter species (non-specific-pathogen-free (non-SPF) conditions). The study characterized a mouse model with both GPx1 and GPx2 genes disrupted (GPX-DKO) with mice that had microflora-associated intestinal cancers which are correlated with elevated intestinal pathology or inflammation (Chu et al. 2004). It was observed that GPX-DKO mice that grew under germ-free
conditions had virtually no pathology and tumours (Chu et al. 2004). However, the study observed that the colonizing of germ-free mice with commensal microflora without any known pathogens, the GPX-DKO mice (<9%) developed tumours in the ileum or colon (Chu et al. 2004). Further observations were that 25% of GPX-DKO mice that were raised under non-SPF conditions from birth or transferred from SPF conditions at weaning stage displayed predominantly tumours in the ileum than the colon (Chu et al. 2004). It must be pointed out that almost 30% of tumours are cancerous, most are invasive adenocarcinomas with a few as signet-ring cell carcinomas (Chu et al. 2004). The study concluded that GPX-DKO mice are highly susceptible to bacteria associated inflammation and cancer and the sensitivity shown by the mice suggested that stress from peroxidative action plays a significant role in ileum and colon pathology and inflammation which often present a platform to tumorigenesis (Chu et al. 2004) as GPx1 and GPx2 are major enzymes that jointly play a role of reducing oxidative stress inducing hydroperoxides in intestinal epithelium (Esworthy et al. 1998, Chu et al. 2004).

Further studies have demonstrated the link between the GPx selenoenzymes and cancer. Cytosolic GPx1 has been implicated in the development of various cancers of the head and neck, lung, breast and colon due to the allelic loss at the GPX1 locus (Hu et al. 2005). The loss of heterozygosity at GPX1 locus was observed as a common event in the cancer of head and neck, breast, lung, and colon (Hu et al. 2005). Hu et al (2005) investigated allelic loss at the GPX1 locus in colon cancer by examining loss of heterozygosity (LOH) in DNA that was extracted from both tumour and the adjacent histopathologically normal tissue with tissue samples obtained from 53 colon cancer patients. Alanine codon repeats and a proline-leucine polymorphism (198P/L), two highly polymorphic markers making up the GPX1 gene were used to examine LOH at the locus and analysis of both polymorphisms identified LOH at GPX1 in a significant percentage of colorectal cancer, 42% (Hu et al. 2005). The findings indicated that the loss of heterozygosity at the GPX1 locus is a prevalent event in the development of cancer and that the GPX1 gene or other tightly linked genes may be involved in the cause of colorectal cancer (Hu et al. 2005).

Bartel et al (2007) states that in contrast to GPx1, the inactivation of gastrointestinal tract specific GPx2 was more detrimental because it was observed that one intact allele was sufficient to inhibit intestinal inflammation therefore showing that it has anti-cancer effects rather than playing a role as an anti-inflammatory. Furthermore, GPx2 plays a role in cell growth and differentiation, suppression of cyclo-oxygenase-2 expression, an enzyme whose inhibition provides relief from inflammation and pain (Banning et al. 2008) and activation by the β-catenin-T cell factor (TCF) complex (Kipp, Banning & Brigeliu-Fohle 2007). The expression of cyclo-oxygenase-2 is also

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regulated by Nrf2, a transcription factor that induces enzymes with cytoprotective and tumour preventive properties (Banning et al. 2005). Studies reveal GPx2 may play dual roles in carcinogenesis because the selenoprotein was observed to be highly expressed in human colorectal adenomas and carcinomas (Mork et al. 2000, Murawaki et al. 2008). However, Banning et al (2008) stated that the beneficial function of GPx2 in carcinogenesis may be dependent on the tumorigenesis stage. During the initiation stage of tumorigenesis, GPx2 is able to protect cells from oxidative damage and also reduce cyclo-oxygenase-2 expression and prostaglandin E2 production by the enzyme (Fiala et al. 1991, Jacobs et al. 2004). It is important to point out that the notable characteristic of selenoproteins (including selenoenzymes) is that their cancer protective effects are more pronounced at the early stage of carcinogenesis (Fiala et al. 1991, Rao et al. 2001, Jacobs et al. 2004).

Although as earlier mentioned, some studies reported that genetic variants of GPx1 were associated with increased colorectal cancer risk and the loss of heterozygosity at the GPx1 locus played a role in malignant progression (Hu et al. 2005), further research is required to determine conclusively whether GPx1 possesses protective effects for the prevention of cancer (Thomson 2004). Hu and Diamond (2003) carried out a study of a GPX1 gene variant that resulted in a leucine or proline at codon 198 and observed that the leucine allele is more frequently associated with breast cancer. In addition, it was observed that MCF-7 cells (breast cancer cell line) transfected with GPX1 Pro198 constructs displayed a higher GPx activity in response to increasing Se concentrations than those transfected with the Leu198 variant (Hu & Diamond 2003). Thus the findings suggest that the leucine variant of GPx1 may be a risk or a contributing factor to the development of breast cancer (Hu & Diamond 2003).

The findings that genetic variants of GPx1 were linked to increased colorectal cancer and loss of heterozygosity at GPx1 locus was involved with malignant progression (Hu et al. 2005) along with the differential expression patterns for GPx1 in tumour v. normal tissues (Hu & Diamond (2003) support the relevance of GPx1 in cancer prevention (Diwadkar-Navsariwala & Diamond 2004, Moghadaszadeh & Beggs 2006). However, gathering evidence from other studies has suggested that GPx1 might not act as the prime chemoprevention mechanism (Bermano et al. 1995, Irons et al. 2006) because the selenoenzyme reached its maximum with adequate Se intake and showed no appreciable changes when the intake of Se was upped to levels 10-fold higher. The very elevated Se levels are required to attain chemopreventive effects in the animal models used (Ganther 1999). Studies reveal that since the intake of Se at supra-nutritional amounts also lowered the risk of colon cancer in transgenic mice that had reduced GPx1 expression, it indicates that the chemopreventive effect of Se may not be dependent on GPx1 expression (Irons et al. 2006) but on
other glutathione peroxidases, other selenoproteins and selenocompounds. Studies by Ran et al (2004) demonstrated that GPx4, a phospholipid hydroperoxidase that protects cells against membrane lipid peroxidation, is essential for mouse survival and deficiency of the selenoenzyme makes cells susceptible to oxidative injury. Yang et al (2014) reports that GPx4 is an essential regulator of ferroptotic cancer cell death, a form of non-apoptotic cell death for which key regulators remain unknown (Yang et al. 2014). The study revealed that GPx4 overexpression and knockdown modulated the lethality of ferroptosis inducing compounds, with sensitivity profiling in 177 cancer cell lines revealing that diffuse large B-cell lymphomas and renal cell carcinomas were particularly susceptible to GPx4 regulated ferroptosis (Yang et al. 2014). To determine the generality of ferroptosis regulation by GPx4, the study treated HT-1080 fibrosarcoma cells with each lethal compound either a ferroptosis inducing compound or a non-ferroptosis compound under a GPx4-inhibited condition (Buthionine Sulfoximine treatment which inhibits GPxs by depleting glutathione) or a GPx4-upregulated condition (by overexpressing GPx4) (Yang et al. 2014). It was observed that GPx4 inhibition using buthionine sulfoximine enhanced ferroptotic death of cells induced by ferroptosis inducing compounds, whereas GPx4 up-regulation (overexpression) suppressed ferroptosis induced by ferroptosis inducing compounds (Yang et al. 2014). The study also observed that when GPx4 was inhibited, cells could not reduce exogenously added phosphatidylcholine hydroperoxide (Yang et al. 2014).

Thioredoxin reductases

Thioredoxin reductase-1 (TrxR-1) plays an important role in antioxidant defence constituting a part of the thioredoxin system. It is however reported to display dual and contradictory effects on tumour development (Moghadaszadeh & Beggs 2006). Al-Taie et al (2004) reports high levels of expression of TrxR-1 in a variety of tumour tissues in humans. Selenium has been reported to influence TrxR-1 expression levels in two ways, with expression levels of the selenoprotein increasing with excess Se intake and continued high levels of Se intake causing a decline in expression (Ganther 1999). Studies by Lu, Chew and Holmgren (2007) reveal that the selenocysteine-dependent TrxR enzymes are important molecular target for anticancer drug development. The study states that blocking the replication of cancer cell DNA and repair and induction of oxidative stress by the inhibition of the thioredoxin and glutathione systems are possible cancer chemotherapeutic strategies (Lu, Chew & Holmgren 2007). The thioredoxin system which comprises of NADPH, TRxRs and thioredoxin and the glutathione system made up of NADPH, glutathione reductase and glutathione supported by glutaredoxin are the two electron donor systems that control cellular proliferation, viability and apoptosis (Lu, Chew & Holmgren
The study revealed that the basis for anti-cancer mechanisms by arsenic trioxide (ATO), an effective cancer therapeutic drug for acute promyelocytic leukemia and with anticancer activity against a wide range of solid tumours, is by targeting thioredoxin reductase (Lu, Chew & Holmgren 2007). The drug is reported to exert its anti-cancer effect mainly through elevated oxidative stress, where it irreversibly inhibits mammalian TrxR where both the N-terminal redox-active dithiol and the C-terminal selenothiol-active site of the reduced state TrxR may react with ATO (Lu, Chew & Holmgren 2007). Arsenic trioxide inhibited MCF-7 cell growth which correlated with the irreversible inactivation of TrxR leading to the oxidation of thioredoxin (Lu, Chew & Holmgren 2007). It was further observed that the inhibition of TrxR by ATO was attenuated by glutathione, and depletion of glutathione by buthionine sulfoximine enhanced ATO-induced cell death (Lu, Chew & Holmgren 2007). Conclusions were drawn from the study that the anticancer activity of ATO was by means of a Trx system-mediated apoptosis (Lu, Chew & Holmgren 2007), a system that consists of TrxR enzymes.

Selenoproteins

Selenoprotein P, Selenoprotein S, Selenoprotein H, Selenoprotein N, Selenoprotein M, Selenoprotein W, Selenoprotein H, 15kDa Selenoprotein,

Selenoprotein P (SeP), serving as the major Se transport protein in blood is one of the non-enzymatic selenoproteins that has been implicated in cancer risk and tumor development (Meyer et al. 2012). Studies by Meyer et al (2012) analysed serum selenoprotein P and serum Se as markers of selenium status in renal cell cancer patients and whether their concentrations correlated with cancer-specific mortality. Serum Se and selenoprotein P concentrations in renal cell cancer patients and controls that displayed no evidence of malignancy were measured by X-ray fluorescence and an immunoassay respectively. The study observed that in the renal cell cancer patients, higher tumour grade and tumour progression at diagnosis correlated with reduced selenoprotein P and selenium concentrations compared to the controls (Meyer et al. 2012). The low selenium and selenoprotein P levels in serum were significantly associated with cancer severity, where higher cancer grade and progression (stage) were characterised by more aggressive cases of renal cell cancer in patients (Meyer et al. 2012). The study observed that low serum selenoprotein P levels of below 2.4mg/l, which represented the bottom tertile of the renal cell cancer patients, were found in patients with higher tumour grade, stage and with metastases (Meyer et al. 2012). Furthermore, renal cell cancer patient survival data indicated that the serum selenoprotein P levels at diagnosis, falling below 2.4mg/l, correlated with a poor 5 year survival rate of only 20% (Meyer et al. 2012). It is important
to point out that the study observed mortality rate to be inversely associated with selenoprotein P concentrations at diagnosis, suggesting that an adjuvant supplementation with selenium to support conventional therapeutic measures may be beneficial in up-regulating selenoprotein P expression and increasing survival rate of renal cell cancer patients deficient in selenium (Meyer et al. 2012). The study thus drew conclusions that selenoprotein P and Se concentrations were of prognostic value in renal cell cancer and may serve as additional diagnostic biomarkers to identify a deficit in selenium in kidney cancer patients that could potentially affect therapy regimen (Meyer et al. 2012).

Studies by Al-Taie et al (2004) have implicated polymorphisms in SeP in colorectal cancer and demonstrated reduced SeP expression in colorectal adenomas. The study investigated the expression of SeP in colorectal cancers in comparison with corresponding normal colon mucosa and also analysed the occurrence of genetic alterations within the SeP gene (Al-Taie et al. 2004). The study observed a significant reduction or loss of selenoprotein P mRNA expression in colon cancer cells indicating a link between a decrease in selenoprotein P expression and colorectal carcinogenesis (Al-Taie et al. 2004).

Epidemiological findings linking selenium intake and selenoproteins expression to colorectal cancer risk are limited. However there is much stronger evidence for an association with adenoma risk (Meplan & Hesketh 2012). Studies have demonstrated an association between a genetic variant in the selenoprotein S gene and colorectal cancer risk (Meplan & Hesketh 2012). It is known that the intake of selenium regulates the expression of selenoproteins S, W, H, M and the 15 kDa selenoprotein (Sep 15) in the colon, and downstream targets such as the endoplasmic reticulum response to stress, oxidative stress and inflammatory pathways (Meplan & Hesketh 2012). The Se regulated selenoproteins mentioned above play a major role in the ability of the epithelial cells of the colon to respond to microbial and oxidative damage and a combination of low selenium intake and single nucleotide polymorphisms in the selenoproteins genes can interfere with their role of mitigating microbial and oxidative stress damage leading to increased risk of pre-neoplastic lesions (Meplan & Hesketh 2012). Reports reveal that selenoprotein S and the 15kDa play roles in the endoplasmic reticulum stress response (Korotkov et al. 2001, Kelly et al. 2009). The genes encoding selenoprotein S and the 15kDa selenoprotein are reported to have single nucleotide polymorphisms that affect the expression of the selenoproteins (Kelly et al. 2009, Korotkov et al. 2001). Studies by Curran et al (2005) reveal that a single nucleotide polymorphism in the selenoprotein S gene (SELS) promoter at position 105 (rs34713741) affects inflammatory markers levels such as tumour necrosis factor-alpha and interleukin and therefore regarded as possessing functionally significant effects. The single nucleotide polymorphism located in the promoter region,
a regulatory region, of the SELS gene has the capacity to change the level of expression of the selenoprotein (Curran et al. 2005).

Genotyping studies have indicated links between a single nucleotide polymorphism (SNP) in the promoter region of the selenoprotein S gene (SELS) and colorectal cancer risk (Meplan et al. 2010, Sutherland et al. 2010). Studies reveal that single nucleotide polymorphisms in the promoter region of the SELS gene were observed to modulate disease risk with the T allele of rs34713741 elevating colorectal cancer risk and displaying an odds ratio of 1.68 (1.16-2.43) in one population under study and carriage of a second variant in close proximity giving an odds ratio of 2.25 for homozygous females in a geographically different population (Curran et al. 2005). The replication of the association between single nucleotide polymorphisms and disease risk in the two geographically distinct populations strongly indicates that other genetic factors, lifestyle and dietary patterns have no bearing in the SNPs in the SELS promoter influence on colorectal cancer risk (Curran et al. 2005). It is reported that rs34713741 influences markers of inflammatory function (Curran et al. 2005) and other reports revealing that the SNP in SELS promoter is linked to gastric cancer risk (Shibata et al. 2009). The studies are in agreement in revealing that SNPs in SELS gene influence colorectal cancer risk and also identifying selenoprotein S as an important selenoprotein of relevance in the understanding of the mechanisms by which selenium protects epithelial cells of the gut from tumourigenesis (Curran et al. 2005, Shibata et al. 2009, Meplan et al. 2010, Sutherland et al. 2010).

Several types of stresses such as nutrient starvation, hypoxia and oxidative stress decrease the protein folding capacity of the endoplasmic reticulum, leading to a condition known as endoplasmic reticulum stress and causing the accumulation and aggregation of misfolded proteins (Li, Zhang & Li 2011). It is reported that severe and prolonged endoplasmic reticulum stress is able to ultimately lead to apoptotic cell death (Szegedi et al. 2006, Verfaillie et al. 2010). Various selenoproteins are expressed in the endoplasmic reticulum, the identified selenoproteins expressed are selenoprotein S, selenoprotein N, selenoprotein M, selenoprotein H and 15kDa selenoprotein (Korotkov et al. 2001, Petit et al. 2003, Curran et al. 2005, Kelly et al. 2009, Reeves et al. 2010). The selenoproteins play roles in endoplasmic reticulum stress response and the removal of misfolded proteins from the endoplasmic reticulum lumen to the cytosol and they influence inflammatory signalling pathways thereby reducing endoplasmic reticulum stress (Korotkov et al. 2001, Petit et al. 2003, Curran et al. 2005, Kelly et al. 2009, Reeves et al. 2010). Curran et al (2005) however states that the sensitivity of the endoplasmic reticulum selenoproteins to selenium levels and the presence of
functional single nucleotide polymorphisms in their genes affecting their expression, reveals that reduced selenium levels and genetic factors can each aggravate endoplasmic reticulum stress.

1.4.0 Selenoproteins; other roles and biological functions

Animal and human studies have demonstrated the role of selenium in cancer prevention, although the precise mechanisms of selenium’s anti-cancer action remain unresolved (Diwadkar-Navsariwala & Diamond 2004, Hu et al. 2010, Hu et al. 2011). The mammalian genome encodes 25 selenoproteins, each containing Se, as an essential component, in the form of the seleno-amino acid selenocysteine (Sec). Although the functions of most have not been determined, a number of these selenoproteins possess antioxidant activities enabling them to scavenge free radicals and perform a wide range of biological functions making the selenoproteins of particular relevance to anticancer mechanisms (Mork et al. 1998, Pagmantidis et al. 2008). The effect of Se on modulating the activities of the selenoproteins is one possible means by which Se suppresses carcinogenesis (Diwadkar-Navsariwala & Diamond 2004).

Reports reveal that almost all of the identified selenoproteins are oxidoreductases with the Sec residue in their active centres (Kasaikina et al. 2012). In all selenoproteins, selenium acts as an active centre essential for the selenoproteins’ biological activity in the formation of a selenol group (-SeH) on the selenocysteine residue in a selenoprotein sequence (Lu & Holmgren 2009, Reeves & Hoffmann 2009). The insertion of Sec into the primary structures of the selenoproteins occurs through an in-frame UGA codon on mRNA in the presence of the Sec insertion sequence (SECIS) element that is located in the 3’ untranslated of selenoprotein mRNAs in eukaryotes (Kasaikina et al. 2012, Ogra & Anan 2012). It is important to point out that for proteins to qualify as ‘TRUE’ selenoproteins, their primary sequence needs to consist of at least one Sec residue, whereas in contrast proteins containing Se in the form of selenomethionine are known as Se-containing proteins but not selenoproteins (Suzuki 2005). It is reported that Se-containing proteins are translated without distinguishing SeMet from methionine (Met), by reading the AUG codon for Met (Ogra & Anan 2012). Reports further reveal that there are no known specific effects of Se-containing proteins as in the case of Sec containing selenoproteins (Ogra et al. 2008).

As earlier mentioned, the human genome is reported to contain 25 selenoprotein genes (Kasaikina et al. 2012). Most of the selenoproteins take part in maintaining cellular redox homeostasis and these selenoproteins include three thioredoxin reductases (TrxR-1, TrxR-2, and TrxR-3), five
glutathione peroxidases (GPx-1, GPx-2, GPx-3, GPx-4 and GPx-6), selenoprotein R(SeR, methionine sulfoxide reductase, MsrB1) and three thyroid hormone (Ts) deiodinases (DI1, DI2, and DI3) (Kasaikina et al. 2012). Although the functions of several selenoproteins have been documented (Table 1), the majority of selenoproteins have their functions not known and documented (Kasaikina et al. 2012). The specific reactions catalysed by the majority of selenoproteins are not known except for the GPxs, TrxRs, Dis, SeR and SPS2. However, the common feature shared by all selenoproteins with identified functions is their involvement in oxidoreductase reactions important in intracellular redox homeostasis and antioxidant defence (Bellinger et al. 2010, Kasaikina et al. 2012). The GPxs and SeP (N-terminal domain) are involved in reducing numerous peroxides (Takebe et al. 2002, Lubos et al. 2011) and the TrxRs and SeR are involved in the reduction of disulfides and methionine sulfoxide residues in proteins respectively (Lee et al. 2009, Lu et al. 2009, Holmgren 2010). Furthermore, selenoproteins are involved in the reductive removal of iodine from the outer ring of the prohormone thyroxine (T4) to produce several forms of thyroid hormones, the reaction is catalysed by the Se dependent thyroid hormone deiodinases (Kohrle & Gartner 2009, Marsili et al. 2011).

There are several other identified but less characterized selenoproteins that are namely Sep15, SelM, SelH, SelS, SelK, SelW, SelT and SelN and reports reveal almost no studies have been conducted on other identified selenoproteins such as SelI, SelO and SelV (Kasaikina et al. 2012). However, reports state the selenoproteins may also serve as oxidoreductases with selenocysteine found in their active cites (Kasaikina et al. 2012). Reports reveal more than half of mammalian selenoproteins are characterized by the thioredoxin-like fold, this fold being a two-layer α/β/α sandwich structure that includes a conserved CxxC motif, two cysteine residues separated by two other residues (Kasaikina et al. 2012). One of the cysteine residues can be substituted with threonine or serine in some cases. The two-layer fold is particularly common for enzymes that catalyze the formation or isomerization of disulphide bonds or carry out other functions that alter the redox state of cysteine residues (Kasaikina et al. 2012). Furthermore, the endoplasmic reticulum lumen has at least 6 out of the 25 selenoproteins residing in it, SelT, SelS, SelN, SelM, SelK, and Sep15 and an additional selenoprotein DI2 associated with endoplasmic reticulum membranes, its catalytic site facing the cytosol (Kasaikina et al. 2012). Numerous secreted selenoproteins pass through the cytosol. The endoplasmic reticulum’s enrichment with selenoproteins suggests the roles of selenoproteins in the endoplasmic reticulum-associated pathways such as protein secretion or modification (SelM and Sep15) (Labunskyy et al. 2007) and endoplasmic reticulum-associated protein degradation (SelK and SelS) (Ye et al. 2004, Shchedrina et al. 2010, Shchedrina et al. 2011).
Table 1. Mammalian selenoproteins, localization and functions (Kasaikina et al. 2012)

<table>
<thead>
<tr>
<th>Selenoprotein</th>
<th>Localization</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutathione peroxidase-1 (GPx-1)</td>
<td>Cytosol</td>
<td>- glutathione-dependent detoxification of H$_2$O$_2$ (enriched in liver, kidney, erythrocytes)</td>
</tr>
<tr>
<td>Glutathione peroxidase-2 (GPx-2)</td>
<td>Cytosol</td>
<td>- glutathione-dependent detoxification of H$_2$O$_2$ (enriched in the epithelium in intestine and lung)</td>
</tr>
<tr>
<td>Glutathione peroxidase-3 (GPx-3)</td>
<td>Plasma</td>
<td>- glutathione-dependent detoxification of H$_2$O$_2$ (synthesized predominantly by kidneys and secreted to plasma)</td>
</tr>
<tr>
<td>Glutathione peroxidase-4 (GPx-4, PHGPx)</td>
<td>Cytosol, mitochondria nucleus (testis-specific)</td>
<td>- has cystolic, nuclear and mitochondrial isoforms - protects lipids from H$_2$O$_2$-mediated oxidation</td>
</tr>
<tr>
<td>Glutathione peroxidase-6 (GPx-6)</td>
<td>Cytosol</td>
<td>- glutathione-dependent detoxification of H$_2$O$_2$ (enriched in the olfactory epithelium)</td>
</tr>
<tr>
<td>Thioredoxin reductase-1 (TrxR-1, TR1)</td>
<td>Cytosol</td>
<td>- reduces the oxidized form of cytosolic thioredoxin</td>
</tr>
<tr>
<td>Thioredoxin/glutathione reductase-2 (TrxR-2, TR2, TGR)</td>
<td>Cytosol</td>
<td>- catalyzes many reactions, specific for thioredoxin systems</td>
</tr>
<tr>
<td>Thioredoxin reductase-3 (TrxR-3, TR3)</td>
<td>Mitochondria</td>
<td>- reduces oxidized mitochondrial thioredoxin &amp; glutaredoxin 2</td>
</tr>
<tr>
<td>Thyroid hormone deiodinase 1 (DI1)</td>
<td>Plasma Membrane</td>
<td>- removes iodine from outer ring of T4 to produce plasma T3 - catalyzes deiodination and thus inactivation of T3</td>
</tr>
<tr>
<td>Thyroid hormone deiodinase 2 (DI2)</td>
<td>Endoplasmic reticulum</td>
<td>- converts T4 to T3 locally in tissues</td>
</tr>
<tr>
<td>Thyroid hormone deiodinase 3 (DI3)</td>
<td>Plasma membrane</td>
<td>- catalyzes deiodination of T4 to T3 in peripheral tissues</td>
</tr>
<tr>
<td>Selenoprotein P (SeP)</td>
<td>Plasma</td>
<td>- Se transport to peripheral tissues and antioxidant function</td>
</tr>
<tr>
<td>Selenoprotein O (SeO)</td>
<td>Mitochondria</td>
<td>- function not determined</td>
</tr>
<tr>
<td>Selenoprotein R (SeR, MsrB1)</td>
<td>Cytosol</td>
<td>- reduces methionine-R-sulfoxide residues in proteins to methionine</td>
</tr>
<tr>
<td>Selenoprotein S (SeS, SEPS1)</td>
<td>Endoplasmic reticulum</td>
<td>- up-regulated upon treatment with pro-inflammatory cytokines and glucose deprivation - endoplasmic reticulum associated protein degradation component</td>
</tr>
<tr>
<td>Selenoprotein T (SeT)</td>
<td>Endoplasmic reticulum and Golgi</td>
<td>- redox regulation, plays a role in cell adhesion</td>
</tr>
<tr>
<td>Selenophosphate synthase 2 (SPS2)</td>
<td>Cytosol</td>
<td>- synthesis of selenophosphate, involved in selenoprotein synthesis</td>
</tr>
<tr>
<td>Selenoprotein V (SeV)</td>
<td>Cytosol</td>
<td>- Trx-like fold, unknown function, expressed in spermatids</td>
</tr>
<tr>
<td>Selenoprotein W (SeW)</td>
<td>Cytosol</td>
<td>- expressed in skeletal muscle and other tissues (unknown role)</td>
</tr>
<tr>
<td>Selenoprotein K (SeK)</td>
<td>Endoplasmic reticulum membrane</td>
<td>- modulates Ca$^{2+}$ influx that affects immune cell division</td>
</tr>
<tr>
<td>Selenoprotein M (SeM)</td>
<td>Endoplasmic reticulum</td>
<td>- protects neurons from oxidative stress, Trx-like fold</td>
</tr>
<tr>
<td>Selenoprotein N (SeN, SEPN1)</td>
<td>Endoplasmic reticulum membrane</td>
<td>- controls redox state of the intracellular calcium release channel, and therefore affects Ca$^{2+}$ homeostasis</td>
</tr>
<tr>
<td>Selenoprotein H (SeH)</td>
<td>Nucleus</td>
<td>- protects cells from H$_2$O$_2$, increases mitochondrial biogenesis</td>
</tr>
</tbody>
</table>
Reports reveal that thioredoxin reductase 1 (TR1) and 3 (TR3) and glutathione peroxidase 4 (GPx-4) are essential for embryogenesis. Studies by Jakupoglu et al (2005) and Bondareva et al (2007) observed embryonic deaths between days 8.5 and 10.5 following knockout of cytosolic TR1 in mice. Soerensen et al (2008) observed that although the cardiomyocyte-specific TR1 knockout mice displayed no anomalies in development, the neuronal system (NS)-specific TR1 knockout mice displayed severe neurological anomalies, showing symptoms such as tremor and ataxia. The NS-specific knockout symptoms were a consequence of abnormal foliation, cerebellar hypoplasia, perturbed lamination and lowered proliferation of granule cell precursors in the cerebellum (Soerensen et al. 2008). The knockout in mitochondrial TR3 caused embryonic lethality between days 13.5 and 15.5 of embryonic development in mice (Conrad et al. 2004). In contrast to those of the controls, embryos of the mitochondrial TR3 knockout mice were smaller, developed anaemia and displayed elevated levels of liver apoptosis (Conrad et al. 2004). Further, although NS-specific TR3 knockout mice developed normally displaying no signs of neurodegeration, cardiomyocyte-specific TR3 knockout mice diet from heart failure a few hours following birth (Conrad et al. 2004). However, mitochondrial TR3 disruptions in B and T cells were observed not to affect functions of the immune cells and viability in mice (Geisberger et al. 2007). As observed in mice, TR3 polymorphism was observed to be associated with dilated cardiomyopathy in human beings, where both nucleotide substitutions were found in the open reading frame and part of the flavin adenine dinucleotide-binding domain (Sibbing et al. 2011).

Another essential selenoenzyme is GPx-4, represented by cytosolic (cGPx-4), mitochondrial (mGPx4-) and nuclear (nGPx-4) isoforms. Studies observed that its homozygous genetic inactivation had lethal consequences in mice embryos by day 7.5 (Yant et al. 2003, Seiler et al. 2008). Synthesis of the isoforms arises from the same gene, by alternative initiation of transcription and only differs by the sequences on their N-terminals. The expression of nGPx-4 is controlled by its own testes-specific promoter lying in the first intron of the cytosolic GPx-4 transcript (Conrad et al. 2007). As a structural component of the mitochondrial capsule of male germ cells, GPX4 is reported to play a role in sperm maturation (Ursini et al. 1999). Studies by Imai et al (2009) observed consequential infertility in mice following spermatid-specific knockout of GPX4. Furthermore, induced inactivation of GPx4 in mice and primary cells is reported to lead to an increase in 12/15-lipoxygenase-derived lipid peroxidation followed by apoptosis triggered by activation of apoptosis-inducing factor (Imai et al. 2009). Studies determined the functions of each
GPx4 isoform by preparing several knockout transgenic mouse models. It was observed that nGPx4 knockout mice developed normally with testicular structure and fertility not affected in any way, however with observations made of delayed sperm chromatin condensation (Conrad et al. 2005). It is important to point out that the sequence between the two alternative translation initiation codons corresponding to mitochondrial and cytosolic isoforms encodes a mitochondrial signal peptide. Therefore, the introduction of the in-frame stop codon between the start codons caused the specific disruption of the mGPx4 isoform without affecting the expression of cytosolic GPx4 (cGPx4) (Kasaikina et al. 2012). Schneider et al (2009) observed that mGPx4 knockout caused infertility in male mice indicating an essential role of mGPx4 in male reproduction. However, studies reveal no effect on viability and fertility following knockout of other GPxs where knockout in GPx1 displayed no significant phenotypes in mouse (Thu et al. 2010). However, GPx1 knockout mice displayed more susceptibility to oxidative stress, viral myocarditis and to reoxygenation damage due to ischemia-reperfusion injury (Thu et al. 2010). In addition, GPx2 mainly expressed in epithelial tissues, has its inactivation affecting the intestinal cells (Florian et al. 2010). Double inactivation of GPx1 and GPx2 in mice is characterized by colon pathology and severe colitis (Esworthy et al. 2001). Studies observed no significant phenotype with GPx3 knockout in mice, however the specific binding of GPx3 to the basement membranes of renal cortical proximal and distal convoluted tubules was observed (Olson et al. 2010).

Studies have also evaluated selenoprotein function in the thyroid with ambiguous findings. The inactivation of both general and liver-specific thyroid hormone deiodinase 1 (DI1) lead to no significant changes in the thyroid hormone axis (Streckfuss et al. 2005, Schneider et al. 2006). Thyroid hormone deiodinase 2 (DI2) expressed in the pituitary gland is reported to be a thyroxine (T4) sensor which it has been established to be a part of the negative feedback loop for thyroid hormone production (Rosene et al. 2010). Inactivation of DI2 displayed pituitary resistance to T4 in mice (Rosene et al. 2010). Furthermore, DI2 is reported to be significantly involved in the conversion of T4 to triiodothyronine (T3) in peripheral tissues where T3 stimulation is paramount for the development of the auditory functions (Ng et al. 2004, Williams & Bassett 2011). Reports reveal the activity of DI2 was increased in wild type mouse cochlea at post-natal day 7 and was observed to decrease at day 10 with the activity correlating with the onset of hearing (Ng et al. 2004). The finding suggests the selenoenzyme (DI2) plays a significant role in the production of local T3 for the proper development of cochlear (Campos-Barros et al. 2000). Ng et al (2004) reports that deficiency in DI2 resulted in delayed differentiation of cochlear giving rise to irreversible deafness observed in DI2 knockout mice. Further, the isoform DI3 responsible for the inactivation of T3 and T4 had its deficiency in DI3 knockout mice displaying signs of central
hypothyroidism indicating the importance of T3 degradation in maintaining the thyroid hormone axis (Hernandez et al. 2007). Ng et al (2009) reports findings of impaired auditory function in DI3 knockout mice similar to DI2 knockout mice, although with different pathogenesis. In contrast with DI2 inactivation, DI3 knockout showed an acceleration in cochlear differentiation which was also observed to lead to deafness (Ng et al. 2009). This observation suggests a critical role played by DI3 in local protection of the developing tissues from premature exposure to T3 and differentiation (Ng et al. 2009).

Non-enzymatic selenoproteins; Selenoprotein P, Selenoprotein R, Selenoprotein N

Several studies have revealed that the major function of selenoprotein P (SeP) is the transport of Se from the liver to peripheral tissues (Hill et al. 2003, Schomburg et al. 2003, Rayman 2009). The presence of SeP is thought to be vital as it influences the expression of each individual selenoprotein in different tissues (Hill et al. 2003, Hoffmann et al. 2007). The transport function of SeP explains its effective influence on other individual selenoproteins in different tissues (Hill et al. 2003). Furthermore, reports reveal SeP knockout mice developed seizures, male infertility and ataxia, all symptoms of general Se deficiency (Hill et al. 2004, Olson et al. 2005). It is reported the Se deficiency symptoms could be reversed by an increase in dietary Se, save for male infertility (Olson et al. 2005). Selenium deficiency is well studied using the SeP knockout model (Schweizer et al. 2005).

It is further reported that mice with knockout of liver-specific Sec tRNA$_{[\text{ser}\text{Sec}]}$, a unique tRNA where selenocysteine (Sec) biosynthesis occurs (liver Trsp knockout), displayed decreased expression and activity of all selenoproteins in peripheral tissues (Schweizer et al 2005). This observation confirmed the transport function of hepatic SeP (Schweizer et al. 2005). However, Se levels in the brain remained unaffected also with no displays of neurological phenotypes with liver-specific Sec tRNA$_{[\text{ser}\text{Sec}]}$ knockout mice, suggesting another essential SeP function in the brain (Schweizer et al. 2005, Kasaikina et al. 2012). Renko et al (2008) reveals that the restoration of expression of liver specific SeP in SeP knockout mice restored Se transport and eliminated the the symptoms associated with Se deficiency. This indicates that hepatocyte-derived SeP provides the major Se supply for the testes, kidneys and the brain (Renko et al. 2008). However, under Se deficiency conditions, SeP overexpression in the liver was unable to eliminate the phenotypes of SeP knockout in mice, indicating the critical need for local SeP production to support selenoprotein biosynthesis under conditions of limiting Se (Renko et al. 2008). Selenoprotein P is made up of two parts, namely the N-terminal region containing a conserved UxxC motif which is part of the domain
characterized by the thioredoxin-like fold and the C-terminal comprised of multiple Sec residues (Burk & Hill 2009). The C-terminal region of SeP plays a role in providing Se for the synthesis of other selenoproteins (Burk & Hill 2009). Burk and Hill (2009) further state that the deletion of the C-terminal part of SeP causes a much milder phenotype in contrast to the knockout of the entire selenoprotein. In addition, Hill et al (2007), states that in overall, the C-terminus of SeP plays a critical role in the transport of Se. On the other hand, the N-terminal region of SeP is involved in the reduction of reactive oxygen species production, lowering of apoptosis in liver immune cells and elevation of parasite clearance capacity of myeloid cells and increased survival (Bosschaerts et al. 2008). Furthermore, infection with African tripanosomiasis showed lower tissue injury in mice without the C-terminus part of SeP compared to the knockout of the whole selenoprotein indicating another role of the N-terminus (Bosschaerts et al. 2008).

Moving on to another selenoprotein, a knockout of selenoprotein R (SeR, MsrB1) was reported not to lead to strong phenotypes in mice (Fomenko et al. 2009). The MsrB1 knockout mice were found to be fertile and viable although their various tissues were found to have decreased level of methionine sulfoxide reductase specific for the S-diastereomer of methionine sulfoxide (MsrA) and elevated levels of malondialdehyde, protein carbonyls, protein methionine sulfoxide and oxidized glutathione (Fomenko et al. 2009). Reduced levels of free and protein bound thiols also characterised the MsrB1 knockout mice, all molecular signs indicating persistent oxidative stress in the mice (Fomenko et al. 2009). Similarly, systematic knockout of selenoprotein K (SeK) did not affect viability and reproduction in mice (Verma et al. 2011) although the mice exhibited elevated susceptibility to viral infections (Verma et al. 2011). Further, due to receptor mediated Ca$^{2+}$ flux, the inactivation of SeK in mice displayed compromised immune cells functioning including T-cell proliferation, T-cell and neutrophil migration, and Fcγ receptor-mediated oxidative burst in macrophages (Verma et al. 2011).

Looking at selenoprotein N (SeN), genetic anomalies of the selenoprotein are characterized by a human disorder known as SEPN1 related myopathy constituting of early onset muscle atrophy, muscle weakness and myotendinous contractures (Moghadaszadeh et al. 2001). The symptoms of the genetic disorder of SeN give rise to spine rigidity, severe scoliosis and a compromised respiratory system (Moghadaszadeh et al. 2001). Rederstorff et al (2011) developed and characterised a SeN deficiency mouse model and observed that although the mice with SeN inactivation displayed normal embryogenesis and growth, they exhibited limited motility and body rigidity during physical exercise. Furthermore, another observation with the SeN knockout mice at four months old, was a reduction in the pool of muscle satellite cells (SC), cells critical for adult
muscle growth and repair. Reports reveal that SeN expression is drastically elevated during regeneration of muscle followed by cardiotoxin-induced injury (Castets et al. 2011). Under similar conditions, SeP knockout mice displayed poorer regeneration that was characterised by reduced injured-to-colateral muscle ratio and excessive loss of satellite cells (Castets et al. 2011). The observations revealed the essential role of SeN in satellite cell homeostasis as consistent with findings that biopsies from patients with SEPN1 related myopathies displayed significant loss in satellite cells (Castets et al. 2011). Several other selenoproteins with unknown functions remain inadequately characterised and the development and characterisation of their respective knockout models would shed more light on the biological roles of the selenoproteins. The selenoproteins are SeW, SeV, SeT, SeS, SeO, SeI and SPS2 (Kasaikina et al. 2012, Castets et al. 2011).

1.5.0 Selenium: The immune system and inflammatory disorders

Selenoproteins and immune function

The precise role of selenoproteins in the immune system remains largely uncharacterised apart from their functions in inflammation. Proper immune physiological functioning requires an initial acute inflammatory response as poorly modulated chronic inflammation enhances the progression of many ailments such as arthritis, cancer, viral infections, autoimmune disorder, cardiovascular, metabolic and neuro-degenerative disorders (Cook et al. 1995, Wyss-Coray & Mucke 2002, Khanna et al. 2007, Hold & El-Omar 2008, Packard & Libby 2008). Inflammatory gene expression is influenced by reactive oxygen species levels (McCord 2000), therefore selenoproteins affect inflammatory responses by regulating the oxidative state of immune cells (Bellinger et al. 2010). In order for the immune cells to function optimally, glutathione peroxidases and thioredoxin reductases are needed, for controlling oxidative stress and redox regulation (Hoffmann 2007). Furthermore, specific selenoproteins have ROS-independent roles in regulating inflammatory responses (Bellinger et al. 2010). Studies reveal that mice with a T-cell specific deletion in tRNA^{Sec}, causing a knockout of all T-cell selenoproteins, display a large reduction in functional T-cells and show moderate to severe atrophy of the thymus, spleen and lymph nodes (Shrimali et al. 2008). In addition, the mice have decreased antigen-specific production of immunoglobulins in vivo, indicating a dysfunctional adaptive immune response (Shrimali et al. 2008). The deficits in selenoprotein-void T-cells are decreased TCR (T-cell receptor)-induced activation and proliferation of T-cells, and reduced TCR-induced interleukin-2 receptor (IL-2R) up-regulation and extracellular-signal-regulated kinase (ERK)
phosphorylation (Shrimali et al. 2008). Studies by Roy et al (1993) point out that IL-2R is increased in selenium supplemented mice, emphasizing the important role of selenoproteins in regulation of IL-2R. Shrimali et al (2008) reveals that most of the defects in T-cells lacking selenoproteins are reversed by applying an antioxidant N-acetylcysteine, this suggests that selenoproteins are needed for proper regulation of ROS during T-cell activation and proliferation (Shrimali et al. 2008).

Dietary selenium and immune function

Reports have stated that selenium deficiency leads to increased pathology from viral infection as a result of an inflated pro-inflammatory immune response in mice (Beck et al. 2001). Prabhu et al (2002) reveal that the inducible nitric oxide synthase (iNOS) and cyclo-oxygenase (COX)2 pro-inflammatory genes are up-regulated in selenium deficient cultured macrophages, this is done by a process dependent on the redox-sensitive transcription factor NF-κB (nuclear factor κB). Furthermore, selenium supplementation suppresses pro-inflammatory gene expression in lipopolysaccharide treated macrophages by elevating the COX1 dependent formation of 15d-PGJ2 (Bellinger et al. 2010). The 15d-PGJ2 is an endogenous inhibitor of IKKβ (inhibitor of NF-κB) that prevents phosphorylation of IκB by IKKβ and thus translocation and activation of NF-κB (Vunta et al. 2007). Furthermore, 15d-PGJ2 is also able to suppress NF-κB mediated inflammatory responses by binding to and activating the nuclear hormone receptor PPARγ (peroxisome proliferator activated receptor γ) (Ricote et al. 1998, Bailey & Gosh 2005). The findings collectively suggest that inflammatory response termination through increasing 15d-PGJ2 and decreasing NF-κB activity, is a selenium dependent and driven process (Bailey & Gosh 2005, Bellinger et al. 2010).

Selenoproteins are reported to affect viruses and the host organism immune responses (Beck et al. 1994). Studies have observed increases in CVB3 viral mutations and virulence with selenium deficiency or inactivation of glutathione peroxidase-1 in mice (Beck et al. 1994). Nelson et al (2001) also reports that RNA virus of influenza A/Bangkok/1/79 has mutations and becomes virulent when inoculated into mice deficient in selenium, compared to selenium adequate mice. Furthermore, supplementation with selenium is reported to inhibit tumour necrosis factor α (TNFα)-induced HIV replication (Hori et al. 1997). This is attributed to selenoenzyme TrxR-1’s action in reducing oxidized cysteine residues of transactivator of transcription (Tat), inhibiting efficient Tat transactivation and replication of the virus (Kalantari et al. 2008). Turto et al (2010) also reveal that Se deficiencies reduce the effectiveness of specific and non-specific components of the immune system, with cell mediated immunity and B-cell function reported to be impaired by deficiencies in Se. Animal studies by Tinggi (2008) have also demonstrated that Se deficiency results in the
disruption of the immune system function that leads to the inability of phagocytic neutrophils and macrophages to destroy harmful antigens. Reports by WHO (1996) state that at certain levels, Se has been shown to be necessary for proper functioning of the immune system. Studies by Terry et al (2000) have shown that selenium is able to inhibit the progression of the human immunodeficiency virus (HIV) to acquired immunodeficiency syndrome (AIDS). In addition, reports by Yu, Zhu and Li (1997) state that dietary selenium has a protective effect against hepatitis B virus infection in animal and epidemiological studies.

Clinical trials have investigated the therapeutic benefit of selenium for preventing excess inflammation. Forceville et al (1998) reveal that severely ill intensive care unit patients with systematic inflammatory response syndrome (SIRS) or sepsis displayed reduced levels of plasma selenium likely to increase morbidity and mortality. However, another study stated that despite the link between low levels of plasma selenium with poor clinical outcome, selenium supplementation in critical patients with sepsis or SIRS produced little improvement in clinical state (Forceville et al. 2007). Berger et al (2008) however showed that antioxidant supplementation including selenium reduced significantly the inflammatory response in major surgery or trauma patients but was not able to prevent organ dysfunction.

Selenoprotein S (SelS) is one selenoprotein identified to be involved in immune system responses, it is also called VIMP, for valosin-containing protein (VCP)-interacting membrane protein (Ye et al. 2004). Valosin-containing protein, also know an p97, is a cytosolic ATPase whose role is to retrotranslocate misfolded proteins from the endoplasmic reticulum, where they are tagged with ubiquitin and shuttled to the cell proteasome (Bar-Nun 2005). Selenoprotein S occurs in the endoplasmic reticulum membrane and is reported to functionally link p97 to another endoplasmic reticulum membrane protein called derlin, reported to aid removal of proteins from the lumen of the endoplasmic reticulum (Bar-Nun 2005, Ye et al. 2004). The secretion of SelS from liver cells and occurrence in human sera have also been revealed (Gao et al. 2007), and the expression of SelS in liver cells is modulated by inflammatory cytokines and extracellular glucose concentrations (Gao et al. 2006, Gao et al. 2007). Furthermore, SelS is reported to possess an anti-apoptotic role and reduces endoplasmic reticulum stress in peripheral macrophages (Kim et al. 2007) and brain astrocytes (Fradejas et al. 2008). A SelS polymorphism such as the change from G to A at position 105 in the SelS promoter has been observed to impair and significantly lower the expression of the selenoprotein (Curran et al. 2005). It is reported that individuals with the polymorphism displayed elevated plasma levels of inflammatory cytokines tumour necrosis factor alpha (TNFα) and IL-1β (interleukin 1β), increasing the risk of various inflammatory diseases (Curran et al. 2005). Reports
reveal that the -105 polymorphisms is linked with increased incidence of stroke in women (Silander et al. 2008), pre-eclampsia (Moses et al. 2008), coronary heart disease (Alanne et al. 2007) and gastric cancer (Shibata et al. 2009). Furthermore, the -105 polymorphism also shows epistasis with a polymorphism at -511 of IL-1β, greatly increases the risk of rheumatoid arthritis in individuals with both polymorphisms (Marinou et al. 2008). However, there was no link of polymorphisms with rheumatoid arthritis alone (Marinou et al. 2008). It must however be pointed out that other studies observed no links between polymorphisms of SelS to stroke (Hyrenbach et al. 2007), autoimmune disorders (Martinez et al. 2008) or inflammatory bowel ailments (Seiderer et al. 2007). Even though SelS polymorphisms are not known to be directly responsible for any particular disorder, they show how altered expression of one specific selenoprotein can increase the risk of multiple disorders (Bellinger et al. 2010). To shed more light, further studies on specific selenoproteins functions in immune responses need to be carried out to clarify the effects of selenium on the immune system and thereby increasing its therapeutic potential.

1.6.0 Macro fungi (mushroom) and mineral accumulation

1.6.1 Exploitation of macro-fungi (mushrooms) as mineral accumulators

Mushrooms as macro fungi are known for their excellent ability to accumulate metals and minerals from the environment in which they grow (Ingrao, Belloni & Santaroni 1992, van Elteren et al. 1998, Das 2005). The ability of mushrooms to accumulate minerals depends on the species, inhabitation, phases of mushroom growth and precipitation quantity and several types of mushroom species belonging to the genus Agaricus are known for their high accumulation of minerals (Vetter 1989). Environmentalists and eco-scientists have exploited mushroom’s ability to take up minerals and metals for use as bio-pollutant markers for trace elements and radionuclides (Fraiture, Guillitte & Lambinon 1990, Ingrao, Belloni & Santaroni 1992, Das 2005). The process of biosorption is one which microorganisms such as fungi, algae and bacteria are used to remove and recover heavy metals from aqueous solutions and has been known for decades and emerged as a low cost promising technology (Volesky 1994, Das 2005). Kapoor and Viraraghavan (1998) state that in the biosorption process, the uptake of elements occurs as a result of physico-chemical interactions of element ions with the cellular compounds of biological species such as fungi. Mushrooms being macrofungi can act as an effective biosorbent of toxic metals. They are able to grow in natural habitat with large, tough texture and other conducive characteristics needed for their development into sorbents thus removing the need for immobilization or deployment of specialized reactor

Mushroom fruiting bodies are made up of the cap (pileus) with a spore-forming part known as the sporophore, and the stipe (stem, stalk) (Das 2005). Mushrooms take up elements from growth substrates through spacious mycelium and therefore age and size of the fruiting body are of less importance (Das 2005). Furthermore, the proportion of metal contents originating from the atmospheric depositions is also of less significance because of the short-life of mushroom fruiting bodies which is normally 10-14 days (Das 2005). The elemental contents of fruiting bodies are significantly affected by the age of mycelium and by the interval between the fructifications, formation of the fruiting body (Das 2005). Thomet et al (1999) reports that elemental distribution within a fruiting body is not even, with the highest levels observed in the spore-forming part, but not in spores, of the caps, followed by the rest of the cap and the lowest elemental content found in the stipe. Vetter (1994) also states that in cultivated A.bisporus and Pleurotus ostreatus mushrooms, the majority of measured mineral elements were found to occur at higher concentrations in the caps than in stipes with few exceptions for iron, chromium, lithium, cadmium, and strontium where concentrations were observed to be roughly equal in the caps and stipes. There was only one element, sodium (Na) that occurred at higher levels in the stipe that cap (Vetter 1994). Elemental uptake by mushrooms is primarily species dependent, with the role of a genus or family being of lower importance along with the nutritional strategy-mycorrhizal, whether saprophytic or parasitic (Das 2005). In addition, the composition of the growth substrate is another important factor in elemental content of mushrooms (Das 2005). There are great differences in the uptake of individual elements. Isildak et al (2004) reported that A.bisporus mushroom absorbs heavy metals such as copper, cadmium, chromium, lead, manganese, iron, zinc and nickel with the copper level significantly higher compared to the rest of the metals. A study by Kalac et al (2004) reveal that cultivated A.bisporus absorbs less cadmium amounts compared to plants growing in the wild. The A.bisporus mushroom is also reported to be very susceptible to increasing mercury content and to a lesser extent of cadmium in growth substrate and accumulates both metals and lead in its fruiting body (Tuzen et al. 1998).

Furthermore, elemental content is observed in the initial harvest stage in the case of cultivated A.bisporus and elemental levels reported, especially metals, in wild growing A.bisporus are much higher than in the fruiting bodies of the cultivated counterparts (Kalac et al. 2004). The explanation does not lie only in different growth substrate composition and contamination, but also in different age of the mycelium, which in nature may exist for several years while only for several months in the cultivated ones (Das 2005).
The mushrooms’ characteristic of good elemental accumulation opened doors for their use as a functional food in exploiting their ability to take up elements, to deliver minerals of biological importance such as selenium. The safe and effective means to deliver selenium to human populations is through dietary selenium supplementation with Se-enriched foods. Selenium-enriched edible mushrooms such as *A. bisporus* are an attractive food source for such a purpose. Studies have in an effort to capitalize on the popularity of functional foods and nutraceuticals, and create a potential new market niche for mushrooms, developed methods to grow high-selenium mushrooms containing up to 1200 µg/g dry weight that could function as a new organic selenium source that can be used as ingredients in functional foods or in the manufacture of dietary Se supplements (Werner & Beelman 2002). Studies have demonstrated that selenium-enriched mushrooms can be cultivated by administering an inorganic form of Se such as sodium selenite salt into growth media or substrate (Turto et al. 2010, Werner & Beelman 2002), other studies have supplemented growth compost with selenized yeast (Gergely et al. 2006). The mushrooms are able to take up the inorganic selenium from the substrate and incorporate it into their own proteins as selenoproteins or Se containing proteins (Gergely et al. 2006, Ogra et al. Ishiwata 2004). We have also previously cultivated Se-enriched *A. bisporus* mushroom where Se was introduced by irrigating the mushroom mycelia and growth compost with a sodium selenite solution and the mushroom chemical characterization also revealed that the inorganic selenium was taken up by the mushroom and incorporated into the mushroom’s proteins (Maseko et al. 2013).

The study by Werner and Beelman (2002) cultivated high-selenium *A. bisporus* mushroom by adding different amounts of sodium selenite (30 to 300 µg/g d.w.) to a commercial compost supplement, added at spawing to *A. bisporus* (J. Lge) Imbach mushrooms grown in deep bags. The study observed a linear increase response in mushroom selenium uptake to the concentration of selenium added to the compost, although with a significant decrease with each flush of the crop cycle (Werner & Beelman 2002). The upper level of selenium attained in the study was 1300 µg/g (d.w) which is comparable to commercially available high-selenium yeast commonly used in the production of selenium mineral supplements (Werner & Beelman 2002). It is reported that selenium-enriched mushrooms comprising of at least 20% of the US RDA could be marketed as an excellent source of dietary selenium, however enriching the mushroom to levels above that for human direct consumption is not currently advisable (Werner & Beelman 2002). It is important to point out that there was a significant negative relationship between crop yield and *A. bisporus* at higher selenium concentrations, up to 33% reduction (Werner & Beelman 2002). However, the crop yield reduction occurred at levels significantly higher than what would be used by growers to produce *A. bisporus* for direct human consumption.
1.6.2 Selenium content in edible mushrooms

Worldwide production of cultivated edible mushrooms amounted to approximately 3 700 000 tons and about 38% of it is accounted for by *A. bisporus*, followed by *Pleurotus ostreatus* at 24.2% reveals Schmidt (1993). Several studies have analysed the mineral composition of different edible mushrooms either cultivated or growing in the wild. A study by Falandysz (2008) reviewed the selenium content of up to 190 species belonging to 21 families and 56 genera of edible mushrooms and observed that most of the species were selenium poor with levels of <1 µg Se/g dry weight. The fruiting bodies of some wild grown edible mushrooms species are reported to naturally rich in Se and their occurrence information is reviewed along with their suitability as a dietary source of Se for humans (Falandysz 2008). The impact of cooking and possibilities of leaching out, the element’s absorption rates and co-occurrence with some potentially problematic elements are also factors taken into consideration when reviewing the edible mushrooms’ selenium levels (Falandysz 2008).

The ‘Goat’s Foot’ mushroom, *Albatrellus pes-caprae* is reported to be one of the leading high selenium mushroom sources of the species surveyed, with an average selenium concentration of around 200µg Se/g dw and with maximum levels of up to 370 µg/g dw recorded (Falandysz 2008). It is further reported that several other members of the genus *Albatrellus* are also rich in selenium (Falandysz 2008). Furthermore, mushroom ‘King Bolete’, *Boletus edulis*, regarded as the most popular edible wild grown mushroom is also considered abundant in selenium, containing 20 µg Se/g dw on average and with maximum levels up to 70 µg/g dw (Falandysz 2008). Several other species of the genus *Boletus* such as *B. aereus*, *B. aestivalis*, *B. pinicola*, *B. appendiculus*, and *B. erythropolis* are also reported to accumulate high amounts of selenium (Falandysz 2008). More edible mushrooms reported to be relatively rich in selenium are the European Pine Cone Lepidella, *Amanita strobiliformis* containing on average 20 µg Se/g dw, reaching maximum levels of 37 µg/g dw and *Macrolepiota* species with average Se levels in the range of 5 to 10 µg/g dw, an exception being *M. rhacodes* with levels < 10 µg/g dw (Falandysz 2008). In addition, *Lycoperdon* species were also amongst edible mushrooms surveyed and displayed an average of approximately 5 µg Se/g dw (Falandysz 2008).

In contrast, several wild grown species of the *Agaricus* genus are reported to display a relatively lower Se content of approximately 5 µg/g dw, however the level being much greater than that of the cultivated Champignon mushroom (Falandysz 2008). These species include *A. bisporus*, the mushroom of interest in our study, *A. bitorquis*, *A. campestris*, *A. cesarea*, *A. edulis*, *A. silvicatus* and *A. macrosorus* (Falandysz 2008). Other reports reveal that the selenium content of *A. bisporus* is considerably variable ranging from 0.90 to 2.97 µg/g dw (Molnar et al. 1995). The variation in Se
levels is attributed to the different growth conditions and the Se state of compost (substrate) used for the mushroom growth (Molnar et al. 1995, Das 2005). In addition, Vetter and Lelley (2004) also evaluated selenium levels of cultivated *A. bisporus* and observed that levels varied with cultivation flushes where selenium concentrations varied between 0.46 and 5.63 µg Se/g and with the average Se content sitting at 2.82 µg Se/g. It must be pointed out that particularly rich in selenium mushrooms, can be obtained from selenium-enriched mushrooms that are cultivated on substrates fortified with selenium either as an inorganic salt (selenite) or selenized yeast as earlier mentioned in text. Reports reveal that the cultivated Se-enriched *A. bisporus* mushroom can contain up to 30 or 110 µg Se/g dw, while the selenium enriched Varnished Polypore, *Ganoderma lucidum*, is reported to contain up to 72 µg Se/g dw (Falandysz 2008). The main chemical species of selenium that make up selenium-enriched mushrooms are largely in the carpophore of the caps and they include organic selenoamino acids selenomethionine, selenocysteine, and Se-methylselenocysteine, inorganic selenite several other unidentified selenocompounds (Ogra et al. 2004, Gergely et al. 2006, Falandysz 2008, Maseko et al. 2013). The proportions of the selenium species vary widely in the selenium-enriched mushrooms mainly due to the Se status of the growth substrates.

1.7.0 Chemical characterization of selenium compounds

1.7.1 Why chemically characterise selenium species in foods

Selenium exists in many oxidation states, mostly occurring at -2, +4 and +6 oxidation states, and in a variety of inorganic and organic compounds as earlier mentioned. The chemistry of selenium is complex in the environment and living organisms, the element is essential at trace levels and toxic at greater levels. It is important to note that there is a narrow range between the selenium toxicity level and beneficial level to health (WHO 1996) with daily consumption of less than 0.1 mg/kg of body weight resulting in selenium deficiency while levels above 1mg/kg body weight are considered toxic (Wada, Kurihara & Yamazaki 1993). This narrow range between the toxicity level and beneficial level of selenium has led to a demand of high accuracy and precision in the analytical identification and measurement of selenium in its various chemical forms and oxidation states. Analysis of this kind falls under the field of elemental speciation analysis. The speciation analysis of an element is defined as the analytical activity of identifying and quantifying the actual chemical form and the chemical species of an element (B’Hymer & Caruso 2006). The speciation of an element usually requires the coupling of two techniques. Firstly, a technique that separates the element’s chemical forms under study is engaged and then a sensitive detection method to give detection of the analyte or species at low concentrations (B’Hymer et al. 2000). Method specificity
is important for selenium speciation analysis and the most usual approach for selenium speciation analysis is the use of a separation technique combined with a specific and sensitive detection system (B’Hymer & Caruso 2006). Selenium speciation studies reveal that the coupling of chromatography or capillary electrophoresis to achieve analyte separation with highly sensitive detector such as inductively coupled plasma-mass spectrometry (ICP-MS) has shown to be an effective hyphenated system (Uden 2002, B’Hymer & Caruso 2006). Various means of detection have been used to conduct selenium speciation analysis; these include ultraviolet, conductametric, inductively coupled plasma-atomic emission (ICP-AES), electrospray ionization mass spectrometry (ESI-MS) and neuron activation analysis (Uden 2002). The ICP-MS detection offers high sensitivity and relative ease of interfacing with common chromatographic and separation techniques. However, reports reveal ESI-MS is the best approach for the identification of selenium compounds (Francesconi & Sperling 2005). Although ICP-MS is elemental specific and highly sensitive with detection the technique does not provide molecular information about a sample (Ogra et al. 2004). High pressure liquid chromatography (HPLC)-ICP-MS offers the best quantitative method when reference compounds are available to compare retention times of samples with those of the commercially available or chemically synthesized reference compounds (Ogra et al. 2004, B’Hymer & Caruso 2006). Selenium species or compounds of interest that are commonly identified and quantified in speciation analytical studies are listed in table 2. The selenium containing proteins and enzymes are also of biochemical interest, these include the GPxs, TrxRs and selenoproteins including selenoprotein P already discussed in earlier text.

Given selenium’s important nutritional and biological functions in living systems as mentioned before, the element’s complete chemical characterization of its forms is crucial to understanding its metabolic processing in the body and its precise contribution to human health. Identifying and quantifying accurately the selenium species present in foods and supplements is of paramount importance as the chemical form and amount ingested determine the micro element’s bioavailability, metabolic processing and most importantly its toxicity in the body (Mahan & Kim 1996, Shiobara, Yoshida & Suzuki 1998). Furthermore, analytical speciation of organic Se species in particular as the more readily bioavailable form (Mahan & Kim 1996) linked to health and disease prevention (Clark et al. 1996, Spolar et al. 1999, Hu et al. 2008) is vital to understanding the bioavailability of the element, nature of the bioactive forms and mechanisms of operation in the biological systems they render their effects. Analytical speciation is also vital because bioavailability and toxicity of an element depend on the element’s chemical binding and properties arising from the binding (Uden et al. 2004). A clear illustration of the importance of the precise selenium species identification and quantification is that of its use in the treatment of diseases.
Studies by Ammar and Couri (1981) revealed that the median lethal dose of organic selenomethionine administered intravenously was 8.8 mg/kg whereas inorganic sodium selenite was four fold more toxic in mice in a cancer chemoprevention experiment. However, metabolic processing of the two Se chemical forms was different in another species, using the rat model; the study observed that selenomethionine was less potent and active in the inhibition of carcinogenesis compared to sodium selenite (Ammar & Couri 1981). Demonstrating that only quantifying the dosage of Se used in the treatment of disease is not enough, as it is also important to determine its bioavailability, toxicity and activity level in inhibiting development and progression of disease. Speciation analysis of Se using techniques such as HPLC-ESI-MC can help satisfy knowledge on all the parameters since it does not only quantify the element but also identifies the actual chemical form and species of the element present.

| Table 2. Inorganic and organic selenium compounds of interest in speciation analysis |
|-----------------------------------|------------------------|------------------|
| **Compound name** | **Chemical form** | **Formula** |
| Hydrogenselenide | inorganic | H$_2$Se (volatile) |
| Selenous acid (selenite) | inorganic | SeO$_2$H$_2$ (SeO$_2$$^-$) |
| Selenic acid (selenate) | inorganic | SeO$_2$H$_2$ (SeO$_2$$^-$) |
| Selenocyanate | inorganic | HSeCN |
| Trimethylselenonium cation | organic | (CH$_3$)$_3$Se$^+$ |
| Dimethylselenide | organic | (CH$_3$)$_2$Se (volatile) |
| Dimethylselenide | organic | (CH$_3$)$_2$Se-Se(CH$_3$) (volatile) |
| Dimethylseleniumsulfide | organic | (CH$_3$)Se-S(CH$_3$) (volatile) |
| Dimethylseleniumdioxide | organic | (CH$_3$)$_2$SeO$_2$ (volatile) |
| Dimethylselenopropionate | organic | (CH$_3$)$_2$Se'CH$_2$CH$_2$COOH |
| Methylselenol | organic | CH$_3$SeH |
| Methylseleninic acid | organic | CH$_3$Se(O)OH |
| Methylselenenic acid | organic | CH$_3$SeOH |
| Selenocysteine | organic | HOOCCH(NH$_2$)$_2$Se-H |
| Selenomethylcysteine | organic | HOOCCH(NH$_2$)$_2$Se-CH$_3$ |
| Selenocystine | organic | HOOCCH(NH$_2$)$_2$Se-CH$_2$CH(NH$_2$)COOH |
| Selenomethionine | organic | HOOCCH(NH$_2$)$_2$CH$_2$Se-CH$_3$ |
| Selenoethionine | organic | HOOCCH(NH$_2$)$_2$CH$_2$Se-CH$_3$ |
| γ-Glutamyl-Se-methylselenocysteine | organic | H$_2$NCH$_2$CH$_2$-CO-NHCH(COOH)CH$_2$Se-CH$_3$ |
| Selenocystathionine | organic | HOOCCH(NH$_2$)$_2$CH$_2$Se-CH$_2$CH(NH$_3$)COOH |
| Selenohomocysteine | organic | HOOCCH(NH$_2$)$_2$CH$_2$Se-H |
| Se-adenoxyxyselenohomocysteine | organic | HOOCCH(NH$_2$)$_2$CH$_2$Se-CH$_2$C$_4$H$_5$C$_3$N$_4$NH$_2$ |
| Selenosugars | organic | various structures |
| Selenoproteins | organic | various proteins and enzymes |

(B’Hymer & Caruso 2006)
CHAPTER 2

The Biochemistry of Selenoproteins

2.0 Biosynthesis of selenoproteins and selenoenzymes

Studies reveal that the production of selenium-enriched mushrooms from an inorganic source such as selenite, the mushrooms absorb up to 20-30% of the inorganic Se from the growth substrate (Du et al. 2007). The inorganic Se is then bio-transformed into organic forms preferentially integrating the Se into 56-61% proteins as either selenoproteins or selenium containing proteins (Du et al. 2007). Fruiting bodies of mushrooms grown on substrates fortified with selenium are made up of protein-bound selenoamino acids selenomethionine and selenocysteine incorporated into the primary structures of the selenoproteins (Falandysz 2008, Maseko et al. 2013). However, it must be pointed out that the family of selenoproteins (including selenoenzymes) is characterized by a unique feature, that of comprising a selenocysteine (Sec) residue providing the active centres (via the selenol group, -SeH) of the molecules, incorporated in a selenoprotein sequence (Lu & Holmgren 2009). The selenocysteine residue is incorporated into the primary structures of the selenoproteins through the nonsense (stop) UGA codon on mRNA, when a special motif the selenocysteine insertion sequence (SECIS) appears in the 3’ untranslated region of mRNA (Ogra & Anan 2012). During selenoprotein biosynthesis, a unique Sec specific selenocysteinyl transfer RNA, tRNA_{Ser}^{Sec} is used to recognize selenocysteine encoding UGA, with the stemloop structure SECIS element used to reinterpret the UGA codon for the insertion of selenocysteine instead of termination of translation occurring (Berry et al. 1993, Walczak et al. 1996). This translation of the UGA as a selenocysteine instead of its use as a termination codon is the crucial restriction point for the regulation of selenoprotein synthesis by selenium (Jameson & Diamond 2004). The selenocysteine residue is called the “21st amino acid” (Squires & Berry 2008) and its incorporation into the growing polypeptide chain of selenoproteins at the position specified by the UGA codon is cotranslational (Sunde & Raines 2011).

Furthermore, the co-translational incorporation of Sec into selenoprotein polypeptides is mediated by a multi protein complex that includes the Sec insertion sequence binding protein 2, SECISBP2 or SBP2 (Schoenmakers et al. 2010). The SBP2 gene is crucial for the co-translational incorporation of selenocysteine into selenoproteins (Schoenmakers et al. 2010) and the stemloop SECIS element in the untranslated region of mRNA serves as the binding site for SBP2 (Schoenmakers et al. 2010).
When SBP2 binds to the SECIS element, it forms a complex with the specific elongation factor for Sec tRNA\(^{[\text{Ser} \text{Sec}]}\), EF\(_{\text{Sec}}\), needed for the incorporation of Sec into selenoprotein in response to the UGA Sec codon (Turano et al. 2011). Failure of the biosynthesis mechanism can lead to miscoding of the UGA as a stop codon and depending on its location in the mRNA, the transcript may undergo ‘nonsense-mediated decay’ (NMD) affecting the translation of selenocysteine (Jameson & Diamond 2004, Schoenmakers et al. 2010). Throughout the multiple cycles of successful selenoprotein translation, the SBP2 remains bound to the non-Watson-Crick base-paired region in the stem of the SECIS element (James & Diamond 2004).

Reports reveal that twenty-five and twenty-four selenoproteins have been identified in human and mouse respectively, by bioinformatics search for SECIS in human and murine genomes (Gladyshev & Kryukov 2001, Lobanov, Hatfield & Gladyshev 2009). As already stated, true selenoproteins consist of at least one selenocysteine residue in their primary structures in a selenoprotein sequence (Lu & Holmgren 2009). However, in contrast to selenoproteins, proteins containing Se in the form of selenomethionine (SeMet) are known as selenium-containing proteins but not selenoproteins (Suzuki 2005). Reports reveal that Se-containing proteins are translated without any special coding mechanism distinguishing selenomethionine from methionine by reading the AUG codon for methionine (Ogra et al. 2008). Furthermore, Thomson (1998) in corroboration with other studies reveals that when selenomethionine is ingested, it can be readily incorporated into body tissues in a nonspecific and unregulated manner and because it is not distinguishable from methionine, it is incorporated into general body proteins. However, there are no known reports on specific biological effects of Se-containing proteins (methionine containing proteins) recognized (Ogra et al. 2008).

### 2.1.0 Selenocysteine

#### 2.1.1 Selenocysteine biosynthesis

Selenocysteine is the selenium analogue of the amino acid cysteine, formed by the removal of sulphur in the latter’s side chain replacing it with selenium (Figure 1). Selenium and sulphur are two closely related elements that belong to the same group (group 16) of the periodic table and as such share similar chemical properties. In eukaryotic cells, the enzymes selenocysteine lyases (SCLs) and cysteine desulfurases (CDs) catalyse the removal of selenium or sulphur from selenocysteine or Cystine and generally act on both substrates (Collins et al. 2012). Selenocysteine is a naturally occurring selenoprotein amino acid, the 21\(^{st}\) amino acid in the genetic code that exists in three domains of life, archaea, eubacteria and eukaryotes (Xu et al. 2007). The biosynthesis of
selenocysteine (Sec) is unique from other common amino acids in that it is the only known protein amino acid biosynthesis in eukaryotes that occurs directly on its own tRNA, designated Sec tRNA$_{[\text{Ser}]\text{Sec}}$ (Xu et al. 2007, Collins et al. 2012). It is reported to be a means of controlling its reactivity (Turanov et al. 2011, Collins et al. 2012). The biosynthesis of selenocysteine commences with the aminoacylation of tRNA$_{[\text{Ser}]\text{Sec}}$ by seryl-tRNA synthetase (SerS) in the presence of serine, ATP and Mg$^{2+}$ (Turanov et al. 2011). In eukaryotes, the enzyme Sec synthase, a pyridoxal phosphate dependent protein (Forchhammer et al. 1991, Forchhammer, Boesmiller & Bock 1991) interacts with O-phosphoseryl-tRNA$_{[\text{Ser}]\text{Sec}}$ arising from the action of the kinase O-phosphoseryl-tRNA$_{[\text{Ser}]\text{Sec}}$ kinase (PSTK) on seryl-tRNA$_{[\text{Ser}]\text{Sec}}$ (Carlson et al. 2004). Sec synthase then acts on O-phosphoseryl-tRNA$_{[\text{Ser}]\text{Sec}}$ to hydrolyze the phosphate group forming the acceptor molecule, converting it to the intermediate dehydroalanyl-tRNA$_{[\text{Ser}]\text{Sec}}$ (Xu et al. 2007). The intermediate dehydroalanyl-tRNA$_{[\text{Ser}]\text{Sec}}$ accepts the active selenium donor also identified as monoselenophosphate (synthesized by selenophosphate synthatase 2) in eukaryotes, and generates selenocysteyl-tRNA$_{[\text{Ser}]\text{Sec}}$ from the dehydroalanyl-tRNA$_{[\text{Ser}]\text{Sec}}$ (Xu et al. 2007) (Figure 4). Selenocysteine synthase then converts the active acceptor to selenocysteine (Xu et al. 2007), completing the biosynthesis of selenocysteine (Figure 4).

To understand the biosynthesis of Sec, studies relied on bioinformatics for the identification of genes involved in Sec biosynthesis and what aided the identification of the genes was that several factors were previously observed in higher vertebrates that were linked with selenium metabolism (Turanov et al. 2011). For instance, in earlier studies, a kinase that phosphorylated a minor rooster liver seryl-tRNA yielding O-phosphoreryl-tRNA (Maenpaa & Bernfield 1970) and a minor mammalian and chicken liver seryl-tRNA that recognized specifically the stop codon, UGA (Hatfield & Portugal 1970) were reported. Studies subsequently showed that the minor seryl-tRNA$_{\text{UGA}}$ formed phosphoseryl-tRNA (Hatfield, Diamond and Dudock 1982) and identified as Sec tRNA$_{[\text{Ser}]\text{Sec}}$ (Lee et al. 1989), and the kinase enzyme originally reported by Maenpaa and Bernfield (2004) was shown to be PSTK. Furthermore, a 48-kDa protein that bound Sec tRNA$_{[\text{Ser}]\text{Sec}}$ in human liver and designated the soluble liver antigen (SLA) was observed to be targeted by antibodies in patients with autoimmune chronic hepatitis (Gelpi et al. 1992). It was observed that SLA occurred in a separate family within a large superfamily of diverse pyridoxal phosphate dependent transferases (Kernebeck et al. 2001). SLA had been proposed to be the selenocysteine synthase in mammals (Kernebeck et al. 2001, Allmang & Krol 2006), and was indeed subsequently identified as selenocysteine synthase (Xu et al. 2007) crucial to the biosynthesis of selenocysteine.
2.1.2 Selenocysteine incorporation into selenoproteins

As earlier mentioned, the inclusion of selenocysteine residue in the primary structure of selenoproteins serves a crucial role as it acts as an essential active centre through the selenol group (Se-H), required for the molecules’ activity (Lu & Holmgren 2009, Reeves & Hoffman 2009). The incorporation of selenocysteine, as the 21st amino acid in the genetic code, into selenoproteins and selenoenzymes polypeptide chains is co-translational and is specified by the UGA codon that is read as a Sec codon, instead of a stop codon, in the presence of other special elements (Berry et al. 1993, Walczak et al. 1996, Ogra & Anan 2012) as discussed earlier under the biosynthesis of selenoproteins and selenoenzymes section. The incorporation of selenocysteine into selenoprotein molecules occurs via its own tRNA, the selenocysteinyl tRNA, Sec tRNA$^{[\text{Ser}]\text{Sec}}$ following its recognition of the specific UGA codon (Lu & Holmgren 2009, Turanov et al. 2011). The mechanisms required for the co-translational incorporation of Sec, responsible for identifying UGA as a Sec codon instead of a termination codon to cease protein synthesis, involve the stem-loop structure appearing in the 3’ untranslated region of selenoprotein mRNA in eukaryotes, the SECIS element (Lu & Holmgren 2009) and the SECIS binding protein (SBP2 (Reeves & Hoffmann 2009).

The SBP2 binds to the SECIS element and forms a complex with the specific elongation factor for Sec tRNA$^{[\text{Ser}]\text{Sec}}$, EF$^\text{Sec}$, enabling the incorporation of Sec into protein in response to the UGA Sec codon (Lu & Holmgren 2009). It is important to point out that since the synthesis of some selenoproteins is terminated by a UGA stop signal, the machinery for incorporating selenocysteine has the capability of differentiating between the UGA for amino acid incorporation and the UGA for termination of protein synthesis (Squires & Berry 2008). Furthermore, the distance between the Sec codon for selenocysteine incorporation and the SECIS element plays an essential role in the distinction (Squires & Berry 2008). Numerous organisms belonging to the 3 domains of life, archaea, eubacteria, and eukaryotes have the ability to use the UGA stop codon as a Sec insertion codon (Turanov et al. 2011). Studies reveal that of the >500 genomes sequenced in eubacteria, only around 20% encode the machinery for the incorporation of Sec into selenoprotein and around 10% in archaea (Zhang & Gladyshev 2009, Zhang & Gladyshev 2010). However, in eukaryotes, the selenocysteine incorporation mechanism has been reported in several lower organisms such as green algae, kinetoplastida and slime moulds and it also occurs widely in animals although absent in yeasts and higher plants (Zhang & Gladyshev 2009, Gladyshev 2010).
2.2. Selenomethionine

2.2.1 Selenomethionine biosynthesis

Selenomethionine is a naturally occurring amino acid containing selenium and the L-enantiomer of selenomethionine, known as Se-Met or SEM, is a common natural food source of selenium and is the predominant form of selenium found in Brazil nuts, cereal grains, soybeans, and grassland legumes (Whanger 2002) and macro-fungi (Ogra et al. 2004). Earlier studies initially identified selenomethionine (SeMet) in plant proteins and the selenoamino acid was also concurrently shown to be synthesized by some fungal strains of *Saccharomyces cerevisiae* and *Candida albicans* that were grown in selenium containing growth media (Schrauzer 2000). In addition, certain bacteria such as *Escherichia coli* and rumen inhabitants and marine algae also grown in Se containing media were also able to synthesize SeMet at considerable levels (Schrauzer 2000). The biosynthesis of selenomethionine occurs through a nonspecific isosteric substitution for sulphur with Se in methionine (Figure 2) and this is consequential (Unrine et al. 2007). This consequence is that proteins whose primary structure contains selenomethionine without at least a single selenocysteine residue, are not regarded as selenoproteins due to the non-specific and non-genetic coding.
substitution of sulphur for Se, and also the non-specific nature of Se utilization in the proteins concerned (Lobanov et al. 2009, Dudkiewicz et al. 2012). Cereals forage crops and fungi convert inorganic Se forms such as selenite and selenate from the soil or growth substrates into selenomethionine (Figure 3) (Schrauzer 2000, Gergely et al. 2006, Maseko et al. 2013). The selenomethionine can be incorporated into selenium containing protein in the place of methionine (Met) because tRNAMet is not able to discriminate between Met and SeMet, the only difference between the two being the identity of a single atom (Schrauzer 2000). The major pathway of SeMet synthesis in plants, marine algae and fungi is illustrated in Figure 3. It must be pointed out that SeMet is not required for growth by eukaryotes, but it is produced along with Met in quantities dependent on the amount of Se available (Schrauzer 2000, Turto et al. 2007). Reports reveal that micro-fungi such as *S. cerevisiae* yeast assimilate up to 3000 µg/g Se (Demirci 1999), with more than 90% of the total Se in the form of L-SeMet (Schrauzer 1998) and only traces of inorganic Se. Mason (1994) reveals that SeMet is synthesized analogously to Met as observed from an experiment where a mutant strain of yeast unable to synthesize methionine also failed to produce selenomethionine when grown in media containing selenium. Furthermore, studies reveal that higher fungi such as mushrooms have a Se accumulation that fairly compares closely to that of yeast. *Lentinula edodes* and *A. bisporus* are reported to contain considerable levels of selenomethionine in fruiting bodies as one of the main selenium compounds when the fungi were grown on compost enriched with inorganic Se (Turto et al. 2007, Savi‘c et al. 2009, Wu et al 2012). Wu et al (2012) states that the Se associated with protein occupied 40% of the total Se taken up by selenium-enriched *L. edodes* mushroom. Wu et al (2012) further reveals that the water soluble proteins of Se, comprised of significant selenomethionine occupying 21.65% of total Se.

2.2.2 Selenomethionine incorporation into selenium containing proteins

The translational incorporation of selenomethionine into proteins is in contrast with that of selenocysteine, in that it is not incorporated into specific locations of specific proteins as directed by the genetic code like selenocysteine. Selenomethionone is incorporated non-specifically and randomly into proteins in the place of methionine (Schrauzer 2000). As earlier mentioned, the transfer RNA of methionine does not discriminate between methionine and selenomethionine (Schrauzer 2000). The physical and chemical properties of SeMet are very similar to those of Met and it does not come as a surprise that the analog can be inserted into primary structures of protein in place of methionine by various strains of eukaryotic organisms and prokaryotes such as *E.coli* which need methionine for growth (Chen & Metzenberg 1974). The extent to which SeMet is incorporated into proteins in mushroom is dependent on some factors; the levels of methionine,
selenium dosage, selenium status and the selenium chemical forms available in the culture or growth media (Turto et al. 2007). The general trend observed is the increase in SeMet synthesis and incorporation into protein correlates with a higher Se content of growth substrates (Turto et al. 2007). It is important to point out that the replacement of Met with SeMet does not significantly change protein structure for various eukaryotic and prokaryotic organisms. It may however, influence the activity of enzymes if SeMet substitutes Met in the vicinity of the active centre (Boles et al. 1991, Schrauzer 2000). This is as a result of the CH$_3$-Se group of SeMet being more hydrophobic than the CH$_3$-S group of Met, thus substrate access may be affected, changing kinetic parameters (Schrauzer 2000). For instance, studies by Boles et al (1991) observed that the SeMet substituted thymidylate synthase in *E.coli* displayed a 40% higher specific activity than the normal enzyme containing no selenium. The same study also compared the normal enzyme’s thermal stability to that of the SeMet substituted thymidylate synthase and observed that the thermal stability of the latter was lowered eightfold and its sensitivity to dissolved oxygen was significantly increased (Boles et al. 1991). However, studies by Mechaly et al (2000) revealed that the incorporation of selenomethionine to an extra-cellular xylanase selenoenzyme produced by *Bacillus stearothermophilus*, did not significantly alter the enzyme’s catalytic activity and substrate specificity. The study demonstrated that both the native form and selenomethionine substituted xylanases exhibited similar $K_m$ values towards the substrate (Mechaly et al. 2000). The Michael’s constant, $K_m$, value characterizes an enzyme’s affinity for a substrate, therefore the $K_m$ in an enzymatic reaction refers to the substrate concentration at which the reaction rate is half its maximum speed. Mechaly et al (2000) studies were also able to show that selenomethionine containing enzyme adopted the structure of the native protein with methionine.

2.2.3 Selenomethionine in organs and tissues

Higher animals have no efficient mechanism to synthesise methionine, consequently, they are also unable to synthesize selenomethionine (Schrauzer 2000). As expected, earlier studies by Olson and Palmer (1976) detected only selenocysteine and not selenomethionine in tissue of rats supplemented with selenite. The findings raised questions as to whether SeMet played a specific essential or beneficial role in higher organisms. It has been revealed that although selenite or selenate may be utilised for the synthesis of selenoproteins, only SeMet is incorporated into body proteins (non selenoproteins) as illustrated in Figure 5. The incorporation of SeMet into body proteins allows for Se to be stored in the organism and reversibly released by normal metabolic processes, thus giving an accessibility advantage over other selenocompounds (Schrauzer 2000). Reports state that the ingested SeMet is absorbed in the small intestine via the Na$^+$-dependent neutral amino acid
transport system (Vendeland et al. 1994). Earlier studies by Hansson and Jacobsson (1966) reveal that SeMet not immediately metabolized is incorporated into organs possessing high rates of protein synthesis such as the liver, kidney, stomach, pancreas, skeletal muscles, erythrocytes and gastrointestinal mucosa in rat. The study observed that the SeMet retention was similar to that observed with $^{35}$S-methionine and $^{14}$C-phenylalanine (Hansson & Jacobsson 1966). Studies by Deagan et al (1987) observed that in rats fed a basal Se-deficient diet with 2 µg/g Se added as SeMet, the concentration of Se in muscle was 10fold that of rats fed the equivalent amount of selenite and selenocysteine. Oster, Schmiedel and Prellwitz (1988) reported that Se levels of human skeletal muscle show accordingly the dietary SeMet intakes and the pattern amongst different populations with different dietary habits had the highest in Japanese adults at 1700 ng/g, then Canadians at 370 ng/g, Americans at 240 ng/g and the lowest found in New Zealand adults at 61ng/g. Red blood cells incorporate SeMet mainly into haemoglobin (Waschulewski & Sunde 1988) and in plasma, SeMet is located mainly in the albumin proteins as was observed with Chinese men of low Se status, who had the albumin fraction containing 20% of total Se but the levels reached 47% for men in a high Se zone (Whanger & Butler 1994).

In addition, it is reported that SeMet is also significantly retained in the brain, possibly indicating that it is the active form of Se for incorporation into proteins of the brain (Groanbaek & Thorlacius-Ussing 1992). It is also reported that supplemental SeMet, in comparison to selenite and selenate has differential effects on lymphocyte proliferation and other immunological variables (Borella, Bargellini & Medici 1995). The average whole body half-lives of SeMet and selenite reported in humans are 252 and 102d respectively, showing that SeMet is used and reuses extensively (Swanson et al. 1991). Studies further reveal that SeMet supplemented animals keep up higher activities of selenoenzymes during Se depletion for much longer extended periods of time than those supplemented with selenite prior to Se depletion (Schrauzer 2000). Michalke and Schramel (1997) have reported that SeMet has also been detected in human milk, and in nursing mothers, SeMet or Se-yeast has been observed to prevent the decrease of plasma Se and GPx activity and the decline of Se in milk (McGuire et al. 1993). It was observed that the Se content of milk of mothers consuming SeMet was significantly greater than those consuming selenite (McGuire et al. 1993, Alaejos & Romero 1995).
Figure 2. Structures of amino acids Methionine (A) and Selenomethionine (B) illustrating the Se substitution.

Figure 3. The biosynthesis of selenomethionine in eukaryotes; plants, marine algae and fungi. (Schrauzer 2000).
Figure 4. Biosynthesis of selenocysteine (Sec) and de novo biosynthesis of cysteine. The biosynthesis of Sec occurs on its tRNA, and the pathway begins with the attachment of serine to Sec tRNA[Ser]Sec by seryl-tRNA synthetase (SerS) in the presence of ATP (Hatfield et al. 2014).
Figure 5. The flow diagram illustrating the metabolism of selenomethionine, selenite and selenate in eukaryotes (Schrauzer 1998).
2.3.0 Biological Activity of Organic Selenium Compounds

As earlier discussed, Se is purported to be a promising cancer chemopreventive agent basing largely on both animal and human intervention studies and human epidemiological evidence (Clark et al. 1996, Hu et al. 2008, Hu et al. 2011). However, the precise biological mechanisms by which Se exerts its chemopreventive action are not well understood. It remains unclear whether Se acts in its elemental form, through incorporation into organic compounds such as amino acids, through selenoproteins or any combination of these. The following text looks at the chemopreventive actions of the different forms of Se. The different Se compounds differ in the way in which the body processes them, and their impact on the level of risk for cancer also varies.

2.3.1 Selenoamino acids incorporated into proteins

Reports have revealed that selenoamino acid selenomethionine is easier for the human body to absorb than inorganic forms of the element and clinical trials have observed that selenomethionine is absorbed 19% more efficient than selenite (Zeng et al. 2012). Selenomethionine has exhibited anticancer activity and it is believed the anticancer activity associated with selenomethionine is due primarily to enzymatic methioninase cleavage to methylselenol (CH₃SeH), the metabolite believed to be the critical molecule involved in anti-cancer action (Zeng, Wu & Botnen 2009, Zeng et al. 2012). Reports further reveal that the human consumption of 200 µg Se per day of yeast in addition to that provided by the traditional diet, reduced mortality and decreased the incidence of all cancers as well as significantly depressing the incidence of colorectal, lung, and prostate cancers (Clark et al. 1996). However, other studies observed conflicting findings; Hu et al (2008) observed no significant effect of selenium yeast (83% Se occurs as selenomethionine, Rayman 2004) on aberrant crypt foci, oncogenesis and K-ras mutations in azoxymethane challenged mice whereas other Se organic forms (selenoproteins) displayed significant suppression of the aberrant crypt foci and oncogenesis. Selenomethionine rich Se-yeast displayed a much lower bioavailability and capacity to suppress oncogenesis compared to dairy Se, occurring as milk proteins (Hu et al. 2008). Furthermore, studies evaluated the anticancer effect of another selenoamino acid incorporated into primary sequences of protein (selenoprotein), selenocysteine. Li et al (2005) evaluated the effects of dietary supplementation with L-selenocysteine and observed that a 15 µg/g dosage of selenocysteine significantly reduced lung adenoma multiplicity in 4-(methylnitrosamino)-1-(3-pyridyl)-1-butane challenged mice. The study observed a reduction from 7.2 tumours per mouse to 4.6 tumours per mouse (Li et al. 2005). Furthermore, Spolar et al (1999) observed a significant reduction in total, anti-3, 4-dihydrodiol-1, 2-epoxide-deoxyguanosine adducts and 7,12-
dimethylbenz[a]anthracene induced DNA adducts in rats fed Se-enriched *A. bisporus* mushroom. It is known that Se-enriched *A. bisporus* is primarily made up of selenocysteine rich selenoproteins with relatively lesser amounts of free selenomethionone (Maseko et al. 2013). Studies by Lane et al. (1990) observed that dietary supplementation with selenomethionine displayed a higher enhanced effect on GPx activity compared to supplementation with selenocysteine at the same dosage in mouse liver, kidney and heart. However, the same study observed that the highest tissue Se concentration was found with selenocysteine dietary supplementation in kidney tissue suggesting that selenocysteine is more effective at tissue Se deposition than other organic Se sources (Lane et al. 1990).

### 2.3.2 Selenoproteins

Selenoproteins have indicated a more enhanced effect against carcinogenesis compared to the selenoamino acids discussed previously. A dairy source of organic Se in the form of milk selenoproteins was reported to have displayed superior bioavailability and capacity to inhibit oncogenesis compared to selenomethionine (Hu et al. 2008). Dairy Se at 1ppm was observed to significantly suppress aberrant crypt foci, colorectal oncogenesis and reduced cell proliferation and frequency of *K-ras* mutations in azoxymethane treated mice (Hu et al. 2008). Furthermore, the Se-enriched milk proteins readily increased plasma Se levels and elevated acute apoptotic response to azoxymethane in the carcinogen challenged mice (Hu et al. 2008). In correlation to studies by Hu et al (2008), McIntosh (2008) also reports a significant chemopreventive effect of Se-enriched milk proteins in an azoxymethane model of colon cancer. The study observed a 29% depression in colon tumour incidence and a 50% reduction in tumour burden (colon tumours/rat), and the effects were not observed with an equivalent Se dosage provided as selenomethionine rich Se-yeast (McIntosh 2008). The study also further observed that the Se-enriched milk proteins displayed a superior bioavailability relative to yeast Se, as indicated by the plasma Se levels, thus explaining the enhanced efficacy in chemoprevention (McIntosh 2008). In addition, McIntosh (2008) revealed that programmed cell death, apoptosis was increased in colon crypts and the height of the crypts significantly reduced with dairy Se dietary supplementation. However, reports reveal that Se yeast is able to inhibit tumorigenesis at much higher levels relative to dairy Se, where a 45% reduction in tumour burden was observed at 8ppm dietary supplementation with yeast Se (McIntosh et al. 2006). However, undesirable side effects including weight loss were reported at these higher levels of Se (McIntosh et al. 2006). The findings from the studies collectively show that dietary selenium supplementation provided as Se-enriched milk proteins is highly effective as it suppresses oncogenesis at much lower dosages compared to other organic sources.
2.3.3 Selenoamino acids not incorporated into proteins and other seleno-compounds

Studies have also associated chemopreventive action with other selenoamino acids and selenocompounds not structurally associated or incorporated into proteins and sodium selenite as well. Non-protein associated selenoamino acids such as naturally occurring methylselenocysteine (MeSeCys) found in the allium family of plants (garlic and onions) (Whanger 2002) and in mushrooms (Gergely et al. 2006, Maseko et al. 2013), Se-propylselenocysteine and Se-allylselenocysteine are reported to possess anticancer properties. A study by Ip et al (1999) evaluated the mammary cancer chemopreventive action of dietary Se-methylselenocysteine, Se-propylselenocysteine and Se-allylselenocysteine in rat treated with methylnitrosourea. All three organic selenocompounds were very well absorbed through the gastrointestinal tract and Se-allylselenocysteine exhibited the highest chemopreventive activity by causing an 86% reduction in total tumour yield (Ip et al. 1999). However, methylselenocysteine (MeSeCys) and Se-propylselenocysteine displayed similar anticancer effects but less effective, producing a decrease of 50% in tumorigenesis (Ip et al. 1999).

Studies report that the action of methylselenocysteine against tumours is its ability to stop new blood vessel formation or angiogenesis (Bhattacharya et al. 2009, Li et al. 2009). This action dramatically depresses tumour growth and improves the delivery of cancer chemotherapy drugs to the tumours (Bhattacharya et al. 2008). It is reported that methylselenocysteine has exhibited synergistic effects with different chemotherapy drugs encompassing drugs used in mitigating prostate and breast cancers (Bhattacharya et al. 2008). Another anti-cancer action of methylselenocysteine is that it down-regulates the expression of Bcl-2, a protein that slows beneficial apoptosis of cancer cells (Lee et al. 2009) rapidly inducing the appropriate destruction of cancer cells. Pan et al (2011) states that MeSeCys is able to shut down the inflammatory response prompted by the master-inflammation-inducing regulator called NF-kappaB. Suzuki et al (2010) reveals that MeSeCys works in a complimentary manner with other selenocompounds in the manner in which they all affect the body’s expression of relevant proteins playing a role in cancer prevention and suppression. Furthermore, the different selenocompounds induce death of cells in various cancer types, however each compound can be better at destroying some cancers over others (Suzuki et al. 2010). Suzuki et al (2010) reveals this difference in action by stating that protein associated L-selenomethionine is able to elevate cancer cell death by apoptosis, for instance only in cells with an intact “suicide” gene known as p53.34. While on the other hand, methylselenocysteine
induces apoptosis in mutated cells that are devoid of the p53.34 “suicide gene” mechanism (Suzuki et al. 2010).

Inorganic sodium selenite has also been reported to kill cancer cells from a variety of cancer types through several mechanisms. One of the reported anti-cancer actions of sodium selenite is through the selective generation of toxic reactive oxygen species and its targeted destruction of mitochondria occurring only in tumour cells but not in healthy tissue (Fu et al. 2011, Chen et al. 2012.). Broome et al (2004) reveal that sodium selenite is able to increase the activity of antioxidant GPx enzymes in healthy tissue thereby giving double protection. Furthermore, sodium selenite enhances the repair of damaged DNA, decreasing the risk of new cancer development (Dziaman et al. 2009) and also improves the responsiveness of the immune system increasing chances for the destruction of abnormal cells (Broome et al. 2004). The vital anti-cancer mechanism of sodium selenite is to decrease Bcl-2, a protein that is abnormally elevated in cancer cells, preventing their natural death by apoptosis (Nemec & Khaled 2008), and as a consequence sodium selenite is able to increase cancer cell death by apoptosis (Chen et al. 2012). It must be pointed out that the use of sodium selenite as a possible adjunct therapy for the treatment of cancer in patients and for use in the prevention of new or recurring cancer has been supported by numerous human studies (Broome et al. 2004, Fu et al. 2011, Chen et al. 2012).

2.4.0 Body fate of selenium

2.4.1 Absorption

The dietary fate of selenium compounds in humans can differ from that of other animals. Selenium exists mostly as oxidation states -2, +4, and +6 and it forms covalent bonds occurring in compounds C-Se and Se-S (Falandysz 2008). The processing, fate and activity of the seleno-compounds depend on their original chemical forms at the time of ingestion (Shiobara, Yoshida & Suzuki 1998, Thomson 1998). Studies have observed that the efficiency of dietary selenium absorption is variable, the variations being as a result of the different chemical forms of Se. It is important to point out that the type of foodstuff also plays a role in influencing its significance as a source of selenium and other essential elements (Falandysz 2008). In natural food, Se largely occurs as selenoaminoacids and in foods of plant origin and mushroom, Se primarily occurs as selenomethionine (Ogra et al. 2004, Falandysz 2008). Foods of animal origin and some Se-enriched mushrooms consist of Se primarily as selenocysteine (bound to protein) (Falandysz 2008, Maseko et al. 2013). The chemical forms of selenium ingested play an important role in determining not only the element’s bioavailability and absorption but also its metabolic fate, distribution, nutritional
importance, accessibility for functional selenoproteins, accumulation and toxicity (Thomson 1998, Suzuki, Doi & Suzuki 2006). The selenocysteine containing proteins of animals and some mushroom previously mentioned are the most readily available food source of selenium to humans (Allan, Lacourciere & Stadtman 1999). Selenium occurring as selenomethionine in solution is actively transported, its mechanism shared with that of methionine, with a yield of above 95% (Thomson 1998). Furthermore, the element is transported passively as inorganic selenate or selenite with yields of above 90% and around 60% respectively (Thomson 1998). It is reported that some dietary factors are able to influence the absorption rate of selenium occurring in some chemical forms, for example vitamin C is known to prevent selenite absorption (Falandysz 2008). Thomson (1998) revealed that selenium in foodstuffs such as wheat, wheat bread, fish or meat could be retained similarly in human beings as was indicated by the enhancement of glutathione peroxidase activity, although the Se absorption rate may somewhat fluctuate. Inorganic selenium compounds are absorbed and excreted much more quickly relative to the organic selenocompounds. However reports reveal that selenium ingested in the form of selenomethionine, has its clearance rate substantially slower as selenomethionine can be readily incorporated into body tissues in a nonspecific and unregulated manner (Thomson 1998). Selenomethionine absorption in humans and animals is not differentiated from that of methionine and is consequentially incorporated into general body proteins right along with methionine (Thomson 1998).

2.4.2 Metabolism

Selenium is unique in its metabolism compared to other essential elements to nutrition such as copper and zinc. Studies have reviewed the body metabolism of selenium from the viewpoint of metabolomics based on speciation studies. Studies state that both inorganic and organic chemical forms of Se able to act as nutritional sources, once absorbed by the body are transformed (reduced) to the common intermediate, hydrogen selenide (Suzuki 2005, Falandysz 2008) as illustrated in Figure 6. Inorganic selenite and selenate are simply reduced to selenide for further utilization and/or for excretion (Suzuki 2005). Hydrogen selenide is reported to be the key Se intermediate that can be used for the synthesis of selenoamino acid selenocysteine and selenoproteins (Whanger 2002, Whanger 2004). Selenium occurring in selenocysteine is almost fully ionized, enabling it to be an efficient catalyst for cells (Combs 2001). The metabolism of selenocysteine has the selenoamino acid directly lysed to selenide, while the metabolic pathway for selenomethionine involves its transformation to selenocysteine via the trans-selenation pathway, similarly to the trans-sulfuration pathway for methionine to cysteine, and then it is lysed by reduction by β-lyase to selenide (Combs 2001, Suzuki 2005, Suzuki 2006). The metabolism of Se-methylselenocysteine on
the other hand, includes the β-lyase reduction to monomethylselenol (CH₃-Se-H) which may be further methylated or demethylated back to selenide (Combs 2001, Suzuki 2006). Furthermore, selenide is reported to be transformed to selenocysteine on tRNA, and the selenocysteynyl residue can then be incorporation into selenoproteins primary structures by the codon specific to selenocysteine, UGA (Suzuki 2005) as earlier mentioned. During metabolism diverse selenium chemicals in foods are recognized as Se species and transformed to selenide and then utilized for the synthesis of selenoproteins as mentioned earlier. Excess Se is methylated stepwise to methylated Se metabolites from the common intermediate selenide, the major urinary metabolite being 1β-methylseleno-N-acetyl-D-galactosamine, a selenosugar (Suzuki 2005).

Furthermore, ingested selenium is able to bind with toxic elements such as mercury and cadmium to inhibit their toxic action and to act antagonistically with other elements such as zinc, copper, arsenic and barium (Falandysz 2008). Yoneda and Suzuki (1997) state that absorbed inorganic mercury ion and selenium form an equimolar complex that binds specifically to plasma protein but less to some other proteins. Burk and Hill (2005) state that selenoprotein P, made up of histidine and up to 10 selenocysteine residues per protein molecule bind certain metallic elements. Thomson (1998) states that selenite and other selenium forms save for selenomethionine, that are used in the biosynthesis of functional selenoproteins are under homeostatic regulation. Selenite and selenocysteine are reported to be readily excreted in the urine when consumed in excess (Thomson 1998). Suzuki, Doi and Suzuki (2006) reveal that hydrogen selenide not utilized for the biosynthesis of selenoproteins is disposed of after being bio-transformed to seleno-sugar or stepwise methylation to monomethylselenol, dimethylselenide, and trimethylselenonium ions in mammals. Trimethylselenonium ion excreted has been recognized as a good urinary metabolite marker of intake of excessive toxic doses of selenium (Suzuki 2005, Suzuki, Doi & Suzuki 2006). In addition, reports reveal that selenomethionine, selenite, selenate, selenoaminoacids and selenocholine are also excreted selenocompounds in the urine and that daily urinary excretion of Se can be as much as half of its daily intake (Thomson 1998).
2.5.0 Selenoprotein hierarchy

It is reported that following its metabolism and absorption, Se influences the expression of endogenous selenoproteins differently. Early studies observed what appeared to be some form of hierarchy in the distribution of $^{75}$Se between tissues and further observed that within a tissue there was a “molecular hierarchy” where GPx1 was recognized to be less labelled than other selenoproteins under Se deficiency conditions (Behne et al. 1988). Reports by Hill and Burk (1992) state that mRNA levels of selenoprotein P and thyroid hormone deiodinase 1 selenoproteins were observed to decrease less than GPx1 mRNA levels under conditions of Se deficiency. The differences in the mRNA expression levels of the different selenoproteins suggested that the varying regulation indicated the selenoprotein hierarchy (Hill, Lyons & Burk 1992). Furthermore, cloning studies have revealed that GPx4 activity in Se deficient rat liver fell to around 40% of Se adequate concentrations, while cytosolic GPx1 activity dropped to only 1% of the Se adequate concentrations.
levels (Lei et al. 1995). In addition, the study also observed that liver GPx4 mRNA was not downregulated by a deficiency in Se, while mRNA levels of GPx1 dropped to around 10% of Se adequate concentrations showing that there are underlying molecular mechanisms leading to the differential regulation of the selenoprotein expression (Lei et al. 1995). Studies by Richardson (2005) in corroboration of this hierarchy reveal that Se supplementation in Se deficient human subjects had GPx1 activity optimized before selenoprotein P concentration. The study findings suggested that transport and plasma protein selenoprotein P is a better indicator of Se nutritional status in humans (Richardson 2005).

2.6.0 Biochemical biomarkers of selenium status

It is known that the expression and activity of the different selenoproteins including selenoenzymes, respond to certain dietary Se levels differently placing some as better indicators of Se status over others. A study by Barnes et al (2009) observed that when weaning rats are placed on a Se deficient diet at 0.005µg Se/g, liver Se concentrations dropped to below 5% of levels in Se adequate rats (0.24 µg Se/g diet). It was observed that supplementation with Se lead to a sigmoidal response in the liver Se levels, with a plateau breakpoint observed at dosages of 0.08 µg Se/g diet and the plateau in the liver Se concentration extending from 0.08 to 0.24 µg Se/g diet (Barnes et al. 2009). However, Se supplementation at supernutritional levels of 0.4 and 0.8 µg Se/g diet had liver Se concentration increasing above the plateau and were observed to be 70% higher at the 0.8 µg Se/g diet compared to rats on the 0.08-0.24 µg Se/g (Barnes et al. 2009, Schriever et al. 2009). Studies by Schriever et al (2009) and Barnes et al (2009) reveal that liver GPx1 activity in rats deficient in Se, also reduced to 2% of Se adequate levels. The studies further observed that with graded Se supplementation the GPx1 activity in the liver increases sigmoidally to a defined plateau with a breakpoint identified at approximately 0.09 µg Se/g diet (Barnes et al. 2009, Schriever et al. 2009).

The earlier studies (Cohen et al. 1985, Weiss et al. 1997) in correlation with proceeding findings from Barnes et al (2009) and Schriever et al (2009) established that a Se requirement of 0.1 µg Se/g diet as the minimum dietary Se needed to achieve plateau levels of GPx1 activity in rat liver. However, in contrast to liver Se concentration, Se supplementation at supernutritional levels above 0.1 µg Se/g diet do not further increase liver GPx1 activity (Schriever et al. 2009). Reports reveal similar Se responses with other selenoproteins, plasma GPx3 activity (Cohen et al. 1985), liver thioredoxin reductase-1 (Hadley & Sunde 2001), selenoprotein P levels (Yang, Hill & Burk 1989), thyroid hormone deiodinase activity (Bermano et. Al. 1995) and selenoprotein W levels (Yeh et al.
where the minimum dietary requirement to achieve plateau levels was 0.1 µg Se/g diet. However, unlike other selenoproteins, liver GPx4 activity only decreased under Se deficiency conditions to 25-40% of Se adequate levels and reached plateau levels at a lesser 0.05 µg Se/g diet (Lei et al. 1995). It must be pointed out that collectively, the regulation of these selenoproteins as traditional biomarkers by Se status in the diet provide excellent tools for assessing Se status and requirements over the range from Se deficiency to Se adequacy.

Studies by Saedi et al (1988) identifies GPx1 as a reliable molecular biology based biomarker of Se status after observing a dramatic drop in mRNA levels in Se deficient rats and a sigmoidal increase in mRNA levels with increasing dietary Se. Findings by Weiss et al (1996) reveal that dietary requirements based on hepatic GPx1 mRNA levels are approximately 0.05 µg Se/g diet for both genders in rats although female rats displayed twice the level of liver GPx1 mRNA and GPx1 activity relative to the males. However, in contrast, GPx4 mRNA levels (Lei et al. 1995), and selenoprotein P mRNA levels (Weiss et al. 1996, Weiss et al. 1997) were observed not to be regulated by Se status in rats. This suggests the blocking of the use of GPx4 and SeP mRNA as molecular biomarkers for assessing accurately the Se requirements (Sunde & Raines 2011).
2.7.0 Why this Project?

2.7.1 Project Rationale

The rationale behind the project is based on the epidemiological evidence that indicates a strong correlation between Se status and cancer at the population level. Intervention studies with both animals and humans have further shown that organic Se taken at exaggerated levels is associated with reduced incidence of certain cancers; colorectal (our main focus), lung, liver and prostate. It is believed that the anti-cancer effect of Se comes about through the action of selenoenzymes and selenoproteins in reducing oxidative stress in the body, where organic Se (selenocysteine) is an essential structural component of the molecules for activity. However, more research into the role of Se in cancer chemoprevention needs to be conducted because the precise contribution of different organic Se sources in their anti-cancer mechanism is contradictory in some cases and still remains ill understood. For instance, rodents or pigs supplemented with the different sources of organic Se, dairy-Se (milk proteins) and yeast-Se in their diet showed differences in cytosolic GPx-1 activity and also displayed differential gene expression of cytosolic GPx-1, gastrointestinal GPx-2, and TrxR-1 selenoenzymes and selenoprotein P, SeP. All selenoproteins are of relevance to the anticancer function of the gastrointestinal tract. There is a need to clearly understand the benefits of Se beyond the nutritional ones and the biological mechanisms by which selenium is beneficial to mammalian health and able to prevent disease. The research findings are of paramount importance to the functional food research community and to the health and medical sectors. There is a considerable potential to provide the health and medical sectors with a food source chemopreventive alternative to treating cancer outside the intensive use of drugs.

2.7.2 Research Gaps

As earlier mentioned that the anti-cancer effect are believed to come about through the action of antioxidant selenoenzymes and selenoproteins in reducing oxidative stress in the body, boosting the level of expression of these molecules is associated with more enhanced protection against oxidative stress. Several studies have biologically evaluated the effect of dietary supplementation with organic Se sources, yeast-Se and dairy-Se on the gene expression and activity of gastrointestinal tract selenoprotein markers GPx-1, GPx-2, TrxR-1 selenoenzymes, and selenoprotein SeP. The two organic Se sources regulated the selenoprotein expression and activity differently using the rodent and pig models. It is clearly deduced from the studies that the organic
Se source is an important factor in determining the bioactivity of Se. These findings will be discussed in detail in the later text.

It is not known how the macro-fungal source of organic Se in the form of selenium-enriched Agaricus bisporus mushroom compares with the other organic Se sources (yeast Se, dairy Se) in terms of regulating the gene expression and enzyme activity of the genes (GPx-1, GPx-2, TrxR-1, and SeP) relevant to the protection of the gut from oxidative stress and cancer. This research program sought to address this gap in knowledge by using the rat model to evaluate the effect of organic Se dietary supplementation in the form of Se-enriched A. bisporus mushroom on the gut antioxidant selenoprotein status markers, GPx-1, GPx-2, TrxR-1 and SeP. The research program also additionally sought to evaluate the effect of further supplementing the Se-enriched diet (from mushroom Se) with α-tocopherol and the effect of hyperthermally induced oxidative stress on the regulation of the selenoprotein status markers in the colon.

2.7.3 Project Justification

The organic Se species of cultivated selenium-enriched A. bisporus mushroom have been chemically characterized and identified by previous studies in literature. However, the mushroom organic Se species have not been biologically evaluated to demonstrate their bioactive effect on the antioxidant selenoprotein status, linked to health benefits and disease prevention in humans. The research program explores the potential for developing a possible market niche for Se-enriched mushroom where commercial mushroom growers produce high Se A. bisporus mushroom for the direct consumption. Alternatively, the research could potentially present a high Se mushroom species with bioactive properties, which could be used as a ‘new’ organic Se source in the manufacture of dietary Se supplements or as a value added ingredient in the formulation of functional foods or nutraceuticals.

2.7.4 Hypotheses

The research hypotheses are that;

“Selenium-enriched A. bisporus mushroom has a significant effect on the antioxidant selenoprotein status in rats”. 
“Dietary supplementation with α-tocopherol to the high Se diet has a significant effect on the regulation of the antioxidant selenoproteins in rats”.

“Dietary supplementation with Se-enriched A.bisprous mushroom in a high Se diet has a significant effect on the regulation of the antioxidant selenoproteins in rats”.

“Hyperthermally induced oxidative stress has a significant effect on the antioxidant selenoprotein status in rats which can be corrected by dietary supplementation with Se-enriched A.bisprous mushroom”.

“Hyperthermally induced oxidative stress has a significant effect on the gut mucosal permeability in rats which can be corrected by dietary supplementation with Se-enriched A.bisporus mushroom.

2.7.5 Objectives

To test the hypotheses, the research program is made up of two main objectives:

1. Chemical characterization of Se and speciation of organic Se species in cultivated Se-enriched Agaricus bisporus white button mushroom

2. Biological evaluation of the effect of the cultivated Se-enriched Agaricus bisporus mushroom on the antioxidant selenoprotein status and gut permeability of mammals (using a rat model);

   a) The effect of Se alone and α-tocopherol supplementation on the Se effect will be evaluated
   b) The effect of Se-enriched A.bisporus on the antioxidant selenoprotein status will be evaluated under thermoneutral and hyperthermally induced oxidative stress conditions
   c) The effect of Se-enriched A.bisporus mushroom on gut mucosal permeability under hyperthermally induced oxidative stress conditions will be evaluated
2.7.6 Aims

To test the hypotheses, the aims of the research project were to;

i. Chemically characterize quantitatively and qualitatively and speciate the organic Se compounds formed in fruiting bodies of cultivated Se-enriched *Agaricus bisporus* mushroom. This involves;

   a. Cultivation of Se-enriched *A. bisporus* mushroom in compost irrigated with sodium selenite solution
   b. Quantification of total Se content expressed as unit Se per dry weight of mushroom and as unit Se per protein mass contained in the mushroom
   c. Speciation analysis to identify and quantify the main selenoamino acids species of biological interest in the cultivated Se-enriched *A. bisporus* mushroom; selenomethionine, selenocysteine, and methylselenocysteine

ii. a. Biologically evaluate antioxidant selenoprotein status in rats supplemented with the Se-enriched *A. bisporus* mushroom in their diet, with or without additional supplementation with α-tocopherol, and subjected to hyperthermally induced oxidative stress.

ii. b. Biologically evaluate the effect of dietary supplementation with mushroom Se on ileum mucosal permeability of rats subjected to hyperthermally induced oxidative stress.

The antioxidant selenoprotein status markers evaluated are as follows;

   a. GPx-1 mRNA expression and GPx-1 enzyme activity
   b. GPx-2 mRNA expression
   c. TrxR-1 mRNA expression
   d. SeP mRNA expression

iii. Biologically evaluate the effects of a high Se diet in the form of the Se-enriched *A. bisporus* mushroom with or without additional α-tocopherol supplementation on gut (ileum) epithelium permeability in hyperthermally induced oxidative stress in rat.
CHAPTER 3

The cultivation of selenium-enriched Agaricus bisporus mushroom and chemical characterization and speciation of organic selenium species in the cultivated selenium-enriched Agaricus bisporus mushroom

Chapter published as;


(Original article attached in Appendix).
Chapter 3 addressed the following **Aim**:

i. Chemical characterization, qualitatively and quantitatively and speciation of the organic Se compounds formed in fruiting bodies of cultivated Se-enriched *Agaricus bisporus* mushroom.

   The aim involved:

   a. Cultivation of Se-enriched *A.bisporus* mushroom in compost irrigated with sodium selenite solution
   b. Quantification of total Se content expressed as unit Se per dry weight of mushroom and as per protein mass contained in mushroom
   c. Speciation analysis to identify and quantify the main selenoamino acids species of interest in the Se-enriched mushroom; selenomethionine, selenocysteine, methylselenocysteine

The interest in the qualitative and quantitative chemical characterization and speciation of the organic Se compounds formed in fruiting bodies of cultivated Se-enriched *Agaricus bisporus* mushroom is due to the awareness that organic Se from different food sources displays differential biological properties. The different organic Se sources show varying bioavailability, effect on the regulation of the antioxidant selenoprotein of interest with our study (GPx-1, GPx-2, TrxR-1 and SeP), anti-cancer, anti-inflammatory and other health properties. Furthermore, the quantities of the organic Se species present in the food sources influence their bioavailability and bioactive effects upon consumption in the diet. For these reasons, there is a need to fully characterise the various organic Se forms present in selenium-enriched foods to understand their potential health effects.

Although literature indicates that previous studies have chemically characterized and identified the organic Se species comprised by various selenized food sources including mushrooms of various species, precise quantifications of each organic Se compound detected were not availed. As such, one of the objectives of our study sought to address the gap in quantifying the organic Se compounds identified in our cultivated Se-enriched *A.bisporus* mushroom. The cultivation of the Se-enriched *A.bisporus* mushroom was carried out using commercial mushroom grower kits that comprised compost inoculated with fungal spawn and peat moss to serve as casing layer and a sodium selenite solution was used for irrigation to deliver the Se to the growing mushrooms. The cultivated Se-enriched mushrooms were subjected to irrigation with differently concentrated sodium selenite solutions. The sodium selenite solution that yielded the highest Se accumulation in the selenized mushrooms was used to produce the Se-enriched mushrooms used in the formulation of rat feeds for the biological evaluation of mushroom Se studies (Chapters 4, 5, & 6).
ABSTRACT

The selenium concentration in *Agaricus bisporus* cultivated in growth compost irrigated with sodium selenite solution increased by 28- and 43-fold compared to the control mushroom irrigated solely with water. Selenium contents of mushroom proteins increased from 13.8 to 60.1 and 14.1 to 137µg Se/g in caps and stalks from control and selenised mushrooms, respectively. Selenocystine (SeCys; detected as [SeCys]$_2$ dimer), selenomethionine (SeMet), and methyl-selenocysteine (MeSeCys) were separated, identified and quantified by liquid chromatography–electrospray ionisation-mass spectrometry from water solubilised and acetone precipitated proteins, and significant increases were observed for the selenised mushrooms. The maximum selenoamino acids concentration in caps and stalks of control/selenised mushrooms was 4.16/9.65µg/g dried weight (DW) for SeCys, 0.08/0.58µg/g DW for SeMet, and 0.031/0.10µg/g DW for MeSeCys, respectively. The most notable result was the much higher levels of SeCys accumulated by *A. bisporus* compared to SeMet and MeSeCys, for both control and selenised *A. bisporus*.

1. Introduction

Selenium is an essential micronutrient required at trace levels for human health (Ponnampalam, Jayasooriya, Dunshea, & Gill, 2009). Se occurs in different chemical forms which determine the element’s nutritional requirement, metabolic processing, bioavailability and toxicity in the body. The inorganic forms are mostly selenite (SeO$_3^{2-}$) and selenate (SeO$_4^{2-}$) salts, while the known organic forms are selenoamino acids, selenopeptides and selenoproteins. Organic Se shows a higher bioavailability as it is readily absorbed in animal digestive tracts and also has higher threshold for toxicity compared to inorganic Se (Uglietta et al., 2008, Amoako, Uden, & Tyson, 2009). Furthermore, the health benefits have been linked to organic Se compounds which constitute a component of the body’s endogenous antioxidant defence system, protecting cellular components such as cell membranes, lipids, lipoproteins and DNA from oxidative damage by free radicals, reactive oxygen and reactive nitrogen species (Ponnampalam et al., 2009). Such oxidative damage is linked to the development of cardiovascular and neurodegenerative diseases, inflammatory ailments, and cancers. Several experimental animal and human intervention studies have shown the anti-cancer effect of organic selenium in the form of selenised yeast (Spolar, Schaffer, Beelman, & Milner, 1999, Hu, McIntosh, Le Leu, & Young, 2010), selenised milk (Hu, McIntosh, Le Leu, Woodman, & Young, 2008, Hu et al., 2010, 2011), selenocysteine (SeCys) and selenomethionine (SeMet) (Uden, Boakye, Kahakachchi, & Tyson, 2004, B’Hymer & Caruso, 2006, Amoako et al., 2009, Hudson et al., 2011).
There are at least 30 selenoprotein species in mammalian systems involved in the Se-antioxidant defense system (Lobanov, Hatfield, & Gladyshev, 2009). The selenoproteins include the biologically important antioxidant selenoenzymes, glutathione per-oxidases (GPx), thioredoxin reductases (TrxR) and iodothyronine 50-deiodinases (IDI). However, the Se health effects are not limited to its antioxidant role with reports indicating that Se deficiencies reduce the effectiveness of specific and non-specific components of the immune system (Tinggi, 2007), and facilitate the progression of human immunodeficiency virus infection to acquired immunodeficiency syndrome (Lobanov et al., 2009). While Se can be acquired from the diet, as it occurs naturally in a wide range of foods such as nuts, eggs, fish, liver, chicken, garlic, and red meat, its abundance in most natural and common processed foods is low. Typical dietary intake of Se in USA is estimated to be between 80 and 120 µg Se/day (Amoako et al., 2009), but intake is substantially less in many regions of the world including Australia (National Health and Medical Research Council, 2006). Concern for potential deficiency diseases associated with a low Se status has led to the establishment of the recommended daily requirements for Se in many countries. The Australian National Health and Medical Research Council (NHMRC) recommends Se daily intakes of 60–70 and 30 µg for Australian adults and children, respectively (NHMRC, 2006). Bio-fortification of selected agricultural foods with Se is an effective strategy to increase Se status in a population that may be at risk of Se deficiency-related diseases with a safe, bioavailable and bioactive organic Se. While Se enriched yeasts are currently commercially available as supplements, fortification of other food sources with Se would help to alleviate Se deficiency in the population, by making more Se-fortified food choices available.

Mushrooms are known to be an excellent accumulator of minerals from the environment in which they grow (Tinggi, 2007). Mushrooms are macro-fungi with distinctive and visible fruiting bodies that may grow above or below ground. The fruiting body or cap (carpophores, mycocarp) is mostly above the ground of higher fungi and formed from spacious underground myella (hyphae) by the process of frutification. Since mushrooms contain significant amounts of proteins ranging from 16.5% to 39% dry weight, it is expected that mushroom protein fractions would contain high levels of organic Se. However, reports indicate that most wild growing and farm edible mushroom species including Agaricus bisporus are poor Se sources with a concentration of less than 1 µg Se/g dw (Turto, Gutkowska, Herold, Klimaszewska, & Suchockl, 2010). This is largely due to the poor Se content of the soil from which the mushrooms are cultivated. However, because of the mushroom’s ability to incorporate large amounts of heavy elements in their fruiting bodies, their Se content can be increased through cultivation on growth substrates fortified with Se either as an inorganic salt or as selenised yeasts. Werner and Beelman (2002) developed a method of growing A.bisporus with a
Se concentration of 1200 µg/g dw to function as a ‘new’ organic source of Se and to serve as an ingredient in the development of functional food products or for use in the manufacture of dietary supplements. Furthermore, interest in fungal (mushroom) organic Se is due to our awareness that organic Se from different food sources shows different bioavailability, anti-cancer and other health properties. For this reason there is a need to fully characterise the various organic Se forms to understand their potential health effects. For example, organic Se in mammalian cow milk is more bioavailable and more effective against chemically-induced colorectal cancer in an animal model than yeast organic Se (McIntosh 2008, Uglietta et al., 2008, Ponnampalam et al., 2009).

We report in this paper on the incorporation of Se in various forms into the most consumed and cultivatable button mushroom, A. bisporus.

2. Materials and methods

2.1. Materials

Acetone (CHROMASOLV® Plus grade), acetonitrile (LC–MS hypergrade), formic acid (LC–MS Ultra grade), hydrogen peroxide (30%, v/v), methyl-selenocysteine hydrochloride (synonym Se-(methyl) selenocysteine hydrochloride, MeSeCys·HCl; Cat No. M6680), nitric acid (69%, v/v), proteinase K (Tritirachium album; Cat No. P8044), sodium selenite (Na$_2$SeO$_3$; Cat No. 214485), selenocystine ([CysSe]$_2$; Cat No. S1650), D,L-selenomethionine (SeMet; Cat No. S3875), trypsin (porcine pancreas, Cat No T4799), Tris-(2-carboxyethyl)-phosphine hydrochloride (TCEP), Tris (hydro-xymethyl) aminomethane hydrochloride (Tris–HCl) and ascorbic acid were purchased from Sigma–Aldrich (Australia). BioRad® Lowry Protein Assay kit was purchased from BioRad (USA). All other reagents used were of analytical grade. 6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) was synthesised according to Cohen, Liu, Sanuda-Pena, Harvey-White, and Kalra (1998). Deionised water (18.2 MO) was produced using a Synergy UV Millipore System (Millipore) and was used throughout.

2.2. Cultivation of Se-enriched A.bisporus

White button mushroom grower kits were obtained from a local commercial mushroom grower for the cultivation of Se-enriched mushrooms. Each mushroom grower kit weighed approximately 2kg and comprised compost inoculated with A. bisporus spawn and peat moss to serve as a casing layer and supporting mycelia and mushroom growth. The mushrooms were grown under controlled
conditions in growth chambers of a glass house. Growth compost was kept moist and humid at all times with water sprays twice a day. Inoculated compost was kept under dark conditions and the temperature was initially held constant at 18 °C with high carbon dioxide levels to encourage mycelia growth and to induce fruiting body formation. Once the fruiting bodies started to appear (visible as small white pin-heads) on the surface of the compost, the temperature was adjusted to 20°C, carbon dioxide levels were reduced and irrigation with 10, 20 or 40 mg Se/l solutions (as Na₂SeO₃ prepared in deionised water) was administered on the surface of the compost as sprays using a spray bottle. The young mushroom ‘pins’ and ‘buttons’ were irrigated with the selenite solution twice daily until they reached maturity for harvesting. A separate mushroom grower kit, to serve as the control, was subjected to similar growth conditions, except for the irrigation process, which was done with deionised water only. Mushrooms were allowed to grow to 200–400 g (cap + stalk) before harvesting. The harvested mushrooms that had been irrigated with the water and the 3 Se concentrations were separated into caps and stalks, frozen at -80°C overnight, and then freeze-dried. The lyophilised caps and stalk samples were grounded separately into fine powders using a commercial blender and stored at room temperature (RT) in moisture free sealable packs until required for chemical analysis.

2.3. Determination of total Se in mushrooms

Nitric acid/hydrogen peroxide digestion of mushrooms was performed according to Gergely, Kubachka, Mounicou, Fodor, and Caruso (2006). Dried mushroom powder (0.1 g) from caps and stalks was weighed separately into digester tubes (10 ml). 1 ml of nitric acid (69%, v/v) was added to digester tubes and the mixture left for 24 h at RT. The tubes were then positioned inside a heating block for digestion at 60°C for 1 h followed by 110°C for 5 h. After cooling, 2 ml of hydrogen peroxide (30%, v/v) was added to the tubes and further heating was carried out for 1 h at 80°C. Reduction of Se (VI) to Se (IV) with concentrated HCl was performed according to Corns, Stockwell, Ebdon, and Hill (1993). Digests were diluted with 4 M HCl to a final volume of 10 ml. Sample blanks of non-irrigated mushrooms were also digested and treated in the same manner. Four replicate determinations were performed for each mushroom caps or stalks sample. Digested samples were further diluted 1:10 or higher with 50% HCl (v:v) prior to analysis. Selenium was analysed by hydride generation atomic fluorescence spectroscopy (PSA 10.055 Millennium Excaliber System, Exeter, UK). The instrument was fitted with a selenium lamp as a radiation source. Standard instrument conditions included a sample delay time of 10 s, analysis time of 40 s and memory time of 60 s. Argon was supplied at 30 psi with the shield gas flow rate of 0.3 l min⁻¹, carrier gas flow rate of 0.3 l min⁻¹ and a dryer gas flow rate of 2.5 l min⁻¹. A calibration curve was
established using solutions of Se (VI) pre-reduced to Se (IV) within a range of 0–20 ng Se/g. Digested samples were pre-treated in the same way as the standard solutions and then analysed. Selenium spiked digest samples and blanks were in-cluded for quality control via spike and recovery. Limit of detection was 0.1 ng Se/g dw.

2.4. Extraction of mushroom proteins

Mushroom proteins were extracted according to Gergely et al. (2006). 0.5 g of cap and stalk powder were weighed and transferred into separate 100 ml conical flasks. Then, 50 ml of 30 mM Tris–HCl (pH 7.5) was added to the samples. The samples were extracted by stirring with a magnetic flea and stirrer for 24 h at RT. The extracts were then centrifuged at 6000 rpm for 100 min and the supernatant transferred into fresh tubes. Solubilised proteins were precipitated with acetone added to 90% (v/v), according to Gergely et al. (2006). Samples were kept at 4 °C for 48 h in a fridge to precipitate high and low MW proteins, and the precipitates were collected by centrifugation at 6000 rpm for 30 min and then dried in a stream of N₂. The precipitated proteins were then solubilised in 2 ml 30 mM Tris–HCl (pH 7.5) and three replicate samples were prepared from each mushroom sample.

2.5. Protein determination

Protein quantitative determinations were performed using BioRad® Lowry Protein Assay kit. Bovine serum albumin was used as a protein standard.

2.6. Determination of the Se content in mushroom proteins

The Se content of the acetone precipitated proteins from mushroom caps and stalks extracts was determined by AFS. Samples were subjected to nitric acid and hydrogen peroxide heat digestion as described in Section 2.3 for mushroom powders. Three replicates determination were performed for each protein sample.
2.7. Organic Se speciation of mushroom proteins

2.7.1. Enzymatic hydrolysis of proteins

To identify selenoamino acid species in mushroom proteins, the acetone precipitated proteins from cap and stalk extracts were subjected to exhaustive enzymatic digestions according to Gergely et al. (2006). To a solution of protein (1 ml, 1.6–2.3 mg protein), 30 mg of trypsin was added and the mixture incubated at 50°C for 24 h, with continuous agitation on a sample shaker. 30 mg of Proteinase K was then added to the mixture which was kept at 50°C for another 24 h with continuous agitation. The digest was then centrifuged at 5000 rpm for 30 min, the supernatant was collected and three replicates were analysed by liquid chromatography–electrospray ionisation–mass spectrometry (LC–ESI–MS) for the identification and quantification of Se amino acid species. The analytical procedures are outlined below.

2.7.2. Amine derivatisation of amino acids

Enzyme hydrolysed protein samples (25 µl) were mixed indi-vidually with 75 µl LC–MS hypergrade acetonitrile in an Eppendorf tube and the solutions were mixed vigorously using a vortex mixer for 2 min. 50 µl of deionised water was then added to each solution which was then centrifuged at 6000 rpm, for 5 min, at room temperature to precipitate insoluble compounds. The amino acids in the supernatants were then subjected to derivatisation using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) according to Boughton et al. (2011), in order to facilitate their retention on a reverse phase LC column. A 10 µl aliquot of each supernatant was mixed with 70µl of 200 mM borate buffer (pH 8.8) containing 10 mM TECP and 1 mM ascorbic acid (to minimise oxidation) in an 1.5 ml Eppendorf tube. The solution was mixed using a vortex mixer for 1 min. A 20 µl aliquot of the derivation reagent (AQC) was added to each sample, mixed vigorously using a vortex mixer, and incubated with agitation using a shaker at 55°C for 10 min. Samples were then centrifuged for 5 min and the clear solutions transferred into glass vials for LC–ESI–MS analysis.

2.7.3. Se and selenoamino acids standards

For total Se determination, Se calibration standards were prepared by serial dilution of a 1.00 mg Se/l stock solution (as Na2SeO3) with 4 M HCl to prepare Se standard solutions of 100, 50, 25, 12.5 and 6.25 µg Se/l. A standard stock solution of selenoamino acids at 2.50 mM each was prepared by
dissolving 41.8 mg SeCys–SeCys, 24.5 mg SeMet and 27.3 mg MeSeCys in 50.0 ml of 1 mM Tris-(2-carboxyethyl)–phosphine hydrochloride (TCEPH) buffer, pH 8.8. Calibration standards containing 100, 50, 10, 5 and 1 µM of each selenoamino acid were made up by appropriate dilutions using deionised water.

2.7.4. LC–ESI–MS

The LC–ESI–MS analytical procedure was carried out according to Boughton et al. (2011). Derivatised samples (1 µl each) were analysed by HPLC using gradient elution with water (A) and aceto-nitrile (B) mobile phases (0 min, 99% A + 1% B; 9 min, 85% A + 15% B; 14 min, 70% A + 30% B; 14.1 min, 99% A + 1% B). Both mobile phases contained 0.1% formic acid. An Agilent Zorbax Eclipse XDB-C18 Rapid Resolution HT 2.1 x 50 mm, 1.8µm column was used and the flow rate was 300µl/min. The temperature was maintained at 30°C.

An Agilent 1200 LC-system coupled to an Agilent 6410b ESI-triple quadrupole MS was used for the detection of the selenoamino acids. The following source conditions were used: sheath gas temperature – 315°C, gas flow – 10 l/min, nebuliser pressure – 45 psi, and capillary voltage – 3800 V. Selenium has a distinct isotope pattern, the most abundant isotope was chosen for quantification (Table 1). Positive ion multiple reaction monitoring (MRM) was used to measure the intensity of the eluting selenoamino acid derivatives (Table 1).

2.7.5. Quantification of selenoamino acids

Quantification of SeMet, (SeCys)₂ and MeSeCys was achieved using the external calibration curve method. Calibration curves within the concentration range of 0.05–5µM were prepared for each of the selenoamino acids, SeMet, (SeCys)₂ and MeSeCys, i.e. SeMet y= 19692x + 1826.5, R²= 0.9999; (SeCys)₂ y= 1280.4x + 383.38, R²= 0.9972; MeSeCys y= 13318x + 34299, R²= 0.9580, where y is peak area and x is concentration (µM).The amount in mass (µg) in the mushroom was then calculated taking into account the molecular weight of the selenoamino acid species, the dried weight of mushroom used, the volume of mushroom sample and the volume analysed by LC–ESI–MS.

2.8. Statistical analysis

Statistical analyses were performed using Genstat for linear and quadratic response to level of Se in irrigation water as well as differences between parts, and by one way ANOVA followed by the
Fisher’s protected Least Significant Difference Test using SAS software version 9.2. Uncertainty of the mean was reported to 2 significant figures according to the European Analytical Chemist guidelines (Ellison & Williams, 2012).

**Table 1**

LC–MS MRM table for the internal standard (2-aminobutyric acid) and Se-amino acids.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Precursor ion (mV)</th>
<th>Product ion (mV)</th>
<th>Fragmentor (mV)</th>
<th>Collision energy (mV)</th>
</tr>
</thead>
<tbody>
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<td>2-aminobutyric acid</td>
<td>274.1</td>
<td>171</td>
<td>140</td>
<td>25</td>
</tr>
<tr>
<td>Se-cysteine</td>
<td>339.1</td>
<td>171</td>
<td>140</td>
<td>20</td>
</tr>
<tr>
<td>Se-cystine</td>
<td>340.1</td>
<td>171</td>
<td>140</td>
<td>20</td>
</tr>
<tr>
<td>Methy-SeCysteine</td>
<td>354.1</td>
<td>171</td>
<td>140</td>
<td>20</td>
</tr>
<tr>
<td>Se-methionine</td>
<td>368.1</td>
<td>171</td>
<td>140</td>
<td>20</td>
</tr>
</tbody>
</table>
3. Results and discussions

3.1. Selenium content of whole mushrooms

Selenium accumulated in both the caps and stalks of cultivated mushrooms. The Se content in control *A. bisporus* grown by irrigation with deionised water only was determined to be 12.2 µg Se/g in dried caps and 9.6 µg Se/g in dried stalks (Fig. 1). Compared to the control mushrooms, there was a significant increase in the Se content in both mushrooms caps and stalks grown by irrigation with 10, 20 and 40 mg Se/l solutions as Na$_2$SeO$_3$. The Se content of the caps increased in a quadratic fashion reaching a maximum of 347 µg Se/g (at 20 mg Se/l irrigation), while the Se content of the stalks increased linearly across the range of Se concentrations investigated reaching 415 µg Se/g (at 40 mg Se/l irrigation) (Fig. 1). This corresponded to a maximum 28- and 43-fold increase in Se content in Na$_2$SeO$_3$ irrigated mushroom caps and stalks, respectively.

Wild mushrooms display variable concentrations of Se depending on the species, compost and growth substrate conditions such as nutrient content and availability. Thus, the Se content in wild growing *Agaricus* species such as *A. bisporus*, *Agaricus edulis*, *Agaricus bitorquis*, *Agaricus silvaticus* and *Agaricus macrosporus* showed considerably variation ranging from 0.9–5 µg Se/g dw (Spolar et al., 1999, Falandysz, 2008). Other edible wild growing mushrooms such as *Boletus edulis* and *Albatrellus pescaprae* were reported to contain 20–70 and 200 µg Se/g dw, respectively (Falandysz 2008). Cultivated mushrooms also display variable concentrations of Se that is dependent on the species and growing conditions. Cultivated *A. bisporus* has been reported to contain from 0.46–5.63 µg Se/g dw (Vetter & Lelley, 2004) to 770.7 µg Se/g dw (Gergely et al., 2006). Cultivated *Lentinula edodes* has been reported to contain 43.0 µg Se/g dw (Gergely et al., 2006). Thus, the Se level of our cultivated *A. bisporus* was at the high end of the literature values for mushrooms.

Studies on the elemental composition and distribution of fruiting bodies of *A. bisporus* not limited to Se alone generally reveal caps as higher accumulators compared to stalks (Vetter & Lelley, 2004). The cap/stalk Se level ratio for our cultivated *A. bisporus* ranged from 0.75–3.05 depending on the Se irrigation level, with the 20 mg Se/l irrigation showing the highest ratio (Fig. 1). However, elemental distribution in *Lentinula*, *Psathyrella* and *Termitomyces* showed higher content for stalks than caps (Alofe, Odeyemi, & Oke, 1996). These differences are most likely due to species variation, compost composition, ‘flush cycle’, and developmental stage (time of harvest) of the mushrooms (Alofe et al., 1996, Vetter & Lelley, 2004).
The protein content of the control mushrooms was 24 mg/g dw for caps and 18 mg/g dw for stalks, respectively (Fig. 2A). The protein yields were lower than what was generally reported in the literature for mushrooms (Van Elteren, Woroniecka, & Kroon 1998, Gergely et al., 2006). This finding can be attributed to the higher biomass of our cultivated mushrooms producing a dilution effect. The protein content of the caps increased linearly with increasing level of Se in irrigation water to 51 mg/g dw at 20 mg Se/l (Fig. 2A). This seems to suggest that at low concentrations of Se in control mushrooms there was an inhibition of protein deposition or conversely high levels of Se stimulate protein deposition in irrigated mushrooms. The increase in cap protein content may also be attributed to the observed retardation in cap size (smaller biomass) corresponded to an increased protein density as compared to the control due to the selenite irrigation during cultivation. The change in protein content in the stalks was affected to a lesser extent compared to the control (Fig. 2A).

The Se concentrations of the cap and stalk proteins in the control mushrooms were similar with values of 13.8 and 14.1 µg Se/g protein, respectively (Fig. 2B). Se irrigation during growth also increased the incorporation of Se into mushroom cap and stalk proteins, and the increase was in a quadratic fashion reaching a maximum of 60.1 µg Se/g protein (at 20 mg Se/l irrigation) for caps and 137 µg Se/g protein (at 40 mg Se/l irrigation) for stalks (Fig. 2B). This corresponded to a 4.3- and 9.7-fold increase compared to control caps and stalks, respectively. However, the proportion of protein-Se to total-Se (organic plus inorganic) in mushrooms decreased from 2.7% and 2.6% in control to 0.90% and 0.36% in Se irrigated caps and stalks, respectively. This indicates that the level of accumulation of non-protein associated Se (which is mainly inorganic Se, see below) is higher than the accumulation of protein associated Se.
**Fig. 1.** Selenium concentration in *A. bisporus* at different Se (as sodium selenite) irrigation levels. Error bars indicate SD of the mean (n= 3). Different letters a–d (caps) or numbers i–iii (stalks) indicate significant differences (P< 0.05).
Fig 2. Concentrations of water soluble proteins in *A. bisporus* mushroom caps and stalks (A), and of Se in these proteins (B) for different Se (as sodium selenite) irrigation levels. Error bars indicate SD of the mean (n= 3). Different letters a–b (caps) or numbers i–iv (stalks) within A or B indicate significant differences (P< 0.05).
Selenium speciation studies have identified SeMet (Ogra, Ishiwata, Encinar, Lobinski, & Suzuki, 2004, Gergely et al., 2006, Huerta, Sanchez, & Sanz-Medel, 2006), SeCys (Yoshida et al., 2005, Gergely et al., 2006, Turto, Gutkowska, & Malinowska 2007) and MeSeCys (Yoshida et al., 2005, Gergely et al., 2006) in Se-enriched mushrooms. However, information on the quantities of the individual selenoamino acids and their distribution in caps and stalks are rather limited. Our analysis of the organic Se species using LC–ESI–MS showed that 3 selenoamino acid species, SeMet, (SeCys)$_2$, and MeSeCys (where the Se hydrogen is replaced with a methyl group; Fig. 3) were accumulated by A. bisporus (Fig. 4). However, (SeCys)$_2$ is likely to be an artefact product of the oxidation of SeCys to its dimer as SeCys was not detected in the mushrooms. A significant increase in the content of selenoamino acids in selenoproteins was observed for Se irrigated mushrooms compared to the control. In addition, stalks generally displayed a higher concentration of selenoamino acids per g protein, compared to the caps across all irrigation treatments. The concentration of selenoamino acids determined in control caps and stalks was 3.3 and 4.5 µg SeMet/g protein, 347 and 593 µg (SeCys)$_2$/g protein and 1.4 and 3.4 µg MeSeCys/g protein, respectively (Fig. 5). The Se irrigation increased the concentration of selenoamino acids in caps and stalks to the following levels: 11.4 and 9.6 µg SeMet/g protein and at 20 and 40 µg Se/l irrigation, respectively; 585 and 1202 µg (SeCys)$_2$/g protein and at 10 and 40 µg Se/l irrigation, respectively; 1.9 and 5.3 µg MeSeCys/g protein at 20 µg Se/l irrigation (Fig. 5). The maximum fold increase of the individual selenoamino acids for caps and stalks as a result of Se irrigation was 3.4-and 2.1-fold for SeMet, 1.6- and 2.0-fold for (SeCys)$_2$ and 1.3-and 1.5-fold for MeSeCys, respectively.

On a dry weight basis caps contained higher levels of selenoamino acids across all irrigation treatments (Table 2). The maximum observed increase in the content of selenoamino acids in dry caps and stalks compared to controls was 7.2- and 2.9-fold for SeMet at 20 µg Se/l irrigation, 2.3- and 2.0-fold for SeCys (calculated using the (SeCys)$_2$ contents) at 10 µg Se/l irrigation, and 3.2- and 2.3-fold for MeSeCys at 20 µg Se/l irrigation, respectively. This study also looked for free selenoamino acids not associated with the acetone protein precipitates and such acids have not been detected (data not shown), thus indicating that the non-protein associated Se in the mushrooms as detected by AFS is likely to be inorganic Se.

The most notable result obtained in the present study was the much higher levels of SeCys (detected as (SeCys)$_2$), accumulated by A. bisporus, compared to SeMet and MeSeCys. This finding differs from the results reported by Turto et al. (2007) and Wu, Ding, Li, Zhang, and Wu (2012) on the
detection of SeMet as the main selenoamino acid in *A. bisporus* and *L. edodes* mushrooms grown on compost enriched with inorganic Se. The reason for these discrepancies is unclear at present, but is likely due to the efficiency of the different methods used in the extraction and recovery of selenoproteins, and in the quantification of the selenoamino acids. Regarding the synthesis of SeMet it is known that Se is converted into SeMet and is then incorporated non-specifically and randomly into proteins in the place of Met because the aminoacyl tRNA synthetase is not able to discriminate between Met and Se-Met (Schrauzer, 2000, Lobanov et al., 2009). However, the isosteric substitution of sulphur with Se in Met could limit the SeMet biological impact, thus proteins whose primary structure contains Se-Met without at least a single SeCys residue are generally not regarded as true selenoproteins (Lobanov et al., 2009). MeSeCys occurs as a free water soluble selenoamino acid not structurally incorporated in selenoproteins, but is associated with the acetone precipitated soluble protein fraction as shown by our study and by others (Gergely et al., 2006).

By contrast, the synthesis of SeCys and its incorporation into proteins is unique in that it is not directly loaded onto its cognate RNA, but is instead synthesised directly as SeCys-tRNA by the modification of Ser-tRNA (Collins et al., 2012). The SeCys residue functions as an active site amino acid in selenoenzymes such as glutathione peroxidase and thioredoxin reductase and provides antioxidant properties in non-enzyme selenoproteins. Both classes of organic Se compounds are of significant biological importance in reducing cellular oxidative stress (Ogra & Anan, 2012). Thus, the high level of SeCys in Se-enriched mushrooms could provide better biological benefits than Se-enriched yeast which contains mainly SeMet (Schrauzer 2000, Turto et al., 2007, Amoako et al., 2009, Behne, Alber & Kyriakopoulos 2009, Wu et al., 2012).
Fig. 3. Structures of selenoamino acids studied.
Fig. 4. LC–ESI–MS chromatograms of (A) Se standards containing Se-Met, MeSeCys and Se-Cystine ((SeCys)₂), and (B) enzymatic digest of acetone precipitated water soluble proteins of A. bisporus. The eluting position of SeCys was determined independently. SeCys was generated by reducing Se-Cystine with TECEP and ascorbic acid.
**Fig. 5.** SeMet (A), Se-Cystine (B) and MeSeCys (C) contents of water soluble proteins of *A. bisporus* grown at different Se (as sodium selenite) irrigation levels. Error bars indicate SD of the mean (n= 3). Different letters (for caps) and numbers (for stalks) within A, B or C indicate significant differences P < 0.05).
Table 2

Total selenoaminoacids in mushrooms

<table>
<thead>
<tr>
<th>Irrigation (µg/g)</th>
<th>Control</th>
<th>10mg Se/L&lt;sup&gt;a&lt;/sup&gt;</th>
<th>20mg Se/L&lt;sup&gt;a&lt;/sup&gt;</th>
<th>40mg Se/L&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Caps</td>
<td>Stalks</td>
<td>Caps</td>
<td>Stalks</td>
</tr>
<tr>
<td>SeMet</td>
<td>0.08</td>
<td>0.081</td>
<td>0.11</td>
<td>0.10</td>
</tr>
<tr>
<td>±0.014</td>
<td>±0.0048</td>
<td>±0.023 ±0.027</td>
<td>±0.25</td>
<td>±0.019</td>
</tr>
<tr>
<td>SeCys</td>
<td>4.16</td>
<td>5.35</td>
<td>9.6</td>
<td>10.8</td>
</tr>
<tr>
<td>±0.15</td>
<td>±0.88</td>
<td>±1.4 ±1.3</td>
<td>±0.22</td>
<td>±3.0</td>
</tr>
<tr>
<td>MeSeCys</td>
<td>0.031</td>
<td>0.061</td>
<td>0.04</td>
<td>0.038</td>
</tr>
<tr>
<td>±0.0084</td>
<td>±0.028</td>
<td>±0.021 ±0.021</td>
<td>±0.022</td>
<td>±0.075</td>
</tr>
</tbody>
</table>

<sup>a</sup> Se concentration (as sodium selenite) in irrigation solution. Control mushrooms were irrigated with deionised water instead.

<sup>b</sup> µg Selenoamino acid/g dried mushrooms. Values and uncertainties were calculated using data from Fig. 5 and % weight of protein in mushroom caps or stalks, and expressed as dried weight of caps or stalks.

<sup>c</sup> Calculated as half of the (SeCys)<sub>2</sub> mass content
4. Conclusion

We showed that SeCys was the main selenoamino acid assimilated into water soluble proteins by *A. bisporus* cultivated in growth compost and that irrigation with up to 40 mg Se/l as sodium selenite solution increased its assimilation into proteins. Beside SeCys, SeMet was also detected in the irrigated mushrooms and assimilation into proteins also increased with selenite irrigation, albeit at a much lower level than SeCys. MeSeCys, a non-protein selenoamino acid, was precipitated with the protein fraction with acetone and also occurred at a lower level. No free non-protein SeCys or SeMet was detected in the mushrooms studied. The high level of SeCys containing selenoproteins raises the possibility that Se-enriched *A. bisporus* might provide a different anti-cancer effect compared to Se-enriched yeast which is known to contain up to 90% of its organic Se as free SeMet.

Acknowledgements

We would like to acknowledge Mr. Jim Fuller (Spawn Lab Team Leader, Mushroom Exchange Pty. Ltd., 45 Cookes Road, Mernda, VICTORIA, AUSTRALIA, 3754) contribution in supplying the mushroom growing kits and advice on the cultivation of the *A. bisporus*. 
CHAPTER 4

The effect of dietary supplementation of selenium in the form of Se-enriched Agaricus.bisporus mushroom on colonic antioxidant selenoprotein status in rat.

Chapter published as;


(Original article attached in Appendix).
Chapter 4 addressed the following **Aim**:

i. Biological evaluation of antioxidant selenoprotein status in the colon of rats whose diet was supplemented with the cultivated Se-enriched *A.bisporus* mushroom, and kept under thermoneutral conditions.

The antioxidant selenoprotein status markers evaluated were as follows:

a. GPx-1 mRNA expression and enzyme activity  
b. GPx-2 mRNA expression  
c. TrxR-1 mRNA expression  
d. SeP mRNA expression

It is known from previous studies that dietary supplementation with organic Se up-regulates the colonic antioxidant selenoproteins that are of particular relevance to anti-cancer function in the gastrointestinal tract (Uglietta et al. 2007, Hu et al. 2010, 2011). It is believed that the anti-cancer effect of the antioxidant selenoproteins comes about through their action of neutralizing reactive oxygen species and nitrogen species, inhibiting them from reacting with cellular components, thus reducing oxidative stress (Gromadzinska et al 2008). However, studies have demonstrated that dietary supplementation with different organic Se sources regulate the expression of the antioxidant selenoproteins differently in different mammalian species. Rodent and pig models from previous studies showed differences in cytosolic (GPx-1), gastrointestinal (GPx-2) glutathione peroxidases, gastrointestinal thioredoxin reductase (TrxR-1) and selenoprotein P (SeP) regulation and level of expression following dietary supplementation with dairy or yeast organic Se (Uglietta et al. 2007, 2008, Hu et al. 2010). Chapter four presents the findings of our evaluation of the effect of macro fungal organic Se in the form of Se-enriched mushroom proteins (chemically characterized in chapter 3) on the status (regulation) of the colonic selenoproteins GPx-1, GPx-2, TrxR-1 and SeP in rat. The chapter seeks to compare and contrast the biological effect of dietary supplementation with mushroom Se on the selenoprotein status to that of dairy Se and yeast Se from previous studies. The chapter addresses the existing gap in knowledge as previous studies have not evaluated the effect of mushroom organic Se on the regulation of the antioxidant selenoproteins linked to health and disease prevention.
ABSTRACT

The effect of dietary supplementation with Se-enriched Agaricus bisporus on cytosolic glutathione peroxidase-1 (GPx-1), gastrointestinal specific glutathione peroxidase-2 (GPx-2), thioredoxin reductase-1 (TrxR-1) and selenoprotein P (SeP) mRNA expression and GPx-1 enzyme activity in rat colon was examined. Rats were fed for 5 weeks with control diet (0.15 µg Se/g feed) or Se-enriched diet fortified with selenised mushroom (1µg Se/g feed). The mRNA expression levels were found to be significantly (P< 0.01) up-regulated by 1.65-fold and 2.3-fold for GPx-1 and GPx-2, respectively, but were not significantly different for TrxR-1 and SeP between the 2 diet treatments. The up-regulation of GPx-1 mRNA expression was consistent with GPX-1 activity level, which was significantly (P< 0.05) increased by 1.77-fold in rats fed with the Se-enriched diet compared to the control diet. The results showed that selenised A. bisporus can positively increase GPx-1 and GPx-2 gene expression and GPx-1 enzyme activity in rat colon.

1. Introduction

Selenium is an essential trace element, a micronutrient required for a number of physiological functions, health and disease prevention in humans (Clark et al., 1996, Flores-Mateo, Navas-Acien, Pastor-Barriuso, & Guallar, 2006). Selenium can be acquired from the diet as it occurs naturally in certain foods such as nuts, eggs, fish, liver, chicken, garlic and some species of mushrooms. The Food and Drug Administration (USA) and the Australian National Health and Medical Research Council (NHMRC) recommend dietary allowances for adults of Se at 55 µg and 70 µg per day, respectively. However, epidemiological and intervention studies have demonstrated that higher Se intake above nutritional recommendations offer enhanced protection against cardiovascular and inflammations ailments, and reduced the incidence of colorectal, lung, prostate and liver cancers (Spolar, Schaffer, Beelman, & Milner, 1999, Diwadkar-Navsariwala & Diamond, 2004, Hu et al., 2011). Se occurs in 2 chemical forms, organic and inorganic. The chemical form of Se is the determining factor of the element’s bioavailability, biological efficacy, metabolic processing and toxicity (Mahan & Kim, 1996, Shiobara, Yoshida, & Suzuki, 1998). Organic Se occurs in foods mainly as free or protein-bound selenomethionine (SeMet), selenocysteine (SeCys) and methylselenocysteine (MeSe-Cys). Inorganic Se occurs in foods mainly as selenite (SeO\(^2\)-) and selenate (SeO\(^4\)-) salts at very lowlevels (Amoako, Uden, & Tyson, 2009). Organic Se forms are preferred for dietary purposes because they have higher bioavailability and bioactivity and a much lower toxicity level compared to inorganic Se (Turto, Gutkowska, Herold, Klimaszewska, & Suchockl, 2010). As
such, the qualitative and quantitative Se speciation analysis in determining the selenium chemical forms of Se-enriched foods for supplement use is important (Maseko et al., 2013).

Organic Se has been linked to beneficial biological effects and disease prevention (Clark et al., 1996, Flores-Mateo et al., 2006). It is believed that the health benefits of organic Se come about through the incorporation of the element into selenoproteins, and the role of these selenoproteins in reducing cellular oxidative stress arising from free radical damage (Holben & Smith, 1999, Gromadzinska, Reszka, Bruzelius, Wasowicz, & Akesson, 2008). There are at least 30 selenoproteins identified in mammalian systems of which a number are reported to be linked to anticancer function in the gastrointestinal tract (Gromadzinska et al., 2008). The selenoproteins include the ubiquitous and biologically important antioxidant selenoenzymes, glutathione peroxidases (GPxs), thioredoxin reductases (TrxRs) and iodothyronine deiodinases (IDIs) (Holben & Smith, 1999). However, the Se health effects are not limited to its antioxidant role as Se deficiencies reduce the effectiveness of specific and non-specific components of the immune system (Tinggi, 2008) and facilitate the progression of human immunodeficiency virus infection to acquired immune deficiency syndrome (Tinggi, 2008).

While epidemiological studies have provided strong correlation between Se status and cancer at population level (Clark et al., 1996), intervention studies with both animals and humans showed that organic Se intake from different Se sources influenced the activity and expression of the enzyme and non-enzyme selenoproteins differently even at similar doses. Studies by Lane, Strength, Johnson, and White (1991) and Behne, Alber, and Kyriakopoulos (2009) reported that liver GPx-1 activity of rodents was significantly increased after intra-peritoneal administration of 3 µg Se-Met/kg or diet supplementation with 1.5 mg Se/kg of a Se-enriched yeast, respectively, and found no such responses with supplementation with SeCys and selenite salt. Hu, McIntosh, Le Leu, and Young (2010) observed no significant changes in cellular glutathione peroxidase (GPx-1) gene expression and activity, but a significant up-regulation of gastrointestinal specific glutathione peroxidase 2 (GPx-2) and a dose dependent up-regulation in selenoprotein P (SeP) gene expression as a response to Se-enriched milk proteins supplementation. In contrast, similar doses of Se-enriched yeast supplementation significantly increased GPx-1 expression and activity but with no effect on GPx-2 (Hu et al., 2010). Human clinical trials further demonstrate differences in the regulation of selenoproteins genes expression from different organic sources. Studies by Hu et al. (2011) report a significantly higher up-regulation in rectal GPx-1 and GPx-2 gene expression as a response to Se-enriched milk proteins supplementation compared to Se-enriched yeast in human subjects. Both Se-enriched milk proteins and Se-enriched yeast significantly increased SeP mRNA
levels in rectum biopsies of human subjects, although only the Se-enriched milk proteins sustained SeP mRNA expression after a ‘wash out’ period by withdrawing Se supplementation (Hu et al., 2011).

We have previously reported on the characterisation of the organic Se species in cultivated Se-enriched Agaricus bisporus and found that it contained high levels of SeCys containing selenoproteins (Maseko et al., 2013), which differed from Se-enriched yeast and Se-enriched dairy proteins. This raised the possibility that Se-enriched mushroom might produce a different biological effect. Selenium enriched mushrooms could compliment the commercially available Se-enriched yeast as an alternative source of functional Se for dietary and health purposes. In this paper, we report on the effect of supplementing Se in rat feeds with our Se-enriched A. bisporus on the expression of colonic GPx-1, GPx-2, TrxR-1 and SeP and the activity of colonic GPx-1 in rats to provide evidence for its potential anti-cancer use.

2. Materials and methods

2.1. Materials

Sodium selenite (Na₂SeO₃; Cat no. 214485), 2-amino-2-hydroxymethyl-propane-1,3-diol hydrochloride (Tris-HCl), ethylenediamine-tetraacetic acid disodium salt (EDTANa₂), glutathione peroxidase 1 (GPx-1; Cat no. G6137), immunoglobulin G (IgG; Cat no. I4506), dithiothreitol and sodium hydrogen phosphate were purchased from Sigma–Aldrich. Deionised water (18.2 MX) was produced using a Synergy UV Millipore System (Millipore) and was used throughout.

2.2. Cultivation of Se-enriched A. bisporus mushroom

Selenium-enriched A. bisporus mushroom was cultivated using white button mushroom grower kits supplied by a local commercial mushroom grower (Mr. Jim Fuller, Spawn Lab Team Leader, Mushroom Exchange Pty. Ltd., 45 Cookes Road, Mernda, Victoria, Australia, 3754). Each mushroom grower kit weighed approximately 2 kg and comprised of compost inoculated with A. bisporus spawn and peat moss to serve as a casing layer and supporting mycelia and mushroom growth. The mushrooms were grown under controlled conditions in growth chambers of a glass house. Growth compost was kept moist and humid at all times with water sprays twice a day. Inoculated compost was kept under dark conditions and the temperature was initially held constant.
at 18°C with high carbon dioxide levels to encourage mycelia growth and to induce fruiting body formation. Once fruiting bodies started to appear (visible as small white pin-heads) on the surface of the compost, the temperature was adjusted to 20°C, carbon dioxide levels were reduced and irrigation with 40 mg/l Se solutions (as Na$_2$SeO$_3$ prepared in deionised water) was administered on the surface of the compost as sprays using a spray bottle. The young mushroom ‘pins’ and ‘buttons’ were irrigated with the selenite solution twice daily until they reached maturity for harvesting. A separate mushroom grower kit was used to produce the control mushroom under similar growth conditions, except irrigation was done with deionised water only. Mushrooms were allowed to grow to 200–400 g (cap + stalk) before harvesting. Mushroom caps were separated from the harvested mushrooms, frozen at -80°C overnight and then freeze-dried. The lyophilised caps were ground into fine powders using a commercial blender and stored at room temperature (RT) in moisture free sealable packs until required for preparation of rat feeds.

2.3. Preparation of control and Se-enriched mushroom rat feed pellets

Rat feed pellets were prepared by Specialty Feeds Co (3150, Great Eastern Highway, Glen Forrest, Western Australia, 6071). Control mushroom feed pellets were prepared by supplementing a low Se rodent feed formulation AIN 93G (20 kg) with 59 g control mushroom caps containing 12.2µg Se/g dried caps to give a final Se content of 0.15 µg Se/g feeds in control feeds. Se-enriched mushroom feed pellets were prepared by supplementing the low Se rodent feed formulation AIN 93G (20 kg) with 59 g of a Se-enriched mushroom caps containing 300 µg Se/g dried caps to give a final Se content of 1 µg Se/g feeds in Se-enriched feeds. The total Se content in dried mushroom caps was determined by subjecting the dried mushroom caps (0.1 g) to a nitric acid (69% v/v) (1 ml) and hydrogen peroxide (30%; v/v) (2 ml) digestion according to Maseko et al. (2013). The digests were made to volume (10 ml) with 4 M HCl and analysed for total Se content using hydride generation atomic fluorescence spectroscopy (PSA 10.055 Millennium Excaliber System, Exeter, UK). The composition of the formulated control and Se-enriched feeds diet are listed in (Table 1).

2.4. Animals

A total of twenty-six 9 week old Sprague Dawley male rats weighing between 217 and 326 g were obtained from the Monash University Animal Services, Melbourne, Australia. The animal experiment protocols were approved by the Melbourne School of Land & Environment Research Animal Ethics Committee, University of Melbourne (ethics approval no. 1112154.1). Rats were randomly divided into 2 experimental groups and housed in cages (3 per cage). The cages had dust
free woodchip and shredded newspaper bedding, which was changed weekly to ensure a clean and healthy cage environment. The rats were housed in an air-conditioned, temperature controlled animal facility with a 12 h light–dark cycle at 21°C. Rats were given free access to food and water at all times.

2.5. Rat feeding and harvesting of colon tissue

Rats were randomly assigned to 2 experimental diets of control feeds (0.15 µg Se/g feeds;n= 12) and Se enriched feeds (1 µg Se/g feeds;n= 14) groups. All rats were at first acclimatised with free access to control diet and water for one week before the animals were given their respective control or Se-enriched diets and water at *ad libitum* for a further period of 5 weeks. Body weights of the animals were recorded at the start of the experiment and continued weekly in order to monitor their growth, which was normal. Their food intake and behaviour were also monitored throughout the experiment, which were also normal. Animals were sacrificed after the feeding period with an initial step of a single intra-peritoneal injection of ketamine and xylazine mix to anaesthetise and an overdose of the ketamine and xylazine mix as the final euthanasia step. Colon tissue was recovered from each rat and a portion was placed in RNAlater® solution at 4°C for 24 h before storage at -80°C until real time PCR analysis. The remaining colon tissue from the rat was rapidly frozen in liquid N₂ and stored at -80°C for GPx-1 activity assay.
### Table 1

Composition of experimental diets.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Control diet (mg/g feeds)</th>
<th>Se-enriched diet (mg/g feeds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein (acid)</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Canola oil</td>
<td>70</td>
<td>70</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Wheat starch</td>
<td>407</td>
<td>407</td>
</tr>
<tr>
<td>Dextrinised starch</td>
<td>132</td>
<td>132</td>
</tr>
<tr>
<td>DL methionine</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>LR calcium carbonate</td>
<td>10.4</td>
<td>10.4</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>2.6</td>
<td>2.6</td>
</tr>
<tr>
<td>AIN93 trace minerals (no added Se)</td>
<td>1.4</td>
<td>1.4</td>
</tr>
<tr>
<td>LR potassium citrate</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>LR potassium dihydrogen phosphate</td>
<td>6.9</td>
<td>6.9</td>
</tr>
<tr>
<td>LR potassium sulphate</td>
<td>1.6</td>
<td>1.6</td>
</tr>
<tr>
<td>Choline chloride (75%)</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>LR magnesium oxide</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>AIN93 vitamins</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Control A. bisporus mushroom (12 µg Se/g dried caps)</td>
<td>0.295</td>
<td>–</td>
</tr>
<tr>
<td>Se-enriched A. bisporus mushroom* (300µg Se/g dried caps)</td>
<td>–</td>
<td>0.295</td>
</tr>
<tr>
<td>Total Se</td>
<td>0.00015 (0.15ppm)</td>
<td>0.001 (1 ppm)</td>
</tr>
</tbody>
</table>

*Se-enriched Agaricus bisporus was cultivated by irrigation of growth compost with sodium selenite solution. The selenoamino acids content of the Se-enriched mushroom used to fortify Se in the feeds were 8.35 µg selenocysteine/g dried weight (DW), 0.31 µg selenomethionine/g DW and 0.08 µg methyl-selenocysteine/g DW (Maseko et al., 2013).* 

### 2.6. Gene expression

#### 2.6.1. RNA isolation

Total RNA was extracted from 30 mg colon tissue from each rat using a commercial kit, QIAGEN RNeasy Mini Kit (QIAGEN, Victoria, Australia), and the extraction was performed in triplicates from each rat colon. The quality (purity) and concentration of total RNA extracted was determined using NanoDrop® ND-1000 UV–Vis spectrophotometer by measuring the absorbance at λ260 nm and 280 nm and determining the 260:280 absorbance ratio. Pure RNA has an A260:A280 ratio of
1.9–2.1, and the extracted RNA samples from the rats’ colon had A260: A280 ratios between 2.04 and 2.13 (data not shown).

2.6.2. cDNA synthesis

The BIO-RAD iScript™ Select cDNA Synthesis Kit (NSW, Australia) was used to synthesise the first strand cDNA (20 µl) from 0.3µg total RNA from each sample. The cDNA product was diluted 1:30 with nuclease free water and used for real-time quantitative PCR.

2.6.3. Real-time quantitative PCR

Real-time quantitative PCR of 4 genes, GPX1, GPX2, SEPP1 and TXNRD1 was performed for each sample on an iQ™ 5 Multicolor Real-Time PCR icycler Detection System (BIORAD, NSW, Australia). Oligonucleotide primers were designed using Primer 3 software v.0.4.0 (Bioinformatics Methods and Protocols: Methods in Molecular Biology, Humana Press, Totowa, NJ, USA), based on sequences obtained from the Genbank database (Table 2). The primers were validated by conventional PCR of cDNA (data not shown). The PCR reagents for quantitative analysis were contained in the iQ SYBR Green Supermix kit from BIORAD. The PCR reactions were determined in a final volume of 20 µl containing 6µl of diluted cDNA (1:30, v/v) and 10 µl iQ SYBR Green Supermix with a 1 x final concentration. Primer concentrations in the reaction mix for each gene was 250 nM for both the sense and antisense primer pairs. Nuclease free water was used to make up to the final volume. The cycling PCR reaction for each sample started with an initial hot start of 95°C for 3 min as the initial denaturation step of 1 cycle, followed by 45 cycles at 95°C for 30 s (denaturation), 60°C for 30 s (annealing), 72°C for 30 s (extension) and completed with a final extension step at 95°C for 1 min. The specificity of the PCR reaction (product) was demonstrated by melting curve analysis post PCR reactions; which showed only one peak present for all PCR products of GPx-1, GPx-2, SeP and TrxR-1 test genes and β-actin and glyceraldehyde 3-phosphate dehydrogenase reference genes. A non-template (without cDNA) reaction was included with each PCR run to serve as a negative control. The real-time quantitative PCR assay was optimised by running serial dilutions of cDNA template and using the results to generate a standard curve. The standard curve was generated by plotting the log of the starting dilution factor of the template against cycle threshold (C_T) value obtained during amplification of each dilution factor (template). The liner regression line and the coefficient of determination (R^2) of the standard curve were used to evaluate whether the qPCR assay was optimised. Amplification efficiency (E) of primer pair for each gene was calculated from the slope of the standard curve. Relative gene expression of each
target gene, in the test and control samples using reference β-actin gene as normalisers was determined. The cycle threshold (Cₜ) values for each target gene were normalised with the reference gene for both test and control samples, and the ΔCₜ method using the reference gene was used to calculate the relative expression values for the test and the control groups. The ratio between the test and control expression values was calculated by dividing the two values and relative expression expressed as fold change.

Table 2

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<td>Antisense</td>
<td>CTTCAGCTGTGGTGTAAGA</td>
</tr>
</tbody>
</table>

2.7. Glutathione peroxidase-1 (GPX-1) activity assay

Glutathione peroxidase-1 specific enzyme activity in rat colon was measured using a Glutathione Peroxidase Cellular Activity Assay Kit (Sigma–Aldrich, cat no CGP1, NSW, Australia). The colon was cut open and the mucosa layer scraped off. The colon was placed in 1 ml of cold (4°C) 50 mM Tris–HCl buffer (pH 8) containing 0.5 mM EDTANa₂ in a 2 ml tube containing 7 ceramic beads (2.8 mm diameter ceramic beads, cat no 1311450, Gene Works,Australia). The tube was placed in a rotor-stator tissue disruptor bead beater (MB8 model, BioSpec Products, DainTree Scientific Pty. Australia) for 5 mins to homogenise the tissue and the homogenate quickly placed in an ice bath to chill. The homogenate was then centrifuged at 14,000g (Beckman Benchtop Centrifuge, Allegra X-22 series, Gladesville, Australia) for 30 mins and the supernatant collected as the solubilised source of GPx-1. Protein concentration in the 100 µl of the supernatant was determined in triplicate using the Lowry protein assay kit (cat no. 500-0112, BioRad, USA). GPx-1 activity in 50 µl of the supernatant was determined in triplicate by adding 950 µl of reaction solution containing 50 mM
Tris HCl buffer (pH 8), 0.5 mM EDTA, 5 mM NADPH, 42 mM reduced glutathione, 10 units/ml glutathione reductase and 30 mM tert-butylhydroperoxide as the substrate. A glutathione peroxidase standard stock solution (100 unit/ml supplemented with 1 mg/ml IgG and 1 mM DDT) was used as a positive activity control. The decrease in NADPH due to its oxidation to NADP⁺ over a 20 min time period was measured by absorbance at λ340 nm using a UV–Vis spectro-photometer (Multiskan spectrophotometer, Thermo Electron, Thermo Fishers Scientific, USA). One unit of glutathione peroxidase is defined as the enzyme reaction that causes the formation of 1µmol of NADP⁺ from NADPH per min in the presence of 40 mM reduced glutathione, 9.5 unit/ml glutathione reductase and 28.5 mM tert-butyl hydroperoxide. The specific activity of GPx-1 was expressed as mU/mg protein.

2.8. Statistical analysis

The mean of triplicate determinations of GPx-1, GPx-2, TrxR-1 or SeP gene expression and GPx-1 specific activity in each rat colon was used to calculate the group mean (control diet or Se-enriched diet) and standard error of group. Statistical analyses of group means by one-way analysis of variance (ANOVA) were performed using GenStat (14 Ed), with P values less than 0.05 considered statistically different. Uncertainty of group means was reported to 2 significant figures according to the European Analytical Chemist guidelines (Ellison and Williams, 2012).
3. Results and discussion

3.1. Glutathione peroxidase-1 gene expression

Glutathione peroxidase is the main enzyme responsible for regenerating ‘spent’ oxidised glutathione, essential reducing molecules that protect cells from oxidative damage by free radicals and other oxidants (Hamanishi et al., 2004). Cytosolic GPx-1 is the most ubiquitous and prevalent isoform of GPxs expressed in almost all tissues of mammalian systems. It contributes to about 70% of the total GPx enzyme activity in the cells of the gastrointestinal tract and therefore plays a significant role in the colon’s antioxidant defense (Drew, Farquharson, Arthur, Morrice, & Duthie, 2005). Assuch, elevation by increased mRNA expression in response to oxidative stress is considered beneficial to cells. Rats fed for 5 weeks on a dietary supplement with Se-enriched mushroom at a Se concentration of 1 µg Se/g feeds significantly (P< 0.01) up-regulated colonic GPx-1 mRNA expression by 1.65-fold, compared to control rats fed with the same amount of control (non Se-enriched) mushroom at a basal Se concentration of 0.15 µg Se/g feeds (Fig. 1A). The effect of Se-enriched mushroom contrasted with the effect of dietary supplement with Se-enriched dairy proteins, which was reported to have no effect on colonic GPx-1 mRNA expression in mouse (Hu et al., 2010) (Table 3). How-ever, dietary supplement with Se-enriched yeast was reported to have up-regulated colonic GPx-1 mRNA expression by 2.7-fold in mice (Hu et al., 2010). Human clinical trials also showed an increase in rectal GPx-1 mRNA expression with dietary supplement of dairy Se, but not with dietary supplement with yeast Se (Hu et al., 2011).

3.2. Glutathione peroxidase-2 gene expression

GPx-2 is an extracellular enzyme exclusively expressed in the gastrointestinal tract, and as such might play a ‘special’ and large role in shielding the gastrointestinal tract from oxidative damage and offer protection from colorectal cancer (Drew et al., 2005). Indeed, cell membrane damage arising from peroxide accumulation in the gastrointestinal tract due to Se deficiency can be prevented by restoring Se in the diet to adequate levels, and GPx-2 expression is more sensitive to changes in Se levels in the diet (Banning, Deubel, Kluth, & Zhou, 2005). Dietary supplement with Se-enriched mushroom was found to significantly (P< 0.01) up-regulate colonic GPx-2 mRNA expression by 2.30-fold in test rats compared to the control rats (Fig. 2B). Colonic GPx-2 mRNA levels have also been reported to be up-regulated by 1.9-fold by dietary supplement with dairy Se in mice (Hu et al., 2010) (Table 3). The same study, however, observed no changes in GPx-2 mRNA expression with yeast Se at similar Se doses (Hu et al., 2010). The effect of yeast Se on colonic
GPx-2 expression in pigs has been shown to be dose dependent; the up-regulation of GPx-2 expression was observed at relatively higher Se concentrations, at 3 ppm (1.38 fold increase) and 9 ppm (1.95 fold increase), and the effect independent of sex (Uglietta et al., 2007). Azoxymethane challenged rats did not respond to dairy or yeast Se dietary supplementation with the expression of GPx-2 when compared to the basal Se diet, but expression was found to have increased with dairy Se when compared to the yeast Se diet (Uglietta et al., 2006). Differential effects were also observed for rectal GPx-2 gene expression in humans, which was increased by diet supplementation with dairy Se but the increase was less with yeast Se (Hu et al., 2011). Up-regulation of colonic GPx-1 and GPx-2 mRNA levels appears to be dependent on the organic Se sources. Yeast Se occurs mainly as free SeMet (70–80%) plus some free SeCys (4%) and other Se containing compounds (Ip, Birringer, Block, & Koterbai, 2003). Dairy Se also consisted of mainly SeMet (83%) plus some SeCys (5%), but these selenoamino acids are protein bound as selenoproteins in contrast with yeast Se (Heard et al., 2007). The organic Se content by mass of the Se-enriched mushroom used in our study consisted mainly of protein bound SeCys (95.5%) and SeMet (3.6%), plus trace amount of protein associated but free MeSeCys (0.9%) (Maseko et al., 2013). In this aspect the Se-enriched mushroom is more similar to dairy Se than yeast Se, in that it is mainly in the form of selenoproteins, yet its biological effect on colonic GPx-1 mRNA expression in rats was similar to that of yeast Se effect in mice.

3.3. Thioredoxin reductase-1 gene expression

Thioredoxin, together with glutathione, are the cells reducing molecules that protect against oxidative damage. Thioredoxin reductases (TrxRs) regenerate oxidised thioredoxin, in a similar manner to GPxs regenerating oxidised glutathione, and are thus vital enzymes in cells (Mustacich & Powis, 2000). The two mammalian isoforms, TrxR-1 and TrxR-2, are uniquely different from TrxRs in other organisms as they contain a penultimate SeCys residue at the carboxyl terminal domains (Mustacich & Powis, 2000). Dietary supplement with Se-enriched mushroom has no significant effect on colonic TrxR-1 mRNA expression in rats fed with a Se-enriched mushroom diet, when compared to the control rats fed with a control diet (Fig. 1C). Dairy and yeast Se sources have previously been reported to have no significant effect on TrxR-1 expression in mice colon (Hu et al., 2010) (Table 3) and human rectum (Hu et al., 2011) (Table 3). These findings suggest that the role of TrxR-1 oxidation protection in colon and rectum might not be as crucial as that of GPx-2, as previously suggested by others (Hu et al., 2010, 2011).
3.4. Selenoprotein P gene expression

Selenoprotein P is an extracellular glycoprotein secreted by most cells, including colonic cells, and is made up of 1 SeCys residue in the N-terminus and 9 SeCys residues in its C-terminal domain sequence in rodents and humans (Burk & Hill, 2009). Its function is not clearly understood, however as a major plasma selenoprotein it is believed to play a crucial role in the transport of Se, and thus influence the expression of other selenoproteins in different tissues (Richardson, 2005). SeP gene knock-out mice are linked to increased carcinogen-induced oncogenesis and colorectal cancer development (Diwadkar-Navsariwala & Diamond, 2004, Richardson, 2005, Hu, McIntosh, Le Leu, Woodman, & Young, 2008). Dietary supplement with Se-enriched mushroom has no significant effect on colonic SeP mRNA expression in rats when compared to the control rats (Fig. 1D). Dietary supplement with yeast Se has previously been reported to have no effect on colonic SeP mRNA expression in mice (Hu et al., 2010) and azoxymethane challenged rats (Uglietta et al., 2006) (Table 3). However, a dose dependent up-regulation of colonic SeP of 1.59 and 2.3 fold were observed in pigs with yeast Se at 3 and 9 ppm Se, and similar to the effect on GPx-2 expression, the effect was independent of sex (Uglietta et al., 2007).

Dairy Se also increased colonic SeP expression in mice in a dose dependent manner, with a 2.56 fold increase at 1 ppm Se (Hu et al., 2010). Up-regulation of human rectal SeP was also observed with dairy Se dietary supplements, but not with yeast Se (Hu et al., 2011). The lack of significant change in SeP expression by dietary supplement with mushroom and yeast Se in rats and mice colons is not surprising, though both Se sources produced a significant up-regulation of GPx-1 and GPx-2. It is known that there is a varying prioritisation of specific selenoprotein expressions and synthesis in response to dietary Se supply, giving different Se levels for the optimisation of SeP and other selenoproteins (Richardson, 2005). Studies (Richardson, 2005) show that Se supplementation in Se deficient human subjects had GPx activity optimised before SeP concentration. Notably, the process of SeCys insertion into seleno-proteins is less efficient than that of other amino acids (Burk & Hill, 2009). SeP contains the largest number of SeCys residues when compared to other selenoproteins, with 10 SeCys inserted into the full length SeP peptide (Burk & Hill, 2009). Evidence has shown that the 2 SeCys insertion sequence (SECIS) elements in the 3’ untranslated region of SeP mRNA serve different functions to facilitate SeCys insertion (Burk & Hill, 2009). SECIS 2 was shown to be responsible for insertion of the single SeCys residue in the N-terminal domain of SeP and is less efficient that SECIS 1 and as a result the process occurs slowly (Burk & Hill, 2009).
Fig. 1. Effects of dietary supplement of Se-enriched Agaricus bisporus on (A) cytosolic glutathione peroxidase-1 (GPx-1), (B) gastrointestinal glutathione peroxidase-2 (GPx-2), (C) thioredoxin reductase-1 (TrxR-1), and (D) selenoprotein P (SeP) gene expression. mRNA levels were measured in triplicate in each rat colon from rats fed with control (0.15 µg Se/g feeds) or Se-enriched (1 µg Se/g feeds) diet and expression level normalised against the β-actin reference gene. Fold change in GPx-1, GPx-2, TrxR-1 and SeP mRNA levels in Se-enriched diet was calculated relative to the control diet which was set as 1. Error bars indicate standard error (SE) of control (n=11) or Se-enriched (n= 13) diet group.

*, Mean values are significantly different (P<0.01) when compared to controls
Table 3

Effect of dietary Se supplement on GPx-1, GPx-2, TrxR-1 and Sep mRNA expression and GPx-1 enzyme activity

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<th>Yeast Se</th>
<th>Dairy Se</th>
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+/-, Positive up-regulation of or no effect on gene expression or enzyme activity.

<sup>A</sup>This study.

<sup>B</sup>Azoxymethane challenged rat; the positive effect of dairy Se was relative to yeast Se.

<sup>a</sup>Hu et al. (2010).

<sup>b</sup>Hu et al. (2011).

<sup>c</sup>Uglietta et al. (2007).

<sup>d</sup>Uglietta et al. (2006).

<sup>e</sup>Uglietta et al. (2008).

<sup>f</sup>Hu et al. (2008).
Fig. 2. Effects of Se-enriched *Agaricus bisporus* on cytosolic glutathione peroxidase-1 (GPx-1) activity. Enzyme activity was determined in triplicates in each rat colon from rats fed with control (0.15 µg Se/g feeds) or Se-enriched (1 µg Se/g feeds) diet using NADPH as co-factor and tert-butyl hydroperoxide as substrate. Error bars indicate standard deviation of control (n= 7) and Se-enriched (n= 11) diet group.

*, Mean values are significantly different (P<0.05) when compared to controls.

3.5. Glutathione peroxidase-1 activity

An increase in GPx-1 mRNA levels must be linked to an increase in biological activity for positive biological effects. Selenocysteine is an essential constituent of the functional domain of the active sites of GPxs, and its incorporation into selenoproteins such as GPxs requires the presence of a UGA codon and the Sec insertion sequence (SECIS) element located in the 3’ untranslated region of selenoprotein mRNAs (Sheppard et al., 2008, Collins et al., 2012). Thus, dietary supplement rich in SeCys could conceivably lead to an increase in GPx enzyme activity derived from a higher expression level of its mRNA. Indeed, the Se-enriched mushroom Se was found to significantly (P< 0.05) increase colonic GPx-1 activity (Fig. 2). The enzyme activity increased by 1.77-fold in rats fed with a Se-enriched mushroom diet (839 ± 101 mU/mg protein), compared to control rats (473 ± 71 mU/mg protein). The increase in GPx-1 activity correlated to the increase in GPx-1 mRNA expression in the rats’ colon (Fig. 1A). Similar positive correlation of GPx-1 enzyme activity and mRNA expression was reported for dietary supplementation with yeast Se in mouse
colon (Hu et al., 2010) (Table 3). Hu et al. (2008) also reported an increase in colonic GPx-1 activity in mice fed with yeast Se although mRNA levels were not determined in the study. However, the mushroom Se (and yeast Se) differs from dairy Se in that dairy Se has no significant effect on colonic GPx-1 activity in mice at a similar Se dose (Hu et al., 2008, 2010). Dietary supplement with dairy Se also did not affect the level of plasma GPx-1 activity in human subjects (Hu et al., 2011).

4. Conclusion

The present study provides further evidence that selenoprotein levels are regulated differently depending on the Se sources. Selenium from Se-enriched A. bisporus seems to be more similar to the Se from Se-enriched yeast than Se-enriched dairy proteins in its regulation of selenoproteins in rat colon, even though its organic Se forms are more similar to dairy Se in that they both exist as protein bound selenoamino acids in contrast to prominently free selenoamino acids in Se-yeast. The differences observed across different Se sources could be due to differences in and varying proportion of organic Se chemical forms (free selenoamino acids and SeMet or SeCys rich selenoproteins). The ease in cultivating Se-enriched A. bisporus and the fact that Se-enriched A. bisporus displayed biological activity relevant to protection against oxidative stress and colorectal cancer would indicate it a viable alternative source of functional organic Se, in addition to Se-enriched yeast and Se-enriched dairy proteins. As mushrooms are a widely consumed commodity, Se-enriched mushrooms could provide an efficient way in delivering functional organic Se of macro fungal origin, and therefore would be commercially beneficial to the mushroom industry.

Acknowledgement

We would like to acknowledge Mr. Jim Fuller’s (Spawn Lab Team Leader, Mushroom Exchange Pty. Ltd., 45 Cookes Road, Mernda, Victoria, Australia, 3754) contribution in supplying the mushroom growing kits and advice on the cultivation of A. bisporus.
CHAPTER 5

The effect of dietary supplementation of selenium in the form of Se-enriched Agaricus.bisporus mushroom on gut permeabilty *ex-vivo* and regulation of GPx-1 and GPx-2 selenoenzymes in ileum of rats exposed to hyperthermal stress.

Chapter published as;

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(Original article attached in Appendix).
Chapter 5 addressed the following **Aim**:

i. The evaluation of gut physiological function and regulation of antioxidant selenoenzymes in the ileum of rats fed Se-enriched *A. bisporus* mushroom and exposed to hyperthermal stress conditions.

The ileum antioxidant selenoenzymes evaluated were as follows:

a. GPx-1 mRNA expression  
b. GPx-2 mRNA expression

Previous studies have shown that dietary selenium supplementation mitigates the adverse effects of heat stress in animals. Dietary supplementation with selenium-enriched probiotics and sodium selenite has been shown to significantly down regulate mRNA levels of heat shock proteins, Hsp70 and Hsp27, bio-markers of heat stress elevated with heat stress, in piglets exposed to heat stress conditions (Gan et al. 2013). The heat shock proteins are involved at a cellular level in a heat shock response to heat stress and other types of cellular injury, offering cell protection from injury and enhancing cellular recovery from heat and other physiological stressors (Welch 1992). The Hsp proteins are consistently induced and up-regulated in cells exposed to heat stress of varying intensities and lengths (Kregel et al. 1995, Skidmore et al. 1995) providing reliable molecular markers of heat stress. More evidence has been provided to show the effect of dietary supplementation with selenium in reducing the impact of high heat load during exposure to hyperthermal stress conditions. Dietary supplementation with Se exhibited noticeable reduction in rectal temperatures and lowered bodyweight loss in sheep exposed to high heat load. Furthermore, exposure to high heat load is reported to decrease weight gain, total immunoglobulin antibodies, phagocytic activity of neutrophils and antioxidant status (Alhidary et al. 2012).

However, the provision of Se in excess of the recommended dietary level was shown to counteract the effects of high heat load by improving immunological response, increasing the eusonophil count and also improving the antioxidative status in sheep (Alhidary et al. 2012). Studies by Marai et al. (2009) also demonstrated the mitigating effects of dietary supplementation with Se following exposure to heat stress. The study observed a significant decrease in rectal temperatures, percentages of dead spermatozoa and sperm abnormalities of heat stressed rams placed on a Se
supplemented diet (Marai et al. 2009). Selenium supplementation also recovered sperm motility, sperm concentration, scrotal and testes lengths of rams exposed to heat stress (Marai et al. 2009).

In light of the previous evidence that linked dietary selenium supplementation with physiological processes involved in the mitigation of the adverse effects of heat stress in animals, Chapter 5 presents findings from our study that showed a link between dietary Se supplementation and gut function during exposure to acute heat stress further providing evidence Se protects against heat stress induced physiological dysfunction. Chapter 5 evaluated the effects of a high Se diet in the form of Se-enriched *A. bisporus* mushroom with or without additional α-tocopherol on ileum epithelium permeability and regulation of the ileum antioxidant GPx-1 and GPx-2 selenoenzyme expression in hyperthermally induced oxidative stress in rat. Chapter 6 further evaluates the protective effect of Se during heat stress. The chapter (six) presents findings on the evaluation of dietary supplementation with mushroom Se with or without the addition of α-tocopherol on the regulation of expression of antioxidant colonic selenoproteins in hyperthermally induced oxidative stress in rat. The use of a small animal model enabled us to measure the molecular markers and physiological status of heat stress with relation to Se and α-tocopherol dietary supplementation, in a controlled reproducible environment.
Abstract: Dietary effects of organic Se supplementation in the form of Se-enriched *Agaricus bisporus* mushroom on ileal mucosal permeability and antioxidant selenoenzymes status in heat induced oxidative stress in rats were evaluated. Acute heat stress (40 °C, 21% relative humidity, 90 min exposure) increased ileum baseline short circuit current ($I_{sc}$; 2.40-fold) and epithelial conductance ($G_e$; 2.74-fold). Dietary supplementation with Se-enriched *A. bisporus* (1 µg Se/g feed) reduced ($P < 0.05$) ileum $I_{sc}$ and $G_e$ during heat stress to 1.74 and 1.91 fold, respectively, indicating protection from heat stress-induced mucosal permeability increase. The expression of ileum glutathione peroxidase (GPx-1 and 2 mRNAs ($P < 0.05$) were up regulated by 1.90 and 1.87-fold, respectively, for non-heat stress rats on the Se-enriched diet relative to the control. The interplay between heat stress and dietary Se is complex. For rats on the control diet, heat stress alone increased ileum expression of GPx-1 (2.33-fold) and GPx-2 (2.23-fold) relative to thermoneutral conditions. For rats on the Se-enriched diet, heat stress increased GPx-1 expression ($P < 0.05$) only. Rats on Se-enriched + α-tocopherol diet exhibited increased expression of both genes ($P < 0.05$). Thus, dietary Se-enriched *A. bisporus* protected against increase in ileum permeability and up regulated GPx-1 and GPx-2 expression, selenoenzymes relevant to mitigating oxidative stress.

1. Introduction

Acute stress and other pathological conditions disrupt gastrointestinal physiology and barrier function making the gut organs vulnerable to various disorders (Kiliaan et al. 1998, Pavlick et al. 2002, Drew et al. 2005, Lu & Holmgren 2009). The gastrointestinal mucosa is made up of the lamina propria and a covering of a single layer of epithelial cells joined together by tight junctions to create a barrier restricting the uptake of material from the lumen. The lamina propria contains immunocytes including eosinophils, neutrophils, macrophages, lymphocytes and mast cells that protect the gut against microorganisms and their toxic products (Furness et al. 2013). However, in leaky gut induced by stress factors such as heat, the tight junction is disrupted causing gastrointestinal barrier dysfunction arising from increased epithelial permeability (Marchiando et al. 2010). This leads to high infection rates and uptake of bacterial endotoxins triggering local inflammation and immune responses (Furness et al. 2013). A “leaky” gut is characterized by elevation in epithelial ionic conductance through the paracellular pathway (Kiliaan et al. 1998, Santos & Perdue 2000). The ion conductance is normally restricted by the tight junctional complex and the relative apposition of basolateral membranes of adjacent epithelial cells that determine the volume of the surrounding aqueous column known as the lateral intercellular space. Stress factors induce a “leaky” gut by disrupting the tight junctional complex causing shedding of the epithelial layer leading to increased permeability (Clark 2009). Epithelial permeability can be determined by
measuring tissue baseline short circuit current (I_{sc}) and conductance (G_e) using an Ussing chamber (Clark 2009). For example, both restraint stress and cold restraint stress have been shown to increase I_{sc} and G_e in the jejunum of Wistar-Kyoto rat using Ussing chamber measurements, and the increased tissue permeability was confirmed by the higher flux of [^{3}H]-mannitol and [^{51}Cr]-labelled EDTA through the tissue (Saunders et al. 1994). Evidence suggested that oxidative stress induced by heat or other factors is characterised by the accumulation of reactive oxygen (ROS) and nitrogen (RNS) species, and that they are significant contributing factors in the pathogenesis of gastrointestinal tract ailments such as inflammatory bowel disease, fibrosis, ulcerative colitis and colon cancer (Pavlick et al. 2002, Chu et al 2004). Selenium is an essential trace element and a micronutrient required for several physiological functions in mammals (Clark et al. 1996, Flores-Mateo et al. 2006). Dietary organic Se has been linked to beneficial biological effects and disease prevention (Clark et al. 1996, Flores-Mateo et al. 2006). The health benefits of organic Se come about through the expression of selenoamino acids containing selenoproteins and selenoenzymes that are involved in mitigating the effects of cellular oxidative stress by inactivating cellular oxidants such as ROS and RNS (Holben & Smith 1999, Rayman 2000, Gromadzinska et al. 2008). These included the antioxidant selenoenzymes glutathione peroxidases (GPxs) and thioredoxin reductases (TrxRs) (Hadley & Sunde 2001, Lu & Holmgren 2009) that play a central role in protecting cells against oxidative injury (Arthur 2000, Rayman 2000). In mouse, supplementation with Se-enriched milk proteins and Se-yeast up-regulated the expression of gut antioxidant selenoproteins, enhancing the capacity for cell protection from oxidative damage (Hu et al. 2010).

The study has previously cultivated Se-enriched 	extit{Agaricus bisporus} mushroom by irrigating growth compost with sodium selenite and chemically characterized the organic Se that is primarily made up of selenocysteine rich selenoproteins (Maseko et al. 2013). We have also demonstrated that dietary Se supplementation with the Se-enriched 	extit{A. bisporus} significantly up-regulated GPx-1 activity, and mRNA expression of GPx-1 and gastrointestinal specific GPx-2, in rat colon (Maseko et al. 2014) genes linked with anti-inflammatory properties and anti-cancer function in the gastrointestinal tract (Chu et al. 2004). In this study, we evaluated the effects of a high Se diet in the form of the Se-enriched 	extit{A. bisporus} with or without additional α-tocopherol on ileum epithelium permeability and regulation of ileum GPx-1 and GPx-2 expression in hyperthermally induced oxidative stress in rat to determine whether there is a link between dietary Se supplementation and gut function.

2. Materials and Methods

2.1. Se-Enriched and Non-Se Enriched Agaricus bisporus Mushroom
Se-enriched *A. bisporus* (button mushroom) was cultivated by irrigation of growth compost with sodium selenite solution using grower kits supplied by a commercial mushroom producer (Mushroom Exchange Pty. Ltd., Mernda, Victoria, Australia) as described elsewhere [19]. Non-Se enriched *A. bisporus* grown under normal mushroom growing conditions was also supplied by Mushroom Exchange Pty. Ltd. Mushroom caps were harvested, frozen at −80 °C, and then freeze-dried. The lyophilised caps were ground into fine powders using a commercial blender and stored at RT in moisture free sealable packs until required for preparation of rat feeds.

2.2. Diets: Preparation of Control; Se-Enriched; Se-Enriched + α-Tocopherol Diets

Rat feed was prepared in the form of pellets by Specialty Feeds Inc. (Glen Forrest, Western Australia). Three diet types including the control were prepared. Control mushroom feed pellets were prepared by supplementing a low Se rodent feed formulation AIN 93G (10 kg; composition detailed in [Hu et al. 2010]) with 20 g control (non-Se enriched) mushroom caps containing 2.22 µg Se/g dried caps to give a final Se content of 0.12 µg Se/g feed in the control diet (Diet 1). Se-enriched mushroom feed pellets were prepared by supplementing the low Se rodent feed formulation AIN 93G (10 kg) with 142 g of Se-enriched mushroom caps containing 62.20 µg Se/g dried caps to give a final Se content of 1 µg Se/g feed in Se-enriched feed (Diet 2). The Se-enriched mushroom feed pellets supplemented with α-tocopherol (0.03% w/w) were similarly prepared as diet 2 but with the addition of α-tocopherol, to give a final Se and α-tocopherol contents of 1 µg Se + 300 µg α-tocopherol/g feed (Diet 3).

2.3. Animals

A total of 48 nine week-old Sprague Dawley male rats weighing 302–426 g obtained from a colony without known adventitious viruses, mycoplasma, enteric pathogenic bacteria and parasites were purchased from the Monash University Animal Services, Melbourne, Australia. The animal experiment protocols were approved by the Melbourne School of Land & Environment Research Animal Ethics Committee, University of Melbourne (ethics approval No. 1312820.1). Rats were randomly divided into three experimental groups and housed two per cage. The rats were housed in an air-conditioned, temperature controlled animal facility with a 12 h light-dark cycle at 21 °C. Rats were given free access to food and water at all times.
2.4. Animal Feeding

Rats were randomly assigned to three experimental diets: diet 1, control (0.12 µg Se/g feed); diet 2, Se-enriched (1 µg Se/g feed); and diet 3, Se-enriched + α-tocopherol (1 µg Se + 300 µg α-tocopherol/g feed). All rats were at first acclimatised with free access to control diet 1 and water for one week before the animals were given their respective diet 1, diet 2 or diet 3 and water ad libitum for a further period of 3 weeks. Body weights of the animals were recorded at the start of the experiment and continued weekly to monitor their growth which was normal. Their food intake and behaviour were also monitored throughout. These were also normal.

2.5. Acute Heat Stress Protocol

After 21 days of feeding of the allocated diets, rats from each diet were randomly allocated to thermoneutral and acute heat stress treatment groups (Table 1) and their body weights determined. Thermoneutral Groups 1, 2 and 3 were exposed to an ambient temperature of 21 °C and had their feed removed but allowed free access to water for a 90 min period. Acute heat stress Groups 4, 5 and 6 were housed individually in cages, exposed to 40 °C and 21% relative humidity (RH) and also had their feed removed and allowed free access to water for a 90 min period in a temperature controlled room. Preliminary observation of rats indicated that they can tolerate 40 °C and 21% RH conditions for up to 90 min but beyond that time point distress symptoms such as hyperventilation and lethargy set in, thus the thermoneutral and acute heat stress condition was limited to 90 min exposure. Animals from both treatments were monitored every 10 min over the 90 min treatment duration for rectal temperatures using a temperature probe (Vicks Speed-Read Digital thermometer, 10 mm × 3 mm probe) and heart rates using a stethoscope (as beats per minute: bpm). Lubricant was used to aid thermometer probe insertion into the rectum. At the end of the treatments, rats were allowed to rest for 20 min at RT before being anaesthetised and sacrificed for tissue excision.

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Treatment (Temperature, °C)</th>
<th>Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (n=8)</td>
<td>Thermoneutral (T21)</td>
<td>Diet 1 (Control; 0.12 µg Se/g feed)</td>
</tr>
<tr>
<td>Group 2 (n = 8)</td>
<td>Thermoneutral (T21)</td>
<td>Diet 2 (Se-enriched; 1 µg Se/g feed)</td>
</tr>
<tr>
<td>Group 3 (n = 8)</td>
<td>Thermoneutral (T21)</td>
<td>Diet 3 (Se-enriched + α-tocopherol; 1 µg Se + 0.3 µg α-tocopherol/g feed)</td>
</tr>
<tr>
<td>Group 4 (n = 8)</td>
<td>Heat stress (T40)</td>
<td>Diet 1 (Control; 0.12 µg Se/g feed)</td>
</tr>
<tr>
<td>Group 5 (n = 8)</td>
<td>Heat stress (T40)</td>
<td>Diet 2 (Se-enriched; 1 µg Se/g feed)</td>
</tr>
<tr>
<td>Group 6 (n = 8)</td>
<td>Heat stress (T40)</td>
<td>Diet 3 (Se-enriched + α-tocopherol; 1 µg Se + 0.3 µg α-tocopherol/g feed)</td>
</tr>
</tbody>
</table>
2.6. Animal Euthanasia and Ileum Tissue Excision

Animals were killed with an initial step of a single intra-peritoneal injection of ketamine and xylazine mix to anaesthetise and an overdose of the ketamine and xylazine mix as the final euthanasia step. Ileum tissue was recovered from each rat, contents flushed out, cut into 2 cm segments and was placed in 37 °C Krebs bicarbonate buffer pH 7.4, aerated with 10% CO₂/90% O₂, containing 25 mM NaHCO₃, 1.2 mM CaCl₂, 10 mM glucose, and 0.01 M nicardipine to prevent muscle contraction.

2.7. Ussing Chamber Analysis

The ileum was cut open along the mesenteric border to expose the mucosa and held open handling only the edges with the aid of pins. The opened ileum segments were lifted with forceps (handling edges only) and carefully without touching the mucosal side mounted on P2311 Ussing Chamber sliders with 0.3 cm² aperture areas. Tissue mounts were secured in place over the slider apertures by pins around the apertures. Excess tissue was removed from around the pins, and silicone grease was applied to both the bottom and top parts of the sliders before mounting on the tissue to ensure a water tight seal. Tissue was kept moist at all times with a few drops of Krebs buffer. Ussing sliders with tissue were inserted into two part chambers (EasyMount Diffusion Chambers, Physiologic Instruments, Navicyte SDR Clinical Technology, 213 Eastern Valley Way, NSW 2068, Australia) that exposed 0.3 cm² of serosal and mucosal surface areas to Krebs bicarbonate buffer (115 mM NaCl, 25 mM NaHCO₃, 2.4 mM K₂HPO₄, 1.2 mM CaCl₂, 1.2 MgCl₂, 0.4 mM KH₂PO₄, pH 7.4) at 37 °C and gassed with carbogen (10% CO₂, 90% O₂). Each chamber half contained 5 mL of the Krebs bicarbonate buffer, with the serosal bath having an additional 10 mM glucose to provide an energy substrate and the mucosal bath containing an additional 10 mM mannitol to maintain osmotic balance across the mucosa on the tissues. Each chamber had a set of four electrodes (two voltage sensing and two current passing electrodes) installed on opposite sides of the tissue and connected to the amplifier through agar bridges. Each hemi-chamber was bubbled with carbogen. A Multichannel Voltage-Current Clamp (Physiologic Instruments, model VCC MC6) linked to the chambers was used to record baseline short circuit current (Iₛₖ). Epithelial conductance (Gₑ) (in mS/cm²) of ileum tissue was determined from the current/voltage relationship. Tissue mounts from all animal subjects were prepared in triplicate and allowed to equilibrate for 30 min in the chambers before measurements were made.
2.8. Gene Expression

2.8.1. RNA Isolation

Total RNA was extracted from RNAlater® solution stabilized ileum tissue (30 mg) using a commercial kit, QIAGEN RNaseasy Mini Kit (QIAGEN, Victoria, Australia), and the extraction was performed in triplicates for each rat ileum. The quality (purity) and concentration of total RNA extracted was determined using NanoDrop® ND-1000 UV-Vis spectrophotometer by measuring the absorbance at λ260 nm and 280 nm and determining the 260:280 absorbance ratio. Pure RNA has an A260:A280 ratio of 1.9–2.1, and the extracted RNA samples from the rats’ ileum had A260:A280 ratios between 1.96 and 2.12 (data not shown).

2.8.2. cDNA Synthesis

The BIO-RAD iScript™ Select cDNA Synthesis Kit (NSW, Australia) was used to synthesise the first strand cDNA (20 µL) from 0.3 µg total RNA from each sample. The cDNA product was diluted 1:30 with nuclease free water and used for real-time quantitative PCR.

2.8.3. Real-Time Quantitative PCR

Real-time quantitative PCR of GPx-1 and GPx-2 genes was carried on an iQ™ 5 Multicolor Real-Time PCR iCycler Detection System (BIORAD, NSW, Australia). Oligonucleotide primers were designed using Primer 3 software v.0.4.0 (Bioinformatics Methods and Protocols: Methods in Molecular Biology, Humana Press, Totowa, NJ, USA) based on sequences obtained from the Genbank database (Table 2). The primers were optimized and validated by conventional PCR of cDNA (data not shown). The PCR reagents for quantitative analysis were contained in the iQSYBR Green Supermix kit from BIORAD. The PCR reactions were performed in a final volume of 20 µL containing 6 µL of diluted cDNA (1:30, v/v) and 10 µL iQ SYBR Green Supermix. Primer concentrations in the reaction mix for each gene was 250 nM for both the sense and antisense primer pairs. Nuclease free water was used to make up the final volume. The cycling PCR reaction for each sample started with an initial hot start of 95 °C for 3 min as the initial denaturation step of 1 cycle, followed by 45 cycles at 95 °C for 30 s (denaturation), 60 °C for 30 s (annealing), 72 °C for 30 s (extension) and completed with a final extension step at 95 °C for 1 min. The specificity of the PCR reaction (product) was demonstrated by melting curve analysis post PCR reactions; which showed only one peak present for all PCR products of GPx-1 and GPx-2 test genes and the β-actin
A non-template (without cDNA) reaction was included with each PCR run as a negative control. The real-time quantitative PCR assay was optimised by running serial dilutions of cDNA template and using the results to generate a standard curve. The linear regression line and the coefficient of determination \( R^2 \) of the standard curve were used to evaluate whether the qPCR assay was optimised. Amplification efficiency \( E \) of primer pair for each gene was calculated from the slope of the standard curve. Relative gene expression for each target gene, in the test and control samples using reference \( \beta \)-actin gene as a normaliser was determined. The cycle threshold \( (C_T) \) values for each target gene were normalised with the reference gene for both test and control samples, and the Pfaffl method (Biorad Real Time PCR Applications Guide: Gene Quantification) was used to calculate the relative gene expression of the target genes in the samples.

### Table 2. Oligonucleotide primers used for real-time quantitative PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene accession number</th>
<th>Primers</th>
<th>Primer sequence 5′–3′</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPx1</td>
<td>NM_030826</td>
<td>Sense</td>
<td>TGAGAAGTGCGAGGTAAGTGAATG</td>
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<tr>
<td></td>
<td></td>
<td>Antisense</td>
<td>CGGGGACCAAATGATGTAC</td>
</tr>
<tr>
<td>GPx2</td>
<td>NM_183403</td>
<td>Sense</td>
<td>TGCCCTACCCTATGACGAC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antisense</td>
<td>TCGATGTGTGGTCCTGGAA</td>
</tr>
<tr>
<td>( \beta )-Actin</td>
<td>NM_031144</td>
<td>Sense</td>
<td>GTCGTACCTGGGCAATTGTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antisense</td>
<td>CTCTCAGCTGGTGTTGGA</td>
</tr>
</tbody>
</table>

#### 2.9. Statistical Analysis

The mean of triplicate determinations was used to calculate the group mean and uncertainty as standard deviation or standard error. Statistical analyses of group means by two-factor analysis of variance (ANOVA) were performed, with a subsequent multiple comparison test by Fisher’s Protected Least Significant Difference (LSD) test using GenStat (14ed). Statistical significance was defined at p values less than 0.05 \( (P < 0.05) \). Uncertainty of group means was reported to two significant figures according to the European Analytical Chemist guidelines (Eurachem 2014).
3. Results

3.1. Heat Stress Physiological Parameters

3.1.1. Rectal Temperatures

The rectal temperatures of rats maintained under thermoneutral conditions (21 °C) were between 34.8 ± 0.3 °C and 35.9 ± 0.3 °C across all diet groups and were not changed over the 90 min duration of the treatment (data not shown). By contrast, there was an increase (P < 0.05) in rectal temperatures in rats exposed to the acute heat stress (40 °C, 21% RH) across all diets (Figure 1A). Rectal temperatures recorded at the start of the heat stress were 35.2 ± 0.3 °C, 35.3 ± 0.3 °C and 35.5 ± 0.4 °C for rats from Diets 1, 2 and 3 respectively. It gradually increased to 39.2 ± 0.3 °C, 38.8 ± 0.6 °C and 39.0 ± 0.5 °C for rats from Diets 1, 2 and 3, respectively, after 30 min of the heat stress condition. The rectal temperature of all heat stress rats reached 40 ± 0.4 °C at the end of the 90 min treatment.

3.1.2. Heart Rates

Heart rates were also used as an indication of heat stress and were measured using a stethoscope. The heart rates of rats maintained under thermoneutral conditions were between 240 ± 3 and 246 ± 3 bpm and were not changed over the 90 min duration of the treatment (data not shown). There was a slight increase (P < 0.05) in heart rate in rats exposed to the acute heat stress (40 °C, 21% RH) across all diets (Figure 1B). Heart rates at the beginning of heat stress were 246 ± 6, 240 ± 3 and 240 ± 6 bpm for rats from Diets 1, 2 and 3, respectively. After 90 min of heat stress, rats recorded heart rates of 282 ± 6, 288 ± 6 and 284 ± 8 bpm for Diets 1, 2 and 3, respectively.

Figure 1. Rectal temperatures (A) and heart rates (B) of rats over time period of exposure to heat stress conditions at 40 °C and 21% RH. Mean value and standard deviation of rectal temperature of rats and heart rates from Diet 1 (n= 7), Diet 2 (n= 7) and Diet 3 (n= 7) were plotted.

*Mean values slightly higher (P<0.05) when compared to controls (non-heat stress rats).
3.2. Ileum Mucosa Permeability Parameters

3.2.1. Baseline Short Circuit Current (I\textsubscript{sc})

There were no significant differences (P < 0.05) in ileum I\textsubscript{sc} for rats maintained under thermoneutral conditions across the three diets, at 17.3 ± 3.5, 20.7 ± 5.0 and 13.4 ± 2.8 µA/cm\textsuperscript{2} for Diets 1, 2 and 3, respectively (Figure 2A). However, heat stress rats displayed significantly (P < 0.05) higher ileum I\textsubscript{sc} than non-heat stress rats across all diet groups. Heat stress rats on control Diet 1 (0.12 µg Se/g) displayed the highest ileum I\textsubscript{sc} of 41.6 ± 6.4 µA/cm\textsuperscript{2}. In addition, it was higher (P < 0.05) than the ileum I\textsubscript{sc} of rats on Se-enriched Diet 2 (1 µg Se/g feed) and Se-enriched + α-tocopherol Diet 3 (1 µg Se + 300 µg α-tocopherol/g feed) at 23.8 ± 3.3 µA/cm\textsuperscript{2} and 18.7 ± 4.7 µA/cm\textsuperscript{2}, respectively. Although there was an observed decrease in I\textsubscript{sc} for heat stressed rats on diet 2 and diet 3, the response was not comparable to the control (T21, diet 1). However, there was no significant (P < 0.05) difference in ileum I\textsubscript{sc} between heat stress rats on Diets 2 and 3.
**Figure 2.** Effect of dietary Se supplementation on ileum (A) baseline short circuit current (Iᵢsc) and (B) epithelial conductance (Gₑ) of non-heat stress and heat stress rats after 30 min of mounting tissue in Ussing Chamber. Number shows mean value and standard error of ileum Iᵢsc or Gₑ for rats on control Diet 1 (0.12 µg Se/g feed; n= 7), Se-enriched Diet 2 (1 µg Se/g feed; n= 7) and Se-enriched + α-tocopherol Diet 3 (1 µgSe/g feed + 300 µg α-tocopherol/g feed; n= 7) that were subjected to thermoneutral (T21 °C) or heatstress (T40 °C) treatments.

* Mean values showing statistical significant effect relative to the control (T21°C, diet 1) (P < 0.05, ANOVA).

a–c Mean values statistically significantly different in Fisher’s protected least significant difference test of multiple comparisons in ANOVA (P < 0.05).
3.2.2. Epithelial Conductance ($G_e$)

Current/voltage relations were determined by setting the clamp voltage at values in the range 1–5 mV. The data was used to calculate $G_e$ (mS/cm²) (Figure 2B). There was no significant (P < 0.05) difference in ileum $G_e$ for rats maintained under thermoneutral conditions across the three diets, which were 39 ± 10, 33.7 ± 7.9 and 32 ± 11 mS/cm² for control diet 1, Se-enriched Diet 2 and Se-enriched + a-tocopherol Diet 3, respectively. Ileum $G_e$ was significantly (P < 0.05) increased in heat stress rats compared to non-heat stress rats. Heat stress rats on Diet 1 displayed the highest ileum $G_e$ of 107 ± 13 mS/cm², and was significantly (P < 0.05) higher than the ileum $G_e$ of heat stress rats on Diet 2 at 56 ± 12 mS/cm² and Diet 3 at 66 ± 16 mS/cm². There was an observed decrease in $G_e$ for heat stressed rats on diet 2 and diet 3, although the response was not comparable to that of the control (T21, diet 1). However, there was no significant (P < 0.05) difference in ileum $G_e$ between Diets 2 and 3.

3.3. Glutathione Peroxidase mRNA Expression

3.3.1. GPx-1 mRNA Expression

There was a significant (P < 0.05) increase in GPx-1 mRNA expression in the ileum for rats maintained under thermoneutral conditions and fed the Se-enriched Diet 2 (1.90 ± 0.67-fold increase) and Se-enriched + a-tocopherol Diet 3 (1.26 ± 0.44-fold increase) compared to the basal level (Figure 3A). The GPx-1 expression level for rats maintained under thermoneutral conditions and fed the Diet 2 was significantly (P < 0.05) higher than those of rats maintained under the same condition and fed on Diet 3. Heat stress alone induced a significant (P < 0.05) increase in GPx-1 expression above basal level (2.33 ± 0.65-fold increase). However, while the heat stress treatment also significantly (P < 0.05) increased GPx-1 expression for rats on Diet 2 (1.51 ± 0.23-fold increase) and Diet 3 (1.58 ± 0.42-fold increase) above basal level, they were significantly (P < 0.05) lower than that of the heat stress rats on Diet 1. There was no significant (P < 0.05) difference in expression of GPx-1 between heat stress rat on Diets 2 and 3.

3.3.2. GPx-2 mRNA Expression

As was observed for GPx-1, there was a significant (P < 0.05) increase in GPx-2 mRNA expression for rats maintained under thermoneutral conditions and fed the Se-enriched Diet 2 (1.87 ± 0.77-fold increase)
increase) and the Se-enriched + α-tocopherol Diet 3 (1.9 ± 1.0-fold increase) compared to the basal level (Figure 3B). The GPx-2 expression levels for rats maintained under thermoneutral conditions and fed the Diets 2 and 3 were not significantly (P < 0.05) different. As was also observed for GPx-1, heat stress alone was found to induce a significant (P < 0.05) increase in GPx-2 expression for rats on Diet 1 (2.23 ± 0.71-fold increase) above basal level. However, while the heat stress treatment also significantly (P < 0.05) increased GPx-2 expression for rats on Diet 3 (1.60 ± 0.37-fold increase), there was no significant (P < 0.05) increase for heat stress rats on Diet 2 (1.07 ± 0.18 fold increase) above basal level. GPx-2 expression level of heat stress rats on Diets 2 and 3 were, as was observed for GPx-1, significantly (P < 0.05) lower than that of the heat stress rats on Diet 1.
Figure 3. Effect of dietary Se supplementation on ileum (A) glutathione peroxidase-1 (GPx-1) and (B) glutathione peroxidase-2 (GPx-2) mRNA expression. mRNA levels were measured in triplicate ileum tissues excised from the ileum of each rat fed with control diet 1 (0.12 µg Se/g feed), Se-enriched Diet 2 (1 µg Se/g feed) or Se-enriched + α-tocopherol Diet 3 (1 µg Se/g feed + 300 µg α-tocopherol/g feed), and expression levels normalized against the β-Actin reference gene. Fold change in GPx-1 and Gpx-2 mRNA levels was calculated relative to a basal level from non-heat stress rats on control Diet 1, which was set at an arbitrary expression level of 1. Number shows mean value and standard error for rats on Diet 1 (n= 7), Diet 2 (n= 7) and Diet 3 (n= 7) that were subjected to thermonutral (21 °C) or heat stress (40 °C) treatments.

* Mean values showing statistical significant effect relative to the basal (T21°C, diet 1) (P < 0.05, ANOVA).
4. Discussion

Relatively few studies have evaluated the protective effects of dietary supplementation against gastrointestinal dysfunction due to oxidative stress. Fasting (Kiliaan et al. 1998) and space restraint induced stress (Saunders et al. 1994) have previously been shown to increase small intestinal permeability in rats. In our study, we showed the correlation between heat induced oxidative stress and gastrointestinal permeability in rats and that exposure to acute heat stress (40 °C, 21% RH, 90 min) alters epithelial physiological function in the small intestine (ileum). The elevation of both rectal temperature and heart rate of rats in the heat stress treatment was consistent with the rats experiencing heat stress. The intestinal physiological changes observed included (1) an increase in baseline short circuit current (Isc) which is a measure of active ion transport across the mucosa (Saunders et al. 1994, Kiliaan et al. 1998), and (Kiliaan et al. 1998) (2) an elevated epithelial conductance (G_e) which is an indicator of gastrointestinal permeability (Saunders et al. 1994, Kiliaan et al. 1998, Santos & Perdue 2000).

The positive correlation between Isc and G_e was expected as an elevated ion transport state correlates with an increased epithelial permeability (Saunders et al. 1994, Santos & Perdue 2000, Forrest et al. 2006). Our study showed a protective effect of dietary mushroom Se supplementation against the increase in ileum permeability induced by acute heat stress. The Isc and G_e of ileum from heat stressed rats placed on Se-enriched Diet 2 for 3 weeks prior to the heat stress treatment were reduced by about half compared to those of the heat stressed rats on control Diet 1. It clearly indicates a reduction of the heat stress induced elevated ion transport across gut epithelium in the rats. Thus, dietary mushroom Se supplementation might have protective effects against hyperthermally induced oxidative stress damage to epithelial functions. It has been suggested that a combination of Se and α-tocopherol may offer better protection against epithelial injury and barrier disruption from induced oxidative stress (Al-Othman et al. 2011). However, we did not observe any enhanced protection against increased leakiness of the gut epithelium from heat stress with supplementation of the Se-enriched diet with α-tocopherol at the dosage evaluated in the study (300 μg/g feed). While heat stress rats on high Se and α-tocopherol containing Diet 3 had lower Isc and G_e than heat stress rats on control Diet 1, they were not significantly different from heat stress rats on Diet 2 with mushroom Se only. The latter Isc and G_e were also lower than that of the control. It may well be that the mushroom Se was already providing maximum protection that a further effect could not be detected with the level of α-tocopherol used in the study.

In order to provide insight into the effect of heat stress on epithelial function at a molecular level, we determined the effect of the mushroom Se supplementation on rat ileum GPx-1 and GPx-2
expression. These are major antioxidant selenoenzymes in the gastrointestinal tract, with GPx-2 the more dominant enzyme and also expressed exclusively in the gastrointestinal tract tissues (Esworthy et al. 1998, Chu et al. 2004). Thus, GPx-2, the more sensitive GPx isomer to changes in dietary Se levels (Drew et al. 2005), is believed to play a larger role in shielding the gastrointestinal tract from oxidative stress, offering protection from oxidative processes-linked inflammation ailments and cancers of the gut (Esworthy et al. 1998, Drew et al. 2005). We observed that regulation of ileum glutathione peroxidases GPx-1 and GPx-2 was influenced differently by the three diets (Figure 3). The effects of mushroom Se and α-tocopherol dietary supplementation on non-heat stress rats GPx-1 and GPx-2 genes are clear. Rats maintained under thermoneutral conditions and fed the Se-enriched Diet 2 showed a markedly elevated expression of ileum GPx-1 and GPx-2 mRNA above the basal level (thermoneutral rats on control Diet 1). Rats maintained under thermoneutral conditions and fed the Se-enriched + α-tocopherol Diet 3 also showed an increase in GPx-1 and GPx-2 mRNA expression over the basal level. It appears that what we are seeing here is a chronic effect of Se on increasing GPx-1 and GPx-2 expression which is often observed (Esworthy et al. 1998, Drew et al. 2005), and that additional supplementation with α-tocopherol at a dosage of 300 µg/g did not impact on the Se effect as the magnitude of the fold increases from the two diets were similar. The mushroom Se-enriched dietary effect on rat ileum was similar to the same diet effect on rat colon, which we have recently shown to significantly up-regulate colonic GPx-1 activity and GPx-1 and GPx-2 mRNA expression above basal (control) level (Maseko et al. 2014). The relations between the Se diet, heat stress, gut permeability and GPxs level is complex. Even though the data did not reveal the roles of the GPx’s in the modulation of the heat-induced permeability increase by Se, it appears that Se induces GPx-1 and 2 gene expression but blunts the increased expression caused by heat exposure.

Heat stressed rats on the control Diet 1 showed a markedly elevated expression of GPx-1 and GPx-2 mRNA above the basal (thermoneutral) level. The adverse effect of heat stress is the over production of ROS in the body which is known to cause oxidative cellular damage (Chu et al. 2004) and reduced intestinal function and integrity (Pavlick et al. 2002, Drew et al. 2005). Heat stress appears to produce an acute effect on the GPx-1 and GPx-2 expression as the animal attempts to counter the heat induced oxidative stress. Although heat stressed rats on the Se-enriched Diet 2 also showed an up-regulation of GPx-1 relative to basal level, the level was significantly lower than that of the heat stressed rats on control Diet 1. In addition, there was no observed up-regulation of GPx-2 of heat stressed rats on Diet 2 above the basal level. While it is recognised that genes expression levels might not be reflective of enzyme activity (Chu et al. 2004, Drew et al. 2005), further studies would be required to clarify their relationships. Other selenoproteins that could possibly have roles
in protection against heat stress were not investigated in this study. The observed effect of α-tocopherol and heat stress on the expression of the GPxs is rather complicated in the Se-enriched dietary regime. While heat stressed rats on the Se-enriched Diet 2 increased GPx-1 expression only, heat stressed rats on Se-enriched + α-tocopherol Diet 3 exhibited increased expressions of both GPx-1 and GPx-2 genes when compared to the basal level. It has been reported that dietary Vitamin E supplementation affected a number of endogenous antioxidants, and that Se is more effective at influencing GPxs up-regulation in carcinogenic malathion challenged rats than α-tocopherol, the latter being more effective at increasing the activity of non-seleno antioxidants such as catalase (Al-Othman et al. 2011). The continued high expression of GPx-2 in rats in the presence of α-tocopherol in the diet points to the special role of the gastrointestinal specific GPx-2 in protection against oxidative stress in the intestine. However, some studies have demonstrated synergism between Se and vitamin E, for example in the genetic inactivation of tRNA[Ser]\(^{\text{Sec}}\) required for selenoprotein expression which leads to diminished cerebral selenoprotein expression and neurodegeneration in mice (Wirth et al. 2010).

5. Conclusions

The present study provided evidence that dietary macro-fungal organic Se from Se-enriched A. bisporus protected the gastrointestinal tract in rats from the effects of heat induced oxidative stress, by restoring epithelial ion transport and barrier functions, and elevating the expression of GPx-1 and gastrointestinal specific GPx-2. Although α-tocopherol supplementation of the organic Se-enriched diet also displayed significant protection of the gastrointestinal tract during heat challenge, it offered no additional benefits to epithelial physiological function and tissue integrity. The relative ease in cultivating Se-enriched A. bisporus mushroom, its unique profile of bioactive Se organic species and its mitigating effects against gastrointestinal injury arising from heat stress in rats, would present the mushroom as a viable and efficient source of functional organic Se with demonstrated biological benefits.

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Author Contributions

Conception and experimental design: Maseko, T; Dunshea, F.R. and Ng, K. Data Collection, analysis and interpretation: Maseko, T; Dunshea, F.R.; Howell, K.; Ng, K.; Furness, J.B.; Rivera, L.R. and Cho, H. Drafting, corrections, critiquing and final revision of the manuscript for important scientific content: Maseko, T.; Dunshea, F.R.; Howell, K.; Ng, K. and Furness, J.B. The final version of the manuscript submitted was approved by all authors.

Conflicts of Interest

The authors declare no conflict of interest.
CHAPTER 6

Effect of dietary selenium supplementation in the form of Se-enriched Agaricus.bisporus mushroom, with or without additional supplementation with α-tocopherol on colonic regulation of antioxidant selenoproteins in rats exposed to hyperthermal stress.

Chapter 6 has been compiled into a manuscript, submitted and under review with British Journal of Nutrition (submitted 01-07-2014).
Chapter 6 addressed the following **Aim:**

i. The biological evaluation of the effect of selenium-enriched *A. bisporus* mushroom, with or without α-tocopherol on antioxidant selenoprotein status in the colon of rats exposed to hyperthermally induced oxidative stress.

The antioxidant selenoproteins evaluated were as follows:

a. GPx-1 mRNA expression and GPx-1 activity  
b. GPx-2 mRNA expression  
c. TrxR-1 mRNA expression  
d. SeP mRNA expression
Abstract

Dietary supplementation with macro-fungal organic Se from Se-enriched A. bisporus mushroom and α-tocopherol regulates expression of colonic GPx-1, GPx-2, TrxR-1 and SeP mRNA in rats differently under thermoneutral and heat stress (40°C, 21% relative humidity, 90 min exposure) conditions. Rats maintained under thermoneutral conditions and fed the mushroom-Se in dietary supplementation showed a significant ($P<0.05$) fold increase in mRNA expression relative to the basal treatment (rats fed the control diet) of GPx-1 (2.98±0.82), GPx-2 (1.8±1.4), TrxR-1 (1.83±0.64) and SeP (2.77±0.92), while supplementation with α-tocopherol alone only affected the expression of GPx-1 (1.83±0.82) and SeP (2.21±0.92). Rats subjected to heat stress treatment and fed the control diet also showed significant ($P<0.05$) fold increase in mRNA expression relative to the basal treatment of GPx-1 (3.15±0.82), GPx-2 (1.6±1.4), TrxR-1 (1.55±0.64) and SeP (1.93±0.92). The expression of GPx-2 (3.2±1.4) and SeP (4.23±0.92) in rats fed the mushroom-Se and expression of TrxR-1 (4.10±0.64) and SeP (7.44±0.92) in rats fed the α-tocopherol diet were higher. GPx-1 activity was increased by 3.8 fold in rats maintained under thermoneutral conditions and fed the mushroom-Se diet compared to rats fed the control diet. Heat stress was observed to have a negative impact on GPx-1 activity which was recovered by mushroom-Se, but not α-tocopherol, in the diet. Thus, Se-enriched A. bisporus mushroom presented an efficient source of functional organic Se with demonstrated biological effects linked to beneficial health properties and disease prevention and protection against heat induced oxidative stress and gut injury.

Introduction

Selenium is an essential trace element and micronutrient required for various metabolic and physiological functions in mammals, and its significance in human health and disease prevention is well documented (Clark et al. 1996, Rayman 2000, Flores-Mateo et al. 2006). Selenium occurs in both organic and inorganic forms, with the organically bound Se present mainly as selenomethionine (SeMet), selenocysteine (SeCys) and methylselenocysteine (MeSeCys) while the inorganic Se exists mainly as selenite and selenate salts in foods (Whanger 2002, Ogra et al. 2004, Amoako et al. 2009). The chemical form of Se is one of the determining factors of the element’s bioavailability, metabolic processing and fate, biological efficacy and toxicity (Mahan and Kim 1996, Shiobara, Yoshida and Suzuki). Moreover, it is the organic Se that has been linked to beneficial biological properties for good health and disease prevention (Clark et al. 1996, Flores-Mateo et al. 2006). This is supported by epidemiological and intervention studies that demonstrated higher Se intake above nutritional recommendations offer enhanced protection against
cardiovascular and inflammations ailments (Flores-Mateo et al. 2006), and reduced the incidence of colorectal, lung, prostate and liver cancers (Spolar et al. 1999, Diwadkar-Navsariwala and Diamond 2004).

Intervention studies have demonstrated that high dietary Se supplementation at 200 µg Se per day in the form of selenised yeast depressed the incidence and mortality of several types of cancers (prostate, colorectal and lung) by 50% over a period of ten years (Clark et al. 1996). Furthermore, dietary Se supplementation with selenised A. bisporus mushroom at 1 µg Se/g feed reduced 7, 12-dimethylbenz-[a]-anthracene (DMBA) induced mammary DNA adducts and decreased the incidence of anti-3,4-dihydrodiol-1,2-epoxide-deoxyguanosine adducts in DMBA challenged rats (Spolar et al. 1999). More recent studies have also demonstrated the protective effect of organic Se against diseases in the form of selenised milk proteins at 1 µg Se/g feed, which suppressed aberrant crypt foci, K-ras mutations and colorectal oncogenesis in azoxymethane challenged mice (Hu et al. 2008).

The biological efficacy and anti-carcinogenic benefits of organic Se are believed to come about through the expression of selenoamino acids containing selenoproteins and selenoenzymes, which are involved in mitigating cellular oxidative stress by inactivating cellular oxidants such as reactive oxygen species (ROS) (Holben & Smith 1999, Rayman 2000, Chu et al. 2004, Gromadzinska et al. 2008). The selenoenzymes, which contain catalytic selenocysteine at the active site, include the glutathione peroxidases (GPxs) and thioredoxin reductases (TrxRs) and are major cellular antioxidant enzymes (Arthur 2000, Rayman 2000, Hadley and Sunde 2001, Lu & Holmgren 2009).

Colorectal cancer (CRC) is reportedly the third most frequent cause of cancer mortality and the fourth most common cause of death in the world (WCRF & AICR 2007). Evidence suggested that oxidative stress induced by heat or other factors is characterised by the accumulation of ROS, and that they are significant contributing factors in gastrointestinal tract dysfunction and the pathogenesis of gastrointestinal tract ailments such as inflammatory bowel disease, fibrosis, ulcerative colitis and CRC (Pavlick et al. 2002, Chu, Esworthy & Doroshow 2004). The over production of ROS causes epithelial cells damage, reduced intestinal barrier function and integrity, and increases CRC risks (Pavlick et al. 2002, Drew et al. a2005).

Human epidemiological studies have shown an inverse relationship between organic Se intake and overall cancer risk, including CRC (Clark et al. 1996). GPx selenoenzyme isoforms include cytosolic GPx-1 which is expressed in almost all tissues, and gastrointestinal tract specific GPx-2, that play a significant role in the antioxidant defence of the gastrointestinal tissues against oxidative injury (Chu, Esworthy & Doroshow 2004, Drew et al. b2005). Several animal and human studies
have demonstrated an up-regulation in expression of selenoproteins and selenoenzymes following dietary Se supplementation. Hu et al. (2010) and Uglietta et al. (2007) showed an up-regulation in mRNA expression and activity of colonic GPxs following dietary Se supplementation with yeast-Se in mouse and pig respectively. Furthermore, human clinical trials showed an increase in expression of rectal GPxs and selenoprotein P (SeP) following dietary Se supplementation with dairy-Se and yeast-Se (Hu et al. 2011).

We have previously shown that Se-enriched *A. bisporus* mushroom cultivated by irrigation of growth compost with sodium selenite contains organic Se mainly as selenocysteine rich selenoproteins (Maseko et al. 2013). We also demonstrated that dietary Se supplementation with the mushroom-Se up-regulated colonic GPx-1 activity and mRNA expression of GPx-1 and GPx-2 in rats (Maseko et al. a2014). We have also demonstrated the protective effects of dietary Se supplementation with the same mushroom-Se against gut barrier dysfunction as indicated by reduced ileum permeability during hyperthermally induced oxidative stress in rat (Maseko et al. b2014). In this study, we evaluated the effects of dietary Se supplementation with the mushroom-Se and with or without additional α-tocopherol on the regulation of the expression of colonic selenoproteins GPx-1, GPx-2, TrxR-1 and SeP in hyperthermally induced oxidative stress in rat.

**Materials and Methods**

*Se-enriched and non-Se enriched A. bisporus mushroom*

Se-enriched *A. bisporus* (white button mushroom) was cultivated using grower kits comprised of growth compost inoculated with *A. bisporus* spawn, supplied by a commercial mushroom producer (Mushroom Exchange Pty. Ltd., Mernda, Victoria, Australia) and irrigation with sodium selenite solution as described elsewhere (Maseko et al. 2013). Non-Se enriched *A. bisporus* were also cultivated under normal mushroom growing conditions without irrigation with sodium selenite but water only. Mushroom caps were harvested from both mushrooms, frozen at -80 °C, and freeze-dried. The lyophilised caps were then grounded into fine powders using a commercial blender and stored at RT in moisture free sealable packs until required for preparation of rat feeds.

*Preparation of control, Se-enriched, α-tocopherol and Se-enriched + α-tocopherol diets*

Rat feed was prepared in the form of pellets by Specialty Feeds Co (Glen Forrest, Western Australia). Four diet types including the control were prepared. The low Se + low α-tocopherol diet serving as the control diet was prepared by supplementing 10 kg of a low Se rodent feed
formulation AIN 93G (composition detailed in Maseko et al. "2014) with 20 g control (non-Se enriched) mushroom caps (containing 2.22 µg Se/g dried caps) with no α-tocopherol supplementation to give a final Se content of 0.12 µg Se/g feed. The high Se + low α-tocopherol diet was prepared by supplementing 10 kg of AIN 93G with 142 g of Se-enriched mushroom caps (containing 62.20 µg Se/g dried caps) with no α-tocopherol supplementation to give a final Se content of 1 µg Se/g feed. The low Se + high α-tocopherol diet was similarly prepared as the control diet but with the addition of α-tocopherol to give a final Se and α-tocopherol contents of 0.12 µg Se/g + 300 µg α-tocopherol/g feed. The high Se + high α-tocopherol diet were similarly prepared as the high Se diet but with the addition of α-tocopherol to give a final Se and α-tocopherol contents of 1 µg Se/g + 300 µg α-tocopherol/g feed.

**Animals**

A total of sixty four 9 weeks old Sprague Dawley male rats weighing 302-426 g obtained from a colony without known adventitious viruses, mycoplasma, enteric pathogenic bacteria and parasites, were purchased from the Monash University Animal Services, Melbourne, Australia. The animal experiment protocols were approved by the Melbourne School of Land & Environment Research Animal Ethics Committee, University of Melbourne (ethics approval no 1312820.1). Rats were randomly divided into 4 experimental dietary groups and housed 2 per cage. The rats were housed in an air-conditioned, temperature controlled animal facility with a 12 h light-dark cycle at 21 °C. Rats were given free access to food and water at all times.

**Animal feeding**

Rats were randomly assigned to 4 experimental diets in a 2 x 2 factorial design: Control diet of low Se + low α-tocopherol; High Se diet of high Se + low α-tocopherol; high α-tocopherol diet of low Se + high α-tocopherol; high Se and α-tocopherol diet of high Se + high α-tocopherol. All rats were initially acclimatised with free access to the control diet and water for one week before the animals were given their respective diets and water ad libitum for a further period of 3 weeks. Body weights of the animals were recorded at the start of the experiment and continued weekly to monitor their growth which was normal. Their food intake and behaviour were also monitored throughout. These were also normal.
**Acute heat stress protocol**

After 21 days of feeding of the allocated diets, rats from each diet were randomly allocated to thermoneutral and acute heat stress treatment groups in a 2x2x2 factorial design (2 levels Se x 2 levels α-tocopherol x 2 levels temperature) and their body weights determined. Rats in the thermoneutral treatments were exposed to an ambient temperature of 21°C and had their feed removed but allowed free access to water for a period of 90 min. Rats exposed to acute heat stress were housed individually in cages, exposed to 40 °C and 21 % relative humidity (RH) and also had their feed removed and allowed free access to water for a 90 min period in a temperature controlled room. Preliminary observation of rats indicated that they can tolerate 40 °C and 21 % RH conditions for up to 90 mins but beyond that time point distress symptoms such as hyperventilation and lethargy sets in, thus the thermoneutral and acute heat stress condition was limited to 90 min exposure. Animals from both treatments were monitored every 10 min over the 90 min treatment duration for rectal temperatures using a temperature probe (Vicks Speed-Read Digital thermometer, 10 mm x 3 mm probe) and heart rates using a stethoscope (as beats per minute: bpm) . Lubricant was used to aid thermometer probe insertion into the rectum. At the end of the treatments, rats were allowed to rest for 20 min at RT before being anaesthetised and sacrificed for tissue excision.

**Animal euthanasia and tissue excision (colon)**

Animals were killed with an initial step of a single intra-peritoneal injection of ketamine and xylazine mix to anaesthetise and an overdose of the ketamine and xylazine mix as the final euthanasia step. Colon tissues were recovered from each rat, and a portion of each tissue was separately placed in RNAlater® solution at 4 °C for 24 h before storage at -80 °C until real time PCR analysis. The remaining portion of the colon tissue from the rat was rapidly frozen in liquid N₂ and stored at -80 °C for GPx-1 activity assay.

**Gene expression**

**RNA isolation**

Total RNA was extracted from RNAlater® solution stabilized colon tissues (30 mg) using a commercial kit, QIAGEN RNeasy® Mini Kit (QIAGEN, Victoria, Australia), and the extraction was performed in triplicates for each rat colon tissue. The quality (purity) and concentration of total RNA extracted was determined using NanoDrop® ND-1000 UV–Vis spectrophotometer by
measuring the absorbance at λ260 nm and 280 nm and determining the 260:280 absorbance ratios. Pure RNA has an A260:A280 ratio of 1.9–2.1, and the extracted RNA samples from the rats’ colon tissues had A260: A280 ratios between 1.94 and 2.13 (data not shown).

cDNA synthesis

The BIO-RAD iScript® Select cDNA Synthesis Kit (NSW, Australia) was used to synthesise the first strand cDNA (20 µL) from 0.3 µg total RNA from each colon sample. The cDNA product was diluted 1:30 with nuclease-free water and used for real-time quantitative PCR.

Real-time quantitative PCR

Real-time quantitative PCR of GPx-1, GPx-2, SeP, and TrxR-1 genes was carried on an iQ® 5 Multicolor Real-Time PCR icycler Detection System (BIORAD, NSW, Australia). Oligonucleotide primers were designed using Primer 3 software v.0.4.0 (Bioinformatics Methods and Protocols: Methods in Molecular Biology, Humana Press, Totowa, NJ, USA) based on sequences obtained from the Genbank database (Table 1). The primers were optimized and validated by conventional PCR of cDNA (data not shown). The PCR reagents for quantitative analysis were contained in the iQ SYBR Green Supermix kit from BIORAD. The PCR reactions were performed in a final volume of 20 µL containing 6 µL of diluted cDNA (1:30, v/v) and 10 µL iQ SYBR Green Supermix. Primer concentrations in the reaction mix for each gene was 250 nM for both the sense and antisense primer pairs. Nuclease free water was used to make up to the final volume.

The cycling PCR reaction for each sample started with an initial hot start of 95 °C for 3 min as the initial denaturation step of 1 cycle, followed by 45 cycles at 95 °C for 30 s (denaturation), 60 °C for 30 s (annealing), 72 °C for 30 s (extension) and completed with a final extension step at 95 °C for 1 min. The specificity of the PCR reaction (product) was demonstrated by melting curve analysis post PCR reactions; which showed only one peak present for all PCR products of GPx-1, GPx-2, SeP, and TrxR-1 test genes and the glyceraldehyde-3-phosphate dehydrogenase, GAPDH, reference gene. A non-template (without cDNA) reaction was included with each PCR run as a negative control. The real-time quantitative PCR assay was optimised by running serial dilutions of cDNA template and using the results to generate a standard curve. The linear regression line and the coefficient of determination (R²) of the standard curve were used to evaluate whether the qPCR assay was optimised. Amplification efficiency (E) of primer pair for each gene was calculated from the slope of the standard curve. Relative gene expression for each target gene, in the test and control
samples using reference GAPDH gene as a normaliser was determined. The cycle threshold (CT) values for each target gene were normalised with the reference gene GAPDH for both test and control samples, and the Pfaffl method of relative quantification normalized to a reference gene was used to calculate the relative gene expression of the target genes in the colon samples (BIORAD Real-Time PCR Applications Guide-Gene Expression).

Table 1: Oligonucleotide primers used for real-time quantitative PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene accession number</th>
<th>Primers</th>
<th>Primer sequence 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPx1</td>
<td>NM_030826</td>
<td>Sense</td>
<td>TGAGAAGTGCGAGGTGAATG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antisense</td>
<td>CGGGGACCAAATGATGTACT</td>
</tr>
<tr>
<td>GPx2</td>
<td>NM_183403</td>
<td>Sense</td>
<td>TGCCCTACCCTTATGACGAC</td>
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<tr>
<td></td>
<td></td>
<td>Antisense</td>
<td>TCGATGTGTGATGGTCTGGAA</td>
</tr>
<tr>
<td>TrxR-1</td>
<td>NM_031614</td>
<td>Sense</td>
<td>CTCTTTCCGCACACACGATA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antisense</td>
<td>CTGTGGGCTCCTGAACAGA</td>
</tr>
<tr>
<td>SeP</td>
<td>NM_001083911</td>
<td>Sense</td>
<td>GGCCGTCTTTGTGTATCACCT</td>
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<tr>
<td></td>
<td></td>
<td>Antisense</td>
<td>GTTTGTCATGGTGCTTGTGG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>NM_017008</td>
<td>Sense</td>
<td>CTCATGACCACAGCTCATGC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antisense</td>
<td>TTCAGCTCTGGGATGACCTT</td>
</tr>
</tbody>
</table>

Glutathione peroxidase-1 (GPX-1) activity assay

Glutathione peroxidase-1 specific enzyme activity was measured using a Glutathione Peroxidase Cellular Activity Assay Kit (Sigma–Aldrich, cat no CGP1, NSW, Australia) in rat colon. The colon was cut open and the mucosa layer scraped off. The colon was then placed in 1 ml of cold (4 °C) 50 mM Tris–HCl buffer (pH 8) containing 0.5 mM EDTA in a 2 mL tube containing 7 ceramic beads (2.8 mm diameter ceramic beads, cat no 1311450, Gene Works,Australia). The tube was placed in a rotor-stator tissue disruptor bead beater (MB8 model, BioSpec Products, DainTree
Scientific Pty, Australia) for 5 min to lyse and homogenise the tissue and the homogenate quickly placed in an ice bath to chill. The homogenate was then centrifuged at 14,000 g (Beckman Benchtop Centrifuge, Allegra X-22 series, Gladesville, Australia) for 35 min and the supernatant collected as the solubilised source of GPx-1.

Protein concentration in the 50 µL of the supernatant was determined in triplicate using the Bradford protein assay kit (cat no. B6916-500, Sigma Aldrich, NSW, Australia). GPx-1 activity in 50 µL of the supernatant was determined in triplicate by adding 950 µL of reaction solution containing 50 mM Tris HCl buffer (pH 8), 0.5 mM EDTA, 5 mM NADPH, 42 mM reduced glutathione, 10 units/mL glutathione reductase and 30 mM tert-butyl hydroperoxide as the substrate. A glutathione peroxidase standard stock solution (100 unit/ml supplemented with 1 mg/mL IgG and 1 mM DDT) was used as a positive activity control. The decrease in NADPH was measured every 30 s over a 10 min time period by absorbance at λ340 nm using a UV–Vis spectrophotometer (Multiskan spectrophotometer, Thermo Electron, Thermo Fishers Scientific, USA). The enzymatic oxidation of NADPH to NADP⁺ was linear over the 10 min time interval. One unit of glutathione peroxidase is defined as the enzyme reaction that causes the formation of 1 µmol of NADP⁺ from NADPH per min in the presence of 40 mM reduced glutathione, 9.5 unit/mL glutathione reductase and 28.5 mM tert-butyl hydroperoxide. The specific activity of GPx-1 was expressed as mU/mg protein.

**Statistical Analysis**

The mean of triplicate determinations was used to calculate the group mean and uncertainty as standard deviation or standard error. Statistical analyses of group means by three-factor analysis of variance (ANOVA), 2 (levels of Se) x 2 (levels of α-tocopherol) x 2 (levels of temperature), were performed, with a subsequent multiple comparison by Fisher’s Protected Least Significant Difference (LSD) test using GenStat (14ed). Statistical significance was defined as P value less than 0.05 (P<0.05) for within group and between group comparisons. Uncertainty of group means was reported to 2 significant figures according to the European Analytical Chemist guidelines (Ellison & Williams 2012).
Results

Glutathione peroxidase-1 mRNA expression

The expression level of GPx-1, GPx-2, TRx-1 or SeP mRNA of rats kept under thermoneutral conditions and fed the control diet is defined as the basal level and is set at an arbitrary level of 1 (Figs. 1 and 2, low Se + low α-tocopherol diet).

Dietary Se (P<0.05) at 1µg Se/g feed and α-tocopherol (P<0.05) at 300 µg/g feed supplementation and acute heat stress (P<0.05) all increased colonic GPx-1 mRNA expression (Fig 1A). However, there was an interaction between both levels of dietary Se and heat stress treatment (P<0.05) such that GPx-1 mRNA expression was increased during heat stress for rats not supplemented with Se. Rats fed the high Se + low α-tocopherol diet and kept under thermoneutral conditions displayed a significant increase in GPx-1 expression at 2.98±0.82 fold above the basal level. A combination of mushroom-Se with α-tocopherol in the high Se + high α-tocopherol diet produced no additional increase in GPx-1 mRNA expression (P<0.05) at 2.40±0.82 fold increase over the basal level (Fig 1A). Acute heat stress significantly increased GPx-1 mRNA expression (P<0.05) across all diets, with rats on the high α-tocopherol + low Se diet displaying the highest GPx-1 expression at 3.52±0.82 fold increase over the basal level. However, there were no significant differences in GPx-1 expression (P<0.05) amongst all diets under acute heat stress conditions.

Glutathione peroxidase-2 mRNA expression

GPx-2 mRNA expression (P<0.05) was significantly increased by dietary Se supplementation and acute heat stress but not by α-tocopherol under thermoneutral conditions. There was an interaction between Se and α-tocopherol for GPx-2 expression (P<0.05). Supplementation with α-tocopherol to the high Se diet, did not show any further enhancement of GPx-2 expression for both thermoneutral and heat stress conditions (Fig 1B) as both diets (high Se and high Se + high α-tocopherol) stimulated the level of GPx-2 similarly under thermoneutral conditions and there was no further improvement in GPx-2 expression (P<0.05) under heat stress conditions. However, unlike GPx-1, rats placed on the high α-tocopherol diet (high α-tocopherol + low Se) and subjected to heat stress treatment significantly increased GPx-2 expression (P<0.05) (2.72±1.4 fold above basal level) further than those on the control diet (low Se + low α-tocopherol) and exposed to heat stress (1.6±1.4) treatment. Furthermore, in contrast with GPx-1 again, rats fed the Se-enriched diet (high
Se + low α-tocopherol), and subjected to heat stress treatment, significantly increased GPx-2 expression (P<0.05) further than those exposed to heat stress treatment alone (Fig 1B).

**Thioredoxin reductase-1 mRNA expression**

TrxR-1 mRNA expression (P<0.05) was increased by dietary Se, dietary α-tocopherol and acute heat stress (Fig 2A). However, there were interactions between dietary Se and α-tocopherol, and between α-tocopherol and heat stress such that heat stress increased TrxR-1 expression (P<0.05) to a much greater level in rats placed on the high α-tocopherol (high α-tocopherol + low Se) diet (4.10±0.64 fold increase above the basal level) than those fed the high Se diet (Fig 2A). Rats kept under thermoneutral conditions and fed the high Se diet significantly increased TrxR-1 mRNA expression (P<0.05) (1.83±0.64 fold increase above the basal level), however there was no increase in TrxR-1 expression (P<0.05) for rats on the high α-tocopherol (high α-tocopherol + low Se) or the mushroom-Se/α-tocopherol combination (high Se + high α-tocopherol) diets (Fig 2A). Furthermore, supplementation with α-tocopherol to the high Se diet did not further enhance the expression of TrxR-1 (P<0.05) for rats exposed to acute heat stress (Fig 2A).

**Selenoprotein P mRNA expression**

It was observed that dietary Se, α-tocopherol supplementation and heat stress significantly increased the expression of SeP mRNA (P<0.05)(Fig 2B). There were interactions between dietary α-tocopherol, selenium and heat stress (P<0.05). Similar to TrxR-1 expression, acute heat stress increased SeP expression (P<0.05) to a much higher level for rats that received the high α-tocopherol (high α-tocopherol + low Se) diet compared to rats fed the high Se diet under similar conditions (Fig 2B). The rats on the high α-tocopherol diet and subjected to heat stress displayed the highest SeP expression at 7.44±0.92 fold increase above the basal level (Fig 2B). However, there was no significant difference in the level of colonic SeP expression (P<0.05) between heat stress rats fed the high Se diet and the high Se/high α-tocopherol combination diet. In contrast with TrxR-1, rats placed under thermoneutral conditions and fed the high α-tocopherol diet displayed a significant increase in SeP expression (P<0.05) at 2.21±0.92 fold increase above basal level and SeP expression for the high α-tocopherol diet was not significantly different from the high Se and high Se/high α-tocopherol combination diets under thermoneutral conditions.
**Glutathione peroxidase-1 activity**

GPx-1 activity was measured in rat colon and the enzyme activity of rats kept under thermoneutral conditions and fed the control diet defined as the basal level enzyme activity which was 9.73±1.55 U/mg protein (Fig. 3-low Se + low α-tocopherol diet).

GPx-1 activity (P<0.05) significantly increased following dietary supplementation with Se but remained unchanged (P<0.05) with dietary α-tocopherol supplementation under thermoneutral conditions. Colonic GPx-1 enzyme activity for rats maintained under thermoneutral conditions and fed the high Se diet was 37.2±1.6 U/mg protein which was a 3.8 fold increase above basal level (Fig 3). There was no further enhancement of GPx-1 activity with the supplementation of α-tocopherol to the high Se diet (high Se + high α-tocopherol) for rats under thermoneutral conditions. GPx-1 activity was markedly reduced for rats subjected to acute heat stress and fed the control (low Se + low α-tocopherol) and the high α-tocopherol (high α-tocopherol + low Se) diets, indicating the failure of α-tocopherol to protect the colon from loss of GPx-1 activity. However, dietary Se supplementation restored GPx-1 activity (P<0.05) under heat stress (8.6±1.5 U/mg protein) to the same level as that of the basal level (low Se + low α-tocopherol diet, under thermoneutral conditions) (Fig 3). Furthermore, although the mushroom-Se/α-tocopherol combination (high Se + high α-tocopherol) diet also restored colonic GPx-1 activity under heat stress, there was no further improvement in GPx-1 activity (P<0.05) relative to the effect of mushroom-Se alone (high Se + low α-tocopherol diet).
Figure 1. Effect of dietary Se supplementation on colon (A) GPx-1 and (B) GPx-2 mRNA expression from rats exposed to thermoneutral (T21°C) or acute heat stress conditions (T40°C, 21% R.H., 90 min exposure). mRNA levels were measured in triplicate colon tissues excised from the colon of each rat fed the control diet (low Se + low α-tocopherol; 0.12 µg Se/g feed); high Se diet (high Se + low α-tocopherol; 1 µg Se/g feed); high α-tocopherol diet (low Se + high α-tocopherol; 0.12 µg Se/g feed + 300 µg α-tocopherol/g feed) or high Se + high α-tocopherol diet (high Se + high α-tocopherol; 1 µg Se/g feed + 300 µg α-tocopherol/g feed), and expression level normalized
against the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reference gene. Fold change in GPx-1 and GPx-2 mRNA levels was calculated relative to a basal level from thermoneutral rats fed the control diet, which was set at an arbitrary expression level of 1. Number shows mean value and standard error of a difference between means (SED) for rats on control diet (n=8), high Se diet (n=8), high α-tocopherol diet (n=8) and high Se + high α-tocopherol diet (n=8) that were subjected to thermoneutral (T21°C) or heat stress (T40°C, 21% RH, 90 min exposure) treatments.

*Mean values statistically significantly different from the control (P<0.05 in ANOVA)
Figure 2: Effect of dietary Se supplementation on colon (A) TrxR-1 and (B) SeP mRNA expression from rats exposed to thermoneutral (T21°C) or acute heat stress conditions (T40°C, 21% R.H., 90 min exposure). mRNA levels were measured in triplicate colon tissues excised from the colon of each rat fed the control diet (low Se + low α-tocopherol; 0.12 µg Se/g feed); high Se diet (high Se + low α-tocopherol; 1 µg Se/g feed); high α-tocopherol diet (low Se + high α-tocopherol; 0.12 µg Se/g feed + 300 µg α-tocopherol/g feed) or high Se + high α-tocopherol diet (high Se + high α-tocopherol; 1 µg Se/g feed + 300 µg α-tocopherol/g feed), and expression level normalized against the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reference gene. Fold change in TrxR-1
and SeP mRNA levels was calculated relative to a basal level from thermoneutral rats fed the control diet, which was set at an arbitrary expression level of 1. Number shows mean value and standard error of a difference between means (SED) for rats on control diet (n=8), high Se diet (n=8), high α-tocopherol diet (n=8) and high Se + high α-tocopherol diet (n=8) that were subjected to thermoneutral (T21°C) or heat stress (T40°C, 21% RH, 90 min exposure) treatments.

*Mean values statistically significantly different from the control (P<0.05 in ANOVA)
Figure 3: Effect of dietary Se supplementation on colon GPx-1 activity from rats exposed to thermoneutral (T21°C) or acute heat stress conditions (T40°C, 21% R.H., 90 min exposure). GPx-1 activity levels were measured in triplicate colon tissues excised from the colon of each rat fed the control diet (low Se + low α-tocopherol; 0.12 µg Se/g feed); high Se diet (high Se + low α-tocopherol; 1 µg Se/g feed); high α-tocopherol diet (low Se + high α-tocopherol; 0.12 µg Se/g feed + 300 µg α-tocopherol/g feed) or high Se + high α-tocopherol diet (high Se + high α-tocopherol; 1 µg Se/g feed + 300 µg α-tocopherol/g feed). Number shows mean value and standard error of a difference between means (SED) for rats on control diet (n=8), high Se diet (n=8), high α-tocopherol diet (n=8) and high Se + high α-tocopherol diet (n=8) that were subjected to thermoneutral (T21°C) or heat stress (T40°C, 21% RH, 90 min exposure) treatments.

**Mean values statistically significantly different (higher) than the basal level (P<0.05 in ANOVA)

* Mean values statistically significantly different (lower) than the basal level (P<0.05 in ANOVA)
Discussion

The study observed an up-regulation in the expression of all colonic selenoproteins examined, following dietary Se supplementation, under both thermoneutral and acute heat stress conditions. However, it was observed that the effects of dietary Se and α-tocopherol supplementation and acute heat stress regulated the colonic selenoproteins uniquely. During acute heat stress, it was observed that SeP, a Se transportation and carrier protein believed to influence the expression of other individual selenoproteins (Richardson 2005, Hoffmann et al. 2007) was expressed at the highest level compared to all selenoproteins, followed by TrxR-1 and then the GPxs. Dietary supplementation with Se but not α-tocopherol increased GPx-1 activity under thermoneutral conditions. However, acute heat stress depressed GPx-1 activity which was restored to the basal level by dietary Se supplementation but not by α-tocopherol.

Dietary Se supplementation with Se-enriched foods is an effective means to deliver Se to the population. Selenised mushrooms are an attractive food source for such a purpose in addition to selenised yeast and selenised milk proteins. However, the organic Se species profiles of yeast-Se, dairy-Se and mushroom-Se are different (Rayman 2004, Gergely et al. 2006, Maseko et al. 2013), and there is increasing evidence that they also produce differential effect on the expression of selenoproteins relevant for Se biological activity such as GPx-1, GPx-2, TRx-1 and SeP (Uglietta et al. 2007, Uglietta et al. 2008, Hu et al. 2010, Maseko et al. 2014). Selenised A. bisporus (button mushroom) that was cultivated by irrigation of growth compost with sodium selenite solution was used in our dietary supplementation experiment to look at the effect of the mushroom organic Se on the expression of selenoproteins in rat colon under thermoneutral and oxidative heat stress conditions.

Rats maintained under thermoneutral conditions and fed a basic control diet supplemented with the mushroom-Se for 3 weeks significantly up-regulated colonic GPx-1 and GPx-2 mRNA expression above the rats that were maintained under thermoneutral conditions but fed only the control diet. This is a chronic effect of dietary mushroom-Se on increasing GPx-1 and GPx-2 expression which we have demonstrated before in the rat colon (Maseko et al. 2014). The chronic effect of Se has also been observed using different organic Se sources although there were different effects on these 2 GPx isoforms. For example, Hu et al. (2010) showed that dietary supplementation with yeast-Se up-regulated colonic GPx-1 mRNA expression but not GPx-2 mRNA in the mouse, while GPx-2 mRNA expression was up-regulated by dietary supplementation with dairy-Se. In contrast, Uglietta et al (2007) showed that colonic GPx-2 mRNA expression was up-regulated by dietary supplementation with yeast-Se in the pig. These findings from animal studies demonstrated that the
type of organic Se species present in the diet determines Se efficacy in influencing the expression of the selenoproteins. Yeast-Se occurs primarily as free selenomethionine (70-80 %) plus some free selenocysteine (4%) (Ip et al. 2003), while dairy-Se is primarily made up of free selenomethionine (83%) and some protein bound selenocysteine (5%) (Heard et al. 2007). By contrast, our cultivated A. bisporus mushroom-Se used in this study consisted mainly of protein bound SeCys (95.5%) and SeMet (3.6%), plus a small amount of protein associated but free MeSeCys (0.9%) (Maseko et al. 2013).

α-Tocopherol is known to affect the expression of some antioxidant enzymes such as GPx-4, a phospholipid hydroperoxide glutathione peroxidase, whose expression level in rats is affected by α-tocopherol deficiency in the diet (Bourre et al. 2000). Thus, we also looked at the effect of α-tocopherol supplementation on colonic selenoproteins genes expression in rats fed the control and the mushroom-Se enriched diets. Indeed, α-tocopherol at 300 µg/g feed influenced the expression of rat colonic GPxs by increasing GPx-1 but not GPx-2 mRNA levels. However, additional α-tocopherol in the mushroom-Se/α-tocopherol combination diet did not produce further enhancement in the expression of colonic GPx-1 or GPx-2 in rats compared to the level induced by mushroom-Se alone, further highlighting the chronic effect of mushroom-Se on these 2 genes. Selenium in its inorganic form (sodium selenite) has also been shown to preferentially affect GPxs expression and catalase activity over that of α-tocopherol in malathion challenged rats (Al-Othman et al. 2011).

There was an acute response in the expression of colonic GPx-1 and GPx-2 in rats to heat stress which induced a markedly elevated expression of these 2 genes. It is known that the adverse effect of heat stress results in the over production and accumulation of ROS in body tissues such as the intestines, which can disrupt intestinal physiological function and reduce mucosal barrier function and integrity (Pavlick et al. 2002, Chu, Esworthy & Doroshow 2004).These conditions are contributing factors in the pathogenesis of gastrointestinal tract ailments such as inflammatory bowel disease, fibrosis, ulcerative colitis and colon cancer (Kiliaam et al. 1998, Pavlick et al. 2002).The elevated mRNA expression of the colonic GPx-1 and GPx-2 in the heat stressed rats indicates an acute effect of heat stress on the GPxs expression as the animals attempted to counter the heat induced oxidative stress in the tissue. This acute effect was observed with the animals exposed to 40 °C heat and 21 % relative humidity for only 90 min. The expression of liver GPx-1 mRNA has also been shown to respond acutely to (cyclic) heat stress (23-38-23 °C) over a 2 h duration in a similar rat strain (Yung et al. 2012).
Heat stressed rats fed the mushroom-Se supplemented diet also exhibited an up-regulation of colonic GPx-1 and GPx-2 mRNA expression. The level of colonic GPx-1 mRNA was similar to that induced by heat stress alone for rats fed the control diet, but, significantly, the level of colonic GPx-2 mRNA was higher. Supplementation with α-tocopherol alone in the diet did not produce enhanced expression of colonic GPx-2 observed with mushroom-Se in heat stressed rats beyond that induced by heat stress. These results supported previous observation that GI specific GPx-2 is more sensitive to changes in Se levels in the diet (Banning et al. 2005), and additionally pointed to the larger role GPx-2 plays in the protection of the colon against hyperthermally induced oxidative stress.

Although dietary supplementation with mushroom-Se enhanced colonic GPx-2 expression in heat stress rats beyond that induced by heat stress alone, the combination of the mushroom-Se with α-tocopherol in the diet failed to induce similar enhancement. It appears that the tissue required a reduced need for GPx-2 with α-tocopherol in diet as α-tocopherol could possibly influence the expression of non-selenium antioxidants such as catalase as the animal attempts to mitigate the effects of induced oxidative stress (Al-Othman et al. 2011).

Up-regulation of GPx-1 mRNA expression in tissues normally correlates with increased GPx-1 activity following Se dietary supplementation. Thus, both GPx-1 mRNA expression and activity were increased in mice colon following yeast-Se and dairy-Se dietary supplementation (Hu et al. 2008, Hu et al. 2010) and in rat colon following A. bisporus mushroom-Se dietary supplementation (Maseko et al. 2014). The effect of the mushroom-Se on GPx-1 activity was confirmed in the present study, with an observed 3.8 fold increase in colonic GPx-1 activity in rats maintained under thermoneutral conditions and fed the A. bisporus mushroom-Se supplemented diet which correlated with a 3.0 fold increase in colonic GPx-1 mRNA expression. Interestingly, while rats fed the α-tocopherol supplemented diet increased colonic GPx1 mRNA expression compared to rats fed the control diet it didn’t produce a corresponding increase in GPx-1 activity. Nonetheless, mushroom-Se in combination with dietary α-tocopherol supplementation produced the same effect as mushroom-Se alone in increasing colonic GPx-1 mRNA expression and enzyme activity.

It is known that an adverse effect of heat stress is the over production of ROS as hydrogen peroxides and superoxides in the body (Chu, Esworthy & Doroshow 2004) and GPxs together with superoxide dismutase are major intracellular enzymatic antioxidant defence against ROS (Ganaie et al. 2013). However the production and removal of various ROS is a dynamic and complex process whose purpose is to attain a stable state of very low concentrations of these oxidants in the cell.
(Toussaint, Houbion & Remacle 1993). Even though heat stress increased colonic GPx-1 mRNA expression in rats, it negatively impacted on the GPx-1 activity level below that of the non-heat stressed rats. Importantly, mushroom-Se supplementation in the diet restored colonic GPx-1 activity level in heat stressed rats back to normal (that is, that of rats maintained under thermoneutral conditions and fed the control diet). In contrast, α-tocopherol supplemented diet failed to restore colonic GPx-1 activity level back to normal. It appears that exposure to acute heat stress might have accumulated ROS overwhelmingly in the colon that depressed GPx-1 activity that was recovered by mushroom-Se, but not by α-tocopherol, in the rats diet.

The role of TrxR is to regenerate thioredoxin from its oxidised form as part of the thioredoxin-peroxiredoxin antioxidant system that complements the glutathione peroxidase antioxidant system in the antioxidant defence of cells (Zhong & Holmgren 2000, Yalcin 2004). Similar to the GPxs, Se availability is the key factor in the expression and activity of TrxRs which included the predominant cytosolic specific TrxR-1 (Mustacich & Powis 2000). Nonetheless, mushroom-Se has little or no observable effect on rat colonic TrxR-1 mRNA expression maintained under thermoneutral conditions with or without additional α-tocopherol in diet, and α-tocopherol itself in the diet also did not increase TrxR-1 expression. The failure of dietary Se in feeds in influencing GI TrxR-1 expression has been observed before in mice colon and human rectum using dairy-Se and yeast-Se (Hu et al. 2010, Hu et al. 2011), and in rat colon using the mushroom-Se (Maseko et al. 2014) in dietary supplementations. The literature consensus is to attribute to the lesser role of the Trx-1 in the thioredoxin-peroxiredoxin antioxidant system compared to the GPxs in the antioxidant defence of colonic and rectum tissues.

SeP is believed to be a Se transportation or carrier protein and as such influences the level of expression of other selenoproteins and also of selenoenzymes required for anti-oxidative defence (Richardson 2005, Hoffmann et al. 2007). Thus, SeP knock-out mice are linked to increased carcinogen-induced cancer development (Diwadkar-Navsariwala et al. 2006, Hu et al. 2008) and significant reduction or loss of SeP mRNA expression is observed in colorectal cancer (Al-Taie et al. 2004, Richardson 2005). However, the literature contains contradictory reports on the effect of dietary Se on SeP expression. For example, dietary supplementation with yeast-Se has been reported to have no effect on colonic SeP mRNA expression in mice (Hu et al. 2010) and in azoxymethane challenged rats (Uglietta et al. 2006). However, a dose dependent up-regulation of colonic SeP was observed in pigs with yeast-Se, and similar to the effect on GPx-2 expression the effect was independent of sex (Uglietta et al. 2007). Dairy-Se was also reported to increase colonic SeP expression in mice in a dose dependent manner (Hu et al. 2010). In the present study,
significant increase in SeP mRNA expression was observed in the colon of rats maintained under thermoneutral conditions across all supplementary diets (mushroom-Se, α-tocopherol, mushroom-Se+α-tocopherol) compared to rats fed the basal diet.

Heat stress produced interesting results on the expression of TrxR-1 and SeP in rat colon across the 4 dietary regimes. Heat stress increased the expression of colonic SeP significantly but had a marginal significant effect on TrxR-1 compared to the thermoneutral level for rats fed the control diet. Mushroom-Se produced a significant increase in the expression of both colonic SeP and TrxR-1 compared to heat stressed rats fed the control diet although expression of SeP was much higher. Furthermore, α-tocopherol was much more effective than mushroom-Se in increasing SeP and TrxR-1 expression compared to heat stressed rats fed the control diet. But this effect was not reproduced in rats fed the mushroom-Se/α-tocopherol combination suggesting a reduced need for high expression of the 2 genes in the presence of α-tocopherol where there is mushroom-Se.

It is known that α-tocopherol influenced the expression of certain selenoproteins such as muscle SePx1, a selenoprotein involved in ca²⁺ homeostasis and schizophrenia (Huang et al. 2011). But cells have varying prioritisation of specific selenoprotein expression and synthesis in response to dietary supply of Se. For example, Se supplementation in Se deficient human subjects had GPx activity optimised before SeP concentration (Richardson 2005). Similarly, the effect of heat stress might cause this varying prioritization of specific selenoproteins expression in response to animals protecting themselves from the effects of hyperthermally induced oxidative stress. Thus, the observation of a much lower expression level of both SeP and TrxR-1 in rat colon of heat stressed rats fed the mushroom-Se/α-tocopherol combination diet compared to heat stressed rats fed only the α-tocopherol diet.
Conclusions

The present study provided evidence that dietary supplementation with macro-fungal organic Se from Se-enriched *A. bisporus* mushroom and α-tocopherol regulates colonic selenoproteins in rats differently under thermoneutral and heat stress conditions. GPx-1, GPx-2, TrxR-1 and SeP mRNA expression were up-regulated following dietary Se supplementation with Se-enriched *A. bisporus* under thermoneutral conditions but additional α-tocopherol did not enhance the mushroom-Se effect. Heat stress treatment also up-regulated GPx-1, GPx-2, TrxR-1 and SeP across all diets, but mushroom-Se increased GPx-2 expression above the effect of heat stress alone indicating its special role in protection against hyperthermally induced gut injury. Mushroom-Se, but not α-tocopherol, increased GPx-1 activity in thermoneutral rats. While heat stress had a negative impact on GPx-1 activity, dietary supplementation with mushroom-Se, but not α-tocopherol, recovered activity to control (thermoneutral) level. Thus, Se-enriched *A. bisporus* presents a viable and an efficient source of functional macro-fungal organic Se that may protect against hyperthermally induced gut injury.
CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

Discussions

There is strong collective evidence from the numerous experimental findings from this dissertation and other studies that dietary supplementation of Se at dosages above the nutritional levels up-regulates selenoproteins of the gastrointestinal tract linked to the mitigation of oxidative stress and anti-cancer mechanisms. An important observation from these different studies including our studies was that dietary Se supplementation with organic Se from different sources had a differential effect on the regulation of the colonic selenoproteins in various mammalian species (Uglietta et al 2006, Uglietta et al. 2007, Hu et al. 2010, Hu et al 2011, Maseko et al. 2014) and on carcinogenesis inhibition (Hu et al. 2008, McIntosh 2008). Furthermore, we have demonstrated with our study that other factors such as the additional supplementation with α-tocopherol (in the presence of high dosages of Se) in the diet and acute heat stress also regulate colonic selenoproteins uniquely in rats. The studies from animal experiments and human clinical trials indicated that the chemical form and species type of Se present in the Se source were the main determinants of the element’s bioavailability, absorption and efficacy in influencing the expression of the selenoproteins and activity of the selenoenzymes (Uglietta et al. 2006, McIntosh 2008, Hu et al 2010, Hu et al. 2011, Maseko et al. 2014) and protection against gut physiological dysfunction during acute heat stress (Maseko et al. 2014).

The knowledge that the chemical form and species type of Se critically influence the element’s biological efficacy is the reason that it becomes important to chemically characterize and use high accuracy and precision techniques to identify and measure Se in its various chemical forms and oxidation states occurring in food sources. This study cultivated Se-enriched A. bisporus mushroom that accumulated a maximum of 347 µg Se/g and 415 µg Se/g DW in caps and stalks respectively. The total Se levels of our cultivated A. bisporus mushroom was at the high end of the literature values for mushrooms with other studies reporting between 20-70 and 200 µg Se/g dw for edible mushrooms Boletus edulis and Albatrellus pes-caprae respectively (Falandysz 2008). In addition, Falandysz (2008) reveals that cultivated A. bisporus is able to assimilate Se concentrations of up to 110 µg Se/g dw. However, studies by Gergely et al (2006) also characterized cultivated A. bisporus and observed much higher levels of Se accumulation relative to our findings at 770.7 µg Se/g dw. It
is expected that cultivated mushrooms will display varying concentrations of Se as the Se levels are influenced by the growing conditions, Se state of the growth substrates and the Se form used to fortify growth substrates (Molnar et al. 1995, Das 2005). As the main focus of our study is organic Se, we quantified the Se associated with the protein fraction of the Se-enriched mushroom which reached maximum levels of 60.1 and 137 µg Se/g protein for caps and stalks respectively. We further performed speciation analysis of the protein associated Se using liquid chromatography-electrospray ionisation-mass spectrometry (LC-ESI-MS), a separation technique combined with a specific and sensitive detection system regarded as the best approach for the identification of Se compounds or species (Francesconi & Sperling 2005). Although other studies state that the inductively coupled plasma-mass spectrometry (ICP-MS) detection system offers the highest sensitivity and the best quantitative methodology, however this is so when reference compounds are available (B’Hymer & Caruso 2006). Our study detected three selenoamino acids accumulated in the Se-enriched cultivated A. bisporus mushroom. Selenocysteine as its dimer form [SeCys]₂, selenomethionine and methyl-selenocysteine were separated and identified by LC-ESI-MS from the water soluble protein fraction of the mushrooms with significant levels of the selenoamino acids observed for the selenised mushrooms compared to the control.

The most notable finding was the accumulation of SeCys (protein associated) by A. bisporus, at much higher levels compared to the other two selenoamino acids SeMet and MeSeCys with MeSeCys accumulated at the lowest level of them all, for both non-selenised and selenised A. bisporus mushroom. Other speciation studies in correlation to our findings also identified the three organic selenoamino acids SeMet, SeCys and SeMeSeCys in cultivated Se-enriched mushroom (Ogra et al. 2004, Gergely et al. 2006, Huerta, Sanchez & Sanz-Medel 2006). However, in contrast to our findings, selenomethionine is detected by others as the main selenoamino acid in Se-enriched A. bisporus and L. edodes mushrooms (Huerta, Sanchez & Sanz-Medel 2006, Turto et al. 2007, Wu et al. 2012). The discrepancy is unclear, however it is likely due to the efficiency of the different methods used in extraction and recovery of selenoproteins and in the quantitative detection of the selenoaminoacids. Studies have also detected inorganic selenite and other unidentified selenocompounds accumulated by Se-enriched mushrooms (Huerta, Sanchez & Sanz-Medel 2006, Gergely et al. 2006) and similarly our study also observed inorganic non-protein associated Se (Maseko et al. 2013). Although Falandysz (2008) states that the selenoamino acids SeMet, SeCys and SeMeSeCys as the main chemical species of Se making up Se-enriched mushrooms are largely found in the carpophore of caps, our findings were in contrast. We observed the stalks to comprise higher levels of the organic selenoamino acids species compared to the caps (Maseko et al. 2013).
However, a different profile of selenocompounds has been reported compared to the mushroom Se, with other sources of selenium such as yeast Se and dairy Se. Reports reveal that yeast Se occurs primarily as non-protein associated free selenomethionine that makes up to 70-80% (Ip et al. 2003) and 83% (Rayman 2004) of the total Se. Also in contrast to the *A. bisporus* mushroom Se, yeast Se contains much lower levels of selenocysteine that is free and not bound to protein like in the case of mushroom Se. Reports reveal that free selenocysteine and other Se containing compounds make up a relatively low 4% of the total Se in yeast Se (Ip et al. 2003) and selenite at 3% is also reported for yeast Se. Furthermore, dairy Se is reported to also be made up of higher levels of selenomethionine, occupying 83% of the total Se, plus lower levels of selenocysteine at 5% of total Se, however these selenoamino acids are protein bound as selenoproteins (milk selenoproteins) (Heard et al. 2007, Rayman 2004). In addition, reports state that dairy Se also contains traces of unknown selenocompounds occupying 4% of the total Se (Rayman 2004). The Se-enriched *A. bisporus* mushroom investigated by us revealed the organic Se content by mass as mainly protein bound SeCys (95.5%) and SeMet (3.6%) plus protein associated but free MeSeCys (0.9%). It is this contrasting organic Se profile existing amongst the different sources of organic Se (Maseko et al. 2013). It is this clear contrast in the organic Se profile existing amongst the different sources of organic Se, yeast Se, dairy Se and mushroom Se that we see differential effects in their biological activity, regulation of the Se dependent selenoproteins expression and activity and the overall efficacy of Se.

As part of the present dissertation, we demonstrated using the rat model, that the expression levels of the colonic antioxidant selenoproteins GPx-1, GPx-2, TrxR-1 and SeP were regulated differently following Se supplementation with mushroom Se. The efficacy of mushroom Se in the regulation of the colonic selenoproteins differed to that of other sources of Se reported by other studies. We observed that dietary supplementation with mushroom Se at 1ppm significantly up-regulated the mRNA expression levels of the major glutathione peroxidases jointly responsible for 70% GPx enzyme activity in the gastrointestinal tract (Esworthy et al. 1998), GPx-1 and GPx-2 in unstressed rats (Maseko et al. 2013). The GPx-1 activity level was also found to be increased consistently with the up-regulated GPx-1 mRNA expression in the rats supplemented with mushroom Se compared to those of the unsupplemented control diet. However, the efficacy of mushroom Se on GPx-1 and GPx-2 observed by us is in contrast with findings from other studies with other sources of Se. Hu et al (2010) reported no significant effect of dairy Se (selenomethionine rich proteins, Ip et al. 2003) on both GPx-1 mRNA expression and GPx-1 activity but observed a dose-dependent increase in GPx-2 mRNA expression in mouse colon. On the other hand, yeast Se (rich in free selenomethionine, Ip et al 2003) significantly up-regulated GPx-1 mRNA expression and activity.
correlating with our findings observed with the mushroom Se (rich in selenocysteine rich selenoproteins, Maseko et al. 2013) (Hu et al. 2010).

However in contrast with our observations with the mushroom Se, yeast Se displayed no effect on the expression of gastrointestinal specific GPx-2 (Hu et al. 2010). It appears the mushroom Se primarily made up of selenocysteine rich proteins (Maseko et al. 2013) has a more influential effect on the expression of the gastrointestinal specific GPx-2 compared to the other sources of Se. It is reported that selenocysteine containing proteins are the most readily available source of Se (Allan, Lacourciere & Stadtman 1999) compared to other selenocompounds. It perhaps explains why mushroom Se supplementation displayed the highest influence of GPx-2 compared to other Se sources. It is useful to also point out that gastrointestinal specific GPx-2 is more sensitive to dietary Se changes (Banning et al. 2005) compared to other GPxs and is thought to play a larger role in the protection of the gut from oxidative stress ailments (Esworthy et al. 1998).

Furthermore, differences in selenoprotein regulation by the different Se sources were also observed with other selenoproteins, TrxR-1 and SeP. In our present study, we observed a significant upregulation in the expression of SeP, a Se transportation protein believed to influence the expression of other selenoproteins in tissues (Hoffmann et al. 2007), for rats supplemented with mushroom Se in their diet (Maseko et al., 2014 manuscript submitted to BJN). However literature reveals contradictory reports where dietary supplementation with yeast Se at the same dosage has been reported to have no effect on colonic SeP expression in mice (Hu et al. 2010) and in azoxymethane challenged rats (Uglietta et al. 2006). Furthermore, Uglietta et al (2007) observed a dose-dependent increase in SeP expression in pigs following dietary supplementation with yeast Se. Similarly, Hu et al (2010) also reported a dose dependent up-regulation in colonic SeP expression following dietary supplementation with dairy Se in mice. However with human subjects, rectal SeP mRNA expression increased significantly following dietary supplementation with both yeast Se and dairy Se although only the dairy Se high in selenomethionine-rich proteins sustained the elevation of SeP after a Se ‘washout’ period (Hu et al. 2011). There is a good literature consensus with colonic TrxR-1, a part of the thioredoxin-peroxiredoxin antioxidant system that’s reported to play a lesser role in antioxidant defence of colonic and rectum tissues, relative to the GPxs. The failure of dietary Se supplementation in influencing gastrointestinal tract TrxR-1 expression has been observed in mice colon and human rectum following dairy-Se and yeast-Se supplementation (Hu et al. 2010, Hu et al. 2011), and in rat colon following mushroom-Se dietary supplementation (Maseko et al. 2014).
We further evaluated whether supplementation with α-tocopherol in the diet improved the efficacy of Se supplementation on the regulation of selenoproteins. We also evaluated whether dietary α-tocopherol supplementation alone in the absence of Se supplementation modulated selenoprotein expression. We observed that α-tocopherol supplementation alone up-regulated the expression of colonic GPx-1, but not of gastrointestinal specific GPx-2 mRNA. This was in correlation with previous studies as Bourre et al. (2000) reveals that dietary α-tocopherol supplementation influences the expression of phospholipid hydroperoxide peroxidase, GPx-4 in rat. As earlier mentioned, we observed an up-regulation in GPx-1 and GPx-2 expression with mushroom Se supplementation. However, we did not observe any further enhancement in the effect of Se, on the expression of colonic GPx-1 or GPx-2 with additional α-tocopherol in the mushroom-Se/α-tocopherol combination diet (Maseko et al. manuscript submitted BJN). This is not surprising as dietary supplementation with α-tocopherol has been reported to preferentially influence the activity of non-selenoenzymes such as catalase whereas Se is more effective at modulating the GPxs in malathion challenged rats (Al-Othman et al. 2011). However, we made an interesting observation where α-tocopherol supplementation alone in the absence of Se also increased the expression of the selenium transport protein, selenoprotein P. Although the mushroom Se supplementation alone enhanced the expression of colonic SeP, the further addition of α-tocopherol in the diet did not further improve the Se effect on SeP expression relative to the effect of mushroom Se alone (Maseko et al. manuscript submitted BJN). However, α-tocopherol supplementation alone did not increase the expression of TrxR-1 and the addition of α-tocopherol in the presence of mushroom Se in the diet did not further improve the effect of Se (Maseko et al., 2014 manuscript submitted BJN).

We also evaluated the effect of acute heat stress on the regulation of the expression of the colonic selenoproteins GPx-1, GPx-2, TrxR-1 and SeP and on colonic GPx-1 activity in rats placed on a high Se diet with or without α-tocopherol supplementation. It was observed that acute heat stress had a differential effect on the modulation of the expression of the colonic selenoproteins. All selenoproteins were significantly up-regulated across all diets of varying Se and α-tocopherol concentrations during acute heat stress, although at varying levels. It was observed that during heat stress, SeP, the transportation protein of Se believed to influence the expression of other individual selenoproteins (Richard 2005, Hoffmann et al. 2007) was expressed at the highest level compared to all the other selenoproteins, followed by TrxR-1 and lastly the GPxs.

Our data indicates that SeP and TrxR-1 making part of the thioredoxin-peroxiredoxin antioxidant defence system are the main selenoproteins of functional significance involved in the protection of the colon during hyperthermally induced oxidative stress. Dietary supplementation with mushroom
Se alone and the combination effect of mushroom Se and α-tocopherol in the diet both up-regulated SeP and TrxR-1 during heat stress although the α-tocopherol addition did not enhance the Se effect for both SeP and TrxR-1. However, it is the dietary supplementation with α-tocopherol alone that showed the highest level of up-regulation of both SeP and TrxR-1 during heat stress, with the expression of SeP as the highest, followed by TrxR-1 expression. Selenoprotein P in addition to its major function as a Se carrier protein is reported to also function as an antioxidant defence and display anti-cancer properties (Al-Taie et al. 2004, Diwadkar-Navsariwala et al. 2006). To present evidence of the involvement of SeP in cancer prevention, studies have demonstrated that SeP knockout mice were linked to increased cancer development (Diwadkar-Navsariwala et al. 2006), a significant reduction or loss of SeP mRNA expression was observed in colorectal cancer subjects (Al-Taie et al. 2004). Studies also observed some genetic variants in SeP associated with human advanced colorectal adenoma (Persson-Moschos et al. 2000). Furthermore, it is important to note that heat stress alone induced the up-regulation of the expression of both SeP and TrxR-1 as observed with rats placed on the control diet (low Se, low α-tocopherol) also displaying enhanced expression of the two genes. The observation is not surprising as the rats not on the ‘special’ Se or α-tocopherol diets were attempting to counteract the effects of hyperthermal stress by up-regulating the endogenous antioxidant selenoproteins.

Furthermore, our study observed that there was an acute response in the expression of colonic GPx-1 and GPx-2 in rats to heat stress which induced a markedly elevated expression of the 2 GPx isoforms. Rats placed on the control diet with lower levels of Se and α-tocopherol and exposed to heat stress displayed an acute increase in the expression of the GPxs. The elevated mRNA expression of the colonic GPx-1 and GPx-2 in the heat stressed rats indicated an acute effect of heat stress on the GPxs expression as the animals attempted to counter the heat induced oxidative stress in the tissue. It is known that the adverse effect of heat stress results in the over production and accumulation of ROS in body tissues such as the intestines, which can damage cellular components, disrupt intestinal physiological function and reduce mucosal barrier function and integrity (Pavlick et al. 2002, Chu, Esworthy & Doroshow 2004). These unpleasant states can act as facilitating platforms to the pathogenesis of gastrointestinal tract diseases such as fibrosis, inflammatory bowel disease, ulcerative colitis and colorectal cancer (Ames, Shigenaga & Hagen 1993, Kiliaan et al. 1998, Pavlick et al. 2002). The family of the GPxs is known to reduce organic and inorganic hydroperoxides thus protecting the cells from the oxidative stress (Arthur 2000). It is reported that DNA damage induced by oxidative stress, is one of the initiators of tumorigenesis (Ames, Shigenaga & Hagen 1993). It is worth noting that the colon is more susceptible to cancer than the small intestine although both tissues have similar anatomy (Bartel et al. 2007). It is therefore a
worthwhile finding by our study to have observed a significant up-regulation in the colonic GPxs known to protect cells from the effects of oxidative stress.

Furthermore, heat stressed rats placed on the mushroom Se supplemented diet also displayed an up-regulation of the colonic GPx-1 and GPx-2, indicating a chronic effect of the mushroom Se in elevating mRNA expression of the 2 colonic GPxs. We observed that the level of colonic GPx-1 mRNA expression for heat stressed rats on the mushroom Se supplemented diet was similar to that induced by heat stress alone for rats fed the control diet. However, the level of colonic GPx-2 mRNA of heat stressed rats on the mushroom Se was much higher than that of heat stressed rats on the control diet. There was an interesting and contrasting finding in comparison to the rats under thermoneutral conditions and supplemented with α-tocopherol. We observed that the heat stressed rats supplemented with α-tocopherol alone, similar to mushroom Se, exhibited an upregulation of both colonic GPxs. However, under thermoneutral conditions, only GPx-1 and not GPx-2 is up-regulated following supplementation with α-tocopherol alone. This finding suggests that supplementation with α-tocopherol alone has no effect on the ‘special’ gastrointestinal specific GPx-2 under thermoneutral conditions.

However during exposure to heat stress, supplementation with α-tocopherol alone in the absence of Se influences the up-regulation of gut specific GPx-2. Although during heat stress we observed an up-regulation in both colonic GPxs expression with α-tocopherol supplementation alone and mushroom Se alone, the addition of α-tocopherol in the mushroom Se supplemented diet did not further enhance the effect of mushroom Se in the expression of both GPxs in heat stressed rats. Furthermore, it is important to also note that supplementation with α-tocopherol alone in the diet did not produce an enhanced expression of colonic GPx-2 observed with mushroom-Se in heat stressed rats beyond that induced by heat stress. These findings support previous observations that gastrointestinal tract specific GPx-2 is more sensitive to changes in Se levels in the diet (Banning et al. 2005), and additionally pointed to the larger role GPx-2 plays in the protection of the colon against hyperthermally induced oxidative stress. Although dietary supplementation with mushroom-Se enhanced colonic GPx-2 expression in heat stress rats beyond that induced by heat stress alone, the combination of the mushroom-Se with α-tocopherol in the diet failed to induce similar enhancement. It appears that the colon tissue required a reduced need for GPx-2 with the presence of α-tocopherol in the diet as α-tocopherol could possibly influence the expression of non-selenium antioxidants such as catalase as the animals attempted to mitigate the effects of induced oxidative stress (Al-Othman et al. 2011).
We observed an up-regulation of colonic GPx-1 mRNA to correlate with increased GPx-1 activity upon Se dietary supplementation. In agreement with previous studies, our study observed that mushroom Se increased colonic GPx-1 activity 3.8 fold under thermoneutral conditions. This correlated with a 3.0 fold increase in colonic GPx-1 mRNA expression. Studies by Hu et al. (2008) and Hu et al. (2010) also revealed an increase in GPx-1 mRNA expression and activity in mice colon following supplementation with yeast Se and dairy Se. An interesting observation was noted under thermoneutral conditions with non-heat stressed rats where the α-tocopherol supplemented diet increased colonic GPx1 mRNA expression compared to rats fed the control diet although a corresponding increase in GPx-1 activity was not observed with α-tocopherol supplementation. However, mushroom Se supplementation in combination with dietary α-tocopherol supplementation also produced the same effect as mushroom Se alone in upregulating colonic GPx-1 mRNA expression and enzyme activity although there was no enhanced Se effect with the addition of α-tocopherol.

On the other hand, an interesting result was obtained with colonic GPx-1 activity under heat stress. Although GPx-1 mRNA expression was increased by heat stress, we observed that heat stress negatively impacted on GPx-1 activity reducing it to below the level of that of non-heat stressed rats. Noteably important, dietary mushroom Se supplementation was able to restore colonic GPx-1 activity level of the heat stressed rats back to normal (non-heat stressed rats on the control diet). Differentially, dietary supplementation with α-tocopherol alone failed to restore colonic GPx-1 activity level back to normal in the heat stressed rats. However, dietary α-tocopherol supplementation in the presence of mushroom Se was able to also restore GPx-1 activity back to normal, although it (α-tocopherol) did not enhance mushroom Se effect. The findings suggest that exposure to acute heat stress might have accumulated ROS overwhelmingly in the colon that depressed GPx-1 activity, which was recovered by mushroom-Se, but not by α-tocopherol in the rats diet. The findings further suggest the heat stressed rats fed the mushroom Se attempted to mitigate the destructive effects of heat stress induced ROS accumulation by elevating GPx-1 mRNA expression and up-regulating the depressed GPx-1 activity back to normal levels. It must be pointed out that GPx-1 expressed in almost all tissues of mammalian systems collectively with GPx-2, contributes to 70% GPx enzyme activity in the gastrointestinal tract (Chu et al. 2004). Known to reduce organic and inorganic hydroperoxides to protect tissue cells from oxidative injury, an initiator of tumorigenesis (Ames, Shigenaga & Hagen 1993) it can be deduced that GPx-1 plays a significant role in the prevention of heat stress related diseases of the gastrointestinal tract. Therefore dietary supplementation with mushroom Se during exposure to acute heat stress is
beneficial as it up-regulates mRNA expression of GPx-1 and restores the depletion of GPx-1 activity in a system overwhelmed by free radical accumulation.

Conclusions

We demonstrated that selenocysteine was the main selenoamino acid species assimilated into water soluble proteins by the cultivated selenium-enriched *A. bisporus* mushroom grown on compost irrigated with sodium selenite solution. The most notable finding with the chemical characterization of the cultivated Se-enriched *A. bisporus* mushroom was the much higher levels of the protein bound selenocysteine accumulated by the mushroom occurring at 95.5% of the organic Se associated with protein. The speciation analysis also detected at relatively lower levels selenomethionine also assimilated into the water soluble proteins, in the selenium-enriched *A. bisporus* mushroom. Assimilation of the selenoamino acids into proteins increased with selenite concentration in irrigation solution although incorporation of selenomethionine into proteins occurred at a much lower level compared to SeCys. In addition, MeSeCys, a non-protein selenoamino acid, was precipitated along with the water soluble protein fraction with acetone and occurred at the lowest level of all selenoamino acids. No free non-protein bound SeCys or SeMet was detected in the mushrooms studied.

Furthermore, we provided evidence from the biological studies that dietary supplementation with mushroom Se composed of selenocysteine rich selenoproteins regulates the colonic selenoproteins differently. Our study observed an up-regulation in the expression of all colonic selenoproteins examined, following dietary supplementation with mushroom Se, under both thermoneutral and acute heat stress conditions. However, it was observed that the effects of dietary Se and α-tocopherol supplementation and acute heat stress regulated the colonic selenoproteins uniquely. We observed that GPx-1, GPx-2, TrxR-1 and SeP mRNA expression were up-regulated following dietary Se supplementation with Se-enriched *A. bisporus* under thermoneutral conditions but additional α-tocopherol did not enhance the mushroom Se effect for all genes under study. An interesting observation under thermoneutral conditions was that dietary supplementation with α-tocopherol alone up-regulated only two selenoproteins, the ubiquitous GPx-1 and the influential Se transport selenoprotein SeP influencing the expression of other selenoproteins within and between tissues. Worth noting under thermoneutral conditions is that the supplementation with α-tocopherol alone did not up-regulate the gastrointestinal tract specific GPx-2. Dietary selenium
supplementation was observed to be more effective at up-regulating gut specific GPx-2 than α-tocopherol supplementation.

However, under heat stress conditions, both mushroom Se and α-tocopherol dietary supplementation significantly up-regulated all colonic selenoproteins under study. However, SeP believed to influence the expression of other selenoproteins was expressed at the highest level compared to all selenoproteins, followed by TrxR-1 and lastly by the GPxs with rats placed on the α-tocopherol diet. The expression levels of SeP and TrxR-1 of rats placed on the α-tocopherol supplemented diet and subjected to heat stress were the highest compared to the expression levels of all selenoproteins observed with other diets under heat stress indicating that the antioxidant thioredoxin system might be engaged at a higher extent than the glutathione system during heat stress. The findings also indicated that during exposure to heat stress, dietary α-tocopherol supplementation is more effective at up-regulating SeP and TrxR-1 expression than the GPxs. Selenium was observed to be more influential in the regulation of the GPxs under heat stress conditions. Although mushroom Se increased mRNA levels of both GPx isoforms under study during heat stress exposure, similar to thermoneutral conditions, the addition of α-tocopherol did not enhance the Se effect.

Furthermore, in correlation with mRNA expression, colonic GPx-1 activity was increased in rats kept under thermoneutral conditions and placed on the mushroom Se diet. Although the mushroom Se increased GPx-1 activity, there was no further improvement in GPx-1 activity observed with additional α-tocopherol supplementation. However, in contrast with GPx-1 mRNA expression, supplementation with α-tocopherol alone did not increase GPx-1 activity under thermoneutral conditions. On the other hand, heat stress was observed to have a negative impact on GPx-1 activity which was recovered to basal levels by mushroom Se, but not by α-tocopherol in the diet.

We also provided evidence that dietary macro-fungal organic Se from cultivated Se-enriched A. bisporus mushroom protected against gastrointestinal dysfunction during heat induced oxidative stress by restoring epithelial ion transport and barrier functions. We further demonstrated that although α-tocopherol supplementation of the organic Se-enriched diet also displayed significant protection of the gastrointestinal tract against increase in epithelial permeability during heat challenge, it offered no additional benefits to epithelial physiological function and tissue integrity.

With the findings observed, therefore all the hypotheses tested are accepted. The relative ease in cultivating Se-enriched A. bisporus mushroom, its unique profile of bioactive Se organic species, its
up-regulating effect of the gut antioxidant selenoproteins linked to anti-cancer mechanisms and its mitigating effects against gastrointestinal injury arising from heat stress in rats, would present the mushroom as a viable and efficient source of functional organic Se with demonstrated biological benefits. Biological effects linked to beneficial health properties and disease prevention and protection against heat induced oxidative stress and gut injury.

**Recommendations of the study**

- The study recommends screening for and further characterization of other selenium species in selenium-enriched *A. bisporus* mushroom or selenium enriched vegetables; i.e. seleno-sugars, seleno-carbohydrates and other seleno-compounds

- Biologically evaluate the effects of seleno-sugars/carbohydrates upon dietary supplementation.

- To evaluate other selenoprotein biomarkers (beyond those looked at by the study) reported to also be linked to the health benefits of selenium (GPx4, selenoprotein O, selenoprotein W, selenoprotein V, selenoprotein 15). They could possibly play a considerable role in protecting the tissues they are expressed in, from oxidative stress.

- Evaluate SeP (as a plasma selenoprotein) expression in the plasma instead of tissues

- Future studies can also evaluate selenoproteins expressed in other tissues outside the gastrointestinal tract.

- Look at the differences in selenoprotein expression across different dietary dosages.
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Appendix

Original copies of published journal articles from chapters 3, 4 and 5 respectively are attached as follows;

1. Chapter 3

2. Chapter 4

3. Chapter 5
Figure 1. Stages of the cultivation of selenium-enriched *Agaricus bisporus* on compost and peat moss casing layer (A); white thread-like mushroom mycelia cells, (B); on-set of ‘pinning’ and fruiting body formation, (C); fruiting bodies, (D); fruiting body enlargement.
**Total Protein Determinations**

Bovine albumin was used as a standard for all total protein determinations.

Sample Calculation;

\[ Y = 0.0079x - 0.0061 \]

\[ 0.343 = 0.0079x - 0.0061 \]

\[ X = \frac{0.343 + 0.0061}{0.0079} \]

\[ X = 44.19 \, \mu g \, protein \]

**GPx-1 Enzyme Activity determinations;**

\[ \Delta A_{340}/6.22 \times DF/V \]

\[ \Delta A_{340}/\text{min (blank)} - A_{340}/\text{min (sample)} \]

\[ 6.22 = \varepsilon \, \text{mM} \, \text{for NADPH} \]

DF=dilution factor of sample

V=sample volume (ml)
GPx-1 enzyme activity expressed as Units/mg protein

Unit definition: 1 unit of glutathione peroxidase will cause the formation of 1.0 µmol of NADP⁺ from NADPH per minute at pH 8.0 at 25 °C in a coupled reaction in the presence of reduced glutathione, glutathione reductase, and tert-butyl hydroperoxide.

GPx-1 enzyme activity calculation sample:

\[ \Delta A_{340}/6.22 \times DF/V \]
-0.0023/(-0.0212)/6.22 x 1x0.05 (50µL)

=0.0189/6.22 x 0.05

=0.061U/ml

**Relative Gene Expression Calculations**

The Pfaffl Method

A method of calculating relative gene expression only when the amplification efficiencies $E$ of the target and normaliser (reference) gene are not the same.

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta CT, \text{target (calibrator-test)}}}{(E_{\text{reference}})^{\Delta CT, \text{reference (calibrator-test)}}}$$

$$E = 10^{-(1/\text{slope})}$$

(Slope value obtained from a standard curve generated from using a 10-fold dilution of the template (cDNA) amplified on the iCycler iQ real-time PCR system.

Amplification efficiencies were determined for ALL genes (target and reference genes).

**Standard Curve Chart: Real Time PCR**
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<th>Fluor</th>
<th>PCREfficiency(%)</th>
<th>Squared</th>
<th>Slope</th>
<th>Y-intercept</th>
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<tbody>
<tr>
<td>β-actin</td>
<td>101.1</td>
<td>0.997</td>
<td>-3.295</td>
<td>15.632</td>
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<tr>
<td>GAPDH</td>
<td>108.7</td>
<td>0.996</td>
<td>-3.130</td>
<td>16.281</td>
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</table>

**PCR Amp/Cycle Chart**

**Standards**

[PCR Amp/Cycle Chart Image]
Samples PCR Quantification Data

Amplification Chart

PCR Base Line Subtracted Curve Fit RFU

Cycle

0 5 10 15 20 25 30 35 40 45

0 100 200 300 400 500 600 700 800 900 1000 1100 1200

Sample: 54.40 54.40
Sample Statistical Analysis

Chapter 6
Below is the ANOVA table showing statistical analysis for the enzyme activity using the 2 x 2 x 2 factorial design (2-levels Se x 2-levels α-tocopherol x 2-levels temperature). Statistical data indicates that only temperature and selenium had a significant effect (P<0.05), probability limit was set at P<0.05. The statistical analysis also shows that there were no interactions between parameters (selenium, vitamin E and temperature), (Fpr. > 0.05).

**Analysis of variance Table**

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<tr>
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</tr>
<tr>
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Author/s:
MASEKO, TEBO

Title:
The chemical characterization and biological evaluation of selenium-enriched Agaricus
disporus mushroom organic selenium species

Date:
2014

Persistent Link:
http://hdl.handle.net/11343/45085

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The chemical characterization and biological evaluation of selenium-enriched Agaricus
disporus mushroom organic selenium species