Proteomic characterization and identification of murine liver and small intestine proteins modulated by tea *(Camellia sinensis)* consumption

By

Salome Smit

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2D	Two dimensional
2DE	Two dimensional gel electrophoresis
4',4''-DiMeEGCG	4', 4''-di-O-methyl-epigallocatechin gallate
4'-O-MeEGC	4'-O-methyl-epigallocatechin
A431	Human epidermoid carcinoma cells
ADME	Absorption, distribution, metabolism, excretion
AFB1	Aflatoxin B1
AIF	Apoptosis inducing factor
AP-1	Activator protein 1
Ave	Average
B[a]P	Benzo-a-pyrine
BAX	Pro-apoptotic protein
Bcl-2	Anti-apoptotic protein
Bis-acrylamide	N,N' – methylenebisacrylamide
C	Catechin
cdk	Cyclin dependent kinase
Cg	Catechin gallate
CID	Collision induced dissociation
cki	Cyclin kinase inhibitor
COMT	Catechol-O-methyltransferase
CV	Coefficient of Variation
CYP	Cytochrome P-450
DNA	Deoxyribonucleic acid
EC	(-)-Epicatechin
ECg	(-)-Epicatechin-3-gallate
EGC	(-)-Epigallocatechin
EGCg	(-)-Epigallocatechin-3-gallate
EGC-O-S	Epigallocatechin-O-sulfate
EGF	Epidermal growth factor
EMSA	Electrphoretic mobility shift assay
Epicatechin-O-G	Epicatechin-O-glucuronide
Epicatechin-O-S	Epiecatechin-O-sulfate
ERK	Extracellular signal-regulated kinase
ESI	Electrospray ionization
FTICR MS	Fourier transform ion cyclotron resonance mass spectrometry
g	Gram
GC	(+)-Gallocatechin
GCg	(-)-Gallocatechin gallate
GC-O-G	Gallocatechin-O-glucuronide
GSH	Reduced glutathione
GST	Glutathione S-transferase
GTP	Green tea polyphenols

H_2O	Water
HCCA	4-hydroxy-α-cyanocinnamic acid
HIV	Human Immunodeficiency virus
HPLC	High performance liquid chromatography
HUVEC	Human umbilical vein endothelial cells
i.g.	Intragastric
i.p.	Intraperitoneal
i.v.	Intravenous
ICAT	Isotope coded affinity tag
IEF	Iso-electrical focusing
IKK	IκB kinase
IL	Interleukin
IMAC	Immobilized metal-ion affinity chromatography
iNos	inducible nitric oxide synthase
IPG	Immobilzed Polyacrylamide gel
K-562	Human leukaemia cells
LC	Liquid chromatography
LC MS	Liquid chromatographic mass spectrometry
LNCaP	Human prostate carcinoma cells
M4	(-)-5-(3',4',5'-trihydroxyphenyl)-γ -valerolactone
M4-O-G	(-)-5-(3',4',5'-trihydroxyphenyl)-γ -valerolactone-O-glucuronide
M4-O-S	(-)-5-(3',4',5'-trihydroxyphenyl)-γ -valerolactone-O-sulfate
M6	(-)-5-(3',4',-dihydroxyphenyl)-valerolactone
MALDI – TOF MS	Matrix assisted laser desorption/ionization time-of-flight mass spectrometry.
MALDI MS	Matrix assisted laser desorption/ionization mass spectrometry
MAPKKK	Mitogen activated protein kinase kinase kinase
ml	Milliliter
MRC	Medical Research Council
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MudPIT	Multi-dimensional protein identification techniques
MW	Molecular weight
n/a	Not applicable
NaCl	Sodium chloride
NF-κB	Nucleur factor – κB
NHEK	Normal human epidermal keratinocytes
NIK	NF-κB inducing kinase
$NO^{\cdot}$	Nitric oxide
NOS1	Nitric oxide synthase gene 1
O-m-EGC-O-G	O-methyl-epigallocatechin-O-glucuronide
O-m-EGC-O-S	O-methyl-epigallocatechin-O-sulfate
OR	NADPH – cytochrome reductase
p.o	*Per os*

P450	Cytochrome P-450
PAGE	Polyacrylamide gel electrophoresis
PAH	Polycyclic hydrocarbons
PC-9	Human lung cancer cells
PDGF	Platelet-derived growth factor
PhIP	2-amino-1-methyl-6-phenyl imidazo-[4, 5-6] pyridine
pI	Iso-electric point
PKC	Protein kinase C
PMF	Peptide mass fingerprint
pRb	Phosphorylated retinoblastoma protein
PTM	Post translational modifications
RBP	Retinoblastoma protein
RIP	Receptor-interacting protein
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RP-HPLC	Reversed phase high performance liquid chromatography
RX	Hydrocarbon
s.c.	Subcutaneous
SA	Sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid)
SAH	S-adenosyl-L-homo-cysteine
SAM	S-adenosyl-methionine
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SE	Standard error
SELDI TOF MS	Surface enhanced laser desorption/ionization time of flight mass spectrometry
SEM	Standard error of the mean
SIB	Swiss Institute of Bioinformatics
SOD	Super oxide dismutase
TEMED	N,N,N’,N’-tetramethyl-ethylenediamine
TF	Theaflavin
TF3’g	Theaflavin-3’-gallate
TFdg	Theaflavin-3,3’-digallate
TFg	Theaflavin-3-gallate
TNF	Tumour necrosis factor
TRADD	TNF receptor associated death domain
Tris	Tris(hydroxymethyl) aminomethane
TTRAF2	TNF receptor-associated factor 2
Ub	Ubiquitinylation
V-79	Lung fibroblast cells
VEGF	Vascular endothelial growth factor
vs	Versus
VSMC	Vascular smooth muscle cells
w/v	Weight per volume

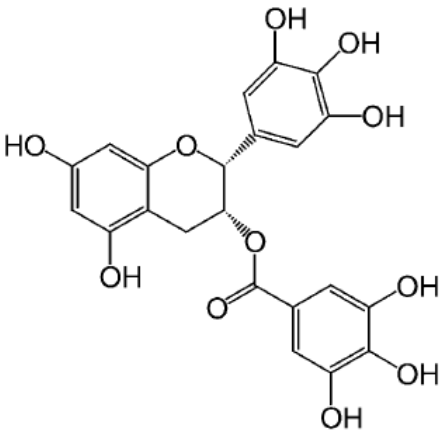
CHAPTER 1

Introduction

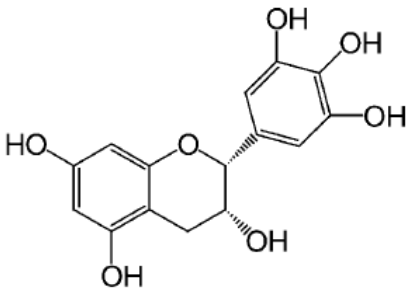
Tea *(Camellia sinensis)* is one of the most popular beverages consumed worldwide. Tea was first discovered for human use 2737 BC when Emperor Sh'eng Nung took the first sip of tea. Since then tea has been used for its medicinal properties for almost 5000 years. Recently the significance of daily tea consumption and its cancer chemo prevention in humans became an important issue. So much so that the Royal Society of Medicine has launched a "tea4health" campaign to urge Britons to consume at least four cups of tea per day to gain maximum health benefits from tea. It has been demonstrated that the oral intake of tea can protect against stroke (Keli, et al., 1996), obesity (Yang, et al., 2003), inflammation (Varilek, et al., 2001; Sang, et al., 2004), arthritis (Haqqi, et al., 1999), coronary heart disease (Hakim, et al., 2003), oxidative damage (Ishige, et al., 2001; Saffari and Sadrzadeh, 2004), DNA damage (Zhang, et al., 2002), inhibit the development of cancer and tumour progression (Sazuka, et al., 1997; Chen, et al., 1998) and can even interfere with HIV-1 infection (Kawai, et al., 2003). The health effects of tea against disease and tumour growth have been attributed to the unique anti-oxidant activities of the tea polyphenols. The most significant properties of tea polyphenols that may affect health, disease and carcinogenesis are the anti-oxidant activities (Ragione, et al., 2002), modulation of carcinogen metabolizing enzymes (Bu-Abbas, et al., 1999 (a)), trapping of ultimate carcinogens (Suganuma, et al., 1999), induction of cell apoptosis and cell cycle arrest (Ahmad, et al., 2000 (a)).

Green tea consists of caffeine and the green tea polyphenols known as the catechins. The catechins comprise 30% of the soluble solids in a cup of green tea. These catechins are (-)-epigallocatechin-3-gallate (EGCg), (-)-epicatechin-3-gallate (ECg), (-)-epigallocatechin (EGC), and (-)-epicatechin (EC). Of these catechins EGCg is the most abundant and the most studied as it is the most biologically active. The familiar yellowish colour of green tea can be attributed to the chlorophyll content and unoxidized catechins. A cup of green tea contains about 300 to 400 mg of green tea polyphenols of which 10 to 30 mg is EGCg (Demeule, et al., 2002).

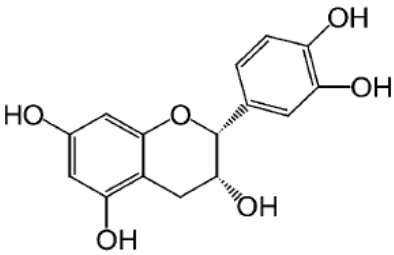
During the fermentation of green tea to produce black tea, the catechins are oxidized by polyphenol oxidase to yield the reddish brown dimeric theaflavins and polymeric thearubigens that are responsible for the characteristic red colour of black tea. As a result of the fermentation process the flavanol content is much lower in black tea, than in green tea (Lunder, 1992). Black tea also contains caffeine, thearubigins and the theaflavins which is produced from the oxidation and polymerization of the green tea catechins. These theaflavins are: theaflavin (TF), theaflavin-3-gallate (TFg), theaflavin-3'-gallate (TF3'g), and theaflavin-3,3'-digallate (TFdg). The structures of the catechins and the theaflavins are shown in Figure 1.1.



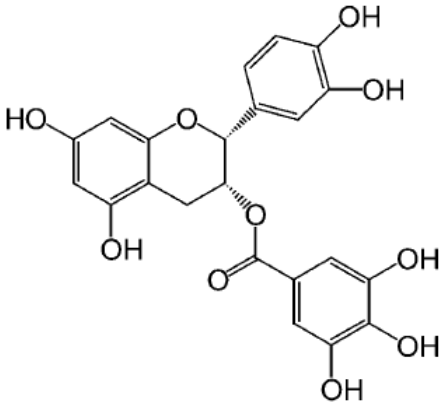
Epigallocatechin-3-gallate (EGCg)



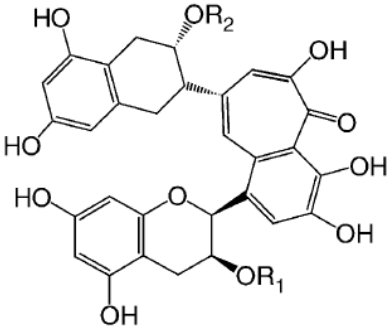
(-)-Epigallocatechin (EGC)



(-)-Epicatechin (EC)



(-)-Epicatechin-3-gallate (ECg)



R1 = R2 = OH	Theaflavin
R1 = OH, R2 = galloyl	Theaflavin-3-gallate
R1 = galloyl, R2 = OH	Theaflavin-3'-gallate
R1 = R2 = galloyl	Theaflavin-3,3'-digallate

Figure 1.1: Structures of the tea polyphenols.

Green tea has been shown to inhibit the growth of many cancers in animal models. Thus for tea and its polyphenols to be effective the polyphenols need to have a broad range of target organs. Or it must act on a critical step common to many cancers like the cell cycle or cell proliferation.

Cancer chemoprevention has been detected in most organs when treated with tea or its catechins. It was found that EGCg induced apoptosis in cancerous cells, but not in normal cells (Chen, et al., 1998). A strategy of chemoprevention would thus be to take advantage of the biochemical differences between cancer cells and their normal counterparts, and to develop an effective way to fight cancer. It is thus also clear that EGCg utilizes different target sites within cancer cells than in the normal cells, and because of this the cancer cell is more susceptible to the action of EGCg, hence causing apoptosis (Chen, et al., 1998). This induction of apoptosis is desirable, as it will destroy the cancerous cells. The Figure below demonstrates how EGCg is able to block tumor induction by inhibiting various different stages during harmful xenobiotic metabolism.

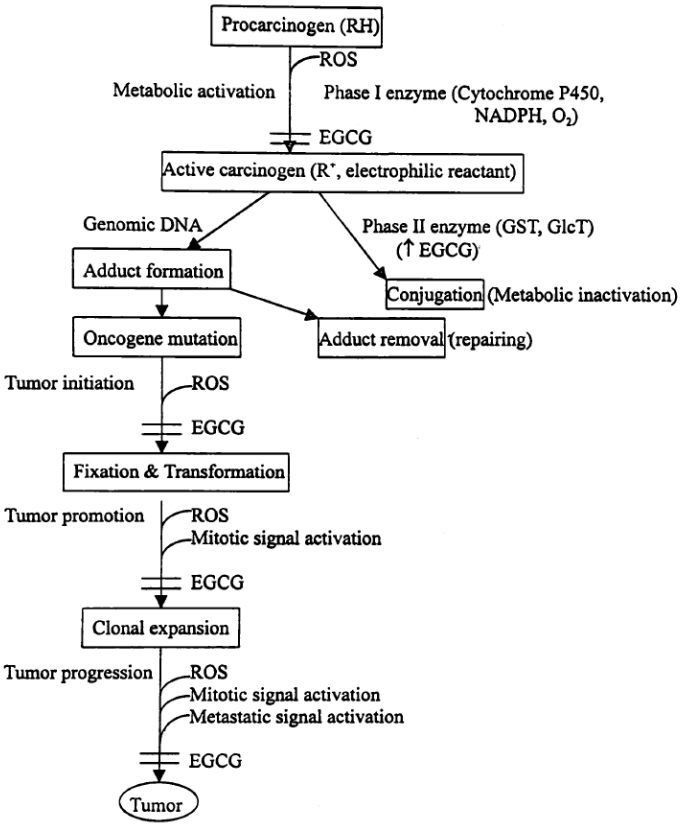


Figure 1.2: Blockade of tumor induction by EGCg (Lin, et al, 1999).

The aim of this introduction is to demonstrate known examples of how tea is able to affect various processes in a cell, from gene expression to protein regulation and degradation, and try to explain how tea does it all!

1.1 Regulation of gene expression

Gene expression is the first step towards regulation of the cell. Gene transcription needs to be regulated as this will later influence the translation of proteins. DNA microarrays are able to measure the expression levels of a large numbers of genes of a specific genome and can play an important role in biology and medicine to study disease from gene expression levels and relate the effect of a disease to a particular gene. A DNA microarray chip is a collection of microscopic DNA spots attached to a solid surface, such as glass, plastic or silicon that forms the array. DNA microarray analysis was done on C57BL/6 Nrf2(-/-) and Nrf2 (+/+) mice to determine the extent of regulation of gene expression in mice by EGCg. Nrf2 genes that are related to cellular defence and detoxification are important in cancer chemoprevention. A loss of Nrf2 function will lead to increased susceptibility to carcinogenesis. It was recently found that EGCg was able to regulate 671 Nrf2 dependent genes and 256 Nrf2 independent genes within the liver (Shen, et al., 2005). For the small intestine EGCg regulated 228 Nrf2 dependent genes and 98 Nrf2 independent genes were identified. From this it is clear that the genes within the liver are more responsive to oral treatment of EGCg than those expressed in the small intestine. Even though oral administration of EGCg could generate high concentrations of EGCg in the intestinal tissue, huge differences between the gene expression pattern of the liver and the small intestine were found. This could be related to the differences in abundance of nuclear transcription factors and other signalling molecules in response to EGCg between the cells of these two tissues. The genes that were regulated were divided into nine categories, based on their functions, these categories are: ubiquitination and proteolysis, electron transport, detoxification, transport, cell growth, apoptosis, cell adhesion, kinases and phosphatases, as well as transcription factors. Some interesting genes regulated by EGCg treatment in the liver were the ubiquitination related genes like ubiquitin specific protease and ubiquitin conjugating enzyme E2I. Xenobiotic metabolising enzymes of both phase I and phase II drug metabolism were found. NOS1 was strongly suppressed in the liver indicating a protective role of EGCg. Apoptosis proteins like Apaf-1 and Bcl-2 were induced in liver and cell cycle control p21 kinase in the small intestine, again suggesting a protective role

for EGCg. EGCg could also block the activation of many signalling pathways like VEGF, EGF, PDGF, NF-κB, and ERK which are all important in cancer chemoprevention.

cDNA microarray analysis was done on human lung cancer (PC-9) cells. Control PC-9 cells and their expression of genes were compared to the PC-9 cells treated with EGCg. It was found that non treated PC-9 cells expressed 163 genes of the possible 588 on the micro array slide, while the EGCg treated PC-9 cells only affected 16 genes in total different to the control. Of these, 12 genes were down regulated (2 fold) and 4 genes were up regulated (2 fold) (Okabe, et al., 2001). Figure 1.3 shows these regulated genes.

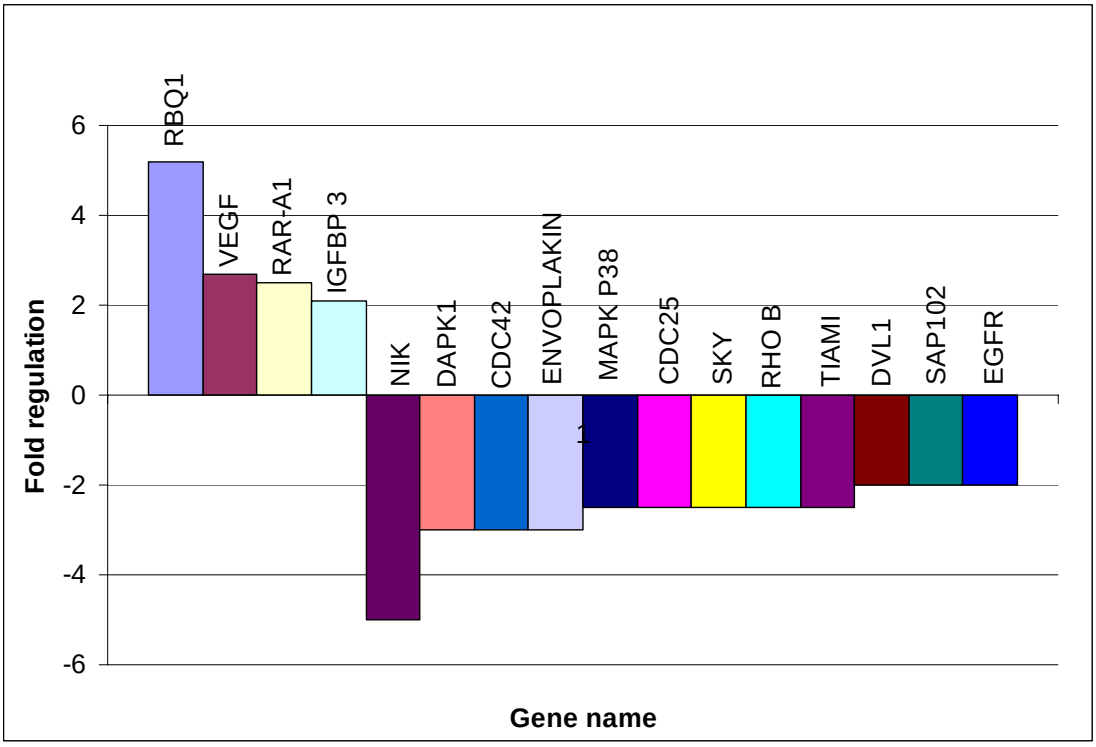


Figure 1.3: Lung cancer genes regulated by EGCg *(Adapted from Okabe, et al., 2001).*

NIK and DAPK1 are two genes connected to apoptosis. NF-κB inducing kinase (NIK) is a member of the mitogen activated protein kinase kinase kinase family (MAPKKK). NIK is usually activated by the interaction of TNF receptor-associated death domain protein (TRADD) with TNF receptor-associated factor 2 (TTRAF2) and receptor-interacting protein (RIP). NIK activates the down stream kinase, IκB kinase (IKK). It is known that IκB is bound to NF-κB. When IκB gets phosphorylated NF-κB is released from IκB and

will then act as a transcription activator (Okabe, et al., 2001). The down regulation of NIK gene expression by EGCg can lead to reduced activation of NF-κB. This would be a desirable scenario since NF-κB is known to be an anti-apoptotic transcription factor. When NF-κB translocates into the nucleus to activate transcription, genes such as Bcl-2, iNOS, TNF-α, and IκB-α, are transcribed. All of these except for IκB-α inhibit apoptosis, thus enabling cellular proliferation. This effect is not always desirable since this effect can cause the promotion and progression of cancerous cells. The inhibition of transcription of IκB-α is also not desirable, as IκB-α inhibit the activity of NF-κB. Thus the fewer IκB-α, the more is NF-κB able to translocate into the nucleus. EGCg thus has a protective effect upon the cell by down regulating the gene expression of NIK, which then indirectly inhibit the action of NF-κB and the anti-apoptotic proteins within the cell.

DAPK is a calcium/calmodulin-dependent serine/tyrosine kinase that carries ankyrin repeats and a death domain. DAPK displays strong tumour suppressive activity, by linking the control of apoptosis to metastasis. The precise relation between DAPK and EGCg and its tumour suppressive activity has not really been established.

Up regulation of the RBQ1 gene had the most alleviated gene expression, this is in contrast to the other retinoblastoma binding protein (RBP2) which was not expressed at all. The function of RBQ1 has not yet been elucidated, but it is known that RBP2 stimulates transcription (Okabe, et al., 2001).

In another experiment EGCg regulated 25 genes in human prostate carcinoma (LNCaP) cells. Of these genes the expression of 16 genes were increased and 9 were reduced by EGCg. The EGCg regulated genes are diverse and do not share a common regulatory pathway (Wang and Mukhtar, 2002). These genes do not correlate with those found by (Okabe, et al., 2001). This could be because different cell lines were used in the two experiments. But still one would expect that some of the genes would be the same since the regulation of the cells would occur in a similar way.

Gene transcription within the cell is just the start of regulation within the cell. From the results shown it can definitely be concluded that one gene does not necessarily produce one protein. Post translational control of the protein is probably also a very important point of

regulation. Various enzymes are also regulated at the gene level but these will be discussed in the next section.

1.2 Regulation of Enzymes.

1.2.1 Drug metabolising enzymes.

The liver is the major site for cytochrome P450 (CYP) activity, and thus has more abundant amounts of CYP than any of the other organ such as the lung, kidney or intestine (Lewis, 2001). The hepatic CYP consists of the CYP isozymes, that are mainly involved in phase I oxidative metabolism of endogenous compounds, drugs and other xenobiotics. These cytochrome P450 enzymes (CYP) are heme-thiolate enzymes that use iron to oxidize xenobiotics as a strategy of the body to dispose of potentially harmful substances by making them more hydrophilic, and thus excreting them from the body. These CYP enzymes generally metabolize chemicals by the insertion of oxygen to produce a hydroxylated (Sheweita, 1999) form of the chemical compound according to the equation:

$$NAPDH + H^+ + \mathbf{RH} + O_2 \rightarrow NADP^+ + \mathbf{R\text{-}OH} + H_2O$$

This hydroxylation step is then often followed by conjugation of the drug to groups such as glutathione by glutathione S-transferase (GST). This conjugation step makes the xenobiotic even more hydrophilic and so the xenobiotic can be easily excreted from the body. This conjugation step is known as phase II drug metabolism.

Differential expression of some CYPs in different organs may also have clinical consequences, especially where the unfortunate side effect of metabolism of a drug is to make a more toxic product. The CYP system participates in the bioactivation of polycyclic aromatic hydrocarbons (PAH's) into their more reactive intermediates. This may also be the situation where it is speculated that some CYPs activate procarcinogens to carcinogens. Anti – oxidants and tea can protect the body against this catastrophe.

CYP requires electrons from NADPH, which is supplied to CYP by NADPH-cytochrome reductase (OR). EGCg inhibit OR and thus electrons cannot be supplied to CYP, consequently inhibiting it. The Figure below demonstrates the catalytic cycle of

cytochrome P450. As can be seen from Figure 1.4, if OR is inhibited, electrons cannot be supplied to the cycle and this will then ultimately inhibit CYP.

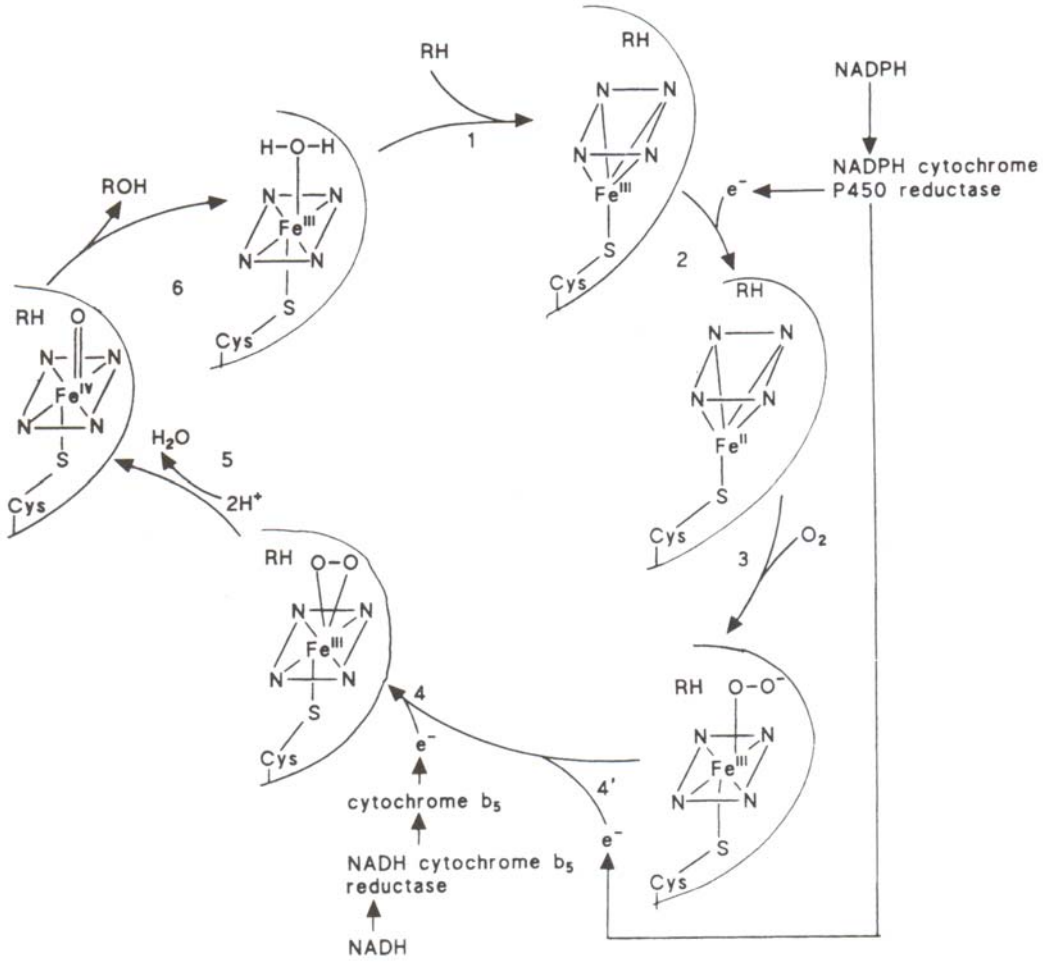


Figure 1.4: The catalytic cycle of cytochrome P450 *(Timbrell, 2000).*

1.2.1.1 Cytochrome P450 regulation by tea.

Green tea has been examined on various human cytochrome P450 subfamilies. These families play an important role in hepatic as well as extra-hepatic metabolism and biotransformation of xenobiotics (Okabe, et al., 2001). Most chemicals require chemical activation as they are not reactive themselves. Cytochrome P450 is one of the major drug metabolizing enzymes involved in activating pro-carcinogens. The CYP1A family are known to catalyse the metabolic activation of polycyclic aromatic hydrocarbons, aromatic

amines and heterocyclic amines. It is known that CYP1A1 activate B[a]P, CYP1A2 activate PhIP, and that CYP3A4 activate AFB1 (Rushmore and Kong, 2002).

Specific substrate probes are used as a measure of the activity of the different CYP isozymes. Green tea enhanced the activities of methoxyresorufin O-demethylase (CYP1A2), Ethoxyresorufin O–deethylase (CYP1A1) (Muto, et al., 2001), pentoxyresorufin O-dependentylase (CYP2B1), and lauric acidhydroxylase. Similar results were found when the cytochromes were treated with black tea (Sohn, et al., 1994; Muto, et al., 2001; Yang, et al., 2003). Immunoblot analysis also confirmed these results when CYP1A2 and CYP4A1 showed a dose dependent increase in protein levels with the addition of green tea, (Bu-Abbas, et al., 1999 (b); Muto, et al., 2001).

Green tea, black tea and decaffeinated tea did not increase the p-hydroxylation of nitrophenol, a selective probe for CYP2E1 or erythromycin N-demethylase (CYP3A). It can thus be assumed that the enhanced hydroxylation of lauric acid is due to ω-hydroxylation, which definitely indicates a huge increase in CYP4A1 activity (Yang, et al., 2003). In contrast to this study another research group found that black tea with its lower flavanol content was the most potent inhibitor of CYP2E1 (Bu-Abbas, et al., 1999 (a); Bu-Abbas, et al., 1999 (b)). They also found that green tea and black tea decreased the activity of CYP3A, which is in contrast to the results found by (Sohn, et al., 1994), as they found that green tea had no effect on CYP2E1 and CYP3A4.

Studies with green tea and black tea have shown that total cytochrome P450 levels stay constant (Sohn, et al., 1994; Bu-Abbas, et al., 1999 (a)). This indicates that the cytochrome P450 isozymes are indeed affected by green tea and black tea but that this modulation of enzyme activity is at the expense of one another. The increase of CYP1A2, CYP4A1 and CYP3B hence occurs at the expense of CYP2E1 and CYP3A when treated with green tea and black tea (Bu-Abbas, et al., 1999 (a)). A similar study was done using the various components of green tea and black tea. It was found that EGCg inhibited the activity of human CYP1A1, CYP1A2, CYP3A4, CYP2A6, CYP2C9, and CYP2E1, after the activation of these cytochromes with their respective mutagens, B[a]P, PhIP and AFB1. These results indicate that EGCg may inhibit the metabolic activation of these procarcinogens. From all the catechins that were tested it was found that the most effective inhibitor of the cytochromes was EGCg, while ECg, EC and EGC inhibited the

cytochromes only weakly. Kinetic studies were done on the cytochrome isoforms and it was found that CYP1A1, CYP2A6, and CYP2E1 displayed mixed type inhibition by EGCg, while CYP1A2, CYP2C9, and that CYP3A4 were inhibited in a non-competitive manner by EGCg (Muto, et al., 2001).

From all the studies that were done using green tea, black tea, decaffeinated black tea, and its components, EGCg, ECg, EC, and EGC, it was seen that all of these definitely had a beneficial effect on detoxification and protection of the body. This protection of the cell is highlighted even more because EGCg is able to regulate the CYP levels depending on the status of the cell.

The mechanism of how green tea, black tea and its constituents protect the body against the harmful onslaught of procarcinogens is still unknown to date. One hypothesis is that the cytochromes require electrons from NADPH via NADPH – Cytochrome reductase (OR) to exert its catalytic activities. EGCg has been shown to inhibit human OR in a competitive manner (Muto, et al., 2001). This means that electrons cannot be supplied to the cytochromes and thus the cytochromes are inhibited indirectly through the action of tea on OR. Thus the inhibition of cytochrome by EGCg is partially due to the inhibition of OR.

1.2.1.2 Phase II drug metabolising enzymes.

Phase II drug metabolism follow phase I drug metabolism. Glutathione S-transferase (GST) is a well-known member of phase II enzymes. It follows CYP by conjugation of the xenobiotic to glutathione (GSH). This creates an even more hydrophilic substance, which is easily excreted from the body. Inducers of GST are considered as protective agents. Figure 1.5 demonstrates phase II metabolism in the body. As can be seen from Figure 1.5 the xenobiotic proceeds through a few steps before it can be excreted from the body as mercapturic acid.

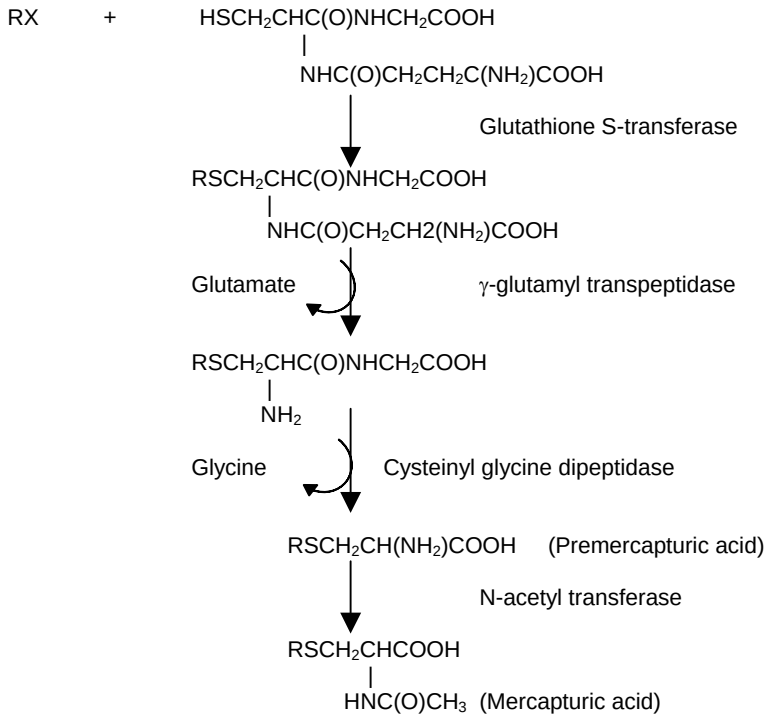


Figure 1.5: Phase II metabolism.

Glutathione S-transferase (GST) activity is enhanced by tea in a dose dependent manner (Bu-Abbas, et al., 1998). GSH is a major cellular antioxidant that participates in the protection against toxic compounds. A general mechanism by which oxygen free radicals induce tissue damage is by lipid peroxidation. GSH plays a central role in preventing lipid peroxidation because it enables GSH peroxidase to scavenge inorganic and organic peroxides, thus helping the cell to cope with oxidative stress (Ahmed, et al., 2002). It was found that EGCg increased GSH levels (Ahmed, et al., 2002), but that glutathione peroxidase activity was unaffected by green tea (Bu-Abbas, et al., 1998). In contrast to these results it was found that EGCg decreased GSH peroxidase activity (Ahmed, et al., 2002). It was also found that EGCg decreased GSH reductase activity (Ahmed, et al., 2002). Glutathione S-transferase, glucuronosyl transferase, epoxide hydrolase activity was enhanced by tea in a dose dependent manner (Bu-Abbas, et al., 1998), as well as the activity of UDP-glucuronyltransferase (Sohn, et al., 1994; Embola, et al., 2002). Catalase was inhibited by black tea and not green tea (Bu-Abbas, et al., 1998). Superoxide dismutase activities were unaffected by green tea (Bu-Abbas, et al., 1998). In contrast to these results, (Kim, et al., 2002), found that EGCg increased both the activity and gene expression of super oxide dismutase (SOD). Super oxide dismutase is known to eliminate

excess oxygen radicals that are produced. The increase in SOD by EGCg would thus exert a protective effect on the cell.

Black tea was more efficient than green tea or decaffeinated black tea in stimulating phase II enzymes (Bu-Abbas, et al., 1998). It can therefore be concluded that neither flavanols nor caffeine is solely responsible for the induction of the hepatic phase II enzymes. It could be due to the theaflavins and thearubigins, which are formed during the fermentation process in which black tea is produced from green tea.

1.2.1.3 Other Enzymes.

Nitric oxide synthase has three isoforms. Isoform I and isoform III produce nitric oxide ($NO^{\cdot}$) at low levels; these two isoforms are usually activated through calcium/calmodulin binding to generate $NO^{\cdot}$ as a signal for physiological function. Inducible nitric oxide synthase (iNOS) is isoform II. This isoform II produces $NO^{\cdot}$ at high levels during inflammation to exert a defence against pathogens. $NO^{\cdot}$ is mutagenic because it can cause deamination of DNA, inactivation of DNA repair enzymes and can also destroy tissue. Besides these actions $NO^{\cdot}$ has also been shown to play a role in inflammatory conditions, sepsis, adult respiratory distress syndrome, arthritis, asthma, inflammatory bowel disease, myocarditis, diabetes, gingivitis and tissue rejection. It is for these reasons that an inhibitor of iNOS would be of great importance. iNOS is mainly regulated at the level of gene expression (Shen, et al., 2005), once the iNOS enzyme is produced it remains activated and constantly produces $NO^{\cdot}$ for the lifetime of the enzyme. It would thus be advantageous if it was possible to control iNOS at both mRNA level and enzymatic activation levels (Chan, et al., 1997).

It was found that EGCg inhibited the gene expression of murine hepatic iNOS by inhibiting the expression of mRNA in a dose dependent manner (Chan, et al., 1997). Murine hepatic iNOS has 80% nucleic acid homology to the human hepatic iNOS and therefore it is concluded that EGCg will have the same effect on human iNOS. iNOS resembles cytochrome P450, and this may be a reason for the inhibition of iNOS by EGCg. EGC, another tea polyphenol was tested, but was ineffective in inhibiting the activity of iNOS. The order of potency found for the various green tea polyphenols on the activity of iNOS was EGCg > ECg > EGC (Chan, et al., 1997). From these results it can be concluded

that the gallate structure is important in inhibiting the iNOS enzyme. Theaflavin-3,3'-digallate (TFdg) a polyphenol from black tea reduced $NO^{\cdot}$ production and inhibited the release of $NO^{\cdot}$ even more than did EGCg. TFdg is thus more effective than EGCg (Lin, et al., 1999). It can be concluded that tea polyphenols alone are important inhibitors but that a whole green tea and black tea extract would probably be more effective than just one of its constituents.

1.3 Regulation/dysregulation of the cell cycle.

Xenobiotics may initiate cell damage or stress, but the cellular proteins that are involved in the control of the cell cycle and apoptosis are the final arbiters of the cell and its final fate. The biochemical pathways that restrain cell cycle transition and induce cell death after stress are known as cell cycle checkpoints. Cells can be arrested at the cell cycle checkpoints temporarily to allow for:

i. Cellular damage to be repaired.
ii. The dissipation of an exogenous cellular stress signal.
iii. Availability of essential growth factors.

Checkpoint signalling may also result in the activation of the pathways leading to programmed cell death if the cellular damage cannot be repaired. Defects in the cell cycle checkpoints can result in gene mutations, chromosome damage, aneuploidy, all of which could contribute to tumourgenesis. The cell cycle is highly organised. Control within the cell cycle is by a cascade of protein phosphorylation that relay a cell from one stage to another and secondly a set of check points that monitor the completion of each step as it is completed, before it can proceed to the next step (Collins, et al., 1997). Cell cycle checkpoints sense flaws in critical events of the cell such as DNA replication and chromosome segregation.

1.3.1 G1/S Checkpoint.

Negative regulation of G1 phase cyclin/cdk complexes plays a key role in the G1/S checkpoint function. Cyclin dependent kinases (cdks) are negatively regulated by a group of functionally related proteins called cyclin dependent inhibitors (cki). These cki falls into two families: the INK4 inhibitors (p16 and p18) and the KIP/WAF inhibitors (p21 and p27). INK4 specifically inhibits cdk4 and cdk6 activities during the G1 phase of the cell

cycle, while KIP can inhibit cdk activity during all phases of the cell cycle. The cdk-cyclin-cki complexes are the workhorses of the cell cycle. p21 is the most important cki as it is seen as the universal inhibitor of all cdks due to its ability to inhibit all the cdks in all the phases of the cell cycle. p21 is also able to regulate the entry of cells into the G1 to S phase of the cell cycle (Kim and Moon, 2005). When normal cells are exposed to genotoxic agents, p21 is induced by p53 dependent transactivation. The elevated p21 binds and inactivates cyclinD/cdk4,6, and cyclin E/cdk2 complexes that result in pRB hypophosphorylation and cell cycle arrest.

Specific cyclin/cyclin dependent kinase (cdk) complexes are activated at different intervals during the cell cycle. Cyclin D1/cdk4 are activated in the mid-G1 phase, whereas cyclin E/cdk2 complexes are required for the G1/S transition, cyclin A/cdk2 for the progression of DNA synthesis, and cyclin A-B/cdk1 for the G2/M transition of mitosis. The activation of cyclin D1/cdk4 and cyclin D1/cdk6 complexes at mid G1 is responsible for the phosphorylation of retinoblastoma protein (pRb). The pRb family form a complex with transcription factors of the E2F family. Phosphorylation of pRb inhibits its interaction with the E2F transcription factor and now permits the expression of the genes necessary for S-phase entry.

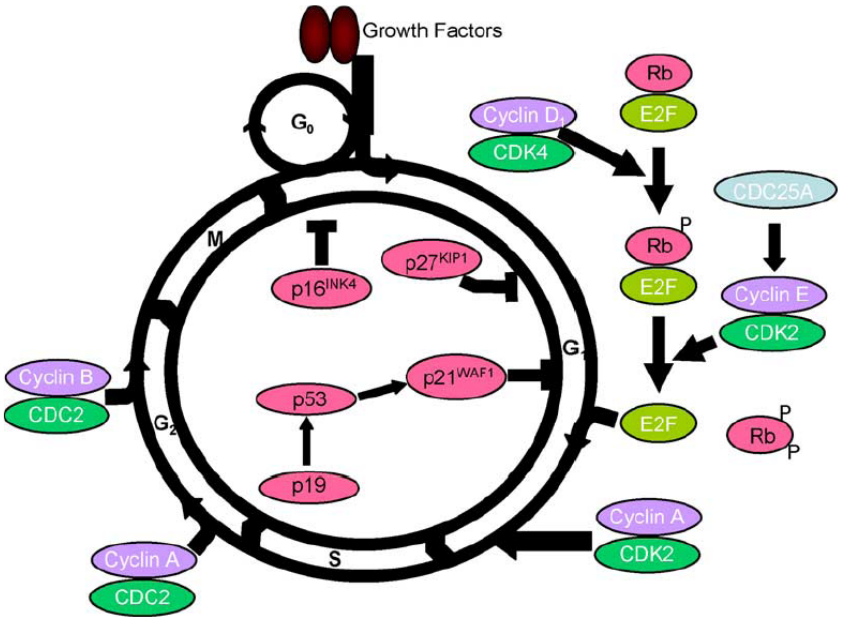


Figure 1.6: Possible regulation of the cell cycle by tea polyphenols *(Hou, et al., 2004).*

1.3.2 Role of p53 in apoptotic signalling.

Early cell changes that occur during apoptosis are associated with mitochondrial changes by members of the Bcl-2 family of protein, including anti-apoptotic protein Bcl-2 and pro-apoptotic BAX proteins. p53 can inhibit Bcl-2 function through transactivation of tBid. tBid initiates a signalling pathway that results in Bcl-2 phosphorylation and inactivation. In contrast to Bcl-2, BAX expression is up regulated during p53 dependent apoptosis. BAX facilitates the release of apoptosis-inducing factor (AIF) and cytochrome c from the mitochondria, thus activating the caspase cascade. Since p53 is mutated or disrupted in the majority of human cancers, any drug that preferentially acts on cells lacking normal p53 is of great interest since it may have tumour cell specificity.

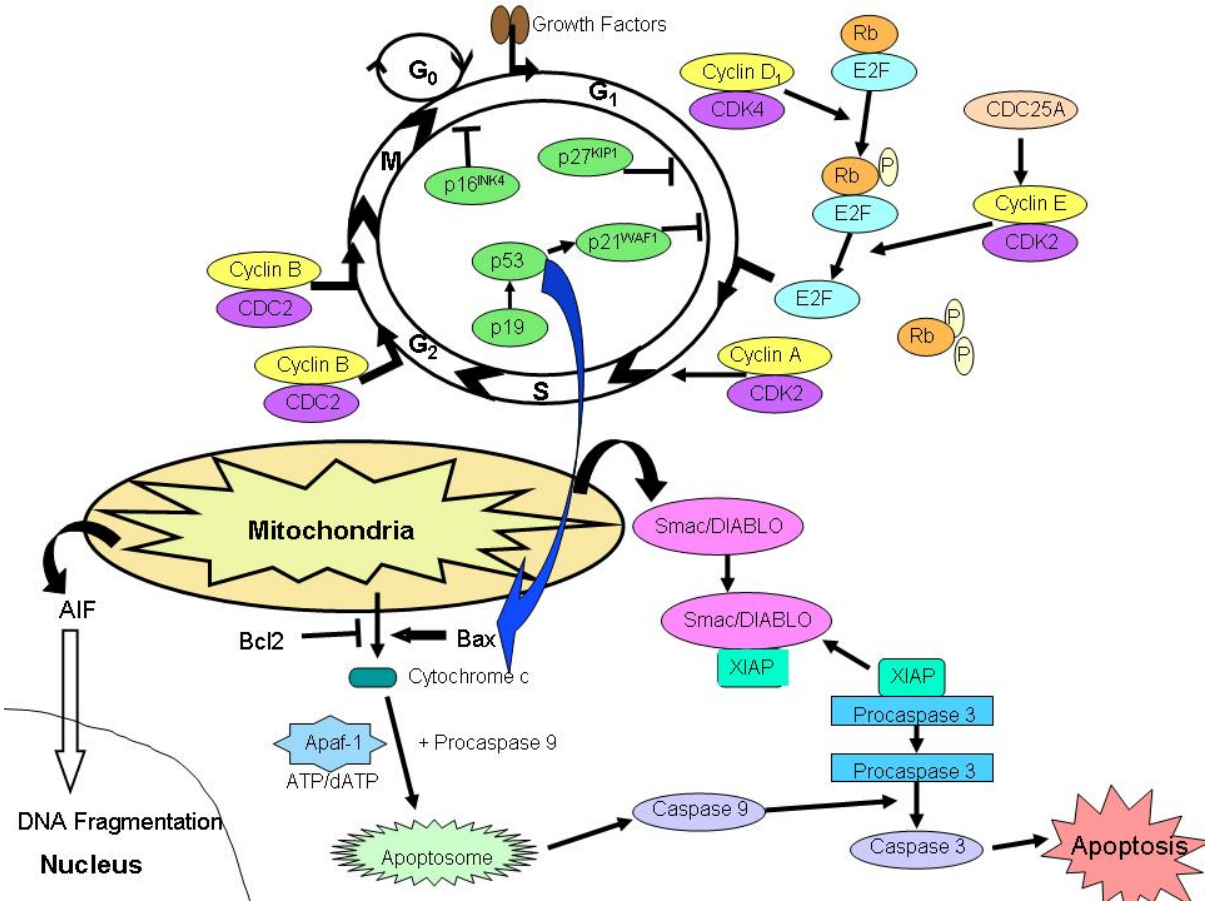


Figure 1.7: The role of p53 in the cell cycle and apoptosis.

This picture indicates how p53 is able to cause cell cycle arrest by activating p21 in the cell cycle, and p53 can activate BAX that will induce apoptosis by the release of cytochrome c from the mitochondria.

The relationship between cancer and the cell cycle is obvious as cancer permits the existence of too many cells. This happens when the cell does not respond to checkpoint signals or p53 dependent transactivation of the checkpoints. Cancer usually develop when mutations occur in the so called tumour suppressor genes, this allows uncontrolled cellular proliferation (Collins, et al., 1997). EGCg has been shown to block the cell cycle and cause cell cycle arrest at various points (Lin, et al., 1999).

1.3.2.1 NF-κB

Nuclear factor-κB (NF-κB) is a widely distributed transcription factor that is present in the cytosol where it consists as a heterodimer with its p50 and p65 subunits as well as its inhibitory unit IκB. The phosphorylation of IκB by NIK results in detachment of IκB-α from NF-κB. IκB-α will then be tagged by ubiquitin and degraded by the proteasome. NF-κB is now activated by the release of IκB-α and thus NF-κB translocates to the nucleus where it activates transcription. NF-κB has a role in inflammation, cellular proliferation and cancer (Lin, et al., 1999; Ahmad, et al., 2000 (b)).

From various experiments that were done it was found that EGCg inhibited the activity of NF-κB in cancerous cells even at doses as low as 10μM that are physiologically attainable. For normal cells it was found that inhibition of NF-κB only occurred at much higher doses (80μM), when tested with electrophoretic mobility shift assay (EMSA) (Lin, et al., 1999; Suganuma, et al., 2000; Ahmad, et al., 2000 (b)). The down regulation of NF-κB also has an effect on iNOS. It was found that by addition of EGCg, NF-κB was down regulated, and the activity of iNOS was inhibited (Lin, et al., 1999). The same results were found for black tea and TFdg (Lin, et al., 1999). The Figure below demonstrates how NF-κB signalling does occur within the cell.

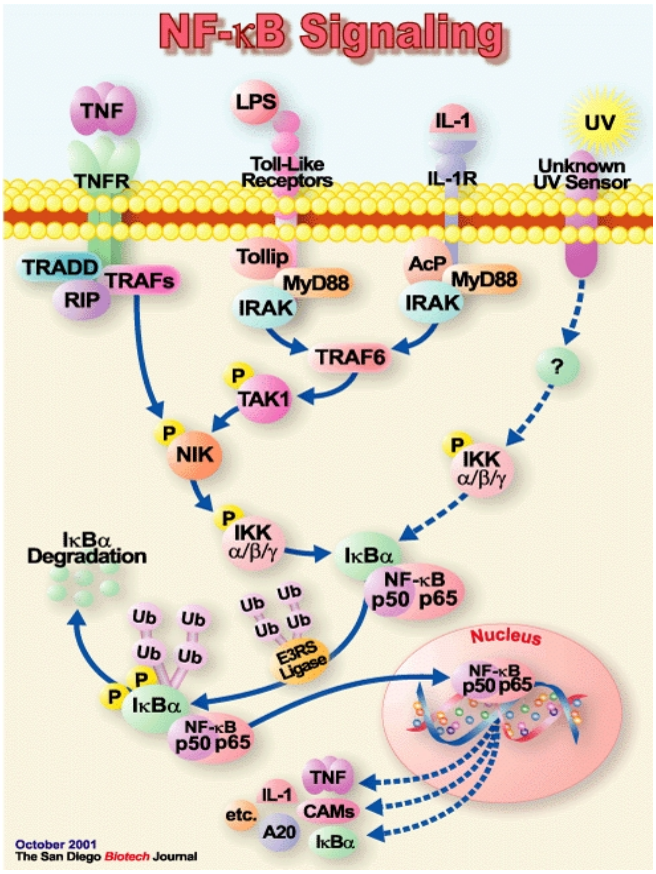


Figure 1.8: NF-κB pathway.

1.3.2.2 TNF-α

TNF-α is inhibited by EGCg treatment in cancerous cells (Ahmad, et al., 2000 (b); Lambert and Yang, 2003 (b)), and transgenic mice (Suganuma, et al., 2000). When TNF is inhibited by EGCg it is unable to bind to NF-κB. EGCg is able to reduce gene expression of TNF-α as well as the activity of TNF-α. TNF-α induced cytokine genes such as IL-1β and IL-10 has also had reduced expression when treated with EGCg. Usually TNF-α signalling results in inhibition of IκBα which will enable NF-κB to translocate into the nucleus to activate transcription and produce even more TNF. EGCg is able to reverse this by inhibiting TNF-α signalling (Lin, et al., 1999; Ahmad, et al., 2000 (b)).

1.3.2.3 p53

Most human cancers have a mutated or inactivated p53. This results in uncontrolled cellular proliferation as p53 controls the cell cycle. The importance of p53 and its ability to induce cell cycle arrest and apoptosis have been demonstrated by various laboratories.

EGCg is able to induce p53 expression and can thus cause cell cycle arrest of cells and ultimately apoptosis of damaged cells (Gupta, et al., 2000; Suganuma, et al., 2000; Ahmad, et al., 2000 (a); Gupta, et al., 2003; Roy, et al., 2005).

It was also seen that black tea induced p53 (Chung, et al., 2003). Experimental results found that caffeine is able to induce transcriptional activation of the p53 promoter as well as increase the protein expression of p53. p53 induced apoptosis by addition of caffeine in p53 wildtype cells but not in p53 deficient cells. Caffeine induced Bax expression and ultimately the protein expression of caspase 3 which is the mediator of apoptosis in p53 wild type cells, but not in p53 deficient cells. Bax can promote the cytosolic release of cytochrome c from the mitochondria, which will activate caspase 3 that will induce apoptosis (He, et al., 2003).

The INK1a/ARF gene is the second most disrupted gene in cancer. It encodes for two tumour suppressor proteins: p16 which can inhibit the phosphorylation of retinoblastoma protein (pRb) by cyclin D dependent kinases and p19, which stabilizes and activates p53 to induce the cell cycle or apoptosis (Inoue, et al., 1999). p53 also has an important role in the regulation of the cell cycle as it is able to induce p21, that can cause G1/S cell cycle arrest (See Figure 1.6 and Figure 1.7). The role of p21 and the other cki, cdk, and cyclins will be discussed next.

1.3.2.4 Cyclins, cdk, and cki

In the G0/G1 phase immunoblot analysis cancer cells revealed that the cyclin kinase inhibitors (cki) (p21/waf1, p27/kip1, p16, and p18) were up regulated in a dose and time dependent manner by treatment with EGCg (Lin, et al., 1999; Ahmad, et al., 2000 (a)). Black tea was also shown to induce p21 (Chung, et al., 2003).

During the cell cycle it is also important that the cki is degraded by the proteasome so that the cdk's can bind to the cyclins and the cell cycle can proceed (Goldberg, et al., 2001).

Induction of cki results in a blockade of G1 to S phase during the cell cycle (Ahmad, et al., 2000 (a); Gupta, et al., 2003). EGCg treatment of LNCaP cells (wildtype p53) resulted in increased expression of p21, p27, p16 and p18, all which are cyclin kinase inhibitors.

Similar results were found with DU145 (mutated p53) (Ahmad, et al., 2000 (a); Gupta, et al., 2003; Kim and Moon, 2005).

These results were further confirmed by using a deleted mutant p21 that does not contain the p53 binding sites. The luciferase activity of the mutant p21 was the same as the wild type p21 luciferase activity (Kim and Moon, 2005). This is in contrast to the proposed mechanism in which it was first thought that p21 is dependent on p53 for up regulation. This suggests that the induction of p21 by EGCg may be independent on p53 activation.

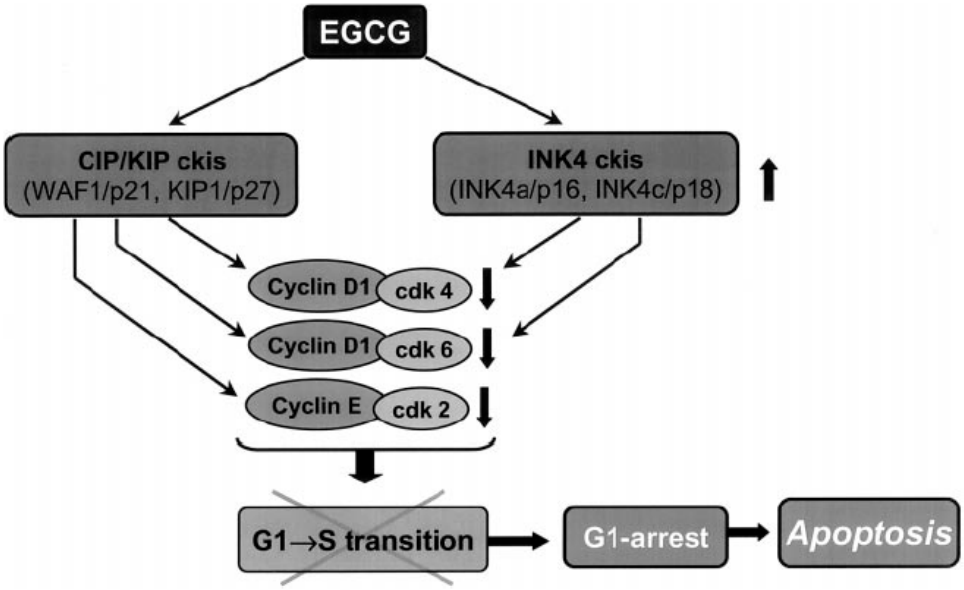


Figure 1.9: The proposed mechanism of how EGCg regulates the cell cycle *(Ahmad, et al., 2000 (a))*.

Cki needs to bind to cdk/cyclin complexes to exert their inhibitory functions. EGCg treatment of cells resulted in down regulation of the protein expression of cyclin D1 and cyclin E (Gupta, et al., 2003; Kim and Moon, 2005). Down regulation of the protein expression of cdk 2, cdk4 and cdk 6 was seen as well as inhibition of the kinase activities of cdk2 and cdk4 (Lin, et al., 1999; Gupta, et al., 2003; Kim and Moon, 2005). In contrast, results found by *Ahmad, et al., (2000 (a)),* showed that cyclin D1 protein expression was down regulated by EGCg but cyclin E protein levels stayed constant. When the kinase activities were tested it was seen that EGCg inhibited the kinase activities of cyclin D and cyclin E. Similarly the same was found for the protein expression levels of cdk4 and cdk6

that was down regulated by EGCg but the protein levels of cdk2 stayed the same. When the kinase activities were measured it was seen that EGCg inhibited the kinase activities of cdk2, cdk4, and cdk6. Thus cdk2 followed the same pattern as cyclin E. These results do verify that the cdk-cyclin-cki complex work as a unit that is either activated by the cdk or inhibited by the cki. The results found by *Ahmad, et al., (2000 (a))*, is probably correct. The most probable explanation for this occurrence where cyclin E and cdk 2 only have increased kinase activity but that the protein expression stays constant is due to the fact that E2F is the transcription factor activating transcription of cdk2 and cyclinE. The mechanism of E2F transcription is explained next.

Cyclin D forms a complex with cdk4 and cdk6, this then triggers phosphorylation of retinoblastoma protein (Rb) in mid- to late G1 phase. In its hypophosphorylated state, Rb prevents G1 exit by binding to the transcription factor E2F, thereby inhibiting its activity. When Rb gets phosphorylated to pRb by the cyclinD-cdk4/6 complex, Rb release E2F, thus enabling E2F to activate the genes that are required for S phase entry (Inoue, et al., 1999). The E2F transcription factor encode genes involved in DNA replication and in cell cycle progression (Hateboer, et al., 1998). The Rb protein changes from a hyper phosphorylated state to the hypophosphorylated phase when cells are treated with EGCg, thus enforcing transcriptional repression on E2F (Lin, et al., 1999). Immunoblot analysis indicated that EGCg decreased the total pRb protein levels in a dose and time dependent manner as well as an increase in the hypophosphorylated form of Rb (Ahmad, et al., 2002).

EGCg inhibit these complexes and are able to cause arrest of cells in the G0 to G1 phase of the cell cycle. Thus it can be concluded that EGCg is capable of restoring proper checkpoint control in human cancer cells during the cell cycle, and that EGCg can induce G0/G1 cell cycle arrest. This is further confirmed by that fact that EGCg resulted in accumulation of cells in the G1 phase of the cell cycle (Kim and Moon, 2005). Arrest is an irreversible process and ultimately the cells have to undergo apoptosis (Ahmad, et al., 2000 (a); Gupta, et al., 2003).

1.3.2.5 Apoptosis and cellular proliferation

One strategy to fight cancer is to take advantage of the differences between cancerous and normal cells (Chen, et al., 1998). EGCg is able to stop the growth and cellular proliferation of cancerous cells as well as induce apoptosis in cancerous cells at doses as low as 40μM,

while even at high doses such as 80μM EGCg the normal cells showed no sign of apoptosis (Ahmad, et al., 2000 (b)). EGCg inhibited the growth and cellular proliferation of human epidermoid carcinoma (A431) cells (Ahmad, et al., 2000 (b)), HTB-94 cells (Islam, et al., 2000), and (VSMC) vascular smooth muscle cells that were stimulated with TNF-α (Kim and Moon, 2005), it also inhibited proliferation in (DU145), (LNCaP) cells (Islam, et al., 2000; Gupta, et al., 2003), PC-9 cells (Suganuma, et al., 2000; Lopaczynski and Zeisel, 2001), lung fibroblast cells (V-79), chronic myelogenic human leukemia cells (K-562) (Roy, et al., 2003), and human breast adenocarcinoma MDA-MB-468 cells (Roy, et al., 2005), but had no effect on the normal human epidermal keratinocytes (NHEK).

Apoptosis is initiated when cytochrome c is released from the mitochondria (Lopaczynski and Zeisel, 2001) (See Figure 1.7). Anti-apoptotic protein Bcl-2 is associated with the inhibition of apoptosis, while the expression of the pro-apoptotic protein Bax is associated with induction of apoptosis. Bax associate with the mitochondria to release cytochrome c that will bind to Apaf-1 in the cytosol, this will then activate procaspase 9, and these caspases will then cleave each other in a caspase cascade to ultimately activate caspase-3. Cleaved caspase-3 is active and the executioner of apoptosis (Roy, et al., 2005). With the onset of cancer Bcl-2 expression is increased, while BAX expression will be inhibited and hence no apoptosis will occur (Frommel, et al., 1999).

EGCg is able to induce the activities of caspase 3 and caspase 8 (Roy, et al., 2003) as well as decrease the protein expression of Bcl-2, while the protein expression of Bax will be increased. EGCg is also able to induce cytochrome c release from the mitochondria and induced Apaf-1 to activate caspase-3 (Roy, et al., 2003; Roy, et al., 2005). Thus it is clear that EGCg is able to take advantage of the differences between normal and cancerous cells and is also able to control signalling pathways in favour of the cell. EGCg would thus be a good candidate for cancer therapy.

1.4 Regulation of protein degradation

The proteasome is a multicatalytic protease complex that degrades most cellular proteins. For this reason the proteasome is typically described as the chamber of doom by A.L. Goldberg, who named the proteasome. A typical cell has about 30 000 proteasomes, each about two million Daltons in size (Goldberg, et al., 2001).

Proteins are marked to be degraded by the proteasome. This marking is usually by the attachment of small ubiquitin molecules that are only 76 amino acids long to the targeted protein. The process of ubiquitination is tightly controlled by various enzymes. The proteasome only recognises the ubiquitinated protein. The proteasome will then unfold and degrade the ubiquitinated protein (Dou, et al., 2003; Smith, et al., 2004).
The 26S proteasome has three subunits. A barrel shaped 20S proteasome with the proteolytic sites on the inside, and a regulatory cap on each side of the barrel. The 19S cap is a highly selective “gatekeeper” that binds to the ubiquitinated protein targeted for destruction. The caps then unfold the protein and inject it into the core were it is cut into pieces (Benaroudj, et al., 2003). Thus the caps regulate the entry of the ubiquitinated protein into the 20S barrel (Dou, et al., 2003).

The ubiquitin pathway makes use of three enzymes and energy (ATP) during the ubiquitination of proteins and is thus the rate limiting step in protein degradation. E1 activates ubiquitin and connects to E2. E3 facilitates the transfer of the activated ubiquitin from E2 to the protein. The process repeats until a polyubiquitin chain is attached to the protein marked for degradation. This then draws the protein to the proteasome. There are hundreds of E3 enzymes that monitor the amino acid sequence of proteins and then mark them for destruction by ubiquitination (Willems, et al., 1996). E3 also have an F-box that acts like a socket and then attach to a specific protein marked for destruction (Goldberg, et al., 2001).

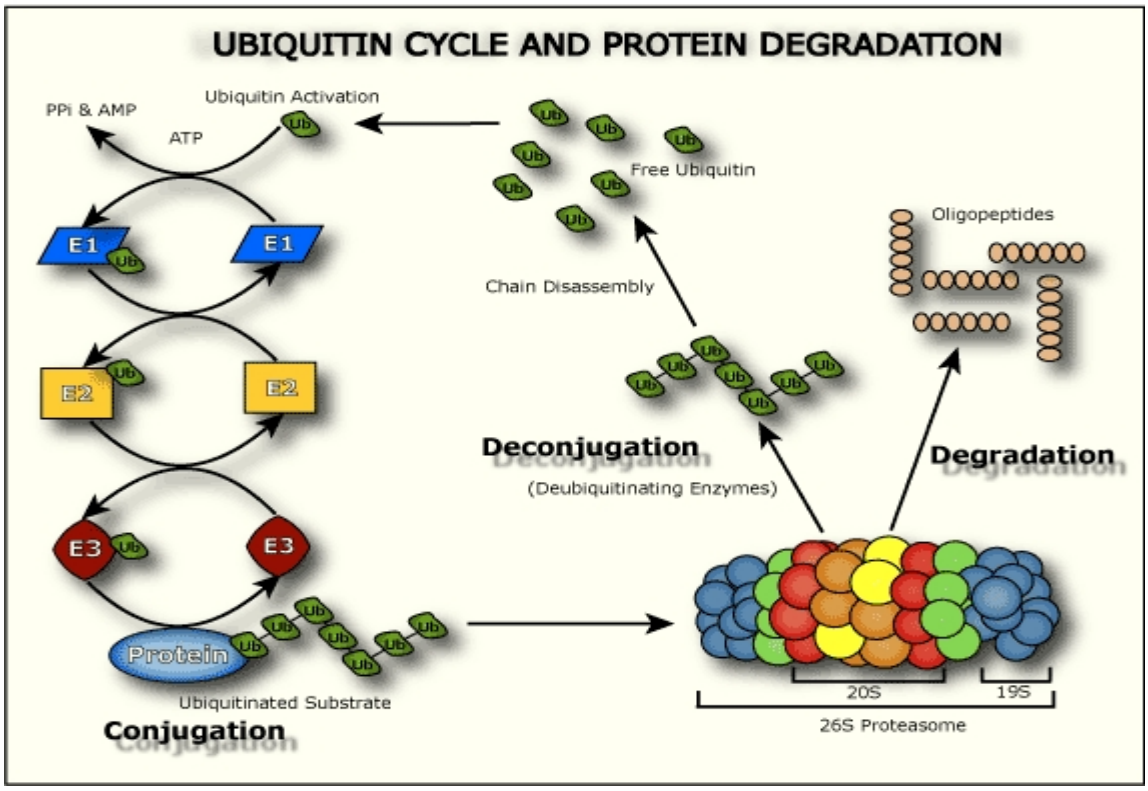


Figure 1.10: The ubiquitin/proteasome recycling pathway.

The proteasome has three proteolytic activities – chymotrypsin-like activity which cleaves after hydrophobic residues, trypsin-like activity with cleavage after basic residues, and caspase like activity, with cleavage after acidic residues (Smith, et al., 2004). Protein degradation is a very important process as it may be needed to stop some biochemical reaction by degrading the active enzyme or it may play a role in activating some pathway needed by the cell. During the cell cycle it is also important that the cki is degraded by the proteasome so that the cdks can bind to the cyclins and the cell cycle can proceed (Goldberg, et al., 2001).

When the proteasome malfunctions either destroying too many proteins or not degrading any protein at all, diseases will appear, as the ubiquitin/proteasome dependent degradation pathway plays an essential role in the up regulation of cell proliferation, down regulation of cell death and the development of drug resistance in human tumour cells. For these reasons the use of a proteasome inhibitor is greatly sought after as this could be a potential anticancer drug (Smith, et al., 2004).

1.4.1 The effect of tea on the chymotrypsin activity of the proteasome.

The research group of *Nam, et al., (2001),* found that EGCg inhibited the 20S proteasome chymotrypsin like activity. Similar results were found with ECg, GCg and Cg – all green tea polyphenols that contain ester bonds. In contrast the tea polyphenols, EC, GC, and C that do not contain any ester bond, had no effect on the 20S proteasome chymotrypsin like activity.

Whole tea extracts were tested on the activity of the proteasome. Green tea extract contains 51.5% EGCg and 14.7% ECg, while black tea contain 19.7% EGCg and 14.9% ECg. Both green tea and black tea strongly inhibited the chymotrypsin like activity of the 20S proteasome (Nam, et al., 2001).

EGCg is able to irreversibly inhibit the chymotrypsin-like activity of the proteasome in both purified proteasome models (Nam, et al., 2001) as well cancer models. On the other hand EGCg has no effect on the trypsin-like activity or caspase-3 like activity of the proteasome (Kuhn, et al., 2004).
From this it can be concluded that the ester bond is essential for the potent inhibition of the 20S proteasome chymotrypsin like activity. Ester bonds have a high nucleophilic susceptibility and therefore they were found to be the more potent inhibitors (Nam, et al., 2001).

1.4.2 The proteasome, the cell cycle and EGCg.

The cell cycle is important in regulating cellular proliferation, while the proteasome has a key role in maintaining the correct balance within the cell cycle. Cyclin B play an important role in mitosis; only when cyclin B has been degraded by the ubiquitin/proteasome pathway can mitosis proceed to the G1 phase of the cell cycle. Cyclin E controls late G1 progression of the cell cycle. Degradation of cyclin E by the ubiquitin/proteasome pathway is essential for entry into the S-phase of the cell cycle. Cdk activation at the G1/S phase of the cell cycle occurs with phosphorylation of retinoblastoma (RB) protein and consequently the release of active E2F, a transcription activator required for expression of the major S phase genes. Cdk inhibitors like p27, p21 and p16, partly regulate the cell cycle. p27 is degraded by the ubiquitin/proteasome pathway, and therefore it is thought that the proteasome may have an important role in

tumour cell survival, as inhibition of the proteasome can lead to an increase in the p27 levels which in turn will lead to cell cycle arrest (Dou, et al., 2003).

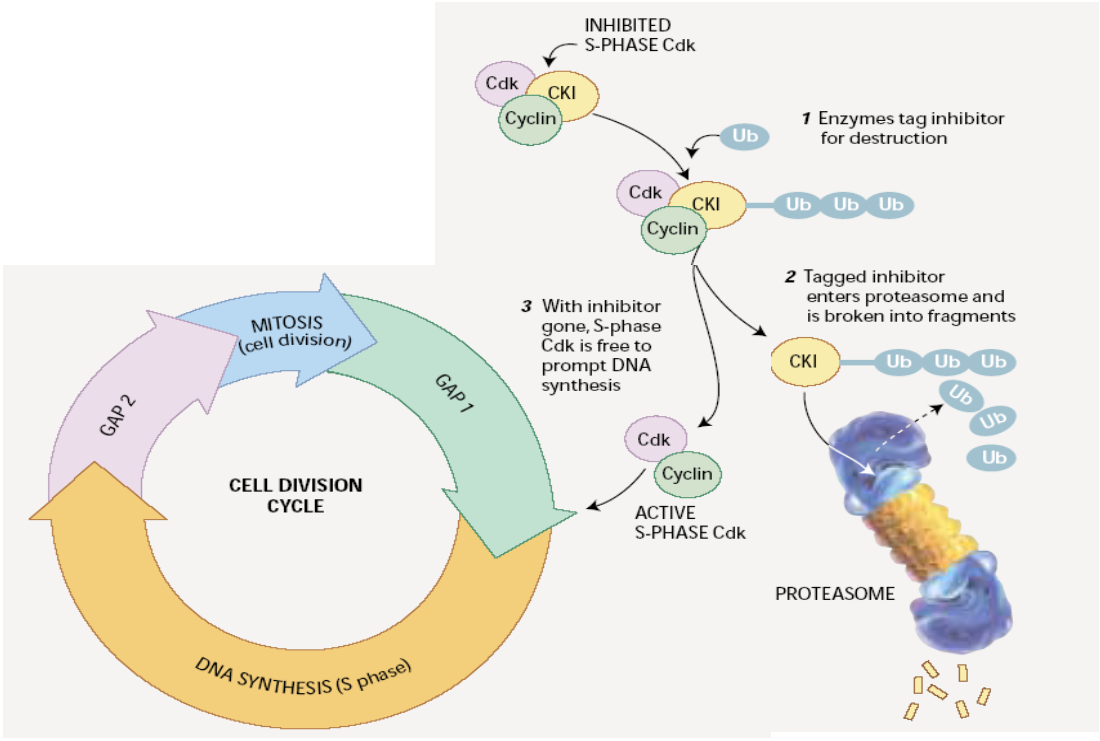


Figure 1.11: Regulation of the cell cycle by the proteasome (Goldberg, et al., 2001).

NF-κB, a transcription factor, is activated by the proteasome. First proteolytic cleavage of p105 occurs, which is a precursor of p50 and p65 of NF-κB. The second process is the degradation of IκB-α, a NF-κB inhibitor, by the proteasome. After activation of NF-κB it translocates to the nucleus to start gene transcription. NF-κB prevents apoptosis and is thus a tumour promoter. Some of the NF-κB transcripts that block apoptosis are Bcl-2, and Bcl-xL (Dou, et al., 2003).

Tea can be seen as a cancer chemopreventitive agent in part because it is able to inhibit the chymotrypsin like activity of the proteasome and had no effect on the trypsin like activity of the proteasome. Proteasome inhibitors induce tumour cell apoptosis; and also trigger programmed cell death in cancerous but not normal cells (Smith, et al., 2004).

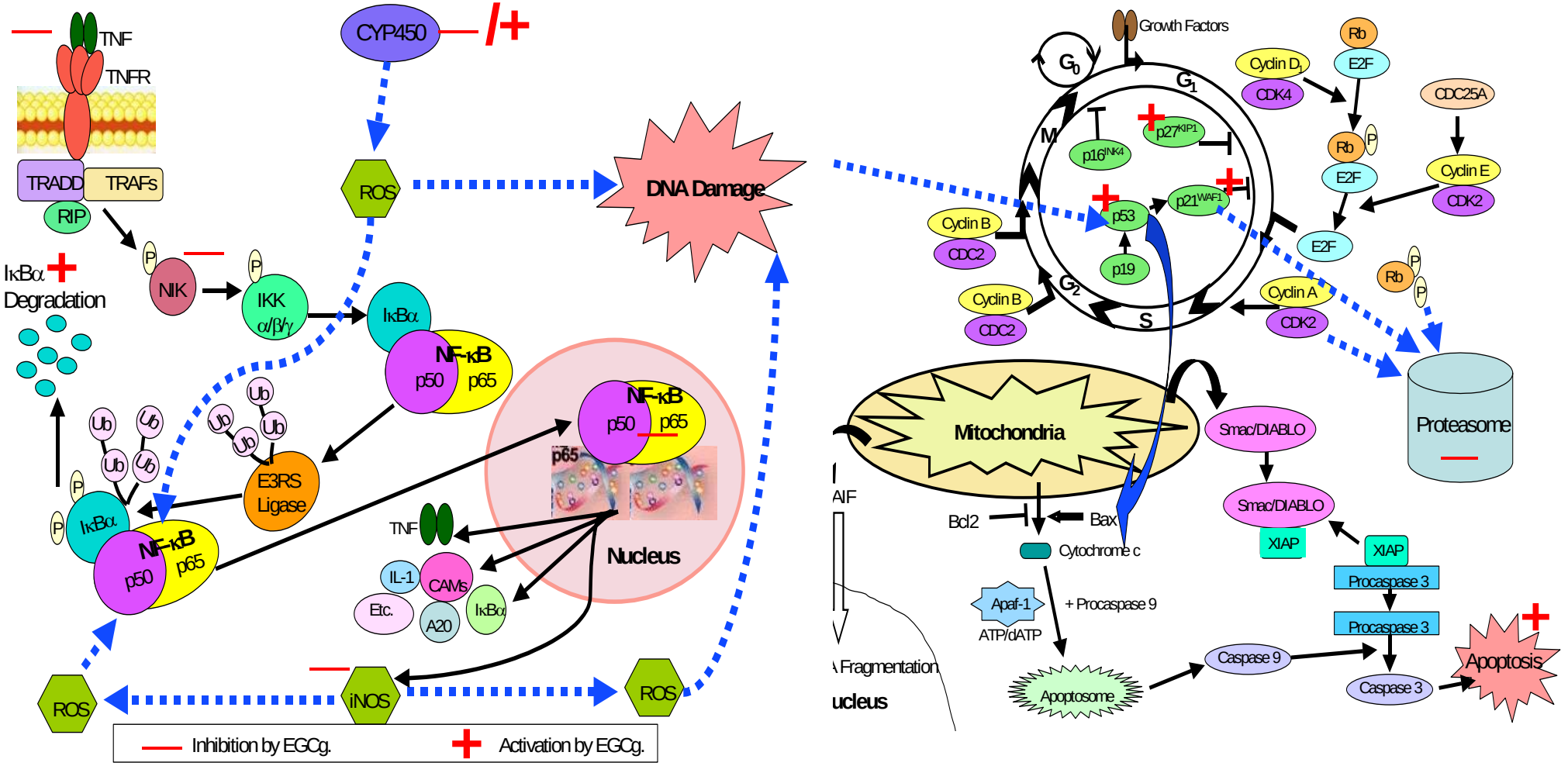


Figure 1.12: Protein regulation by EGCg (See text for explanation).

Green tea, black tea, and some tea components all show protective effects on the well being of the cell. Tea is able to regulate gene expression, regulate enzymes, regulate the cell cycle, cause cell cycle arrest, induce apoptosis, and inhibit the proteasome. Even though a lot of research has been done on especially the green tea polyphenol EGCg that has shown a lot of beneficial effects on the cell, there is probably merit in more research of the whole green tea and -black tea extracts. The protective mechanism of tea is demonstrated in Figure 1.12. From micro array gene expression experiments that were done few genes were modulated by the addition of EGCg. From these experiments it was concluded that protein regulation and post translational modifications is probably a more important level of control than gene expression. The NIK gene expression was reduced by EGCg. The down regulation of NIK gene expression by EGCg can lead to reduced activation of NF-κB. This would be a desirable scenario since NF-κB is known to be an anti-apoptotic transcription factor. When NF-κB translocates into the nucleus to activate transcription, genes such as Bcl-2, iNOS, TNF-α, and IκB-α, are transcribed. All of these except for IκB-α inhibit apoptosis, thus enabling cellular proliferation. This effect is not always desirable since this effect can cause proliferation of cancerous cells. The activity of NF-κB and TNF-α was also inhibited by EGCg. In the cell TNF-α activates NIK by phosphorylation, NIK then phosphorylates IKK, which then phosphorylates IκB-α. The phosphorylation of IκB-α then frees NF-κB from IκB-α, enabling NF-κB to translocates into the nucleus, to activate its various genes. When IκB-α is released from NF-κB, it gets marked by ubiquitin and are degraded by the proteasome. EGCg protect the cell against this catastrophe. First EGCg is able to inhibit the gene expression of NIK, then EGCg is able to inhibit the activity of NF-κB by IκB-α, as IκB-α is not phosphorylated and thus NF-κB are not able to translocate into the nucleus. This whole process is regulated by EGCg at both the gene level as well as the protein level. EGCg is also able to regulate the cell cycle either dependently on p53 or independently of p53, by directly interacting with p21. This is contradictory to the previous proposed mechanism that stated that p21 activation is dependent on p53. The mechanism was described in section 1.4. Cell cycle arrest by EGCg is beneficial to the body as dysfunctional cells will be destroyed by apoptosis. Cancer is usually the result of an inability of the cell to exert cell cycle arrest. Once again all the processes are linked as demonstrated in Figure 1.12. The cdk-cyclin-cki complexes work as a unit in controlling the cell cycle. These cdk-cyclin-cki complexes are also degraded by the proteasome to ensure that the cell will proceed from one stage to

another in the cell cycle. EGCg inhibits the chymotrypsin action of the proteasome. This will then also ensure that the cdk-cyclin-cki complexes are not degraded by the proteasome in an attempt to ensure that cell cycle arrest and apoptosis of faulty cells do occur.

1.5 Aim

The goal of this study is to discover new proteins that may be modulated in mice drinking tea in contrast to control animals that receive only water. It is hoped that some of the known proteins that are modulated as highlighted within this review may also be detected by our proteomic approach. These newly discovered modulated proteins could become new targets for cancer therapy. This will confirm that the proteomic approach does detect some of the hepatic proteins that are known to be modulated by tea for example CYP2E1, GST mu-1 and iNOS. It is hoped that the new proteins that are discovered in this approach may shed new light on the mechansism of tea thearapy in mice bearing tumours.

1.6 Hypotheses

- **Hypothesis I:** Tea drinking is able to modulate the proteome in normal cells from various organs.
- **Hypothesis II:** Some of the modulated proteins will be ones that have already been discovered by other approaches for example by substrate probes for specific CYP isozymes.
- **Hypothesis III:** Some of the modulated proteins will be new discoveries that have never been shown before to be modulated by tea drinking.

CHAPTER 2

Data analysis of C57BL/6 male mice consuming black tea *(Camellia sinensis)* over a six week period

2.1 Introduction

Absorption, distribution, metabolism and excretion (ADME) of tea and its constituents to date are largely unknown. Information regarding ADME is important to fully understand the biological mechanism of tea and its constituents. Tea has been reported to play various important protective roles within experimental subjects as well as a role in weight maintenance. Various studies on humans, mice and rats have been done to date to try and elucidate the full mechanism of metabolism of EGCg and tea. Animal models are used for several reasons. Mice and humans seem to have very similar metabolism (Li et al., 2001; Lambert et al., 2003 (a)) of tea and its constituents, even bioavailability between humans and mice seem very similar (Lambert et al., 2003 (a); Chen et al., 1997).

The rate of absorption of xenobiotics is an import issue. It was determined that oral administration *(p.o)* of green tea and EGCg provides better bioavailability when compared to *intravenous (i.v.), intraperitoneal (i.p.)* and *intragastric (i.g.)* administration (Chen et al., 1997). After absorption the xenobiotic is distributed throughout the whole body. Green tea and EGCg administration revealed that the small intestine absorbed the most EGCg, followed by the stomach (Suganuma et al., 1998; Chen et al., 1997). The mechanism by which catechins are absorbed in the small intestine remains largely unknown. Probably catechins enter the enterocytes by passive diffusion (Donovan et al., 2001) were it is further metabolized. The blood also showed high levels of EGCg and tea after administration. This resulted in mainly the detection of unconjugated catechins like EGC, EC, EGCg, and ECg as well as some of their metabolites 4'-O-MeEGC, M4, M6 (Lee et al., 2002). The blood distributes the EGCg to the various organs where the EGCg will then be absorbed and metabolized. The liver absorbed the third highest amount of EGCg followed by the brain, lung, kidney, uterus and ovary, heart, skin, bone, pancreas, and spleen (Suganuma et al., 1998; Chen et al., 1997). Detection of EGCg immediately after consumption by HPLC and LC/MS/MS has detected EGCg mainly in the unconjugated

form in the liver, lung, spleen, small intestine, colon and kidney (Lambert et al., 2003 (a)) indicating slow metabolism of EGCg.

The lack of information on the bioavailability and biotransformation of tea catechins is a huge limitation in the understanding of tea and its biological activities. The chemical structure of a compound will affect its biological properties such as bioavailability, antioxidant activity, specific interactions with cell receptors and enzymes and other properties. The biological activity of a compound will also be dependent on the bioavailability of that specific compound (Scalbert and Williamson, 2000).
To date poor bioavailability of EGCg has been reported in humans, mice and rats (Chen et al., 1997; Lambert et al., 2003 (a); Sang et al., 2005; De Vries et al., 1998). The bioavailability of EGCg in rats has been reported to be the worst (Lambert et al., 2003 (a); Meng et al., 2002). In rats a maximum of 0.45% of ingested EGCg is available in tissues and the blood (Lambert and Yang, 2003 (c)), while 77% of EGCg is excreted in bile and 2% in urine (Kohri, 2001).

Following absorption and distribution of green tea and its constituents, metabolism in the form of methylation, glucuronidation and sulfation in the small intestine, liver, kidney and other organs does occur (Meng et al., 2002). The small intestine is the major site for glucuronidation and methylation of catechins. A catechin can thus be glucuronidated in the small intestine and then methylated or sulfated in the liver and excreted through the bile (Donovan et al., 2001). Mice seem to be more similar to humans than rats to humans in the glucuronidation of EGCg and EGC (Lu et al., 2003), (Li et al., 2001). This point was further illustrated by the fact that the catalytic efficiency for (-)-EGCg-4’’-O-glucuronide formation was determined, and found to be: first mouse intestine, then mouse liver, human liver followed by rat liver and rat small intestine. (-)-EGCg-4’’-O-glucuronide is formed from EGCg by UDP-glucuronosyltransferase (Lu et al., 2003). COMT is found in the liver, kidney, and gastrointestinal tract, where it catalyses the transfer of methyl groups from S-adenosyl-methionine (SAM) to one of the hydroxyl groups of catechols in the presence of magnesium, and produces O-methylated catechol and S-adenosyl-L-homo-cysteine (SAH). EGCg is methylated by COMT to form 4’, 4’’-di-O-methyl-EGCg both in vitro and in vivo (Chen et al., 2005). Glucuronidation and sulfation are import phase II metabolic reactions in which xenobiotics are conjugated to make a more hydrophilic xenobiotic that can be excreted in the urine (Li et al., 2001).

Upon excretion a large part of EGCg is in the conjugated form in the urine, while in feces nearly all EGCg is in the unconjugated form (Lambert et al., 2003 (a)). Monoglucuronides and monosulfates are of the major conjugates that can be found in urine. Metabolites for green tea consumption in mouse and human urine have been identified. These include GC-O-G, EGC-O-S, Epicatechin-O-G, Epicatechin-O-S, O-m-EGC-O-G, O-m-EGC-O-S, M4-O-G, M4-O-S (Li et al., 2001), M4, M6 (Lee et al., 2002), 2'-cysteinyl EGCg and 2''-cysteinyl EGCg (Sang et al., 2005). Similarly EGCg consumption in mice yielded the following metabolites in urine: 4',4'' – DiMeEGCg, four monomethylated EGCg products and four dimethylated EGCg products (Meng et al., 2002).

Several methylated metabolites have been detected in feces, these include: 4''-O-methyl-EGCg, and 4',4''-di-O-methyl-EGCg (Lambert et al., 2003 (a)). Intestinal microflora may be responsible for the formation of ring-fission products. This step may cause potential re-absorption of the metabolites (Meng et al., 2002; Williamson et al., 2000).

The metabolic fate of tea catechins is not fully understood. To date this Figure is the best demonstration for the metabolism and bioavailability of tea and its constituents.

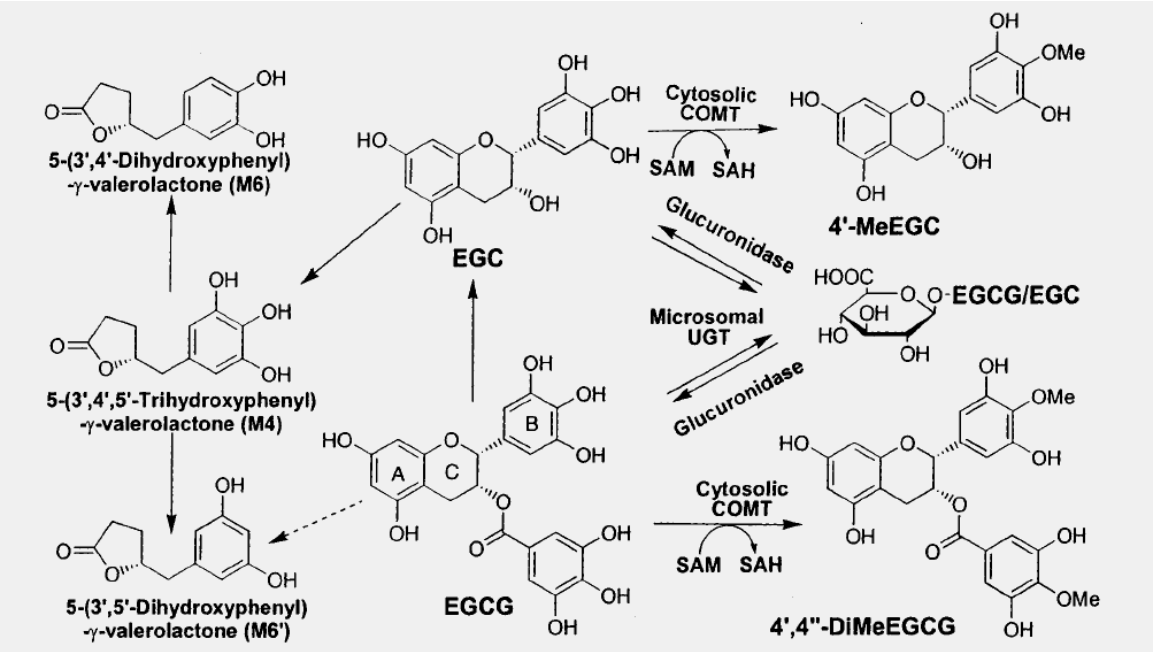


Figure 2.1: Proposed metabolic pathways of EGCg. *(Meng et al., 2002)* *SAM,S-adenosyl-L-methionine; SAH, S-adenosyl-L-homocysteine; UGT, UDP-glucuronosyltransferase. (See text for details)*

Obesity is an increasing problem of the developed world. The incidence of obesity is on the rise occurring in adults as well as children. Obesity itself is not dangerous but is unfortunately associated with increased risk of developing serious diseases such as hypertension, cardiovascular disease, non-insulin dependent diabetes mellitus, gout and osteoarthritis (Kovacs et al., 2004). Green tea and its constituents have been speculated to play a role in weight loss. Although the mechanism of action of weight loss still remains unknown, it has been reported by various groups that weight loss does occur with the consumption of tea or its constituents.

Reports from (Fiorini et al., 2005) demonstrated that mice consumed similar amounts of food and water or EGCg. The average liquid consumption of EGCg per day was 85 mg/kg/day/mouse. At the start of the study a significant decrease in the body weight of the animals treated with EGCg was detected when compared to control animals. Both oral and *i.p.* administration of EGCg achieved similar results. EGCg were able to reduce hepatic fat levels (Fiorini et al., 2005; Klaus et al., 2005). Similar results were found for C57BL/6 male mice treated with 2% green tea (Yang et al., 2003) and tea catechins (Tokimitsu 2004). The green tea and catechins significantly decreased body weight as well as body fat, while liver weight as well as relative liver weight showed an increase with green tea treatment. Green tea has similar effects on rats. When EGCg was administered *i.p.* to rats significant weight loss occurred. When the EGCg treatment was stopped, the rats regained the lost weight (Kao, 2000). Rats consuming black tea also had decreased body weight when compared to control animals. The relative liver weight of rats consuming black tea was lower when compared to the control (Sohn et al., 1994).

In contrast various groups demonstrated that green tea, black tea and EGCg had no influence on food consumption, body weight loss, weight maintenance, or relative liver weight in rats (Raederstorff et al., 2003; Marnewick et al., 2003) or humans (Kovacs et al., 2004). The exact mechanism of how tea is able to influence weight loss still needs to be identified.

The aim of this chapter is to provide experimental data on the mice that were treated with black tea *(Camellia sinensis)* over a six week period.

2.2 Materials and Method

2.2.1 Materials

All materials were of analytical grade and double distilled deionised water from Millipore Milli Q system, (Millipore Corporation, Bedford, MA., USA) was used in all experiments. Tris(hydroxymethyl) aminomethane (Tris) was obtained from Merck, (Darmstadt, Germany). Protease inhibitors (Aprotinin, Pepsatin A, Pefabloc©SC, Leupeptin), were obtained from Sigma Chemical Company (St Louis, MO, USA). Liptons Yellow Label tea was bought from a local Supermarket, and is widely available all over the world. Forty-eight six week old male C57BL/6 mice were obtained from National Health Laboratory Services (Johannesburg, South Africa).

2.2.2 Animals

The experimental procedures were approved by the Institutional Ethics Committee from the University of Pretoria. Forty eight male C57BL/6 mice were housed at the animal facilities of the Medical Research Council (MRC), Pretoria, South Africa.

2.2.3 Treatment of Animals

The animals were randomly divided into three groups containing sixteen animals per group. Eight animals were housed together. The day night cycles were regulated at 12 hours each and the room temperature was kept constant at 25 ºC and the relative humidity kept at 20%. The treatment duration was 6 weeks. The three different groups of animals were as follow: Group 1 (control) received only vehicle (sterilized deionised water), group 2 (low dose) received only 0.5% (w/v) tea, while group 3 (high dose) received only 2% (w/v) tea. The tea and water were freshly prepared every three days. The tea was prepared by boiling deionised water for 5 minutes to remove gasses. The appropriate amount of tea was added to the boiling deionised water and boiled for a further 5 minutes. The tea solution was cooled to room temperature and then filtered through sintered glass to remove any tea debris (Sohn, et al., 1994). All animals received a standard mouse chow (Epol, RSA), *ad libertum*.

2.2.4 Statistical Analysis of Data

Body and organ weight were analyzed with the Statistical Analysis Software (SAS) version 9.1 (SAS Institute, Cary, NC). The univariate procedure was used to obtain descriptive statistics of each group (mean and standard error of the mean) as well as to check for normality of the data and possible outliers. The TTest ($p < 0.05$) was used to determine significant differences between the various groups, and to compare the three groups to each other. A $p < 0.05$ was considered as statistically significant.

2.3 Results

The mice were weighed at the onset of the six weeks of treatment and weekly thereafter except week 5. The final weight measurement was taken on the day of sacrifice.

Tables 2.1 to 2.3 contain all the weight measurements for the first thirty one days of treatment.

Table 2.1: Weekly body weight (g) of the control group of mice drinking only sterilized deionised water.

Control	Week 0	Week 1	Week 2	Week 3	Week 4	Week 6
Ave (g)	23.0	24.4	25.3	26.0	26.4	27.4
N	16	16	16	16	16	8
SD	1.4	1.3	1.3	1.1	1.4	1.5
Range	1.9	1.7	1.6	1.3	2.0	2.3
p-value	0.0925	0.1617	0.7508	0.9887	0.5719	0.6452
%CV	6.03	5.34	5.02	4.40	5.31	5.55
SEM	0.35	0.33	0.32	0.29	0.35	0.54
Ave weight gain (g)	n/a	1.43	0.86	0.70	0.40	1.0

The data for the Student's t-test are for the control group compared to the low dose group.
The p-value given is calculated by the Student's t-test, assuming unequal variance. * Indicates values p < 0.05. % CV is the Coefficient of Variation and is given as percentage. SD is standard deviation. SEM is standard error of the mean.

Table 2.2: Weekly body weight (g) of the low dose group of mice drinking only 0.5 % (w/v) tea.

Low dose	Week 0	Week 1	Week 2	Week 3	Week 4	Week 6
Ave (g)	22.2	23.8	25.1	26.0	26.7	27.1
N	16	16	16	16	16	8
SD	1.4	1.3	1.4	1.3	1.3	0.6
Range	1.9	1.7	1.9	1.7	1.6	0.3
p-value	0.2936	0.0775	0.1450	0.0830	0.0387*	0.21
CV	6.15	5.51	5.47	5.07	4.71	2.11
SEM	0.34	0.33	0.34	0.33	0.31	0.20
Ave weight gain (g)	n/a	1.61	1.38	0.86	0.66	0.4

The data for the Student's t-test are for the low dose group compared to the high dose group.
The p-value given is calculated by the Student's t-test, assuming unequal variance. * Indicates values p < 0.05. % CV is the Coefficient of Variation and is given as percentage. SD is standard deviation. SEM is standard error of the mean.

Table 2.3: Weekly body weight (g) the high dose group of mice drinking only 2 % (w/v) tea.

High dose	Week 0	Week 1	Week 2	Week 3	Week 4	Week 6
Ave (g)	21.5	22.6	24.1	24.7	25.2	25.8
N	16	16	16	16	16	8
SD	1.9	2.1	2.3	2.4	2.3	2.7
Range	3.5	4.5	5.3	5.9	5.1	7.4
p-value	0.0180*	0.0076*	0.0907	0.0773	0.0970	0.1704
CV	8.68	9.41	9.51	9.80	8.93	10.57
SEM	0.47	0.53	0.57	0.61	0.56	0.96
Ave weight gain (g)	n/a	1.1	1.5	0.6	0.5	0.5

The data for the Student's t-test are for the control group compared to the high dose group.
The p-value given is calculated by the Student's t-test, assuming unequal variance. * Indicates values $p < 0.05$. % CV is the Coefficient of Variation and is given as percentage. SD is standard deviation. SEM is standard error of the mean.

From the above tables it can be seen that there were statistical significant differences ($p < 0.05$) between the high dose group and the control group for the first two measurements. The average weight for the first day of measurement for the control group is 23.0g while for the high dose group it is 21.5g. This significance is ignored because on day 1 the mice only started with their treatment, and there should not be any differences, thus the observed differences is merely coincidental and not due to the treatment. The average weight gain for the high dose group during the first week was 1.1g, compared to the control group and the low dose group that both gained 1.4g and 1.61g for the first week. A significant difference was detected between the low dose group and the high dose group during week 4. The average weight for the low dose group at week 4 is 26.7g, while for the high dose group this is 1.5g lower with the average weight being 25.2g. The control group at the same time had an average weight of 26.4g. The initial differences between the control and high dose group of 1.5 g remained throughout the duration of experimental period. Figure 2.2 below demonstrates the average weight gain for the full duration of treatment.

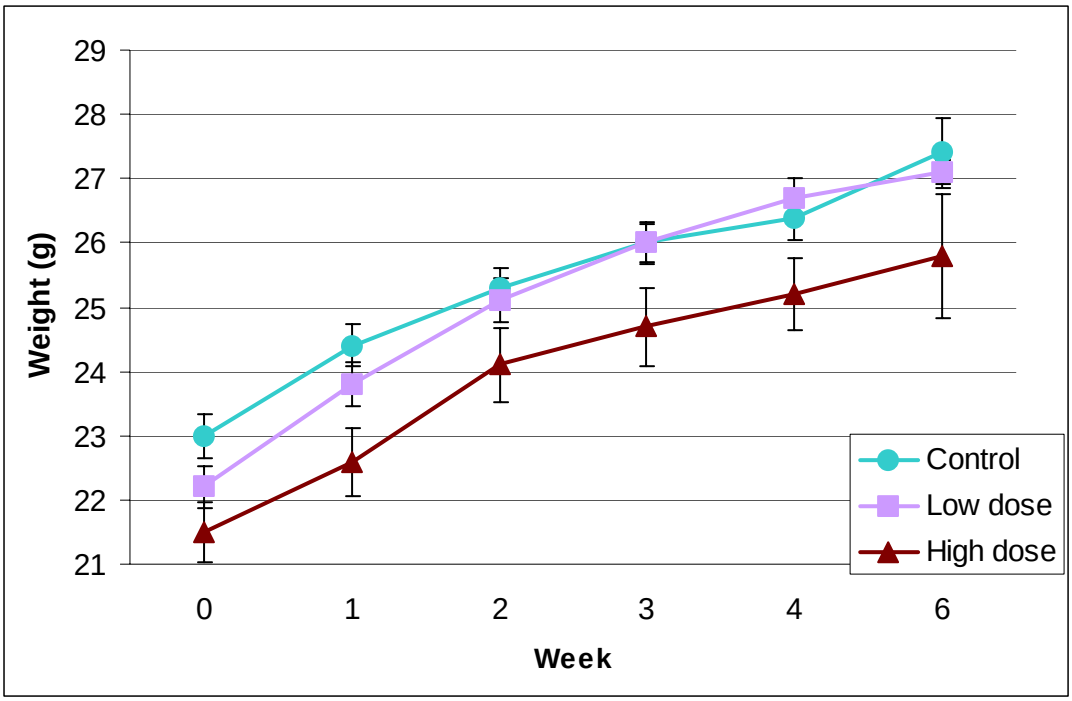


Figure 2.2: Average body weight increase for the C57BL/6 male mice during the six week period of treatment. Symbols represent the mean and error bars represent SEM. For full details see table 2.1 – 2.3.

The daily liquid consumption of the mice is shown in Figure 2.3 below. The amount of liquid consumed per day per 16 mice. The high dose group consumed only 3.3 ml of tea per day per mouse. This is in contrast with the low dose group that consumed 4.3 ml of tea per day per mouse. The control group consumed 3.9 ml of water per day. The liquid consumption between the various groups shows significant differences when compared to each other. It is interesting to note that the low dose group consumed more liquid than the control or high dose group. The average amount of tea ingested by the mice at the start of the study was 1.56 mg tea/mouse/day for the low dose group and 3.13 mg tea/mouse/day for the high dose group. This increased gradually to 2.97 mg tea/mouse/day for the low dose group and 4.38 mg tea/mouse/day for the high dose group at the end of the study. Thus even though the high dose group consumed less liquid than the low dose group the amount of tea ingested for the high dose group is more.

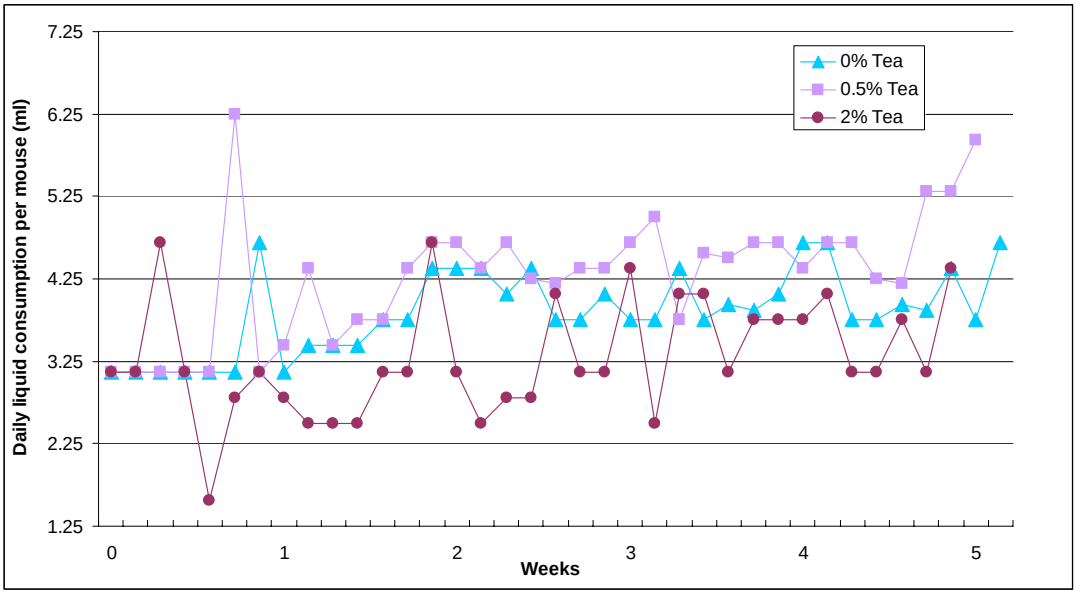


Figure 2.3: Daily liquid consumption of the C57BL/6 male mice over the six week period of the treatment. Average liquid consumption per day per mouse for control group 3.9 ml, low dose 4.3 ml, and high dose 3.3 ml. Control SD ± 7.956, low dose SD ± 12.415, high dose SD ± 11.268. The p-value given is calculated by the Student's t-test, assuming unequal variance. * Indicates values $p < 0.05$. Control vs low dose $p = 0.00592$*, control vs high dose $p = 0.000281$*, low vs high dose $p = 0.0000003$*. % CV is the Coefficient of Variation and is given as percentage. Control CV is 12.83%, low dose CV is 17.99% and high dose CV is 21.26%.

Previous results indicate the data captured while the mice were being treated. After six weeks of treatment the forty eight male C57BL/6 mice were sacrificed and the following results were obtained. The mice were weighed on the day of sacrifice before sample preparation started. The mice were cut open and perfused with saline solution. The target organs were harvested and weighed. After weighing, the organs were placed in 40mM Tris, pH 8.0 containing specific protease inhibitors. For the liver, small intestine, lung, kidney, pancreas, stomach, spleen, colon, esophagus and testis the following results were found regarding the organ weight.

Table 2.4: The organ weights as measured for the control group.

Control	Body Weight	Liver	Small intestine	Lung	Kidney	Testis	Pancreas	Stomach	Spleen	Colon
Average (g)	27.39	1.34	1.02	0.42	0.37	0.21	0.10	0.21	0.09	0.21
N	8	8	8	8	8	8	8	8	8	8
SD	1.521	0.207	0.16	0.054	0.045	0.017	0.021	0.04	0.03	0.03
Range	2.31	0.0429	0.025	0.0029	0.0021	0.0003	0.0004	0.001	0.001	0.001
p-value	0.6452	0.0321*	0.8118	0.1375	0.4694	0.1025	0.1605	1.0000	0.4793	0.1499
% CV	5.55	15.50	15.62	12.92	12.15	7.85	21.46	17.68	30.38	14.52
SEM	0.538	0.073	0.056	0.019	0.016	0.006	0.007	0.013	0.010	0.011

The data for the Student's t-test are for the control group compared to the low dose group. The p-value given is calculated by the Student's t-test, assuming unequal variance. * Indicates values $p < 0.05$. % CV is the Coefficient of Variation and is given as percentage. SD is standard deviation and SEM is standard error of the mean.

Table 2.5: The organ weights as measured for the low dose group.

Low dose	Body Weight	Liver	Small intestine	Lung	Kidney	Testis	Pancreas	Stomach	Spleen	Colon
Average (g)	27.12	1.58	1.00	0.38	0.39	0.23	0.11	0.21	0.08	0.24
N	8	8	8	8	8	8	8	8	8	8
SD	0.572	0.198	0.15	0.039	0.035	0.023	0.016	0.02	0.01	0.05
Range	0.327	0.0393	0.022	0.0016	0.0012	0.0005	0.0003	0.000	0.000	0.002
p-value	0.2108	0.0236*	0.1042	0.6183	0.6061	0.1804	0.0020*	0.7200	0.1164	0.9539
%CV	2.11	12.57	14.95	10.34	8.91	9.86	14.58	10.10	12.20	19.46
SEM	0.202	0.070	0.053	0.014	0.012	0.008	0.006	0.007	0.004	0.016

The data for the Student's t-test are for the low dose group compared to the high dose group. The p-value given is calculated by the Student's t-test, assuming unequal variance. * Indicates values $p < 0.05$. % CV is the Coefficient of Variation and is given as percentage. SD is standard deviation and SEM is standard error of the mean.

Table 2.6: The organ weights as measured for the high dose group.

High dose	Body Weight	Liver	Small intestine	Lung	Kidney	Testis	Pancreas	Stomach	Spleen	Colon
Average (g)	25.77	1.28	1.12	0.37	0.38	0.21	0.08	0.20	0.09	0.24
N	8	8	8	8	8	8	8	8	8	8
SD	2.72	0.27	0.11	0.03	0.04	0.04	0.02	0.02	0.01	0.04
Range	7.42	0.0718	0.012	0.0008	0.0017	0.0019	0.0004	0.000	0.000	0.001
p-value	0.1704	0.6175	0.1846	0.0562	0.8202	0.6613	0.0649	0.8032	0.8193	0.0925
CV	10.57	21.02	9.89	7.56	10.85	21.34	25.21	10.09	14.86	16.06
SEM	0.963	0.095	0.039	0.010	0.014	0.015	0.007	0.007	0.005	0.014

The data for the Student's t-test are for the control group compared to the high dose group. The p-value given is calculated by the Student's t-test, assuming unequal variance. * Indicates values $p < 0.05$. % CV is the Coefficient of Variation and is given as percentage. SD is standard deviation and SEM is standard error of the mean.

The above data can be demonstrated by the following graph.

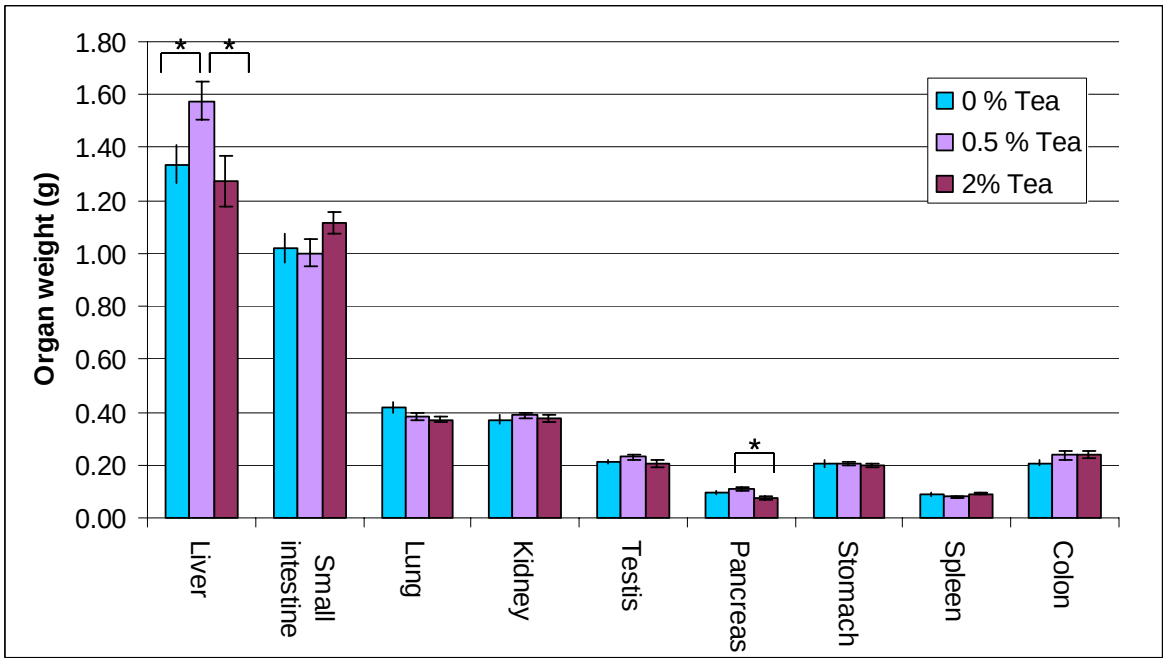


Figure 2.4: The organ weights measured for the various organs.

* Indicates values $p < 0.05$. Error bars represented by standard error of the mean.

Significant differences were found for the liver and pancreas. The liver of the low dose group had an increased weight of 1.58 g compared to the control which was measured as 1.34g and the high dose group that were measured as 1.28g. The pancreas of the high dose group (0.08g) was significantly less than that of the low dose group (0.11 g). The control group (0.10 g) was very similar to the low dose group. To eliminate possible errors that may occur due to body weight and size of the different mice all the organs were expressed as a percentage of the body weight. This gives a more accurate reflection of organ weight changes. The results are given in tables 2.7 – 2.9 below.

Table 2.7: The organ weights expressed as a percentage of the total body weight for the control group.

Control	Liver	Small intestine	Lung	Kidney	Testis	Pancreas	Stomach	Spleen	Colon
Average (%)	4.87	3.72	1.53	1.36	0.78	0.35	0.75	0.32	0.76
N	8	8	8	8	8	8	8	8	8
SD	0.60	0.53	0.15	0.12	0.06	0.07	0.15	0.10	0.12
Range	0.35	0.28	0.02	0.01	0.00	0.00	0.02	0.01	0.01
p-value	0.01*	0.93	0.10	0.25	0.07	0.11	0.94	0.53	0.14
CV	12.23	14.35	10.11	8.88	7.74	19.97	19.83	29.94	15.60
SEM	0.21	0.19	0.05	0.04	0.02	0.02	0.05	0.03	0.04

The data for the Student's t-test are for the control group compared to the low dose group. The p-value given is calculated by the Student's t-test, assuming unequal variance. * Indicates values $p < 0.05$. % CV is the Coefficient of Variation and is given as percentage. SD is standard deviation and SEM is standard error of the mean.

Table 2.8: The organ weights expressed as a percentage of the total body weight of the low dose group.

Low dose	Liver	Small intestine	Lung	Kidney	Testis	Pancreas	Stomach	Spleen	Colon
Average (%)	5.82	3.70	1.40	1.43	0.85	0.41	0.76	0.30	0.88
N	8	8	8	8	8	8	8	8	8
SD	0.72	0.57	0.12	0.11	0.08	0.05	0.08	0.04	0.17
Range	0.52	0.33	0.01	0.01	0.01	0.00	0.01	0.00	0.03
p-value	0.05*	0.02*	0.48	0.51	0.37	0.00*	0.50	0.08	0.51
CV	12.37	15.52	8.70	7.97	9.88	13.23	10.54	13.20	19.19
SEM	0.25	0.20	0.04	0.04	0.03	0.02	0.03	0.01	0.06

The data for the Student's t-test are for the low dose group compared to the high dose group. The p-value given is calculated by the Student's t-test, assuming unequal variance. * Indicates values $p < 0.05$. % CV is the Coefficient of Variation and is given as percentage. SD is standard deviation and SEM is standard error of the mean.

Table 2.9: The organ weights expressed as a percentage of the total body weight of the high dose group.

High dose	Liver	Small intestine	Lung	Kidney	Testis	Pancreas	Stomach	Spleen	Colon
Average (%)	4.93	4.35	1.46	1.47	0.79	0.29	0.79	0.36	0.93
N	8	8	8	8	8	8	8	8	8
SD	0.90	0.36	0.16	0.12	0.14	0.06	0.09	0.08	0.17
Range	0.81	0.13	0.03	0.01	0.02	0.00	0.01	0.01	0.03
p-value	0.87	0.02*	0.39	0.09	0.74	0.10	0.60	0.44	0.03*
CV	18.27	8.27	11.19	8.06	17.28	20.02	11.33	21.12	17.91
SEM	0.32	0.13	0.06	0.04	0.05	0.02	0.03	0.03	0.06

The data for the Student's t-test are for the control group compared to the high dose group. The p-value given is calculated by the Student's t-test, assuming unequal variance. * Indicates values $p < 0.05$. % CV is the Coefficient of Variation and is given as percentage. SD is standard deviation and SEM is standard error of the mean.

The above data can be seen in the Figure below.

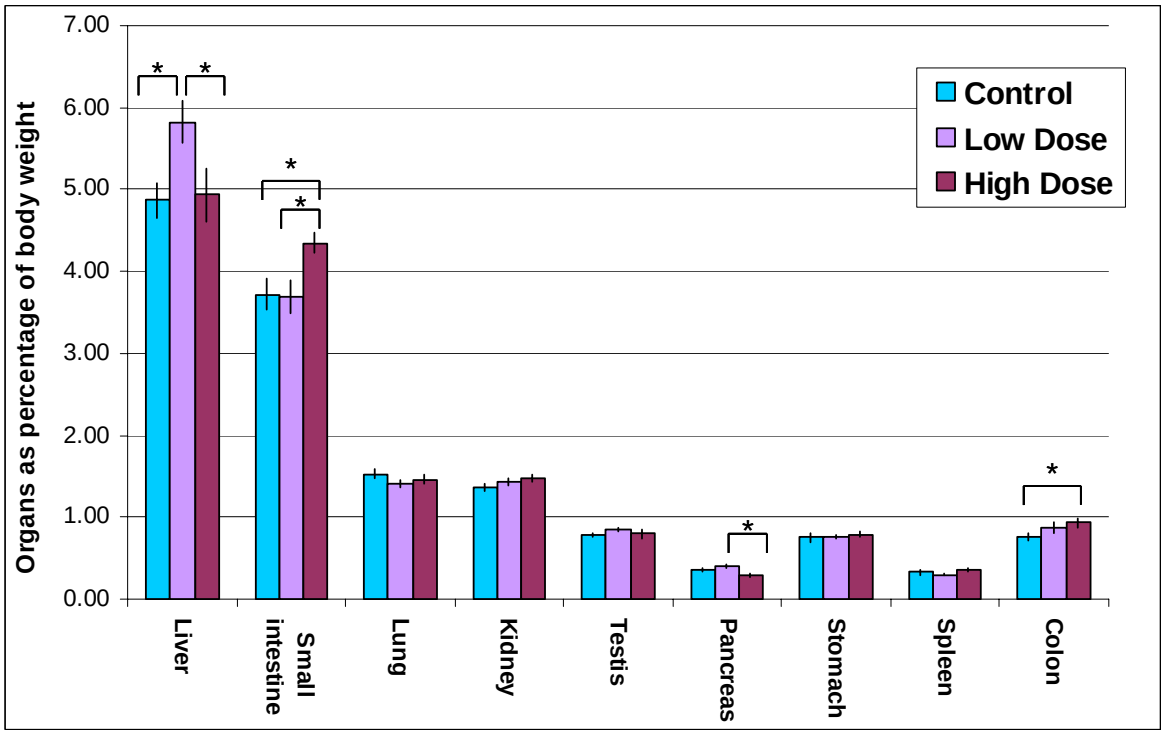


Figure 2.5: The organ weights expressed as a percentage of the body weight for the various groups. Height of the histogram represents the mean. * Indicates values $p < 0.05$. Error bars represented SEM.

When the various organs were expressed as a percentage of the body weight more significant differences did arise. These differences can be expected to be more accurate than only differences based on weight measurements. For the liver it was seen that the low dose group (5.82%) is significant compared to the high dose (4.93%) and control group (4.87%). Significant differences were also found for the small intestine, colon and pancreas when they were expressed as a percentage of the body weight. The pancreas of the low dose group (0.41%) was larger than that of the control (0.35%) and the high dose (0.29%) groups. The colon of the high dose group (0.93%) was heavier than that of the low dose (0.88%) and controls (0.76%) groups. Similarly the small intestines of the high dose group (4.35%) were heavier than that of the low dose (3.7%) and control (3.72%) groups. The small intestine and colon were washed and cleaned with 0.9% saline before weight measurements were taken. Thus these results are only based on the small intestine and colon and not the contents thereof.

2.4 Discussion

Forty eight, six week old male C57BL/6 mice were used in the experimental procedure over the six week period of treatment. The most important reason for using mice rather than rats for the experiments was the fact that various groups have reported that the metabolism and bioavailability in mice is similar to that of humans especially during the metabolism of tea (Li et al., 2001; Lambert et al., 2003 (a); Chen et al., 1997). Three groups were chosen to determine the effect of various tea concentrations. A normal tea bag contains about 2 g of dry tea leaves. Thus a normal cup of 200 ml consumed by humans would be a 1 % (w/v) tea solution. This falls into the range of tea we have chosen for our experiments. We chose 0.5% and 2% (w/v) tea as this is half and double the normal concentration drunk by humans. These doses of tea are normally used in the literature for short-term effects. The daily tea intake per kilogram of mouse body weight is five to twenty times higher than human consumption on a kilogram body weight basis. But humans take tea over many years rather then the short six weeks period. It is interesting to note that even at these twenty times higher doses, no toxicities are seen over the six week period.

Liquid consumption was measured daily for each group of mice. The high dose group consumed less liquid than the control group. This could be because the tea might have a bitter taste for the mice. This did not influence the amount of tea ingested per mouse. The high dose group started with 3.13 mg tea/mouse/day (125.2 mg/kg/day) and ended at 4.38 mg tea/mouse/day (175.2 mg/kg/day). This was higher than that of the low dose group even though the low dose group consumed more liquid. The low dose group started with 1.56 mg tea/mouse/day (62 mg/kg/day) and ended at 2.97 mg tea/mouse/day (118.8 mg/kg/day). This could explain some of the differences that were seen in body weight and organ weight.

Weight gain was measured on a weekly basis for all the groups. The average weight during the first week of treatment seemed to differ significantly between the high dose group and the control group. The high dose group as well as the low dose group weighed less than the control. The significance between the three groups during the first week was ignored because all the mice should have weighed more or less the same. Thus this difference was considered merely coincidental. Both tea groups gained less weight than the control but

this was not significantly different. The control group gained 1.01 g during the last two weeks before sacrifice. On the other hand the low dose group gained only 0.41 g and the high dose group gained 0.50 g during the same time. In total the high dose gained only 4.27g over the six week period compared to the 4.39g gained by the control group. Although from these results it would be easy to conclude that tea does have an effect on weight gain and obesity, it is not that clear cut. These results are not significant and thus agree with similar results that concluded that tea has no influence on body weight or organ weight (Raederstorff et al., 2003; Marnewick et al., 2003). On the other hand it does seem that tea concentration may to play a very important role, not only in weight gain but also in organ weight.

The liver of the low dose (1.58 g) group was significantly larger than that of both the control (1.34 g) and high dose (1.28 g) group. The same was seen when the liver was expressed as a percentage of the body weight. This was done to remove body weight errors. The low dose (5.82 %) liver was extremely larger than the other two groups (C: 4.87 % and H: 4.93 %). Similar results were seen by (Yang et al., 2003 and Raederstorff et al., 2003). They found that the liver weight of rats increased with 0.5% EGCg, and decreased again with 1% EGCg. This increase in liver weight is not believed to be due to a toxic effect of the tea as the mice only ingested 2.97mg tea/mouse/day for the low dose, which is much lower than the 4.38 mg tea/mouse/day of the high dose group. Probably the increase in liver weight could be due to enhanced enzymatic activity within the liver and expansion of the hepatocytes. It could be that the dose is optimal for the mice and that this dose gives the greatest response to tea, hence when the dose start to exceed 0.5% (w/v) tea the optimal concentration is lost. On the other hand the low liver weight of the high dose group could be due to the anti-obesity effect of tea. It could be possible that the hepatic fat levels were reduced in the liver of the high dose group, as previously reported by (Fiorini et al., 2005). Unfortunately this is all speculation for our data, as we did not test for hepatic fat or enzyme assays.

Similar trends were seen for the pancreas of the high dose (0.08g) group that weighed significantly less than that of the control (0.10 g) and low dose (0.11 g) groups. This was elevated even further when expressed as a percentage of the body weight. The pancreas of the low dose (0.41 %) group was significantly larger than that of the control (0.35 %), while the high dose (0.29 %) group weighed less. The pancreas functions to control

glucose levels by secreting insulin to lower glucose levels and secreting glucagon to increase glucose levels. Thus it may seem that tea may play a role in glucose regulation and may therefore play a role in diabetes. It has been reported by (Vinson and Zhang, 2005 and Tsuneki et al., 2004) that tea has an effect on diabetes mellitus. It was also reported by (Suganuma et al., 1998) that the pancreas absorb EGCg and therefore the concentration effect seems to be very important. In another study on diabetes it was reported by (Fajas et al., 2004) that E2F1 has a role in diabetes and pancreatic function. E2F1 is critical in the cell cycle and regulates pancreas cell proliferation and therefore also regulates insulin secretion and production. Reduction in the pancreatic size may be due to a reduction in islet numbers within the pancreas, but this may also bring about a decrease in insulin secretion. EGCg is able to decrease total pRb protein levels in a dose and time dependent manner as well as increase the number of hypophosphorylated Rb (Ahmad et al., 2002). Retinoblastoma protein (Rb) has a central role in the cell cycle. CyclinD forms a complex with cdk4 and cdk6, this then triggers phosphorylation of retinoblastoma protein (Rb) in mid- to late G1 phase. In its hypophosphorylated state, Rb prevents G1 exit by binding to the transcription factor E2F, thereby inhibiting its activity. When Rb gets phosphorylated to pRb by the cyclinD-cdk4/6 complex, Rb release E2F, thus enabling E2F to activate the genes that are required for S phase entry. Thus it may be possible that the high dose group were able to activate the cell cycle by allowing Rb to bind to E2F1 within the pancreas hence causing transcriptional arrest. This may be a reason why the pancreas of the high dose group was so small. For the low dose group it may be possible that the tea concentration is not high enough to affect the Rb protein phosphorylation and therefore do not have an effect on cellular proliferation within the pancreas.

The opposite effect was seen in the small intestine and the colon. In both organs the high dose seemed to increase the weight of the small intestine and the colon. The small intestine of the high dose was measured as (4.35 %), that was significantly larger than both the low dose (3.70%) and the control (3.72%) of the body weight. The high dose group (0.93%) of the colon was significantly higher than the control (0.76%) and the low dose (0.88%) of the body weight. This is a strange finding, especially since the liver and pancreas of the high dose group tended to be smaller than the other groups. Both the small intestine and the colon play a central role in metabolism. The small intestine is the major route of absorption for compounds in the body. It has a good blood supply and very large surface area due to extensive folding and the presence of villi. Thus the small intestine is well

adapted for absorption. It was reported by Suganuma et al., (1998) and Chen et al., (1997) that the small intestine absorbed the most EGCg and tea catechins. Thus it may be possible that the small intestine is able to absorb most of the tea and its components. The small intestine is the major site for glucuronidation and methylation of catechins and it was determined that most catechins found in the small intestine immediately after ingestion of tea were unconjugated. Unfortunately the precise mechanism of catechin absorption in the small intestine is still unknown.

The colon and the kidney were the only two organs that showed a positive dose dependent response in their weights relative to total body weight. The liver showed a hyperbolic dose dependent response in its relative weight. The other organs showed varying dose dependant responses.

No significant effect was found for the body weight. This implies that under this short term trial, tea has no toxic effect on the mice. It was also determined that the same dose of tea has various dose dependent responses on the various organs. These data suggest that a greater range of doses should be tested to determine what effect tea drinking has on organ weights.

CHAPTER 3

Proteomic characterization of modulated proteins from the liver and small intestine

3.1 Introduction

"Two D, or not two D: that is the question:
Whether 'tis nobler in the mind to suffer
The streaks and blobs of intractable proteins
Or to take chips against a sea of genes
And by comparing, find them that
hold the bitter taste of disease and death."

A very noble and lyrical description of proteomics and two dimensional gel electrophoresis in particular by Fey and Larsen, (2001). Two dimensional gel electrophoresis was first introduced in the mid '70's by (O'Farrell, 1975). At first the technique has remained more or less the same and was more a form of art and luck than a technique assessable to all. With time and with the introduction of IPG strips in 1988 by (Gorg et al., 1988), 2DE has evolved and has become a classical technique of choice. 2DE has several properties that have made it difficult to replace for proteome analysis. Unfortunately as with any new and interesting technology the expectations far exceed the reality (Harry et al., 2000), and this is also the case for 2DE, that is far from perfect. Currently there are no other techniques that can deliver the same separation resolution, accuracy, and detailed and rich information on a particular protein including its quantity, post translational modifications, pI, MW, and solubility (Lopez, 2000). Even though there is at present no substitute in sight for 2DE, there are a number of areas that leave room for improvement for example sample preparation and detection. New detergents with better solubilizing power have been developed to improve solubilization of proteins. Urea and thiourea are nonionic chaotropes (a chemical agent that denatures proteins) used for protein solubilization. CHAPS is a zwitterionic detergent that improves solubilization of the proteins and helps to keep the proteins soluble during IEF. Other solubilization agents have recently been developed by

various companies to try and optimize solubilization, these include: TBP, CHAPSO, SB 3-10, and ASB-14. Detection and quantification is also a major problem. Every protein exhibits different staining behavior due to structural and compositional properties (Harry et al., 2000). Coomassie blue is the stain of choice for abundant proteins as it is only able to detect 40ng, but has the advantage of ease of use and being MS compatible. Silver staining is a very sensitive detection method that can detect spots of up to 1ng. The silver binds to the amino acid side chains, usually the sulfhydral and carboxyl groups of the proteins. This may cause protein modifications and different proteins stain with varying success. In general silver staining uses glutaraldehyde as a fixative, which makes it a non-MS compatible detection method. Various new MS compatible silver stains and silver stain kits have been produced during the last few years. The first MS compatible silver stain was developed by (Shevchenko et al., 1996). This method did not use gluteraldehyde, but did suffer from reduced sensitivity. This method was later replaced by (Yan et al., 2000) that developed a silver stain without the need for gluteraldehyde and only one formaldehyde step during development of the gels. Another MS compatible silver stain method is by the removal of the silver ions and washing of the gel bands (Gharahdaghi et al., 1999). The gel is destained prior to enzymatic digestion with potassium ferricyanide and sodium thiosulfate to remove all the silver ions that may interfere with MS. This method has been reported to increase the detection of peptides, improved resolution, increased sensitivity and better identification. Unfortunately silver staining still remains labor intensive, has a limited dynamic range, and has poor reproducibility. Sypro ruby is a fluorescent ruthenium-based stain that binds non-covalently to proteins in gels. It is able to detect proteins at low nanogram (1 ng) levels and is MS compatible. DIGE was developed to overcome gel variability. By using DIGE dyes the number of gels is reduced as up to three samples can be run on one single gel, and this also eliminates experimental and gel to gel variation. DIGE dyes have more accurate detection of proteins as an internal standard can be used and are also able to be used for the quantification of data. Advantages of this stain are that it has good sensitivity, is photo stable, spectrally distinct, and has an increased dynamic range. Unfortunately the three DIGE dyes are extremely expensive and specialized scanners and software are needed for the detection and quantification of the DIGE dyes. Radioisotopes and ICAT are also some of the detection methods used for 2DE and mass spectrometry (Hunter et al., 2002).

Disadvantages associated with the use of 2DE include: the fact that it is a very time consuming technique, it has a limited dynamic range, it does not work well with hydrophobic proteins and is not really quantitative. The cell has an enormous dynamic range of about $1\text{-}10^6$, which is out of range for 2DE that has a maximum dynamic range of 10^4 (Rabilloud, 2002). Proteins less than 8kDa and larger than 200kDa are usually not seen on 2DE (Harry et al., 2000). Membrane proteins are not easily detected because they have low abundance, usually have a pI that is very alkaline and does not fall in the range of normal IPG strips and they are poorly water soluble (Santoni et al., 2000). At low loads high abundance proteins can be easily detected, but low abundance proteins are not detected. Therefore it can be said that proteins not represented in 2DE gels is usually low abundance proteins, hydrophobic, basic and very small or very large proteins. Despite these disadvantages 2DE still remains the method of choice for displaying proteins as a front end of a proteomics project because it can be used to visualize a very large number of proteins simultaneously and can be used in different display formats (Hunter et al., 2002). 2DE will remain as the "workhorse" of proteomics for a long time as it is able to provide valuable insight into the presence or absence of proteins and post translational modifications of proteins within the cell with high separation power and bags full of information on a specific protein spot. As a matter of fact, "2D or not 2D, that is not the question. The real question is rather when and why to go for 2DE gels or avoid them" (Rabilloud, 2002).

Proteomics is the global analysis of gene expression and complex mixtures of proteins for the main purpose of qualitative, quantitative as well as functional analysis of all proteins within a specified sample. This is usually done by using a combination of techniques to resolve, identify, quantify, and characterize. Proteomics was first coined in 1995, with the big "omics" boom. In short proteomics was baptized as a technique for large scale characterization of an entire protein complement of a cell line, tissue or organism. The main goal of proteomics is to obtain a more global picture and integrated view of biology by studying all the proteins of a cell rather than each of the proteins individually (Graves and Haystead, 2002). Proteomics therefore aims to understand function, interactions, modifications, localization and regulation of all proteins expressed by the cell. The analysis of proteins is an important issue because it is mainly proteins that perform most of the reactions that are necessary for the maintenance of the cell and living organisms.

A major issue with establishing proteome characterization is defining the proteome in question. The genome is static, while the proteome is dynamic. A single genome can give rise to an infinite number of qualitative and quantitative different proteomes, depending on variables such as the stage of the cell cycle, growth, nutrient condition, temperature, stress response, pathological conditions, strain differences and many more (Hunter et al., 2002). It can therefore be said that proteomic techniques such as 2DE provide only a "snapshot" of the proteome at a specific time and condition.

Even though two dimensional gel electrophoresis is usually assumed to be the main proteomics mainstay, this is not the case. Various types of proteomics exists each with its own function. Functional proteomics is a broad term that can be used for many proteomic applications. It is the global characterization of the functional features of proteins that are important to better understand events that constitute metabolism, replication and stress response by the cell. Characterization of these proteins can provide important information about protein signaling, disease mechanisms and protein drug interactions. Qualitative proteomics is mainly comparative 2DE, which is used to look for the presence or absence of spots. Protein expression proteomics is a quantitative study of protein expression between samples that differ by some variable. Protein expression is compared between samples and used to identify novel proteins. Structural proteomics is used to map the structure of protein complexes, identify all proteins and determine their location and characterize all the protein-protein interactions. The information gathered by structural proteomics then helps to piece together the overall architecture of cells and explain how the expression of certain proteins gives a cell its unique characteristics. Bottom-up or shotgun proteomics is mainly MS based. It is when all the proteins in a sample are digested before separation. The peptides are then separated by chromatography and analyzed by tandem MS. The advantage is that the peptide analysis is very sensitive, and thus more proteins can be found. It is also quantitative with the use of ICAT, but gives little information on the structure and what the intact protein looks like (Baxevanis and Ouellette, 2005). In top-down proteomics the analysis begins with MS measurements of the intact protein, before any fragmentation or digestion. After measuring the intact mass, the protein is fragmented. This technique is more challenging because it is not as easy as for peptides. The data analysis is also difficult (Baxevanis and Ouellette, 2005).

For drug discovery various types of proteomics also exits. These include: Chemical proteomics parallels classical functional proteomics, except that a tagged small molecule is used to isolate a single protein from an entire proteome. Reverse proteomics use the transcriptome of the organism as the starting point for the proteomic study and not the protein extract. The yeast two hybrid system is the most popular system of reverse proteomics. It involves the reconstruction of a transcription factor, which is then used to determine protein–protein interactions. The advantage of reverse proteomics over classical proteomics is that it does not require protein manipulation as everything is performed at the gene level. The disadvantage is that the protein-protein interactions must occur within the nucleus (Piggot, 2004). Reverse chemical proteomics also uses the transcriptome of the phenotype of interest as the starting point. Currently there is only one type of reverse chemical proteomics and that is display cloning. Display cloning involves the cloning of the transcriptome of interest into a bacteriophage vector (phage display system). The phage then displays the protein on its own surface. A tagged small molecule can then be used to isolate phages displaying the proteins (Piggot, 2004).

Another proteomic technique includes chromatographic procedures. Chromatographic techniques separate proteins on their physiochemical properties such as size, charge and hydrophobicity (Hunter et al., 2002). A popular technique that has emerged is the use of LC/LC/MS/MS (MudPIT) – multi-dimensional-protein identification technique (chromatography). MudPIT has the advantages that it is not time consuming, it can run fully automatically, can use two dimensions of chromatographic separation that increase the number of peptides that can be identified from complex mixtures, it has a wide dynamic range and does not have solubility problems. A disadvantage associated with MudPIT is mainly the post – experimental data processing that can be difficult and laborious (Hunter et al., 2002).

Protein micro-arrays are mainly used to determine protein-protein interactions (Sauer et al., 2005). Functional protein micro-arrays use purified proteins that can separately be spotted on the surface of a glass slide and then analyzed for activity. This approach has huge potential for rapid high through-put analysis of proteomes and other large collections of proteins. The proteins spotted on the slide are usually not denatured, which is in contrast with 2DE. Analytical protein micro-arrays have the potential to monitor protein expression on a proteome wide scale and in medical diagnostics. Ligands such as antibodies and

antigens are usually used to determine the interactions (Phizicky et al., 2003). The major difference between proteomic chip technologies and 2DE can be summarized in the following table.

Table 3.1: Differences between 2DE and proteomic chip technologies (Lopez, 2000).

Proteomics chip technologies	**2DE and MS**
Best with defined ligands	No prior knowledge of sample needed.
Not quantitative	Quantitative
High throughput, general screening	Analytical
Target detected indirectly	Targets detected directly
No specific information about the protein target	Specific info such as pI, MW, quantity, amino acid sequence can be obtain in combination with MS.

Overall proteomics aims to elucidate the function of every protein within a cell, and to know how this function changes in different environmental states, cellular locations, interactions, and modification states. Proteomics will thus provide the blueprint for understanding the proteomes of all organisms in future (Phizicky et al., 2003).

3.2 Materials and Method

3.2.1 Materials

All materials were of electrophoresis or analytical grade and double distilled deionised water (Millipore system Q, Milipore, USA) was used in all experiments. Acetic acid, ammonium persulphate, ethanol, and methanol were obtained from SAARChem, (Muldersdrift, South Africa). 'Electran' acrylamide, glycerol, glycine, N,N,N',N'-tetramethyl-ethylenediamine (TEMED), N,N' – methylenebisacrylamide (Bis-acrylamide), sodium thiosulphate were obtained from BDH Laboratories, (Poole, England). Silver nitrate, sodium acetate, sodium carbonate, sodium chloride (NaCl), sodium dodecyl sulphate (SDS), Tris(hydroxymethyl) aminomethane (Tris) were obtained from Merck, (Darmstadt, Germany).

Agarose, β-mercapto-ethanol, bovine serum albumin (BSA), ethylene diamine tetra acetic acid (EDTA), gluteraldehyde, were obtained from Sigma Chemical Company (St Louis, MO, USA). Iodoacetamide (IAA), and tris(hydroxymethyl) aminomethane (tricine), were obtained from Fluka. CHAPS, and 1,4 dithiothreitol (DTT) were obtained from Roche Diagnostics GmbH (Mannheim, Germany). Quick Start™ Bradford Protein dye, Coomassie Brilliant Blue R-250 (electrophoresis purity grade) were obtained form Bio-Rad, (Hercules, United States of America). Microtitre plates were obtained from Bibby Sterilin, (UK). Immobiline Drystrip gels (IPG strips), 13 cm, pH 3-10 NL, Immobiline Drystrip gels, 7cm, pH 3-10 NL, IPG buffer pH 3-10 NL, low molecular weight marker proteins, PlusOne thiourea, and PlusOne urea, were purchased from Amersham Biosciences, (Uppsala, Sweden).

3.2.2 Sample Preparation from Animal Organs and Protein Extraction

The mice were killed by cervical dislocation, as this would not interfere with any protein regulation. The thoracic cavity was cut open and the left and right femoral arteries were severed. Ice cold 0.9% saline solution was used to perfuse the mice by injecting a needle (21 gauge, 25 ml syringe) into the right chamber of the heart, and pushing the saline solution slowly through the mouse. This was done to remove excess blood from the organs. The various organs were then removed, weighed and placed into ice cold 10 ml (40 mM Tris-HCl, pH 8.0), solution containing a mixture of protease inhibitors Aprotinin, Pepsatin

A, Pefabloc©SC, Leupeptin. The organs and buffer were homogenized using a Potter-Elvejhem homogeniser. The samples were centrifuged at 100 g for 15 minutes using a BHG Hermle Z320 centrifuge to remove major cell debris. Samples were sonicated at 30% duty cycle, setting 3, for 30 seconds using a Branson Cell Disruptor B-30 Sonifier with a microtip. This was followed by centrifugation at 9000g for 15 minutes to obtain the S9 fraction. The S9 fraction is the fraction containing most cytochrome P450 enzymes (Maron and Ames, 1983). The samples were divided into 1.0 ml aliquots in cryotubes and snap frozen using liquid nitrogen. All aliquots of samples were stored at -80°C. During the whole procedure of sample preparation all the samples were kept on ice.

3.2.3 Protein quantification

Total protein of the organs was determined with the method of Bradford, (1976), using the Quick Start™ Bradford dye (Bio-Rad, Hercules, USA). BSA was used as standard reference protein. Organ sample protein was serially diluted. One hundred and fifty microlitre of the standard protein (2.5 μg/ml to 25 μg/ml) and sample were pipetted into a microtitre plate well and 150 μl of the Quick Start™ Bradford dye was added. The microtitre plate was shaken for 10 minutes before reading the absorbance at 595 nm with a Multiskan Ascent spectrophotometer (Thermo Labsysytems). Determinations were performed in duplicate. A standard curve was drawn and this was used to determine the total protein concentration of the organ samples and the amount of protein to be used in the experiments to follow.

3.2.4 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Organ proteins were analyzed using a Laemmli SDS-PAGE system (Laemmli, 1970). A 12.5% T, 2.6% C separating gel (0.375M Tris-HCl, 0.1% SDS, pH 8.8), and 4% T, 2.6% C stacking gel (0.125M Tris-HCl, 0.1% SDS, pH 6.8) were prepared from 30.8% T, 2.6%C acrylamide (30% 'Electran' acrylamide, 0.8% N',N'methylene bisacrylamide) stock solutions. These solutions were degassed for 15 minutes by using a vacuum. The acrylamide was polymerized by addition of 0.05% ammonium persulphate and 0.033% TEMED. Electrophoresis was carried out by using a SDS electrophoresis running buffer (0.25M Tris-HCl, pH 8.3, 0.1% SDS, 192mM Glycine).

Three samples from each group of sixteen animals were chosen at random. Each sample was prepared individually as follows. Sample protein was diluted 1:4 in reducing buffer (0.06M Tris-HCl, 2% (w/v) SDS, 0.1% (v/v) glycerol, 0.05% (v/v) β - mercaptoethanol and 0.025% (v/v) bromophenol blue, pH 6.8), and boiled at 94 °C for 5 minutes. Protein determination was performed on each sample according to the Bradford, (1976) method. The soluble organ proteins from each animal were loaded separately into wells on the gel. The same amount of total protein was loaded in each well of the gel (25 μg of protein for 10 x 8 cm x 1 mm spacer Biometra gels and 50 μg for 16 x 18 cm x 1 mm spacer, Hoefer SE 600 gels). Low molecular weight markers were used in the mass determination (Amersham Biosciences, Uppsala, Sweden) and loaded into one of the wells of the gel. Electrophoresis was carried out with buffer (0.25M Tris-HCl, pH 8.3, 0.1% SDS (w/v), 192mM glycine) using a Biometra electrophoresis system (Biometra, GmbH) with an initial voltage of 30 V until the front reaches the separating gel, then a voltage of 100 V until the bromophenol blue front reaches the bottom of the gel. For each mouse a separate gel were done. Thus each gel is an independent experiment.

3.2.5 Tricine- Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (Tricine SDS-PAGE)

Organ proteins were also analyzed using a tricine SDS-PAGE system (Schagger H. and von Jagow G., 1987) that is suitable for resolution of proteins in the range of 1-100 kDa.
A 16% T, 3% C separating gel (1M Tris-HCl, 0.1% (w/v) SDS, pH 8.45), 10% T, 3% C spacer gel (1M Tris-HCl, 0.1% (w/v) SDS, pH 8.45) and a 4% T, 3% C stacking gel (0.75M Tris-HCl, 0.075% (w/v) SDS, pH 8.45) were prepared from 49.5% T, 3% C acrylamide (48% acrylamide, 1.5% N',N'methylene bisacrylamide) and gel buffer (3M Tris-HCl, 0.3% (w/v) SDS, pH 8.45) stock solutions. These solutions were degassed for 15 minutes by using a vacuum and polymerized by addition of 0.05 % (v/v) ammonium persulphate solution and 0.033% (v/v) TEMED.

Three samples from each group of sixteen animals were chosen at random. Each sample was prepared individually as follows. The sample proteins were diluted 1:4 in reducing buffer (0.06M Tris-HCl, 2% (w/v) SDS, 0.1% (v/v) glycerol, 0.05% (v/v) β-mercaptoethanol and 0.025% (v/v) bromophenol blue, pH 6.8), and boiled at 94 °C for 4

minutes. Protein determination was performed on each sample according to the Bradford, (1976) method. The soluble organ proteins from each animal were loaded separately into wells on the gel. The same amount of protein was loaded for each sample (25 μg of protein for 10 cm x 8 cm x 1 mm spacer gels, BioMetra). Peptide mass markers were used in the mass determination. Peptide mass markers are fragments from a cyanogen bromide digestion of myoglobin to give a ladder of masses as indicated in the results. Electrophoresis was carried out with anodal buffer (0.2M Tris-HCl, pH 8.9 with no addition of NaOH) and cathodal buffer (0.1M Tris-HCl, 0.1M Tricine, 0.1% (w/v) SDS, pH ~ 8.2 with no adjusting of pH) using a BioMetra electrophoresis system (BioMetra, GmbH) with an initial voltage of 30 V for 30 minutes and increasing to 100 V until the bromophenol blue front reaches the bottom of the gel.

3.2.6 Staining of the SDS-PAGE gels

Proteins were visualized by staining in 0.1% (w/v) Coomassie Brilliant Blue G 250 (40% methanol, 10% acetic acid) overnight, and were then destained in excess destaining solution (50% methanol, 10% acetic acid), until the background of the gel was clear and the protein bands were clearly visible (about 3-4 hours).

3.2.7 Analysis of SDS PAGE gels

For the analysis of the one dimensional SDS PAGE and SDS Tricine PAGE gels of the various samples the Quantity One™ Software Package from BioRad (Hercules, USA) was used. All the gels were scanned in using the VersaDoc 4000 image scanner (Bio-Rad, Hercules, USA) and the appropriate software from the Quantity One™ Software package. The software was then used to analyze the gel lanes and the gel bands. Each gel was for an individual mouse and thus each gel is representative of an independent experiment.

3.2.8 Statistical analysis of SDS PAGE bands

Data that were obtained by the Quantity One™ Software Package were exported into Microsoft ™ Excel ™ format. This data was analyzed with the Student's t-test for all the bands. Bands with ($p<0.05$) between the different groups were considered as statistically significant.

3.2.9 Preparation of gel bands for HPLC analysis

Gels were washed twice with milliQ water. The appropriate bands were then cut from the gels using a clean sterile scalpel. Each specific band was then further cut into $1mm^3$ cubes and placed into a clean microcentrifuge tube. The gel pieces were washed with 100 µl water:acetonitrile (1:1) for 15 minutes and repeated twice. The liquid was removed by pipette and replaced with 100 µl acetonitrile. The acetonitrile was left for about 5 minutes until the gel pieces had shrunk and appeared white. The acetonitrile was removed and replaced with 100 µl 0.1M ammonium bicarbonate. After 5 minutes an equal volume of acetonitrile was added to the ammonium bicarbonate and left to incubate for 15 minutes. All liquid was then removed and the gel pieces were dried in a vacuum centrifuge (SpeedyVac). Once the gel pieces were dry the gel pieces were reswollen in 100 µl of 10 mM dithiothreitol (DTT) and left to incubate for 45 minutes at 56ºC. The gel pieces were left to cool to room temperature and the liquid was removed. This was then quickly replaced with 100 µl of 55 mM iodoacetamide (IAA), 0.1 M ammonium bicarbonate and incubated in the dark at room temperature for 30 minutes. The liquid was removed and the gel pieces were washed with acetonitrile and left to shrink until the gel pieces appeared white. Acetonitrile was removed and replaced with 100 µl of 0.1 M ammonium bicarbonate and left to incubate for 5 minutes. An equal volume of acetonitrile was added and left to incubate for 15 minutes. All the liquid was then removed and the gel pieces were dried in a vacuum centrifuge (SpeedyVac). Twenty five microlitres of 25 mM ammonium bicarbonate was added to the dried gel pieces and left to incubate for 15 minutes. The same amount of acetonitrile was added and left to incubate for 15 minutes. The supernatant was recovered and placed in a clean microcentrifuge tube. The extraction of the gel supernatant was repeated twice with 0.1 % TFA, 50% acetonitrile. All the extracts were pooled and then dried in a vacuum centrifuge (SpeedyVac). Prior to HPLC the dried proteins were redissolved in 50 µl of 0.1% TFA, 50% acetonitrile.

3.2.10 HPLC Running conditions

A Waters 600 Controller and Pump was used for the HPLC procedures. A Waters 996 Photodiode Array detector was used to detect proteins at a wavelength of 280nm. The samples was separated by a reversed phase C18 Phenomenex Jupiter 5 micron C18, 300Å HPLC column. The column temperature was kept constant at 24ºC. Total run time was 100 minutes for each sample. Two blanks were used. One blank was only water while the other

blank was a clear piece of acrylamide gel that was cut and processed like normal sample. Solvent A was 0.1% TFA, H_2O and solvent B was 0.1% acetonitrile. The run started with solvent A for 1 minute. For the next 60 minutes solvent B increased to 60%. This then further increased to 95% during the next 15 minutes and then decreased to 5% and was kept constant for 10 minutes. During the last 14 minutes the column was re-equilibrated by keeping the column at 99.9% of solvent A.

3.2.11 Isoelectric Focussing

For proteins extracted from the liver:

The Ettan IPGphore Isoelectric Focusing Unit (Amersham Biosciences, Uppsala, Sweden) was used to separate the proteins according to their isoelectric (pI) points. For the liver samples of the individul animals, 150 μg of protein were loaded onto a 13 cm Immobiline Drystrip, pH 3-10 NL (Amersham Biosciences) together with the rehydration buffer (7M urea, 2 M Thiourea, 4% (w/v) CHAPS, 0.5% (v/v) IPG Buffer (pH 3-10 NL), 0.002% bromophenol blue). The IPG strip was then rehydrated with the sample and rehydration buffer overnight in the IPG Reswelling Strip Holder at room temperature. The rehydrated IPG strips were then placed into the IPG electrode strip holders and left to focus on the IPGphor at 20 ºC. The IEF process involves 4 steps as given in the table below and used to achieve a total of about 17 000 Vhrs.

Table 3.2: The IEF focusing steps used for the liver using 13cm IPG, pH 3-10 NL strips.

Step	Voltage limit (V)	Time or Volt hour (Hours) or (Vhr)	Gradient
1	30 V	0:30 hr	Step `n hold
2	500 V	1:00 hr	Step `n hold
3	1000 V	1:00 hr	Step `n hold
4	8000 V	15 500 Vhr	Step `n hold

The total isoelectric focusing time was about 4 hours and always allowed to proceed to a total of 8000 Vhrs.

For proteins extracted from the small intestine:

The Ettan IPGphore Isoelectric Focusing Unit (Amersham Biosciences) was used to separate the proteins according to their isoelectric (pI) points. For the small intestinal samples of the individual animals, 60 µg of protein were loaded onto a 7 cm Immobiline Drystrip pH 3-10 NL (Amersham Biosciences) together with the rehydration buffer (7M urea, 2M thiourea, 4%(w/v) CHAPS, 0.5% (v/v) IPG Buffer (pH 3-10 NL), 0.002% bromophenol blue). The IPG strip was placed face down onto 125 µl of sample and rehydration buffer. The IPG strip was finally covered with Drystrip cover fluid and left to rehydrate in the IPG reswelling strip holder overnight at room temperature. The following morning the IPG strip was removed from the reswelling chamber and rinsed with MilliQ water to remove any oil. The IPG strip was then placed with the gel side towards the electrodes into the IPG electrode strip holders and once again covered with Drystrip cover fluid. The ceramic IPG strip holders were placed in the Ettan IPGphor and left to focus according to a specified program. The IEF process involves 5 steps.

Table 3.3: The IEF focusing steps used for the small intestine using 7cm IPG, pH 3-10 NL strips.

Step	Voltage limit (V)	Time or Volt hour (Hours) or (Vhr)	Gradient
1	30 V	0:30 hr	Step `n hold
2	300 V	0:40 hr	Step `n hold
3	1000 V	0:30 hr	Gradient
4	5000 V	1:30 hr	Gradient
5	5000 V	3000 Vhr	Step `n hold

3.2.12 Two Dimensional Polyacrylamide Gel Electrophoresis

Before the IPG strips were placed onto the SDS PAGE gels the strips were placed in SDS equilibration buffer (50mM Tris-HCl, pH 8.8, 6M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 0.002% bromophenol blue, 1% DTT) for 15 minutes and then placed in another SDS equilibration buffer for 15 minutes in which the 1% DTT was replaced with 2.5% iodoacetamide. After this the strips were briefly rinsed in SDS electrophoresis running buffer (0.25M Tris-HCl, pH 8.3, 0.1% SDS, 192mM Glycine). The equilibrated IPG strips were placed on top of the SDS PAGE gel and covered with 1% agarose (50mM tris-HCl, pH 6.8) to seal of the IPG strip.

Organ proteins were analyzed using a Laemmli SDS-PAGE system. A 12.5% T, 2.6% C separating gel (0.375M Tris-HCl, 0.1% SDS, pH 8.8), and 4% T, 2.6% C stacking gel (0.125M Tris-HCl, 0.1% SDS, pH 6.8) were prepared from 30.8% T, 2.6% C acrylamide (30% acrylamide, 0.8% N',N'methylene bisacrylamide) stock solutions. These solutions were degassed for 15 minutes and polymerized by addition of 50 µl of 10% ammonium persulphate and 5 µl TEMED. Electrophoresis was carried with buffer (0.25M Tris-HCl, pH 8.3, 0.1% SDS, 192mM Glycine). For the liver the 13 cm pH 3-10 NL IPG strips were run on a 16 x 18 cm x 1 mm gel (Hoefer SE 600) for the second dimension, while the small intestine 7 cm pH 3-10 NL IPG strips were separated on a 10 x 8 cm x 1mm gel (BioMetra, GmbH)

3.2.13 Silver staining of 2 D gels compatible with MS

Method adapted from Jensen et al, (1999). The 2D gels were removed from the glass plates and fixed in a closed container with 45% methanol and 5% acetic acid for 1 hour. The gels were rinsed with MilliQ water and left overnight in MilliQ water to allow proper equilibration of the gels and to minimize background staining. All the gels were sensitized for 2 minutes using 0.02% sodium thiosulphate and then rinsed twice with MilliQ water for 2 minutes respectively. The rinsed water was discarded and replaced with a chilled 0.1% silver nitrate solution and left at 4ºC for 30 minutes. After 30 minutes of staining in silver nitrate the gels were washed twice with MilliQ water. The gels were developed by adding fresh 0.04% formaldehyde to 2% sodium carbonate just prior to adding this solution to the gels. When sufficient development took place the sodium carbonate mixture was discarded and replaced with 1% acetic acid to stop further development of the gels. All gels were stored in 1% acetic acid at 4ºC in airtight containers until use for MS.

3.2.14 Image Analysis of 2 D gels

Analysis of the two dimensional SDS PAGE gels of both the liver and small intestine extracts were done using PD Quest™ Package from BioRad (Hercules, USA). All the gels were scanned in using the VersaDoc 4000 image scanner (Bio-Rad, Hercules, USA) and the appropriate software from the PD Quest™ Software package. The software was used to analyze the spots and identify spots that were modulated as well as spots that were statistically significant ($p<0.05$) between the various groups. Individual spots were

detected by the software with the default settings. The density of each spot was determined and added together to obtain the total density of each gel. The gels were normalized to the gel with the greatest total density. The density of each spot was automatically adjusted with the same correction factor. A master image was created for each organ. The master image contains all the spots that were detected on the control, low dose and high dose gels.

3.2.15 Statistical Analysis of 2 DE gels

The PD Quest software package was used to identify proteins spots on the low and high dose gels that were regulated more than two fold compared to the control. This package also identified proteins whose normalized density was statistically significantly different ($p < 0.05$) between the control and the low or high dose groups.

3.3 Results

3.3.1 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE) of the small intestine and liver

The first step towards the proteomic approach that we followed was to start by separating the organ homogenates with SDS PAGE. Various organs were removed and prepared for analysis as was described in chapter 2. The choice of first starting with the proteomic analysis of the small intestine and liver was also explained in chapter 2. In chapter 2 it was seen that the liver showed significant increases in liver weight between the groups as did the small intestine. It was also described in chapter 2 that the liver and small intestine contain much of the absorbed tea and therefore they were expected to show modulation of proteins. The liver and the small intestine are important in cancer research. Thus the ultimate goal of this study will be to find novel modulated proteins within the liver and small intestine, which can become new targets for cancer therapy.

The small intestinal proteins were prepared according to Laemmli (1970) and a standard SDS PAGE procedure was followed. The protein concentration was determined and then the appropriate amount of protein was diluted 1:4 with a SDS reducing buffer. The same amount of protein was loaded for each group and on each of the gels that were done. These gels were visualized with Coomassie as described in the Methods section. The gels were then scanned and analyzed using Quantity One™ from Bio-Rad to determine if statistical differences ($p<0.05$) are present between the groups. The gel below is an example SDS PAGE gels of proteins extracted from the small intestine. Three different bands were found to be statistically significant different between the groups. These bands are marked on the gel in Figure 3.1 below and are also enlarged in Figure 3.2. Band nr 1 is a 66 kDa band that seems to have an extra band for the control and high dose group when compared to the low dose group. Band nr 2 is a 45 kDa band and is significantly lower in expression in the low and high dose groups when compared to the control group. Band nr 3 is a 10 kDa band. For this band the low dose group has increased expression when compared to the control and high dose groups.

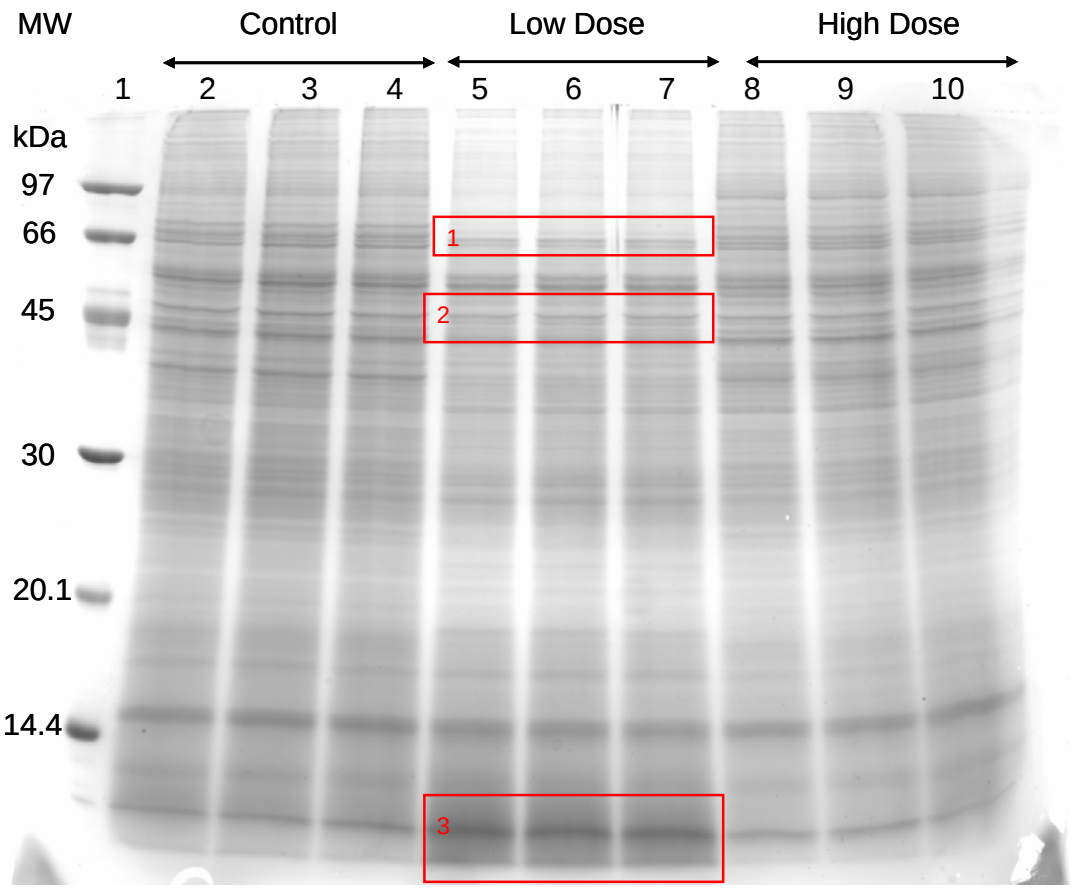


Figure 3.1 SDS PAGE gel of the proteins extracted from the small intestine.

50 μg protein loaded in each well. Lane 1: MW markers, Lane 2-4: control, Lane 5-7: low dose, Lane 8-10: high dose. Gel size is 160 x 180 x 1 mm (Hoefer SE 600).

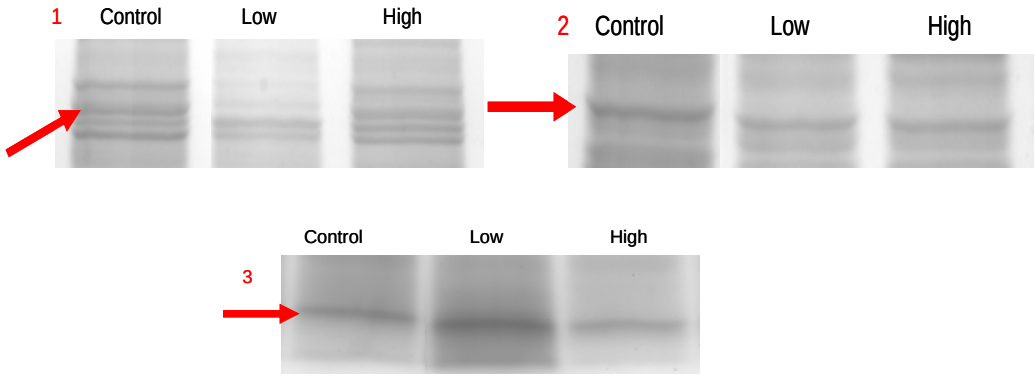


Figure 3.2: Enlargement of the regulated bands found in protein extracts from the small intestine.

For the liver extracts the same approach as for the small intestine extracts were followed. Figure 3.3 below shows an example of proteins extracted from the liver separated on SDS PAGE. For the liver extracts three statistically significant ($p<0.05$) bands were also found as determined by Quantity One™ software. The three significant bands are enlarged in Figure 3.4. Band nr 1 is a 110 kDa band. It has reduced expression of the low dose when compared to the control and high dose. Band nr 2 is a 66 kDa band that has increased expression of the low dose band when compared to the control and high dose groups. Band nr 3 is a 14 kDa band and was the most distinctly and most visible modified band. The low dose group had increased expression of this band when compared to the control group. On the other hand the high dose group had reduced expression of this particular band when compared to the control.

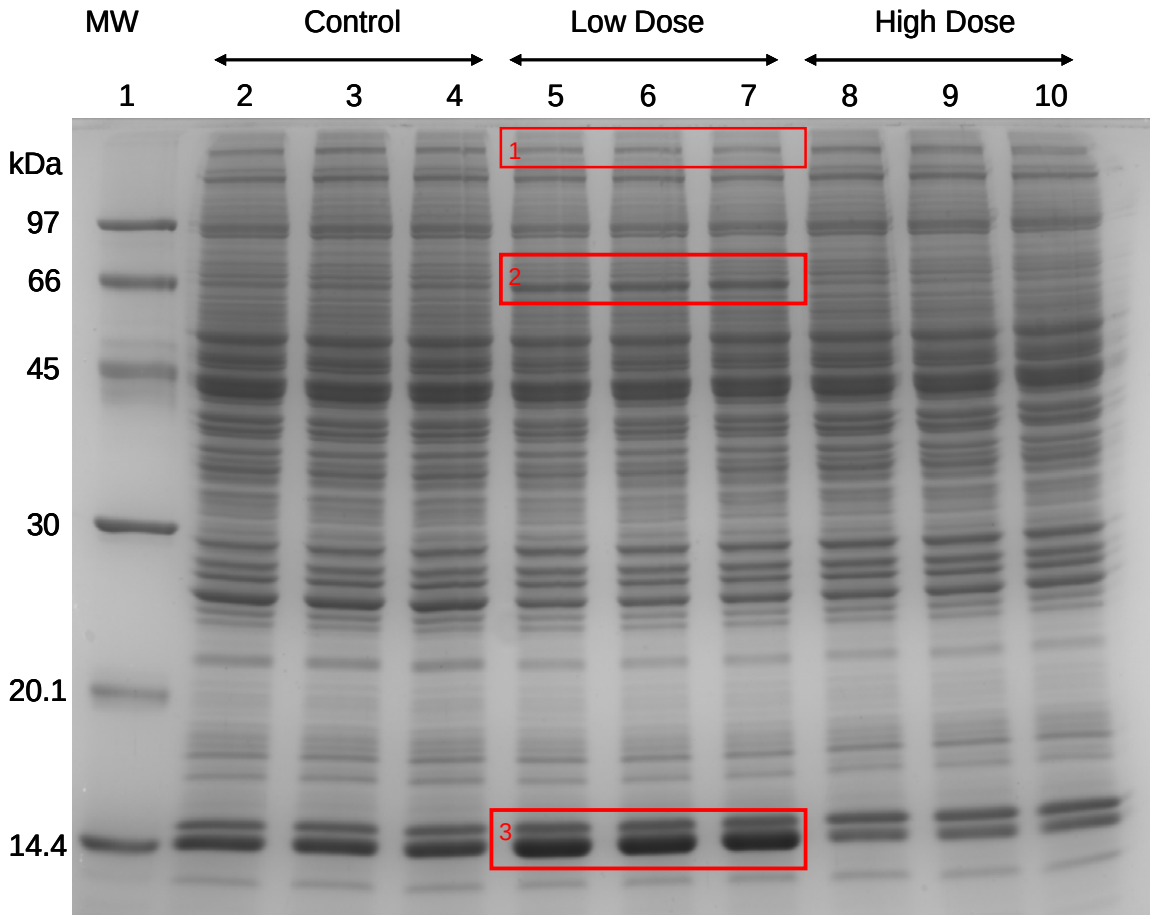


Figure 3.3 SDS PAGE gel of the proteins extracted from the liver.

50 μg protein loaded in each well. Lane 1: MW markers, Lane 2-4: control, Lane 5-7: low dose, Lane 8-10: high dose. Gel size is 160 x 180 x 1 mm (Hoefer SE 600).

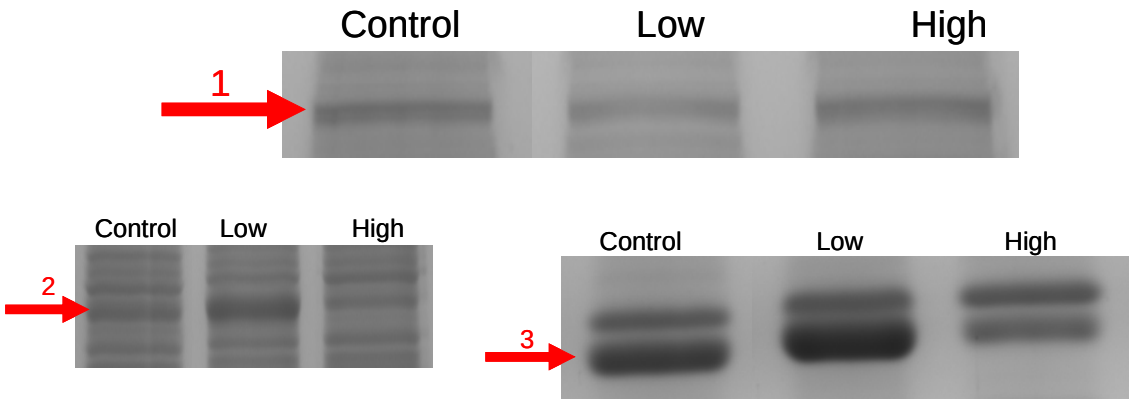


Figure 3.4: Enlargement of the regulated bands found in protein extracts from the liver.

The results that were obtained from SDS PAGE gels from the liver extracts, it was decided to separate two bands from the liver extracts further with HPLC, to determine if the bands are only one protein that is modulated or if the band consists of more than one proteins all with the same molecular weight.

3.3.2 HPLC analysis of the liver protein extract bands and MALDI TOF MS

Band nr 2 and band nr 3 were the most visibly modulated bands on the liver extract SDS PAGE gels. The protein concentration of these bands was high and therefore these bands were separated by C_{18}/RP HPLC to determine how many proteins were present within the single band. The specific bands were cut from the Coomassie stained SDS PAGE gels. The bands were washed and the proteins were extracted from the polyacrylamide gel matrix, and then subjected to HPLC analysis. For HPLC analysis a blank was used. The blank consisted of MilliQ water. A negative gel control was also added. The negative gel control was a clean piece of gel that was cut from the SDS PAGE gel. This was to determine if the polyacrylamide gives rise to peaks during the HPLC process. Control, low dose and high dose bands were subjected to HPLC. The chromatogram (Figure 3.5) below demonstrates the full separation of the 66 kDa band over the 60 minute separation time.

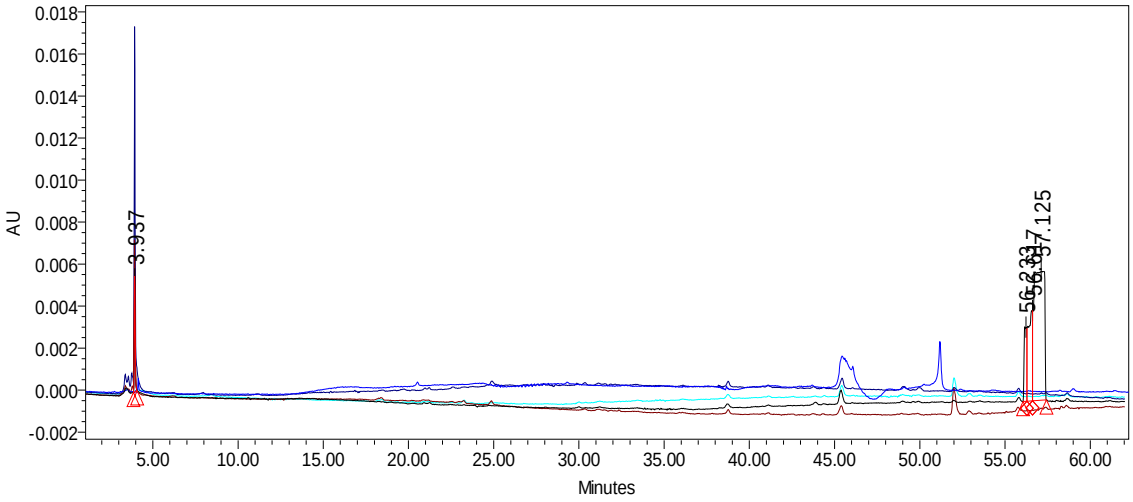


Figure 3.5: HPLC chromatogram of the 66 kDa band (band nr 2) obtained from the liver extracts.

Figure 3.6 demonstrates a peak that was found for the 66 kDa band. It can be seen that the low dose group peak has a higher intensity (1248 µV) than the control (864 µV) and high dose (184 µV) group. This is similar to the results found with SDS PAGE. The control group has almost half the intensity of the low dose group. The high dose group has only a very small peak that is barely detectable. The blank and negative control showed no increase in peak intensity and therefore this can be seen as a real peak for band nr 2. This also seem to be the only real peak for the 66 kDa band as all the other peaks that were detected had the same peak intensities for both the blank and the negative gel control. Therefore it is likely that band nr 2 consists of only one protein that is regulated by tea.

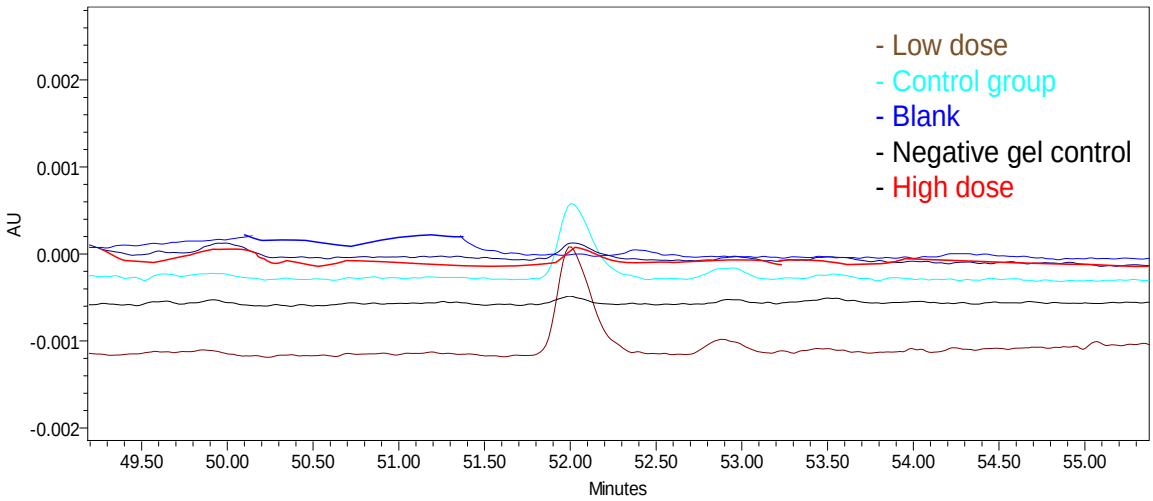


Figure 3.6: HPLC analysis of the 66 kDa band (band nr 2) obtained from the liver extracts.

The other band that was subjected to HPLC was band nr 3 which is the 14 kDa band. Figure 3.7 demonstrates the full chromatogram for band nr 2.

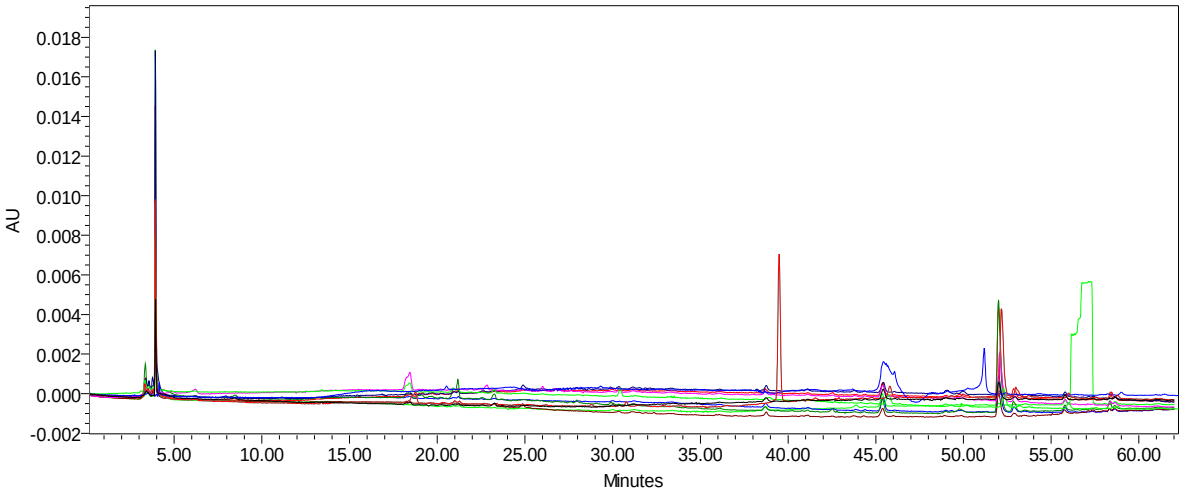


Figure 3.7: HPLC chromatogram of the 14 kDa band (band nr 3) obtained from the liver extracts.

The first real peak that was detected for the 14 kDa was after 39 minutes. It can be seen that the low dose group has extremely high peak intensity. The control and high dose groups have no indication of a peak at this specific time point. The blank and negative gel control also shows no peak at this time point. It can thus be assumed that this is a real peak that is unique to the low dose group. To the left of this unique peak is a series of peaks that are found in all the samples including the negative control and is thus a similar contaminant in all the samples and is thus not a protein peak.

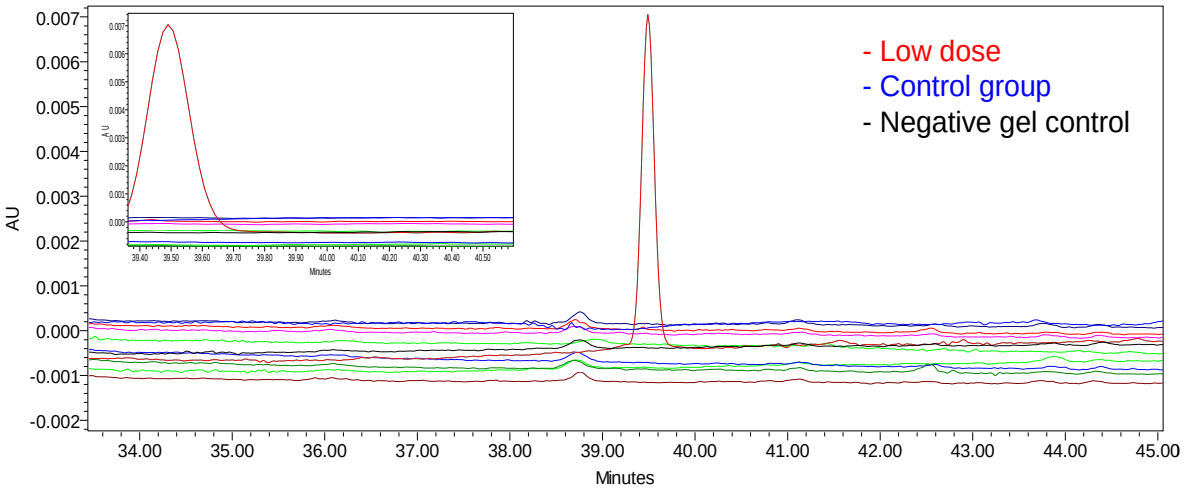


Figure 3.8: HPLC analysis of the 14 kDa band (band nr 3) obtained from the liver extracts.

Another peak for the 14 kDa band was found at about 52 minutes. In this chromatogram it can be determined that the low dose group has increased expression of this specific protein. Three different low dose samples were run. The peak of one of the low dose samples eluted 30 seconds later than the other peaks. This is probably due to the fact that the samples were run at different days. The control groups (2428 μV) had about half the peak intensity of the low dose (4459 μV) groups, while the high dose group had a very small peak (948 μV) that also eluted 10 seconds later. Once again the inclusion of the negative gel control confirms that these differences are real and that the peak is a real protein. These results also confirm the results found on the SDS PAGE gels of the liver. It is also clear that band nr 2 consists of possibly two proteins that both have the same molecular weight as these peaks were detected at 39 and 52 minutes.

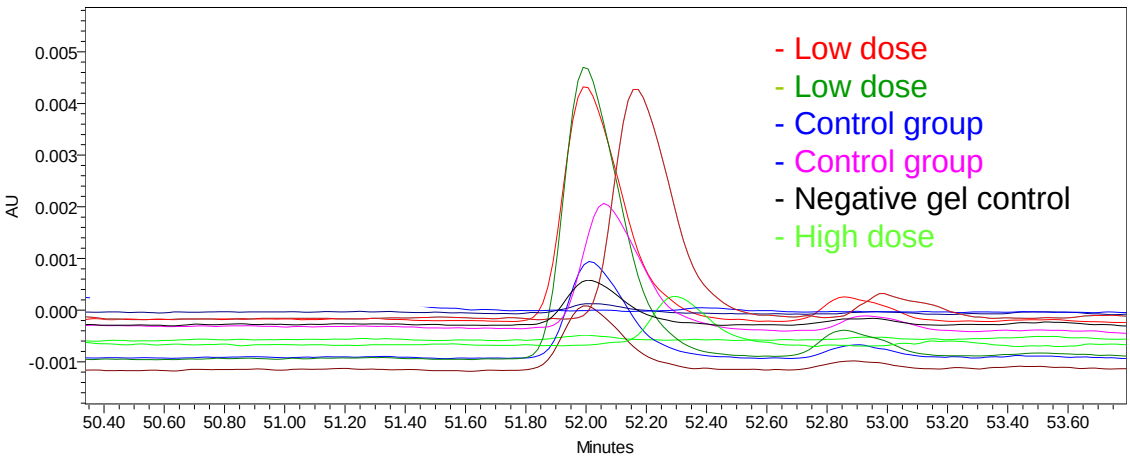


Figure 3.9: HPLC analysis of the 14 kDa band (band nr 3) obtained from the liver extracts.

The 14 kDa band was subjected to MALDI TOF MS in an attempt to identify the proteins that are modulated by tea within this band. The results for the MALDI TOF MS can be seen below.

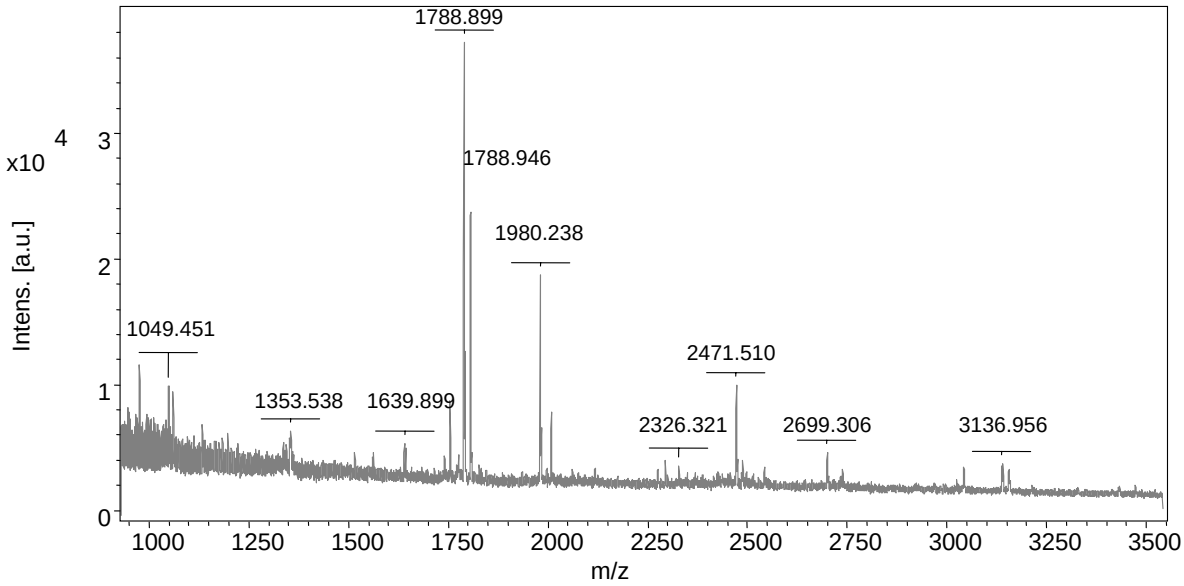


Figure 3.10: MALDI TOF MS spectra of the 14 kDa band obtained from the liver extracts.

The 14 kDa protein was identified by using ProFound as this database enables the user to specify the amount of proteins present within a sample. For the 14kDa band this option were specified as two proteins present within the sample as this was previously detected by HPLC. The proteins identified by the database were Hypothetical protein XP_358319 (Accession nr XP_358319) with a molecular weight of 15 190 Da, and Immunoglobulin α chain (Accession nr AAA38305) with molecular weight of 13 140 Da. Even though the two proteins that were identified fall in the mass range of 14 kDa, these results will still need to be confirmed by LC MS.

3.3.3 Tricine SDS PAGE of protein extracted from the liver and small intestine

Tricine SDS PAGE gel electrophoresis is a gel separation technique that is used to detect extremely small proteins and peptides that may elute on the front on normal SDS PAGE. The proteins extracts of the liver and small intestine were prepared according to the method of (Schagger H. and von Jagow G., 1987) and then subjected to tricine SDS PAGE. Figure 3.12 below is an example of the tricine gels obtained for the proteins extracted from the

small intestine. No significant differences ($p<0.05$) between the three groups were detected for the small intestine.

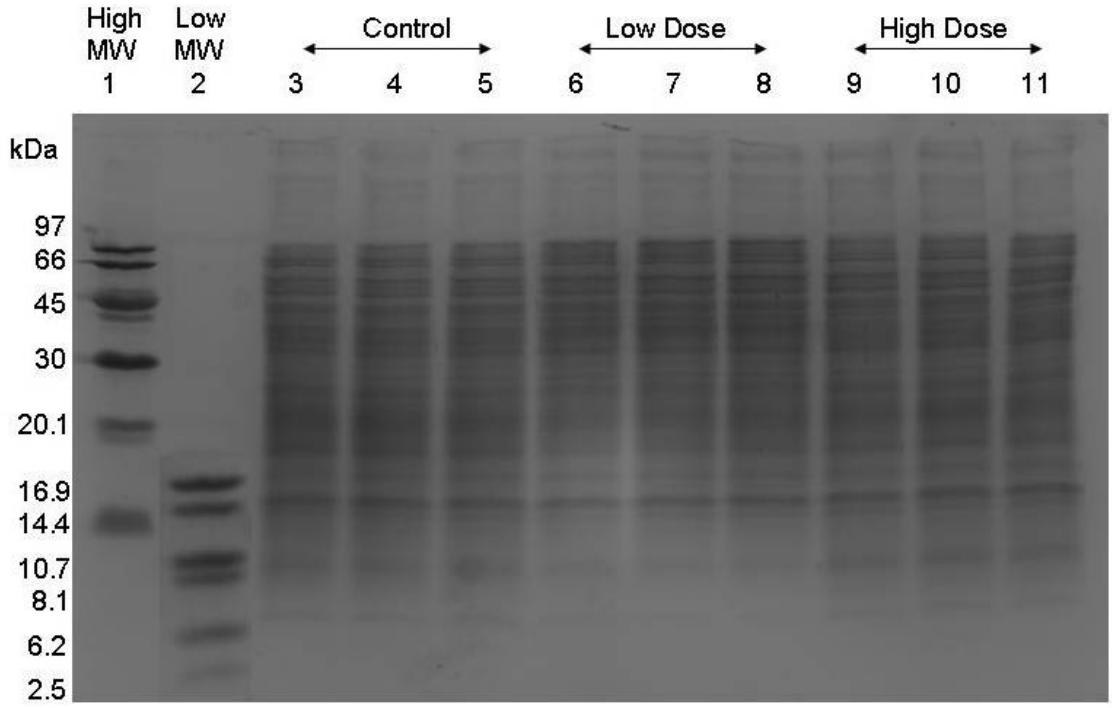


Figure 3.12: Tricine SDS PAGE of the protein extracts of the small intestine.

25 μg protein loaded in each well. Lane 1: MW markers, Lane 2-4: control, Lane 5-7: low dose, Lane 8-10: high dose. Gel size is 100 x 80 x 1 mm (BioMetra, GmbH).

The same was done for the liver. Figure 3.13 shows an example of a liver extract tricine SDS PAGE gel. As for the small intestine extracts no significant differences were found for any of the bands of the various groups.

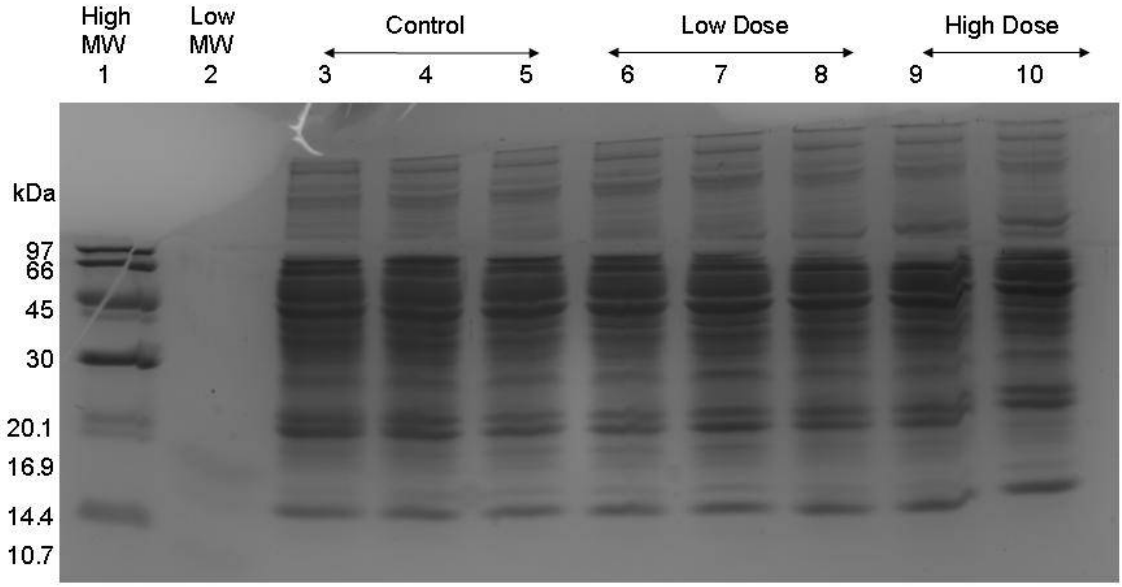


Figure 3.13: Tricine SDS PAGE gel of the protein extracts of the liver.

25 μg protein loaded in each well. Lane 1: MW markers, Lane 2-4: control, Lane 5-7: low dose, Lane 8-10: high dose. Gel size is 100 x 80 x 1 mm (BioMetra, GmbH).

3.3.4 Two dimensional gel electrophoresis of the protein extracts of the small intestine

SDS PAGE of protein extracts from the small intestine and liver was performed and only three bands for each of the organs were found that were modulated. SDS PAGE has limited separating power as the proteins are only separated according to size. And as has been demonstrated by the 14 kDa band of the liver it is possible that more than one protein can exist in one single band seen on the gel. To increase separating power, two dimensional gel electrophoresis (2DE) was done for the proteins extracts of the small intestine and the liver. The proteins are thus now separated according to their iso-electric point (pI), and according to size. Figures 3.14 – 3.16 below demonstrate typical examples of the gels obtained for the protein extracts of the small intestine of individual mice for each of the three dosage groups.

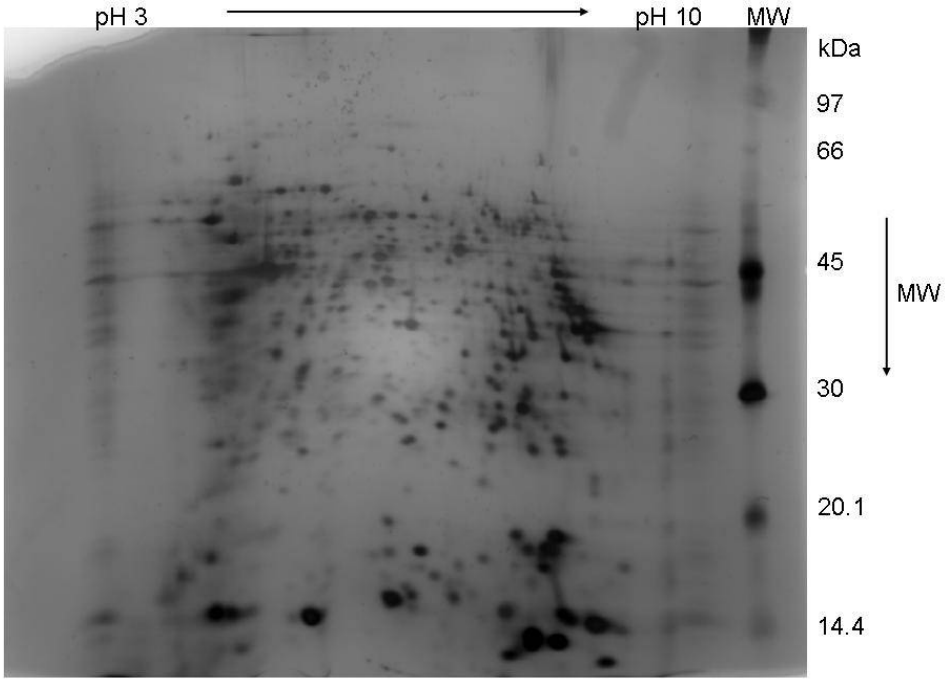


Figure 3.14: Typical two dimensional gel of the protein extracts from the small intestine of an individual mouse from the control group. *(n = 5)*

60 μg protein loaded on 7cm pH 3-10 NL IPG strip. MW markers are indicated on right hand side of gel. Gel size is 100 x 80 x 1 mm (BioMetra, GmbH). N = 5 is the number of individual 2DE gels done for the control group.

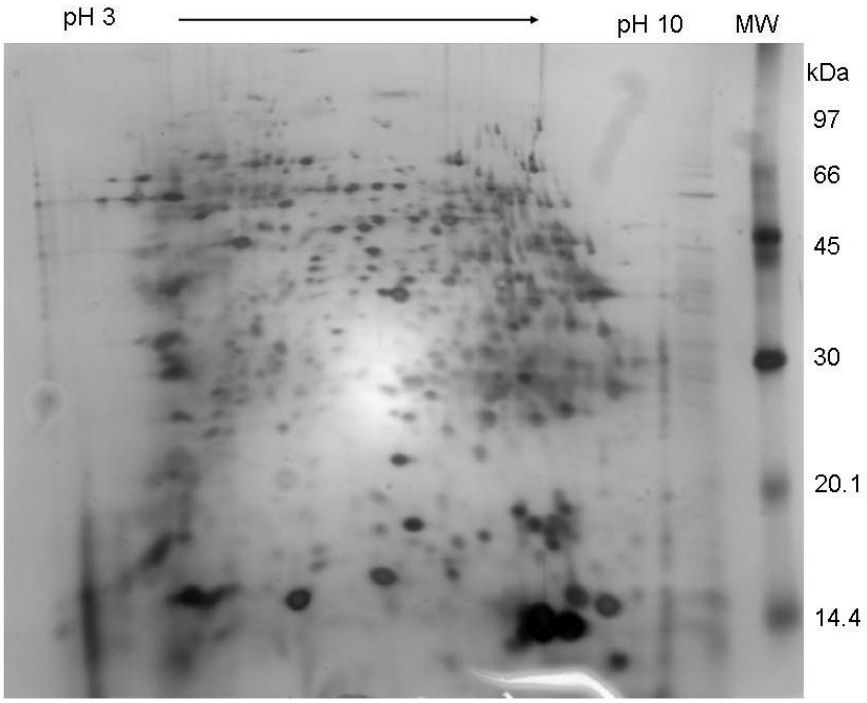


Figure 3.15: Typical two dimensional gel of the protein extracts from the small intestine of an individual mouse from the low dose group. *(n = 6)*

60 μg protein loaded on 7cm pH 3-10 NL IPG strip. MW markers are indicated on right hand side of gel. Gel size is 100 x 80 x 1 mm (BioMetra, GmbH). N = 6 is the number of individual 2DE gels done for the low dose group.

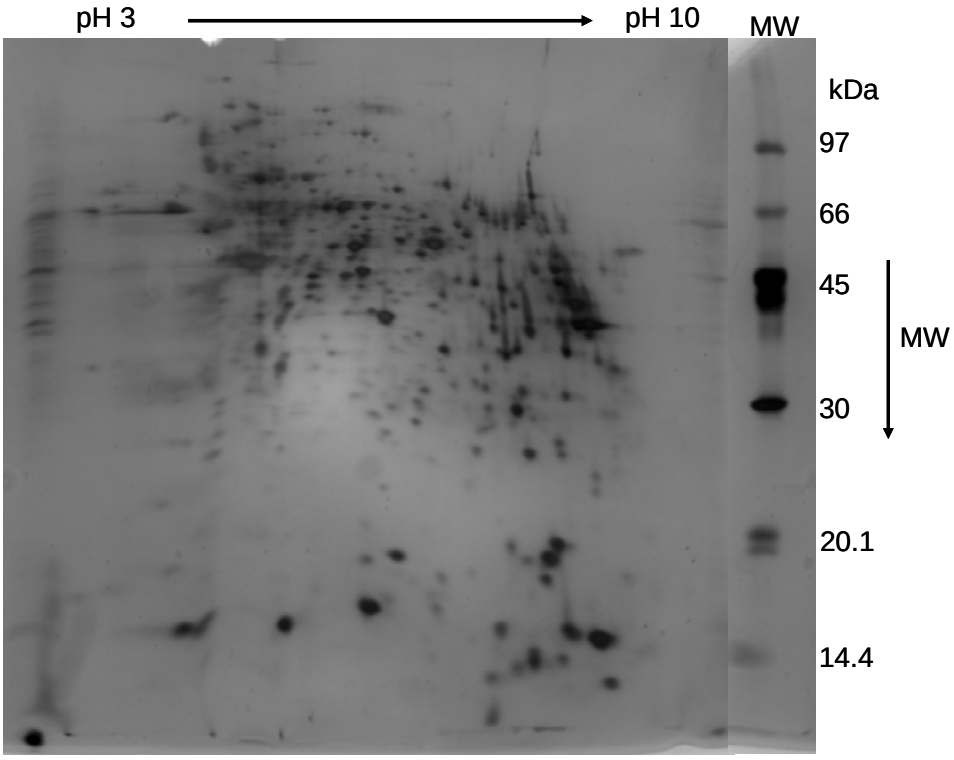


Figure 3.16: Typical two dimensional gel of the protein extracts from the small intestine of an individual mouse from the high dose group. *(n = 5)*

60 μg protein loaded on 7cm pH 3-10 NL IPG strip. MW markers are indicated on right hand side of gel. Gel size is 100 x 80 x 1 mm (BioMetra, GmbH). N = 5 is the number of individual 2DE gels done for the high dose group.

3.3.5 Image Analysis of the protein extracts from the Small intestine

For the protein extracts from the small intestine image analysis was done using PD Quest™ Software from Bio-Rad. Two approaches were followed for the identification of unique spots. First spots were identified between the groups that showed statistically significant differences as determined by the software. The table below shows the number of spots determined to be significant between the groups.

Table 3.4: Statistically significant (t-test) modulated spots of the protein extracts from the small intestine.

Statistical Student's t-test ($p<0.05$)	**Nr of spots**
Control compared to low dose	6
Control compared to high dose	7
Low dose compared to high dose	9

The table below is spots that were identified by PD Quest™ that demonstrated at least two times modulation between the compared groups.

Table 3.5: 2x modulated spots of the protein extracts from the small intestine.

2 x regulation	Nr of spots
Control compared to low dose	6
Control compared to high dose	12
Low dose compared to high dose	9

By using PD Quest ™ software a master image for the statistical and regulation differences were made. The master image of the statistical differences detected in all three groups of the small intestine extracts can be seen in the Figure 3.17 below. The spots that were statistically significant between the groups are shown in Figure 3.18 to Figure 3.20. Part per million (PPM) are indicated on the Figures as a measure of intensity for each spot.

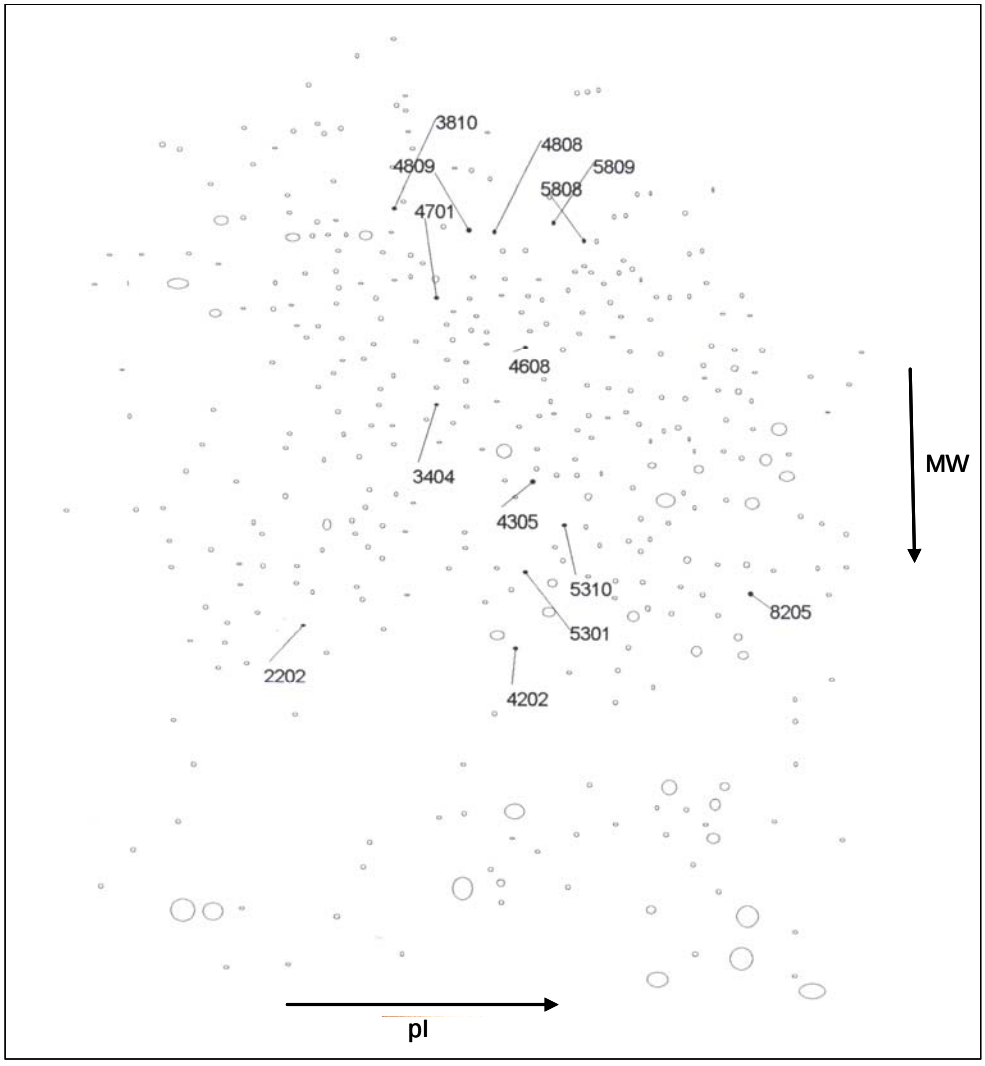
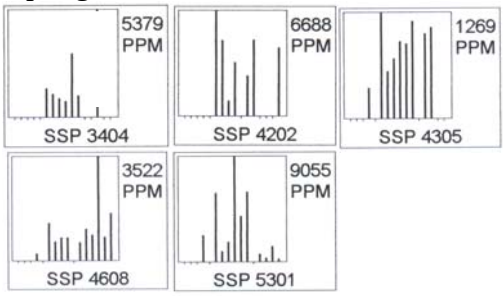


Figure 3.17: Master image for statistical significant differences of all the groups of protein extracts from the small intestine.

When the low dose group was compared to the control group five spots were detected that showed up regulation. Only one spot was identified that was down regulated in the low dose group when compared to the control group.

Up regulation



Down regulation

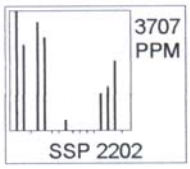


Figure 3.18: Statistical modulation of the low dose group compared to the control group of the small intestine extracts.

Seven spots were up regulated in the high dose group when compared to the control group of the proteins extracted from the small intestine.

Up regulation

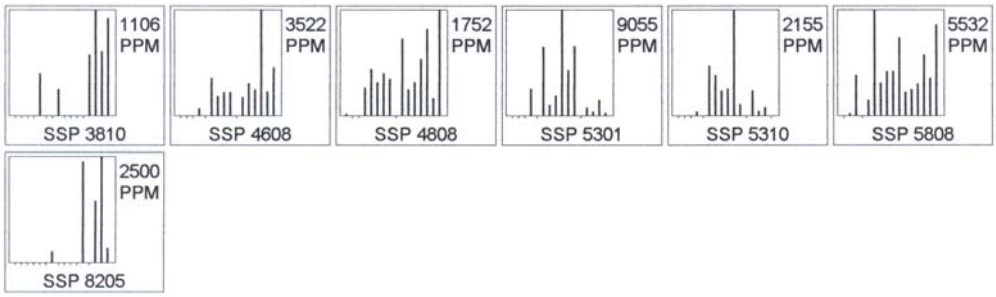


Figure 3.19: Statistical modulation of the high dose group compared to the control group of the small intestine extracts.

Four spots were up regulated in the high dose group when compared to the low dose group. Five spots were down regulated in the high dose group when compared to the low dose group of the proteins extracted from the small intestine.

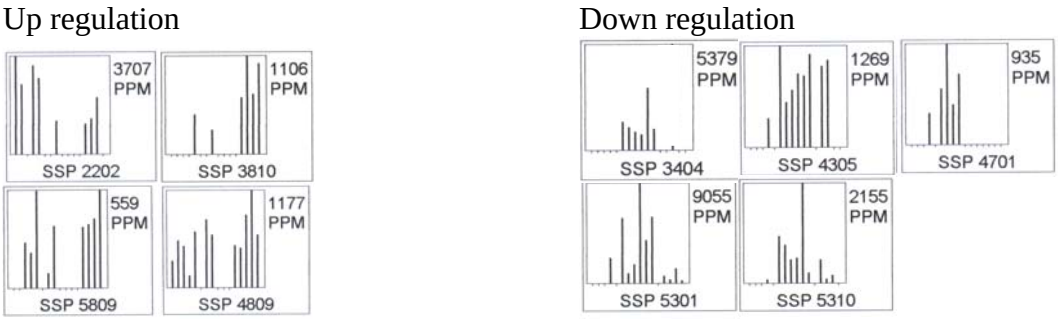


Figure 3.20: Statistical modulation of the high dose group compared to the low dose group of the small intestine extracts.

Spots that showed at least two times up regulation between groups were also determined. The Figure below is an indication of the master gel for regulation.

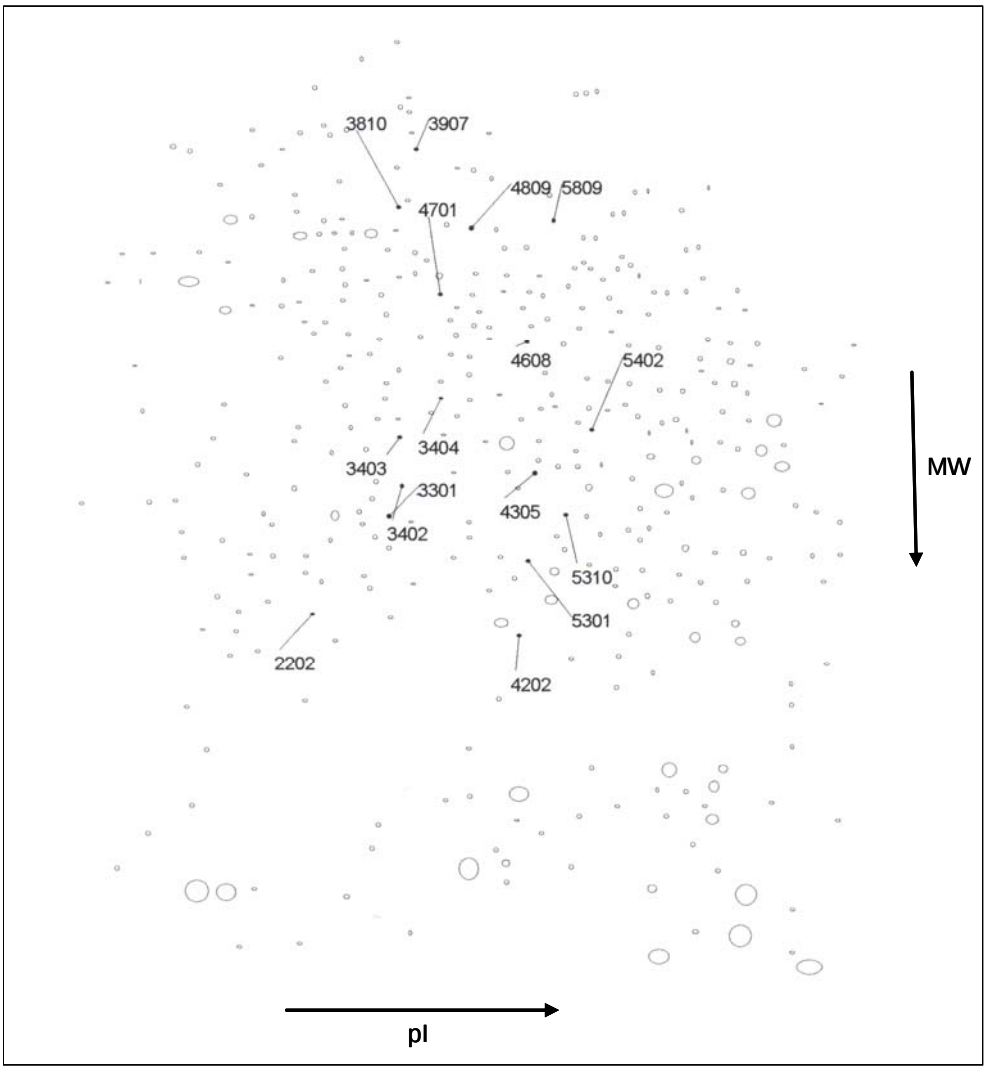


Figure 3.21: The master image gel for at least two times regulation between the three groups of the protein extracts from the small intestine.

Five spots were detected that were up regulated in the low dose group when compared to the control group. Only one spot was down regulated in the low dose group when compared to the control group.

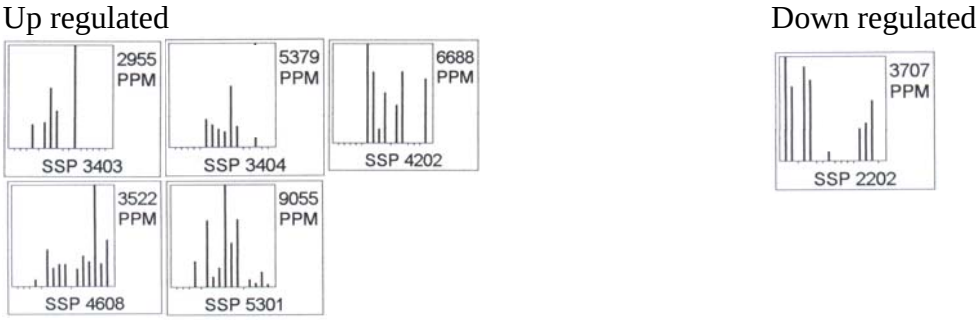


Figure 3.22: Modulation (2x) of the low dose group as compared to the control of the small intestine extracts.

Eight spots were up regulated and four spots were down regulated in the high dose group when compared to the control group.

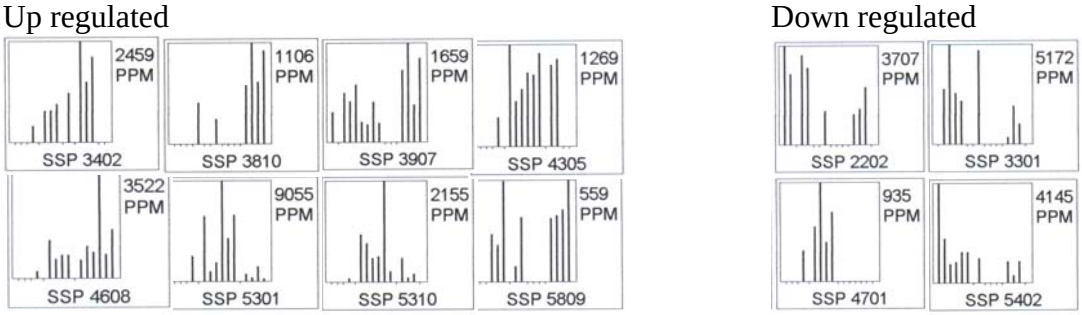
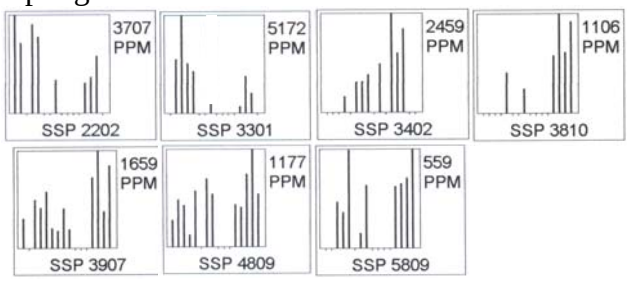


Figure 3.23: Modulation (2x) of the high dose group compared to the control of the small intestine extracts.

Seven spots were up regulated and only two down regulated in the high dose when compared to the low dose group of the small intestine extracts.

Up regulation



Down regulation

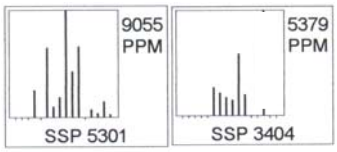


Figure 3.24: Modulation (2x) of the high dose compared to the low dose group of the small intestine extracts. Regulation shown is for the high dose group.

3.3.6 Two dimensional gel electrophoresis of the liver extracts

For the liver, sample preparation had to be optimized. At first a standard rehydration buffer was used that consisted of: 8M Urea, 2% CHAPS, and 18 mM DTT. Isoelectric focusing was done using 13 cm, pH 3-10 NL, IPG strips. The IPG strips were focused by using three steps for focusing. The first step was 500 V for one hour; this was followed by 1000 V for one hour. The last step used a maximum of 8000 V, and the strips was focused for up to 16 000 Vhrs. This resulted in about 300 spots that were visible on the silver stained 2DE gel. To increase the number of spots, the rehydration buffer was modified to: 7M urea, 2M thiourea, 4% CHAPS and 50 mM DTT. This rehydration buffer has extremely strong solubilizing power. The addition of thiourea also helps to keep the proteins soluble. The amount of DTT was increased to 50 mM to increase the reducing power. The iso-electric focusing steps were also adapted. The first step was 30 V for 30 minutes, then 500 V for one hour, followed by 1000 V for one hour. The last focusing step used a maximum of 8000 V, and were left to focus until a total of 17 000 Vhrs were reached. The number of spots on the silver stained 2DE gels increased from about 300 with the first method to about 600 spots detected using the second method. The Figure below demonstrates the differences seen between the two different methods used.

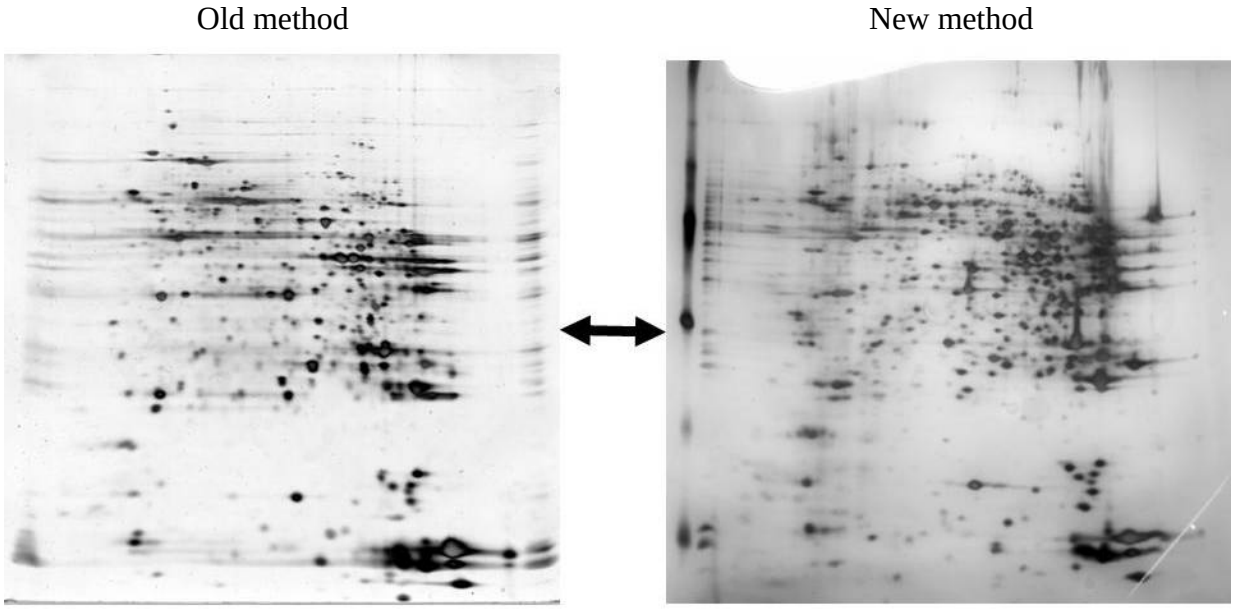


Figure 3.25: Comparison of the methods used for optimization of the protein extracted from the liver 2D gels.

After optimization of the liver samples the samples for all three groups were subjected to 2DE. The 2DE gels for the control, low dose and high dose groups can be seen in Figure 3.26 to Figure 3.28 below.

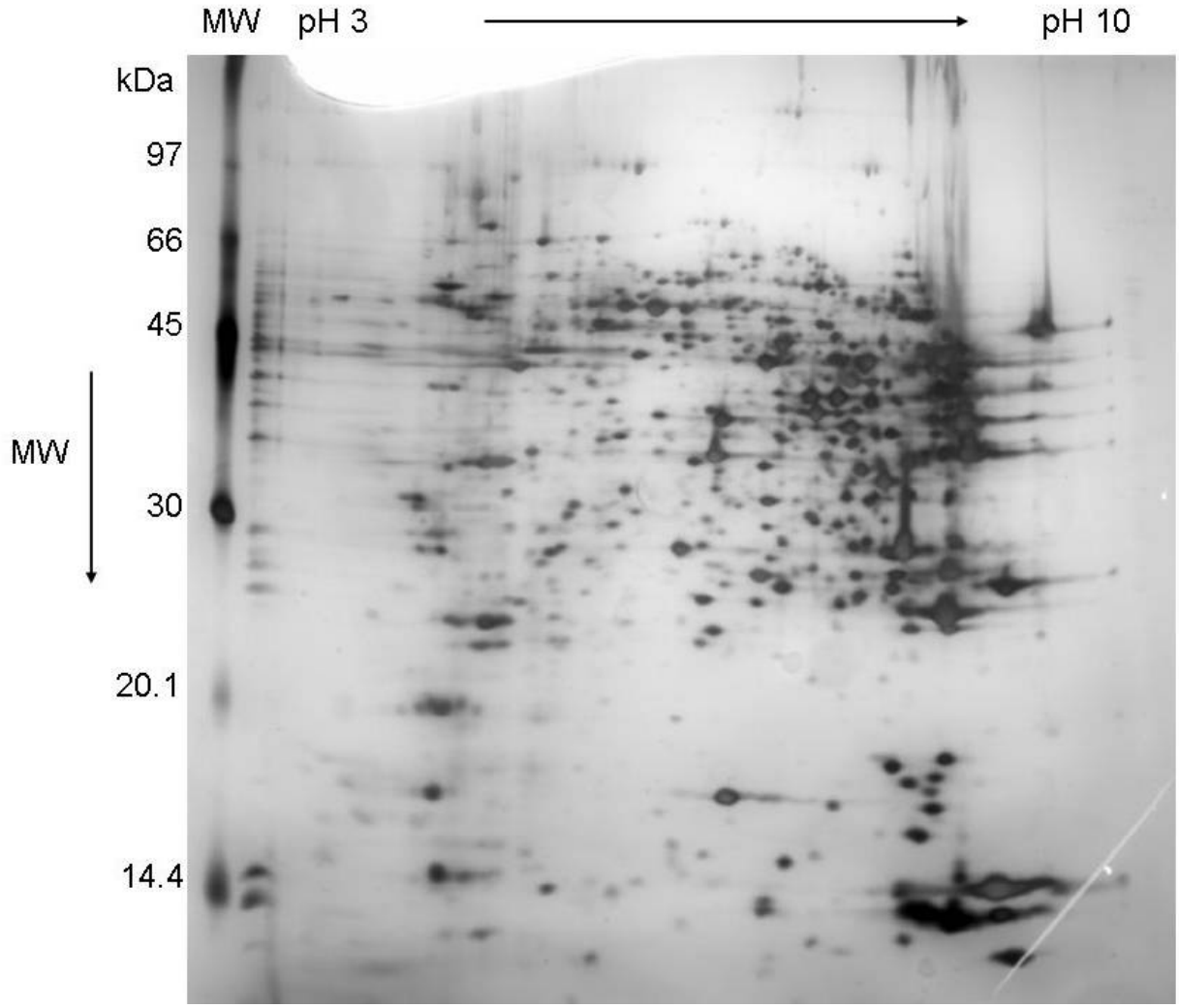


Figure 3.26: Typical two dimensional gel of the proteins extracted from the liver of an individual mouse from the control group *(n = 5)*.

150 μg protein loaded on 13cm pH 3-10 NL IPG strip. MW markers are indicated on right hand side of gel. Gel size is 160 x 180 x 1 mm (Hoefer SE 600). N = 5 is the number of individual 2DE gels done for the control group.

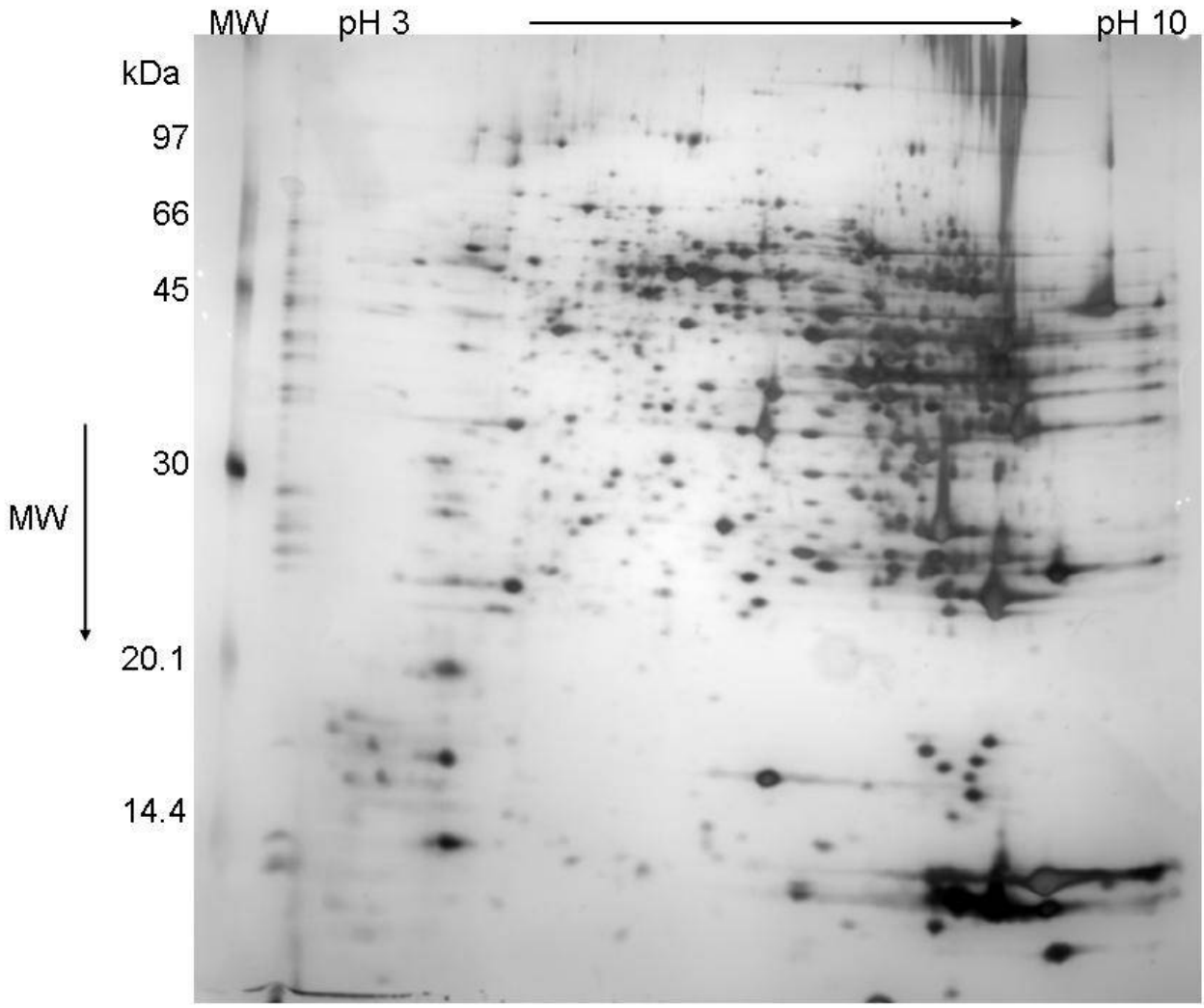


Figure 3.27: Typical two dimensional gel of the protein extracts from the liver of an individual mouse from the low dose group *(n = 6)*.

150 μg protein loaded on 13cm pH 3-10 NL IPG strip. MW markers are indicated on right hand side of gel. Gel size is 160 x 180 x 1 mm (Hoefer SE 600). N = 6 is the number of individual 2DE gels done for the low dose group.

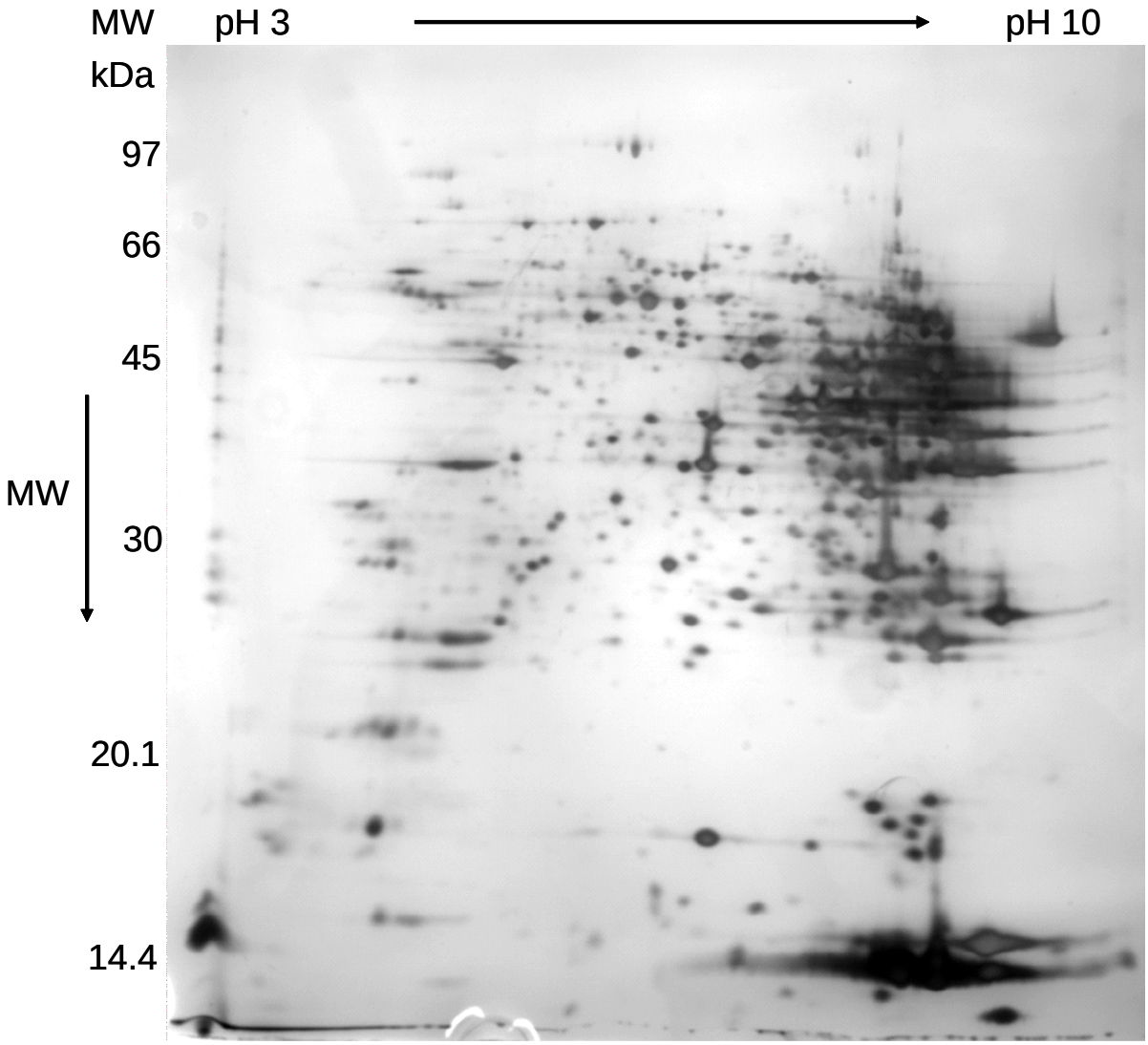


Figure 3.28: Typical two dimensional gel of the protein ectracts from the liver an individual mouse from the high dose group *(n = 6)*.

150 μg protein loaded on 13cm pH 3-10 NL IPG strip. MW markers are indicated on right hand side of gel. Gel size is 160 x 180 x 1 mm (Hoefer SE 600). N = 6 is the number of individual 2DE gels done for the high dose group.

3.3.7 Image analysis of the protein extracts from the liver

As for the small intestine protein extracts spots that showed statistically significant differences between the groups and spots that showed at least two times modulation were identified. The tables below show the amount of spots identified.

Table 3.6: Statistically significant (t-test) modulated spots of the liver protein extracts.

Statistical Student's t-test ($p<0.05$)	**Nr of spots**
Control compared to low dose	4
Control compared to high dose	9
Low dose compared to high dose	22

Table 3.7: 2x regulated spots of the liver protein extracts.

2x regulation	**Nr of spots**
Control compared to low dose	3
Control compared to high dose	1
Low dose compared to high dose	7

The Figure below is an indication of the master image of the statistical significant differences detected between all the groups.

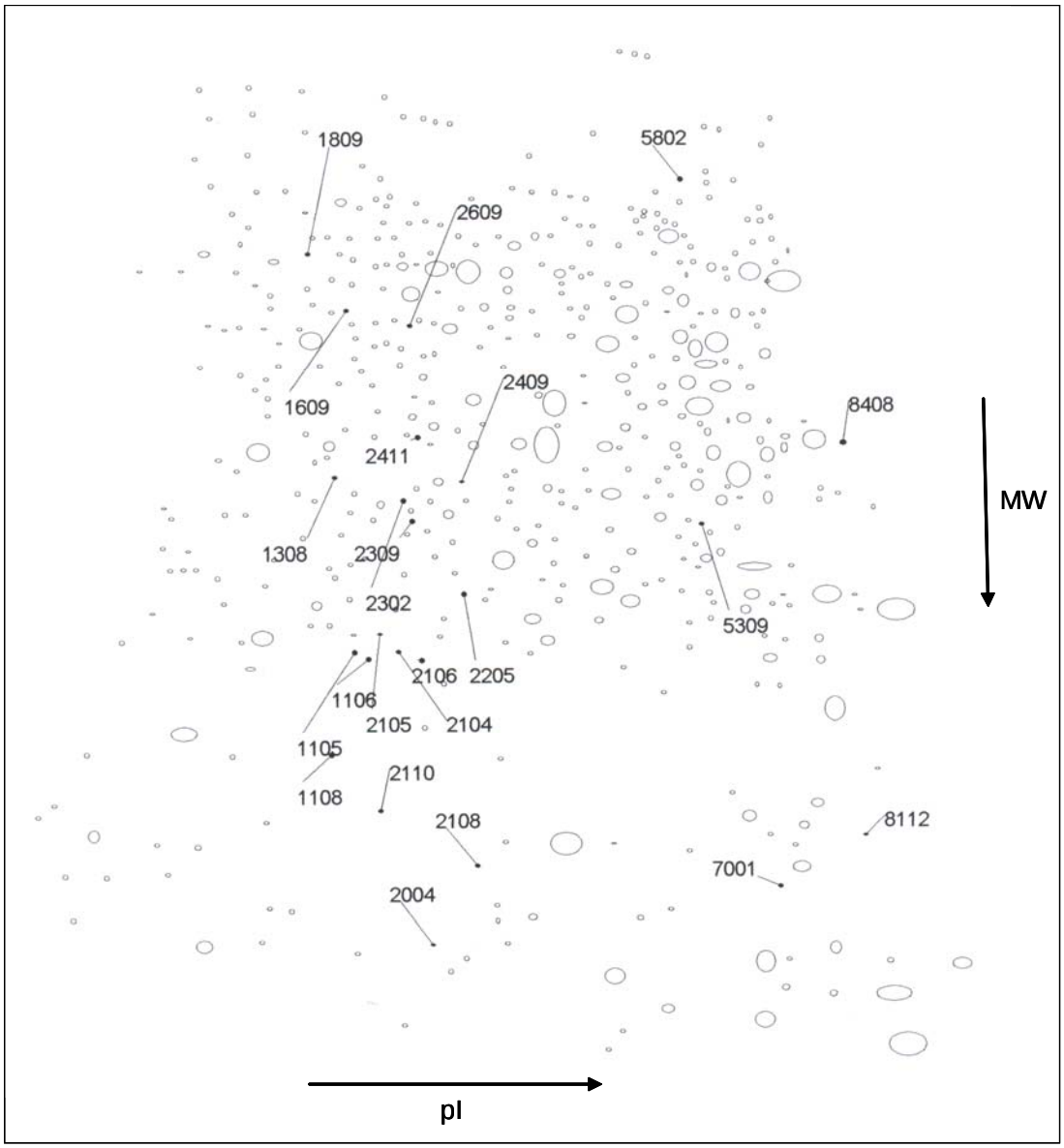


Figure 3.29: Master image of statistical significant differences detected for the protein extracts from the liver.

Four spots were identified that were up regulated in the low dose group when compared to the control group of the liver. No down regulated spots were found for the low dose compared to the control group.

Up regulation

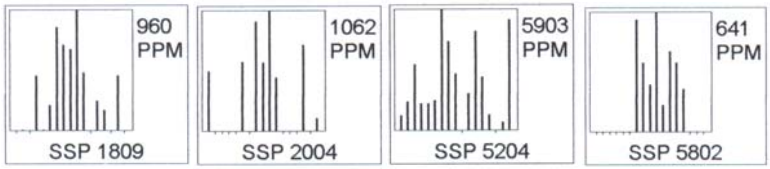


Figure 3.30: Statistical modulation of the low dose group compared to the control control group of the liver extracts.

Eight spots were up regulated and only one down regulated for the high dose group when compared to the control group.

Up regulation

Down regulation

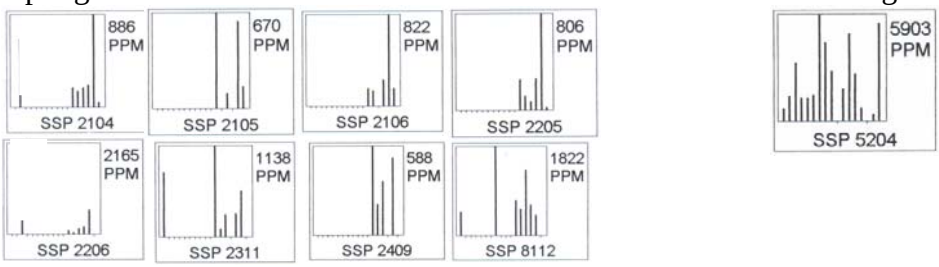


Figure 3.31: Statistical modulation of the high dose group compared to the control group of the liver extracts.

Sixteen spots were up regulated and six were down regulated in the high dose group when it was compared to the low dose group of the liver protein extract gels.

Up regulated

Down regulated

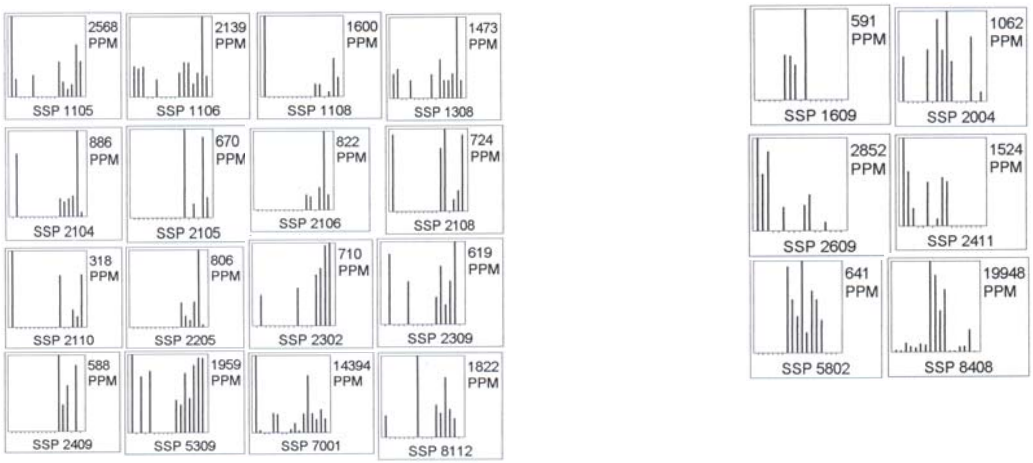


Figure 3.32: Statistical modulation of the high dose group compared to the low dose group of the liver extracts.

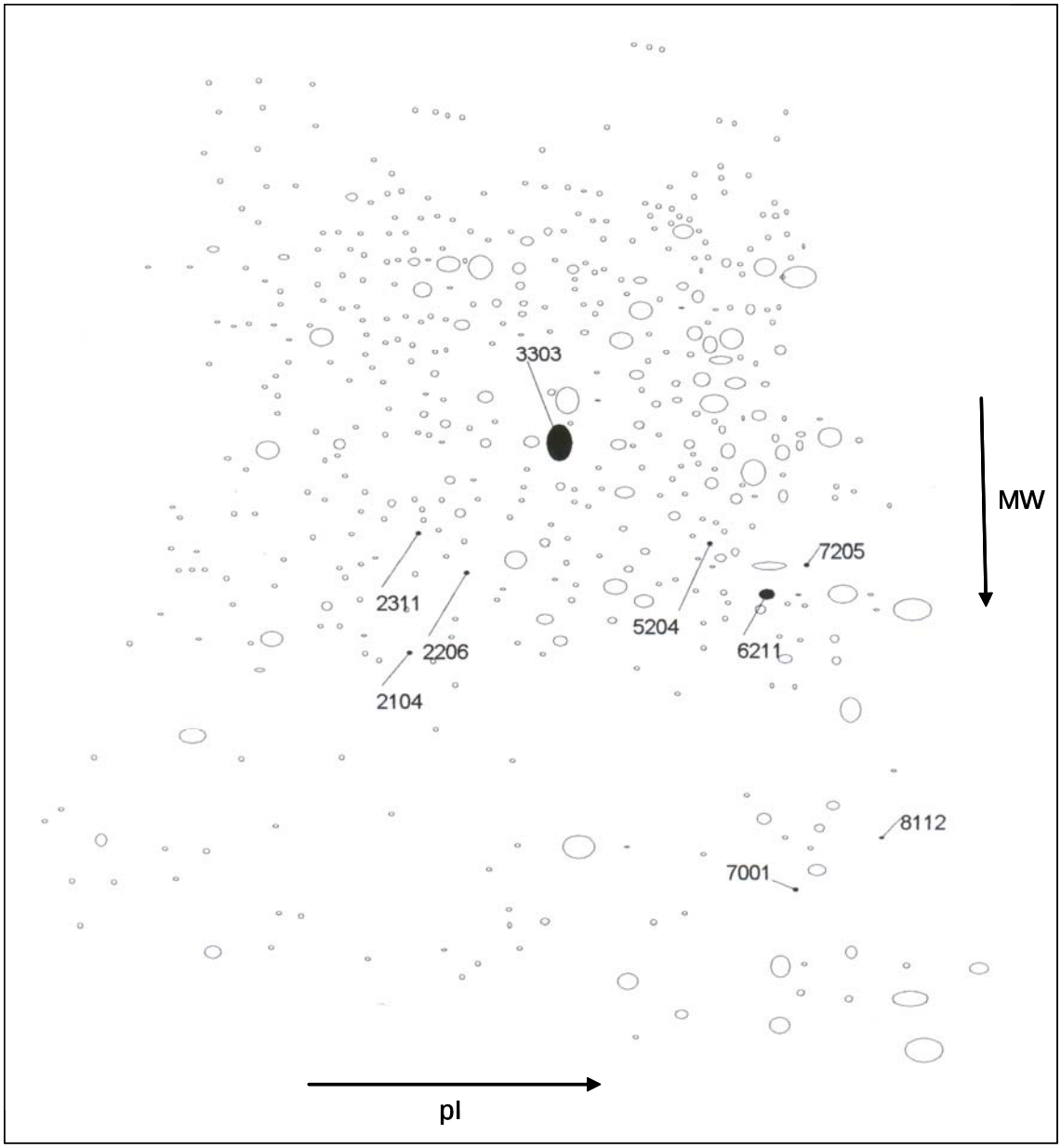


Figure 3.33: Master image at least two times regulation between the different groups of the protein extracts from the liver.

For the low dose group three spots were up regulated at least two times when compared to the control group.

Up regulation

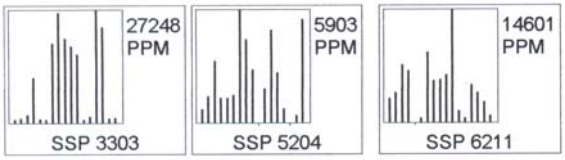


Figure 3.34: Modulation (2x) of the low dose group as compared to the control group of the liver extracts.

Only one spot was up regulated by the high dose group when it was compared to the control group.

Up regulation

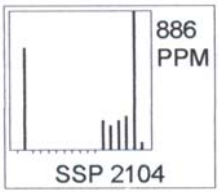
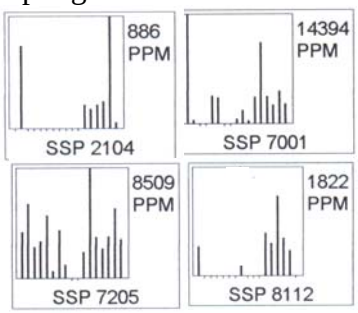


Figure 3.35: Modulation (2x) of the high dose group as compared to the control group of the liver extracts.

Four spots were up regulated and three spots were down regulated in the high dose group when compared to the low dose group.

Up regulation



Down regulation

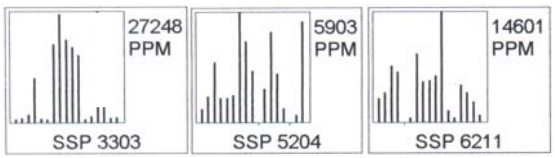


Figure 3.36: Modulation (2x) for the high dose group as compared to the low dose group of the liver extracts.

3.4 Discussion

In chapter 2 the various organs of the mice were harvested and prepared for further analysis. In this chapter the small intestine and liver were subjected to different proteomic separation techniques to try and identify possible regulation that may occur between the proteins of the three different groups. The aim of this study is to identify proteins that may be modulated by tea when compared to control groups by a proteomic approach. At first a simple but easy separation technique was used to separate the proteins of the small intestine and the liver. The proteins of both organs were separated by SDS PAGE gel electrophoresis. For the small intestine three bands were detected by SDS PAGE that showed significant differences between the various groups as determined by Quantity One™ from Bio-Rad. The first band that was detected to have difference was a 66 kDa band. This band seemed to have an extra band for the control and high dose group when they were compared to the low dose group. It could thus be that the low dose protein showed significant down regulation, or it could be that the high dose group and the control have some novel protein that is not present within the low dose group. Probably the absence of the band is due to down regulation of the band by the low dose tea. This could be because the small intestine of the high dose group had an increased weight when compared to the other groups (see chapter 2). The second band that was regulated was a 45 kDa band that had significantly lower expression in the low dose and high dose groups when compared to the control. The third band detected to have significant regulation was a 10 kDa band. This band was up regulated in the low dose group.

For the liver the same approach was followed as for the small intestine. Separation of the liver proteins of the three groups by SDS PAGE yielded three bands that were significantly different between the three groups. The first band is a 110 kDa band. This large protein was down regulated in the low dose group. The second significant band that was detected was a 66 kDa band. This band had increased expression of the protein for the low dose group when compared to the control and high dose groups. In protein extracts from the small intestine a 66 kDa band was also detected to be regulated. But for the small intestine down regulation occurred for the low dose group which is in contrast to the liver 66 kDa band that were up regulated. The probability of these two bands of the small intestine and the liver being the same is very small. One would expect regulation of specific protein to

be the same independently of the tissue. The third band that was detected in the liver was the most prominent band of the low dose. This 14 kDa band had increased expression in the low dose, and reduced expression in the high dose group when compared to the control. Various proteins with a molecular mass of about 14 kDa are known that might be regulated by tea. Two of these proteins are cytochrome b5 that has a molecular weight of 15 110 Da, the other protein may be superoxide dismutase [CuZnSOD] that has a molecular weight of 14 537 Da. Cytochrome b5 is a membrane bound hemoprotein that function as an electron carrier for several membrane bound oxygenases. It is also involved in several steps of the sterol biosynthesis pathway, and plays a role in the cytochrome P450 pathway. In the CYP pathway cytochrome b5 is able to transfer electrons to the CYP's. Therefore up regulation of the CYP pathway will result in an increase of cytochrome b5. SOD plays an important role in protecting living cells against toxicity and mutagenicity of reactive O_2 species by scavenging O^-_2. Oxidative stress can cause lipid peroxidation and reactive oxygen species will also cause DNA damage and damage to proteins. It was found that EGCg increased both the activity and gene expression of super oxide dismutase (SOD) (Kim et al., 2002). Because super oxide dismutase is known to eliminate excess oxygen radicals that are produced, an increase in SOD by EGCg would exert a protective effect on the cell, and we would thus expect an increase in the protein expression of our gels. To determine the identity of the 66 kDa band and the 14 kDa band of the liver, these two bands were subjected to HPLC analysis to determine how many proteins are present within each single band. During the HPLC analysis of the two bands of the liver two important controls were used. The one control was water only being injected and the other control was an extract from a blank piece of gel. This negative gel control was handled as the normal sample during sample preparation. This is used to determine if contaminants are present within the gel and what the influence of the polyacrylamide will be on the HPLC chromatogram. The HPLC analysis of the 66 kDa band of the liver showed that only one protein was present within this band as only one protein was detected on the HPLC. From the HPLC of the 66 kDa band it was determined that the low dose group had higher peak intensity than that of the high dose and control groups. This is similar to the results of the SDS PAGE gels that determined that the 66 kDa band was up regulated in the low dose group. This confirms that there is probably only one protein present in the 66 kDa band and that this protein is up regulated by the low dose group. Two different peaks were identified during HPLC for the 14 kDa band, thus indicating probably the presence of two proteins within the single band. The first peak that was identified was at 39 minutes. This peak has increased

expression of the low dose group as the peak intensity of the low dose group was higher than any of the other two groups. A second peak at 52 minutes was identified that also had increased peak intensity in the low dose group. The high dose group had much lower peak intensity than that of the control for the 52 minute protein. Once again this is similar to the results found by SDS PAGE. After the confirmation of the HPLC and SDS PAGE the 14 kDa band was subjected to MALDI TOF MS. The peptide mass fingerprints were subjected to database searching. Protein Prospector was used as the search engine using the NCBI database. Protein Prospector allows the user to specify the number of proteins present within a peptide mass finger print. This parameter was specified as two for the 14 kDa band. The band was digested with trypsin and also treated with DTT and iodoacetamide and thus fixed modifications were set to carbamidomethyl. The protein mixture was identified by the program as hypothetical protein XP_358319 (Accession number XP_358319) with a molecular weight of 15 190 Da and immunoglobulin α chain (Accession number AAA38305) with a molecular weight of 13 140 Da. This was not expected. Even though the molecular weights of the two identified proteins match that of the 14 kDa band the identification of these proteins will need to be confirmed by LC MS.

Tricine SDS PAGE gel electrophoresis is a gel separation technique that is used to detect extremely small proteins and peptides that may get lost on normal SDS PAGE. The proteins of the liver and small intestine were prepared according to the method of Schagger and von Jagow, (1987) and then subjected to tricine SDS PAGE. No significant differences were detected for the small intestine or the liver protein extracts.

SDS PAGE of the protein extracts from the small intestine and liver was done and only three bands for each of the organs were found that were modulated. SDS PAGE has limited separating power as the proteins are only separated according to size. And as has been demonstrated by the 14 kDa band of the liver extracts, it is possible that more than one protein can exist in one single band on the gel. To increase separating power, two dimensional gel electrophoresis (2DE) was done for the small intestine and the liver protein extracts. The proteins are thus now separated according to their iso-electric point (pI), and according to size. For the small intestine protein extracts sixteen 2DE gels were ran in total. These gels were then analyzed using PD Quest Software from Bio-Rad. A master image was put together of all the gels. From the master image the differences between the various dosage groups could be determined. Two different parameters were

used for the determination of spots that were regulated. The first was the identification of spots that show statistically significant ($p<0.05$) differences between the three groups of gels as determent by the program. The second identification step was for spots that were regulated at least two fold or more between the three groups. For the small intestine protein extracts a total of 22 spots were significantly different between the three groups, while 27 spots were determined to have at least two times regulation between the groups.

For the liver protein extracts the 2DE sample preparation first had to be optimized. A standard sample preparation technique was used for the first runs of the 2DE gels. The first step of 30V was added to ensure that high molecular weight proteins enter the IPG strip and mainly to remove salt contaminants that may cause streaking and increase the conductivity of the IPG strip. This can create a problem as the IPG strip may spark, or it may increase focusing times. The focusing voltages were increased gradually over time. The final step used a maximum of 8000 V until the IPG strip reaches a total of 17 000 Vhrs. This is to ensure that all the proteins within the IPG strip have reached their isoelectrical points (pI) and stay focused at this particular point. By controlling the number of volt hours between the gels reproducibility between gels was also increased. By these modifications the number of spots on the silver stained 2DE gels increased from about 300 with the first method to about 600 spots detected using the second method. Once the sample preparation procedure was optimized for the liver the gels could be run for reproducible results. In total seventeen gels were run with liver extracts. From these 17 gels a master image was prepared in a similar way as for extracts of the small intestine. From the master image it was determined that a total of 35 spots were significantly different between the three groups. Eleven spots were determined to be regulated at least two times between the three groups.

Some of these spots were identified to determine their identity and function in the next chapter.

CHAPTER 4

Mass spectrometric and bioinformatic identification of proteins extracted from the liver and small intestine

4.1 Introduction

Proteomics not only consists of various separation techniques, but also depends on successful identification of individual proteins. During the last decade with the big "omics" boom a vast number of regulated proteins were found. Just like separation techniques in proteomics have expanded exponentially, so have identification techniques. Mass spectrometry has exploded onto the proteomics scene. Previously Edman degradation was used to sequence proteins. Edman degradation has a limited use these days because of its inability to analyze peptides that lack free N-termini. These days de novo sequencing is used for proteins by fragmenting peptides by MS. Mass spectrometry (MS) offers sensitivity and speed for the identification of proteins and is currently the method of choice for post gel identification of proteins. MS was developed by Sir J.J. Thomson in the early 1900s, when he obtained the mass spectra of small gaseous ions. The principle of MS relies on the measurement of the mass-to-charge ratio (m/z) of gas phase ions. MS offers a new dimension to determining protein molecular weights. SDS PAGE can determine mass accuracy up to 5 to 10% of the real molecular weight, but MS has the ability to produce mass accuracy of ppm and ppb (Jonsson, 2001). MS is a widespread technique with key functions in a number of fields including biochemistry, biotechnology, pharmacology, microbiology, organic chemistry and the proteomics and functional genomics field.

Matrix assisted laser desorption/ionization mass spectrometry (MALDI MS) was first introduced in the late 1980s by F. Hillenkamp and his group. MALDI MS is a "soft" ionization technique and is therefore compatible with large biomolecules like proteins. This "soft" ionization method allows these large molecules to be transferred to the gas phase without fragmentation. The analytes are mixed with a saturated solution of ultra-violet- absorbing matrix. Most common matrices that are used are α-cyano-4-hydroxy-cinnamic acid (HCCA) for small peptides and 3,5-dimethoxy-4-hydroxy-cinnamic acid

(sinapic acid, SA) is used for larger peptides. The matrix and analytes are mixed and then applied on a target plate. The solvent evaporates and the matrix and the analytes co-crystalize on the target plate. A laser beam (usually nitrogen at 337nm) provides the light that is absorbed by the aromatic matrix molecules. Energy is subsequently transferred to the analyte that becomes desorbed into the gas phase (Jonsson, 2001). MALDI is usually coupled to a Time of flight (TOF) mass analyzer, which is a high-speed mass analyzer, but has limited resolution. The principle of TOF is to measure the time of flight of ions accelerated out of the ion source into a field free drift tube to a detector, ions will then separate according to their m/z ratio. Light ions will arrive at the detector first and heavy ions later. MALDI TOF MS is an instrument of choice and is most commonly used with 2DE for identification; this is mainly due to the fact that MALDI TOF MS usually produces singly charged ions and is thus good for the analysis of complex biological mixtures such as protein digests.

Advantages of MALDI TOF MS are: ease of operation, rapid results, produces mostly singly charged ions $(M + H)^+$, the mass range of the analytes are between 500 Da and 300000 Da, it has good resolution, has accurate mass measurements when an internal standard is used, good sensitivity for peptides (< 10fmol), is mainly used for the specific analysis of peptides and proteins, low purchasing cost, and reasonable running costs. MALDI MS is also able to provide information on the molecular weight of a protein with high mass accuracy, and identify protein modifications (Schwartz, et al., 2003). MALDI is less sensitive to salts than some of the other available mass spectrometers, but salt contamination will still cause peak broadening with the formation of adducts. This will lower sensitivity and mass accuracy. Another disadvantage is that the interpretation of data requires a degree of expertise (Jonsson, 2001). Factors that influence MALDI response is peptide polarity and basicity, as highly acidic peptides often yield a poor response in MALDI (Cohen and Chait, 1996). Sensitivity of MALDI depends on the chemical characteristics of the sample, sample preparation procedures followed, the amount of sample loaded, the desorption/ionization method used, and the mass analyzer that is used (Schwartz, et al., 2003).

An improvement on MALDI-TOF MS is the introduction of a reflectron MALDI-TOF MS. The ions within the MALDI move at different kinetic energies. By reflecting the ions back

onto their path, ions with the same kinetic energies will move together. If an ion has a high kinetic energy it will penetrate the reflectron deeper and thus take a longer time to reach the mass analyzer. On the other hand ions with low kinetic energies will reflect faster as it will not penetrate the reflectron that deeply and thus reach the mass analyzer faster. A reflectron MALDI-TOF MS has the advantage of improved resolution.

Surface enhanced laser desorption/ionization time of flight mass spectrometry (SELDI TOF MS) is used for protein profiling and biomarker identification. The purpose of SELDI is to determine various interactions and then use MS to identify these components. For SELDI TOF MS chemically modified MALDI target plates are used to retain a group of proteins. Examples of these target plate coating interactions are: hydrophobic interactions, receptor-ligand interactions, DNA-protein interactions, and antibody-antigen interactions.

Wax coated MALDI target plates have recently been suggested by (Terry, et al., 2004) to improve the quality of mass spectra. His group suggested that by using a wax coated target plate this will enable the recovery of the tryptic peptides from the MALDI plate for further LC/MS/MS to obtain the amino acid sequence. Direct proteomic analysis of tissue sections is also emerging. (Schwartz, et al., 2003) published a method for sample preparation to allow fast and reliable tissue analysis. By using this technique signals can be localized to a specific tissue region.

Another soft ionization method is electrospray ionization mass spectrometry (ESI MS). ESI MS is an atmospheric pressure ionization method that produces small charged droplets from a liquid medium under the influence of an electric field. It is ideal for combining chromatographic methods with MS. The flow of liquid is passed through a thin conducting needle at a high voltage (3-4 kV). The mist then breaks up into small highly charged droplets as they are moving towards the counter electrode. Both a potential and pressure gradient will direct the charged droplets toward the inlet of the MS (Jonsson, 2001). ESI MS has good ion transmission and produces multiply charged ions that are ideal for molecular weight determinations.

Nanospray has evolved from electrospray. For nanospray MS the sample is sprayed from a metal coated capillary with an opening of 1-10 μm. This results in a flow rate of 20 -50 nl/min, and thus small sample volumes are used. An added advantage is that nanospray MS

has higher ionization efficiency than a conventional electrospray source due to production of smaller droplets. Nanospray MS is also less sensitive to salts than ESI MS.

Liquid chromatography mass spectrometry (LC MS) has the advantage that it is able to separate peptides with the same mass but different polarities, therefore there will be a gain in sensitivity, and all the processes can be automated. MudPIT may replace 2DE. The first LC separation step is usually ion exchange chromatography to separate the peptides on charge. The fractions will then be desalted on a short trap column, and then proceed to be analyzed with C18-RP column to separate the peptides on hydrophobicity and size. After separation the peptides will directly be injected into the MS for identification. Complex mixtures can use three dimensional LC separations by first being separated by affinity chromatography, then ion exchange followed by reverse phase chromatography before being identified by MS. Isotope coded affinity tag (ICAT) is a quantitative method, which uses a cysteine modification with an isotope labeled biotin tag. ICAT makes use of the incorporation of hydrogen (H) or deuterium (D) ions into the peptide. ICAT can also be modified to incorporate nitrogen isotopes like ^{15}N into the sample medium and ^{14}N for the control medium. These nitrogen isotopes will then be taken up by the organism. Another method is by the use of ^{18}O and ^{16}O. Two oxygens will be incorporated into the carboxyl termini of all tryptic digests, this will cause a difference of 4Da when analyzed by MS.

MALDI-TOF-TOF MS aim to produce tandem MS with high resolution and mass accuracy while maintaining the same speed and mass range of MALDI. To date MALDI TOF-TOF has poor sensitivity for high mass product ions, this thus limits its ability to provide complete sequence information.

MALDI Q-TOF MS has a broad application in protein research. It combines the high speed and throughput of MALDI peptide mass fingerprinting with high mass accuracy and excellent resolving power in MS and MS/MS functions. A Q-TOF provides the advantage of tandem mass spectrometry to identify peptides by de novo sequencing, a peptide sequence tag can be used and post translational modifications can be identified. A peptide can thus be identified by its peptide mass fingerprint (PMF) or MS/MS spectra generated by Collision induced dissociation (CID) of the precursor ions. Collision induced dissociation (CID) occurs when the cell contains an inert gas into which the precursor ions

which has been selected by the first mass analyzer, collides and fragments. An added advantage is that both PMF and MS/MS can be carried out on the same target plate and the sample will not be destroyed within minutes as it stays on the target plate.

Fourier transform ion cyclotron resonance mass spectrometry (FTICR MS) is probably the most sensitive MS technique to date. It offers high mass accuracy, high mass resolution, and has MS/MS capabilities. It can detect peptides in the sub femtomole region up to a limit of about 10 amole. At the same time it also offers resolution of up to 0.1 ppm (Martin, et al., 2000).

The genes are the starting points within the cell for protein expression. Information contained within the genes is able to predict what can happen within a cell, while the mRNA predicts what might happen. Translation predicts what is likely to happen within the cell but only after proteins have undergone post translational modifications can there be predicted what may actually happen in the cell. Post translational modifications (PTM) is very important in modern protein chemistry as PTM's determine the function of all proteins. More than three hundred post translational modifications are already known and the number of PTMs is still on the rise as new PTMs are still being discovered.

PTM mapping can be done by four different approaches (Mann and Jensen, 2003). These are: 2DE, affinity based enrichment of modified proteins like immunoprecipitation combined with MS, identification of PTMs in complex mixtures by LC-MS/MS, and derivatization and affinity based methods like IMAC. Phosphorylation is one of the well known modifications that occurs in proteins. Phosphorylation may function as a molecular switch in functions like enzyme regulation, signaling processes and subcellular localization (Reinders, et al., 2004). Phosphorylation is easier to analyze than glycosylastion due to its simple structure. There are four major types of phosphorylation that can be identified by MS. The first is O-phosphates that occur on serine, threonine, and tyrosine residues, they are stable under acidic conditions. Second is N-phosphates (arginine, lysine, and histidine), they are stable under alkaline conditions, third is acylphosphates (aspartic acid, and glutamic acid), they are very reactive and labile in acid as well as alkali. Last is S-phosphates (cysteine), they have moderate stability under acidic and alkali conditions (Reinders, et al., 2004). A common technique to isolate phosphopeptides is by immunoprecipitation with phosphospecific antibodies. Another approach is by immobilized metal affinity chromatography (IMAC). The use of IMAC was first proposed in 2000 as Zip™ tips, and is used to selectively purify phosphopeptides that bind to the

chelated trivalent metal ions. This method was not selective enough because carboxyl (COO^-) groups from the carboxy termini bound to the tips. This was also true for negatively charged amino acids like glutamic acid and aspartic acid that were able to bind non-specifically to the tips. A modification by *Ficarro et al., (2002),* solved the problem. He proposed that after the tryptic digestion of the peptides that the carboxyl groups should be converted to methyl esters with methanolic hydrochloric acid. The phosphate groups will retain their negative charge and thus bind to the IMAC tip. The phosphorylated peptides can be identified by a mass shift of 98Da and 80Da for phosphoric and phosphorous acid that are attached to the peptides.

Glycosylation is the addition of sugar moieties to the polypeptide backbone. Glycosylation is present in the cell in the cytoplasm, the inner subcellular compartments and the extracellular matrices. The most analyzed form of glycosylation in eukaryotes is N-linked glycosylation. N-linked glycosylation forms β bonds between the N-acetylglucosamine of the reducing glycan termini and the amino groups of asparagines side chains (Reinders, et al., 2004). Glycopeptides are difficult to study because of their bulky structures and due to the fact that they ionize poorly in MS. LC-MS is usually a better approach to identify glycopeptides.

Biological data is currently being produced at a phenomenal rate. The use of computers is of extreme importance in biology to manage data, and this is usually done by the use of bioinformatics. Bioinformatics is defined as the application of computational techniques to understand and organize the information associated with biological macromolecules (Luscombe, et al., 2001).

The aims of bioinformatics are (Luscombe, et al., 2001):

1. Organize data and make the data accessible as well as allow the addition of new entries.
2. Develop tools and resources that aid in the analysis of the data being generated.
3. Use tools to analyze the data and interpret the results in a biological meaningful manner.

The primary sources of data for bioinformatics come from DNA or protein sequences, macromolecular structures, and the results of genomics experiments. Bioinformatics should

be able to integrate multiple sources, manage large volumes of data, group together meaningful similarities and identify the related ones. Computational methods must also be combined with good statistical analysis for objective analysis (Luscombe, et al., 2001).

For mass spectrometry the generation of data is sometimes easy. A huge bottleneck during MS is identification of the protein from PMF, or MS/MS data obtained, and to interpret the data. Usually databases are used to identify the proteins, but search algorithms can only identify known proteins within the database. Search parameters may have an influence on successful identification and must thus be applied with caution and knowledge and usually benefit from experience. Another problem is that all the algorithms in databases are heuristically based, meaning that they do not produce the perfect answer every time, they only provide the best fit. The choice of the database used also has an impact on the results, as data input differ between databases, scoring functions differ, and parameters that are excepted differ.

Factors that are important for successful identification include (Baxevanis and Ouellette, 2005):

- What is the mass accuracy expected from the MS used?
- What modifications have been introduced?
- Are contaminants present?
- Are multiple proteins present?
- What is the charge and isotopic state of the data?
- Was the signal to noise ratio good?

The answers to these questions must be known to adapt parameters for successful identification of the proteins.

A popular search engine used for PMF, MS/MS or partial amino acid sequence searches is the MASCOT search engine that was developed and maintained by Matrix Science. Mascot provides a choice of databases that include Swiss-Prot, NCBI, and MSDB. Mascot provides flexible searching with several functions that can be used separately or in combination. It makes use of the MOWSE (molecular weight search) scoring algorithm to report if an identified protein is significant ($p<0.05$) or not. This score will also depend on the amount and quality of the input data as well as the amount of proteins in the specified database. The standard parameters provided by the program include: a choice of the enzyme used, number of missed cleavages expected, search tolerance and the database to

be searched. Fixed modifications are also available. A fixed modification is used when every residue should have a modification, like cysteine modifications to prevent disulfide bridges.

PepIdent and ALdente are available from the ExPASy server that is maintained by the Swiss Institute of Bioinformatics (SIB). These search engines compare experimental peptide masses with theoretical masses generated from proteins in the Swiss-Prot or TrEMBL databases. Various parameters can be chosen by the user these include: enzyme used, number of missed cleavages, modifications, and type of mass (average or monoisotopic). The scoring function used by this program is simply the ratio of the theoretical peptides matched to the protein to the total number of peptide masses submitted. The strength of the result and the percentage coverage is important, as well as the difference between the first and second hits. The results include a summary of the search parameters that include basic information on the proteins matched, the Swiss-Prot/TrEMBL accession number, the protein sequence, matched modifications, percent coverage, and the score.

Other protein identification tools using MS data include ProFound, PeptideSearch, Sherpa, MassSearch, *Pep*Mapper and Lutefisk.

The aim of this chapter is to demonstrate how mass spectrometry and various bioinformatic techniques have been used to successfully identify the regulated proteins in the liver and the small intestine and to speculate on the function that these proteins may have on health. It is hoped that some of these identified proteins may play a role in cancer chemoprevention.

4.2 Materials and Methods

4.2.1 Materials

All materials were of analytical grade and double distilled deionised water (Millipore system Q, Millipore, USA) was used in all experiments. α-cyano-4-hydroxycinnamic acid was obtained from Sigma Chemical Company (St Louis, MO, USA). Ammonium bicarbonate was obtained from Merck (Darmstadt, Germany). Iodoacetamide (IAA), was obtained from Fluka. CHAPS, and 1, 4 dithiothreitol (DTT) and trypsin (bovine pancreas, sequencing grade) were obtained from Roche Diagnostics GmbH (Mannheim, Germany).

4.2.2 Sample preparation of MS samples from 2 D gels

Gloves were worn during all sample preparation procedure and great care was taken to avoid keratin contamination. Gels were washed twice with MilliQ water. The appropriate bands were then cut from the gels using a clean sterile scalpel. Each specific band was then further cut into 1mm^3 cubes and placed into a clean microcentrifuge tube. The gel pieces were washed with 100 µl water:acetonitrile (1:1) for 15 minutes and repeated twice. The liquid was removed by pipette and replaced with 100 µl acetonitrile. The acetonitrile was left for about 5 minutes until the gel pieces have shrunk and appeared white. The acetonitrile was then removed and replaced with 100 µl 0.1 M ammonium bicarbonate. After 5 minutes an equal volume of acetonitrile was added to the ammonium bicarbonate and left to incubate for 15 minutes. All the liquid was then removed and the gel pieces were dried in a vacuum centrifuge (SpeedyVac). Once the gel pieces were dried the gel pieces were reswollen in 100 µl of 10 mM dithiothreitol (DTT) and left to incubate for 45 minutes at 56ºC. The gel pieces were then left to cool to room temperature and the liquid was removed. This was then quickly replaced with 100 µl of 55 mM iodoacetamide (IAA), 0.1 M ammonium bicarbonate and incubated in the dark at room temperature for 30 minutes. The liquid was removed and the gel pieces were washed with acetonitrile and left to shrink until the gel pieces appeared white. Acetonitrile was removed and replaced with 100 µl of 0.1 M ammonium bicarbonate and left to incubate for 5 minutes. An equal volume of acetonitrile was added and left to incubate for 15 minutes. All the liquid was then removed and the gel pieces were dried in a vacuum centrifuge (SpeedyVac). Cold

digestion buffer containing 50 mM ammonium bicarbonate, 5 mM calcium chloride and 12.5 ng/µl trypsin sequencing grade (Roche Applied Sciences, Mannheim, Germany) was added to the dried gel pieces and left to incubate for 45 minutes on ice. The supernatant was then removed and replaced with 20 µl of digestion buffer but without trypsin (50 mM ammonium bicarbonate, 5mM calcium chloride). The gel pieces were incubated at 37ºC overnight. Twenty-five microlitres of 25 mM ammonium bicarbonate was added to the dried gel pieces and left to incubate for 15 minutes. The same amount of acetonitrile was then added and left to incubate for 15 minutes. The supernatant was then recovered and placed in a clean microcentrifuge tube. The extraction of the supernatant was repeated twice with 0.1 % TFA, 50% acetonitrile. All the extracts were pooled and then dried in a vacuum centrifuge (SpeedyVac). For MALDI-TOF MS the dried peptides were redissolved in 10 µl of 0.1% TFA, 50% acetonitrile. This was then sonicated briefly. Then 1 µl of sample was mixed with 1 µl of a solution containing 5mg/ml α-cyano-4-hydroxycinnamic acid, 50% acetonitrile, 0.1% TFA. Two microlitre (2 µl) was spotted onto the MALDI-TOF sample plate and left to dry. The peptide mixtures were analyzed by a Matrix assisted laser desorption/ionization time of flight mass spectrometer (Bruker MALDI-TOF MS) Peak analysis was done using Bruker Autoflex software. The peptide mass fingerprints were submitted to databases like Mascot (www.matrixscience.com) and Aldente (www.expasy.com) for protein identification from the peptide mass fingerprints.

4.3 Results

In chapter 3 the statistically significant and two times regulated spots were determined for the liver and small intestine by using PD Quest™ Software from Bio-Rad. For the small intestine the following spots shown in Figure 4.1 were excised for MALDI TOF MS and identification.

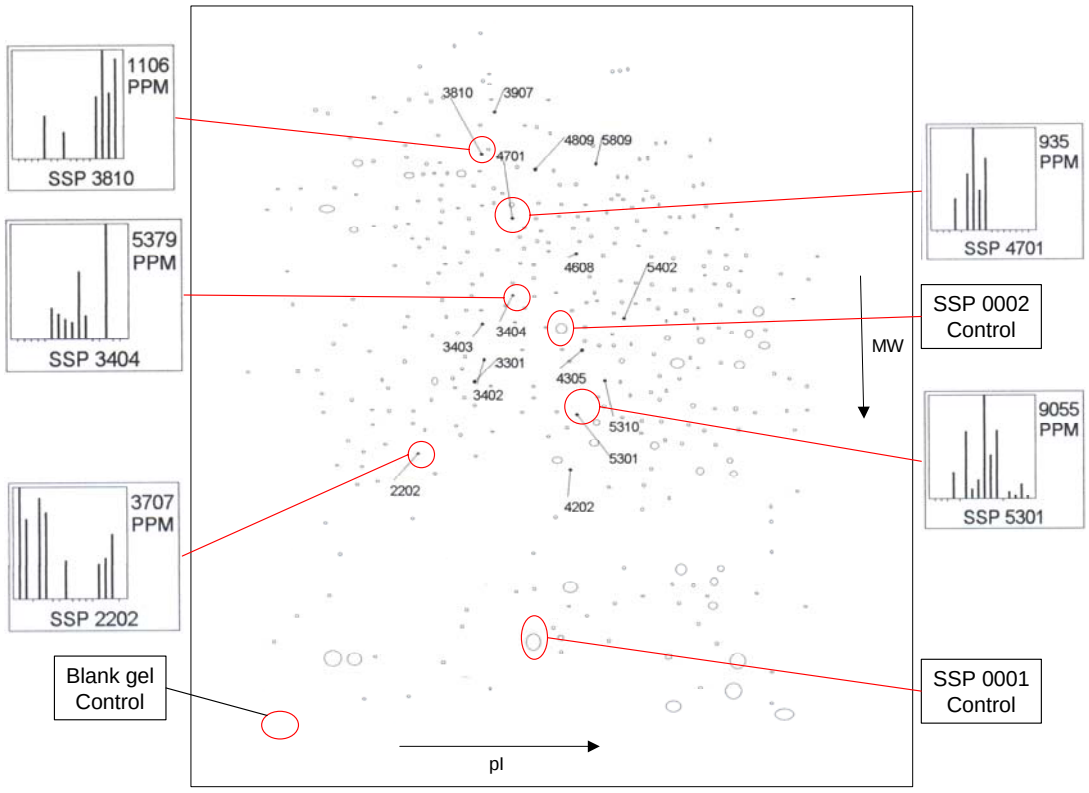


Figure 4.1: Spots excised from the small intestinal protein extract gels for MS identification.

The Figure 4.2 below shows the spots that were excised for the liver and subjected to MALDI-TOF MS for identification.

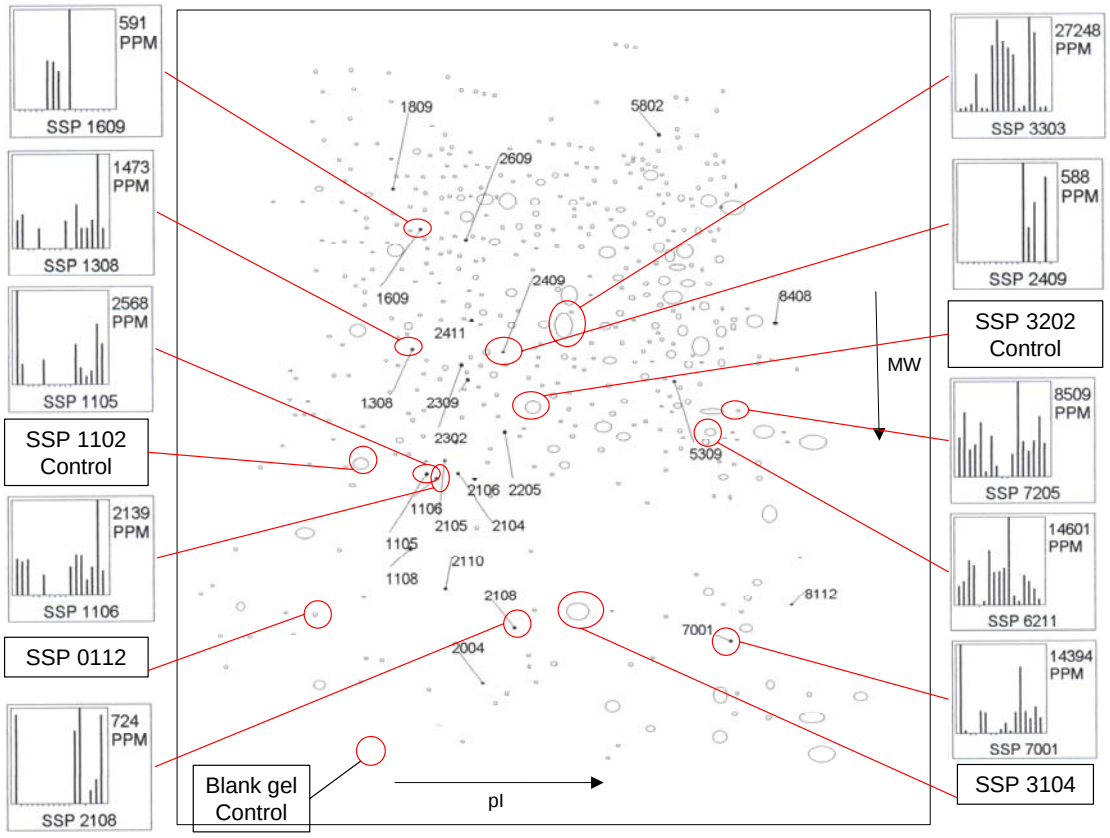


Figure 4.2: Spots excised from the liver protein extract gels for MS identification.

After excision of the specified spots the peptide mass fingerprint of each spots was determined by using a MALDI-TOF MS. Figure 4.3 below is for the negative control used during MALDI-TOF MS.

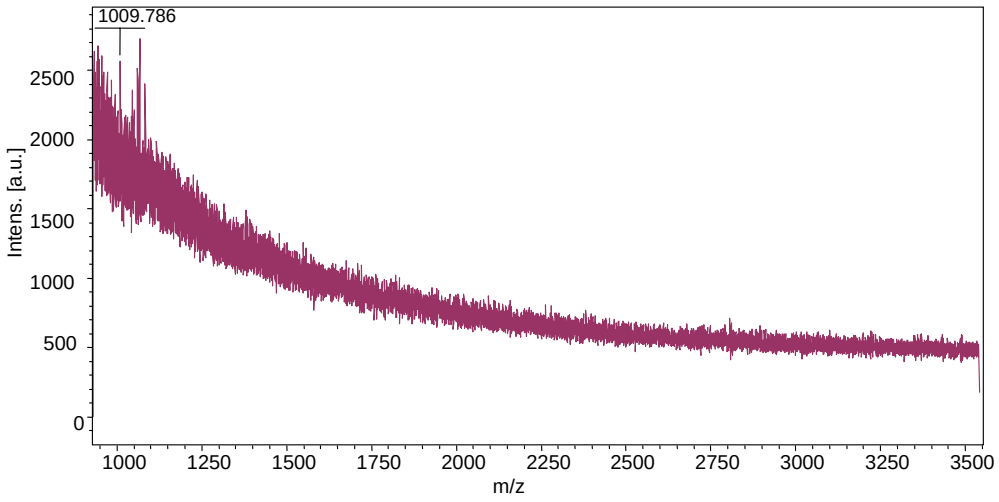


Figure 4.3: The MALDI TOF MS spectra of HCCA matrix only.

This spectra of Figure 4.3 is the negative control and is for the matrix only. The peak intensity is very low with no obvious peaks. This is an indication that little contamination exists. Another blank control was used during the MS determinations. The blank used was a clear piece of polyacrylamide that was cut from the gel where no protein was evident and processed as a regular sample. This is to determine the existence of contaminating products and to determine the self cleavage of trypsin. If trypsin autolyses itself this will be detected in the blank sample.

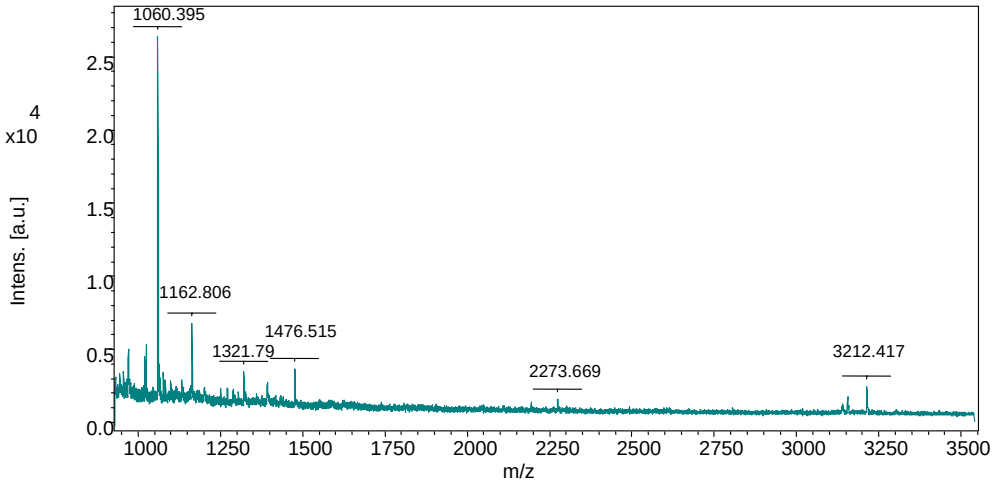


Figure 4.4: MALDI TOF MS spectra of the blank control gel used for MS.

The peak intensity of the blank gel sample shown in Figure 4.4 is very low, which is a good indication that very little contamination does exist. Peak $[M+H]^+$ 2273.669 could be a trypsin autolysis product, while $[M+H]^+$ 1476.515 could be due to skin keratin contamination. Some of the bovine tryptic autolysis products are expected to be $[M+H]^+$ 1020.50, 1111.55, 1153.57, 2193.99 and 2273.15.

For the small intestine extract ten spots were cut from the gel to be identified. For the liver extract twenty spots were cut from the gel to be identified. Some examples of the MS spectra are shown below.

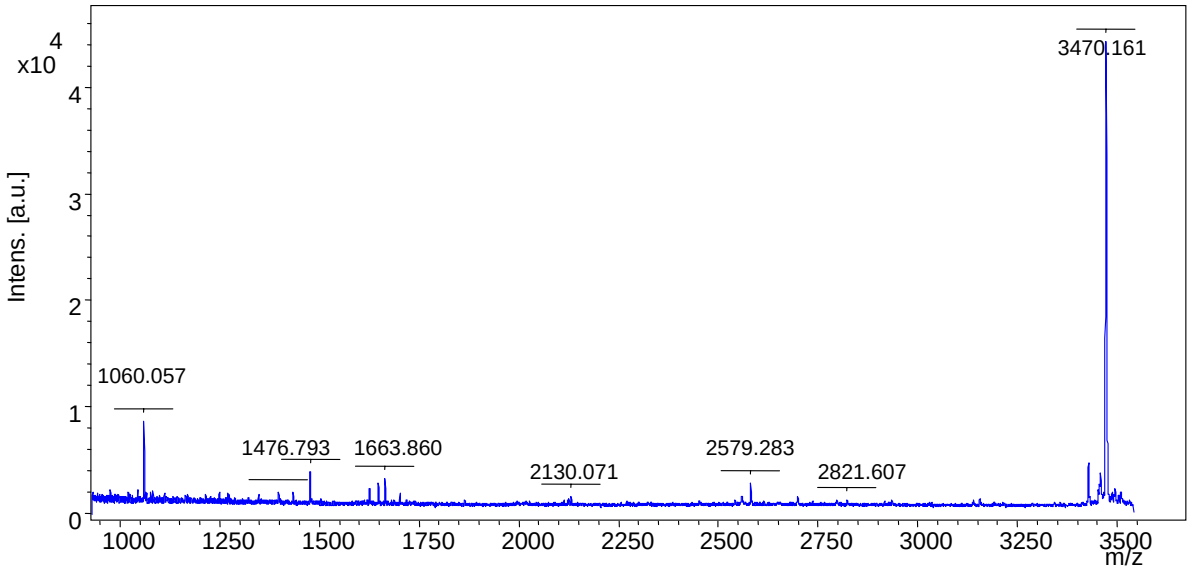


Figure 4.5: MALDI TOF MS spectra for SSP 0001 of the small intestine extract.

The peak intensity obtained for the small intestinal spot; SSP 0001 shown in Figure 4.5 was on the low side. It could be that the spots do contain little protein, or that some of the peptides were lost during sample preparation. The spectra is still good enough for a positive identification. The spectra of Figure 4.6 to Figure 4.8 is from the liver extract spots. The spectra for the liver extract samples show better intensity than that of the small intestine extracts.

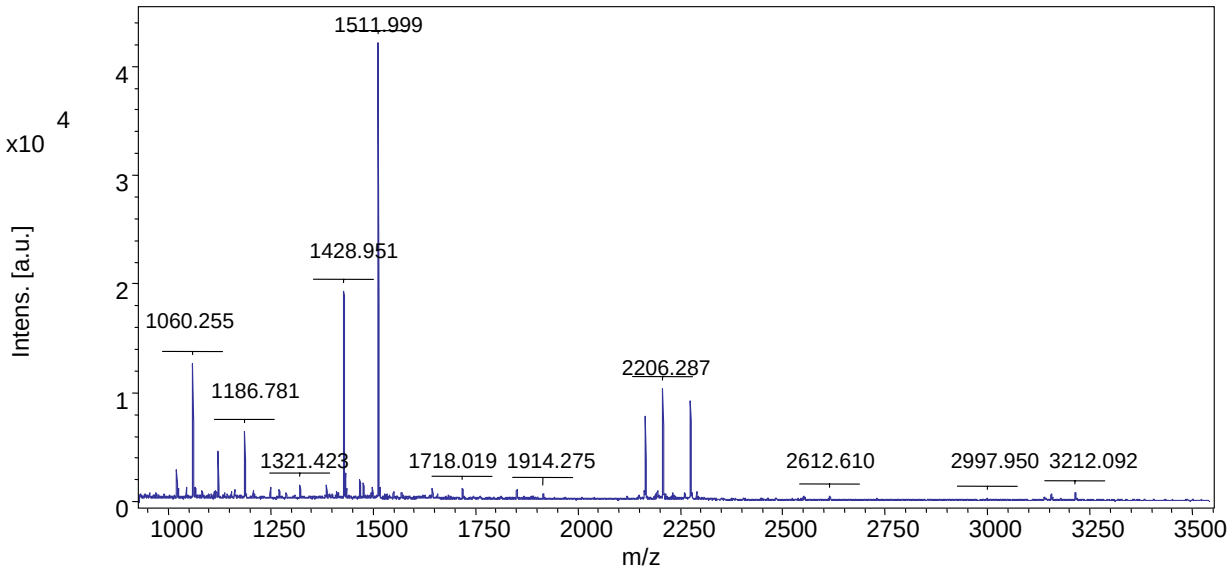


Figure 4.6: MALDI TOF MS spectra of SSP 0112 for the liver extract.

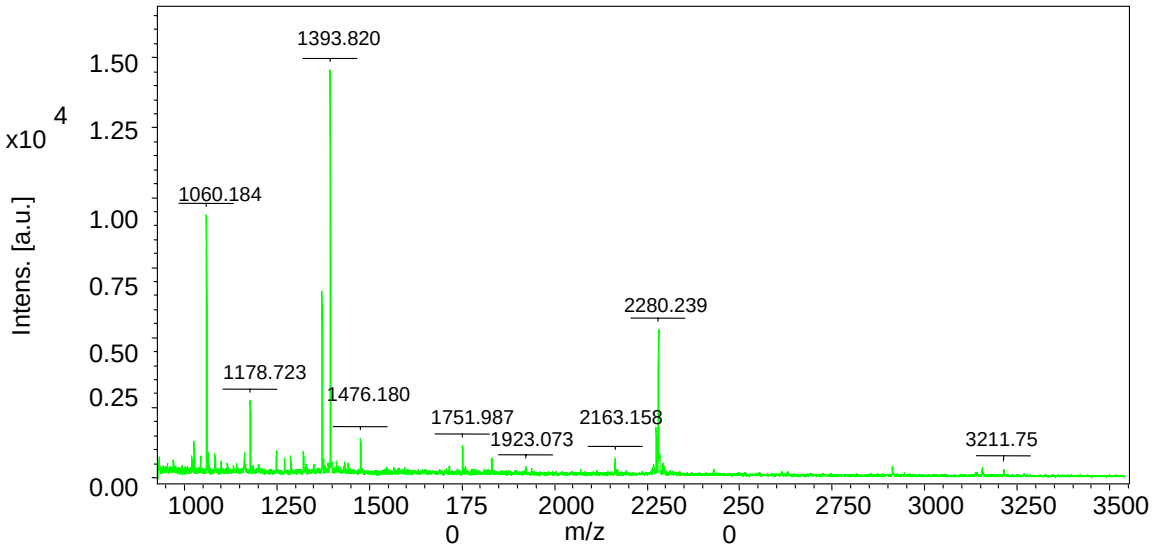


Figure 4.7: MALDI TOF MS spectra of SSP 3303 for the liver extract.

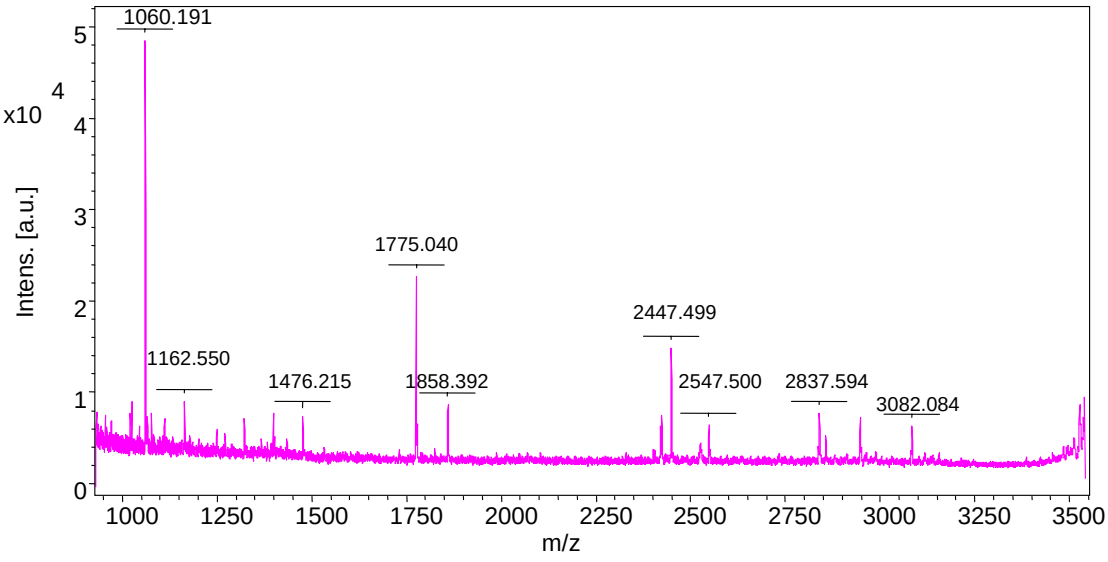


Figure 4.8: MALDI TOF MS spectra of SSP 3202 for the liver extract.

The spectra obtained from the MALDI-TOF MS was subjected to database searching and the following proteins were identified for the small intestine and the liver. Ten small intestine proteins were submitted for database searching. Seven proteins were identified as shown in table 4.1. For the small intestine very few proteins were positively identified, but with a low identification confidence. However for four of the identified proteins, good correlation was observed between the data based on pI and MW and the results obtained in these gels. A problem that did arise with the identification of the small intestinal proteins was that there is no current 2DE map in the literature. These proteins will thus need to be identified by *de novo* sequencing for absolute positive identification. Twenty liver proteins were subjected to database searching but only fifteen liver proteins could be identified with high identification confidence and good correlation to the gel data based on pI and MW. These proteins are shown in Figure 4.2.

Table 4.1: Modulated small intestine proteins that were identified by MALDI TOF MS.

SSP	Gene name	Swiss Prot Accession nr	Name	pI	MW (Da)	Regulation by tea. L	H	Literature	New
0001	SKP1A	Q9WTX5	S-phase kinase associated protein p19	6.0	18.70	Control			✓
5301		XP_903753	Hypothetical protein XP_903753				Up		✓
2202		Gi/74140771	Unnamed protein product			Down	Down		✓
3810	CAP2	Q9CYT6	Adenylyl cyclase associated protein 2 (CAP2)	8.68	63 319		Up		✓
4701	ZCCHC11	Q9EQN6	Developmental control protein fragment				Down		✓
0002	LYS	CAA40485.5	Lysosomal acid phosphatase	6.6	48 720	Control			✓
3404	CYP2D13	AAH18344.1	Cytochrome P450, family 2, subfamily d, polypeptide 13	6.3	57 000		Up	Muto, 2001	

L is for the low dose compared to the control. H is for the high dose compared to the control.

Table 4.2: Modulated liver proteins that were identified by MALDI TOF MS.

SSP	Gene name	Swiss Prot Accession nr	Name	pI	MW (Da)	Regulation by tea. L	H	Literature	New
0112	CYB5	P56395	Cytochrome b5	4.97	15 110	Up		Liu, 2003	
1102	GLO	Q9CPU0	Lactoylglutathione lyase (EC 4.4.1.5)	5.2	21 000	Control		Control	
1105	ITP	Q9D892	Inosine triphosphate pyrophosphatase	5.6	21 897		Up		✓
1106	GPX1	P11352	Glutathione peroxidase 1 (EC 1.11.1.9)	6.74	22 282	Down	Up	Ahmed, 2002	
1308	ANXA4	P97429	Annexin A4 (IV)	5.44	35 858		Up		✓
1609	KRT8	P11679	Keratin, type II, cytoskeletal 8	6.16	36 345	Up			✓
2108	UBA2	P61089	Ubiquitin-conjugating enzyme	6.13	17 138		Up		✓
2409	KHK	P97328	Ketohexokinase (hepatic fructokinase) (EC 2.7.1.3)	5.8	32 750		Up		✓
3104	SOD1	P08228	Superoxide dismutase [CuZnSOD] (EC 1.15.1.1)	6.14	14 537	Up		Lin, 1998	
3202	INMT	P40936	Indolethlamine N-methyltransferase	6.0	30 068	Control		Control	
3303	MDH1	P14152	Malate dehydogenase (cytoplasmic) (EC 1.1.1.37)	6.16	36 345	Up			✓
6211	TPI1	P17751	Triosephosphate isomerase (EC 5.3.1.1)	7.09	26 581	Up			✓
6211	GSTT1	Q644471	Glutathione S-transferase, theta 1 (EC 2.5.1.18)	6.85	27 245	Up			✓
7001	MYG	P04247	Myoglobin	7.22	16 938		Up		✓
7205	GSTM1	P10649	Glutathione S-transferase, mu1 (EC 2.5.1.18)	7.71	26 067		Up	Bu-Abbas, 1998	

L is for the low dose compared to the control. H is for the high dose compared to the control.

4.4 Discussion

Regulated spots were identified using PD Quest 2D gel analysis software. These spots were then cut from the gels and subjected to MALDI-TOF MS. The appropriate controls were used during the MS procedure to ensure the validity of the results that were obtained. The proteins were identified by subjecting the MS spectra to databases and only proteins with significant scores were used for positive identification. None of the small intestinal MS spectra gave strong identification data. Thus the protein with the highest score was taken as a positive identification. The molecular weight and pI of the proteins was then matched to the spot position for further identification.

Lysosomal acid phosphatase (CAA40485, X57199.1, EC 3.1.3.2) was used as a control.

S-phase kinase-associated protein 1A (Q9WTX5) is also known as Cyclin A/CDK2-associated protein p19 (p19A). p19A is an essential component of the SCF (SKP1-CUL1-F-box protein) ubiquitin ligase complex, which mediates the ubiquitination of proteins involved in cell cycle progression, signal transduction and transcription. This enzyme thus plays a role in the third step of ubiquitin conjugation. p19A is able to induce p53 which is the regulator of the cell cycle. p19A is also able to interact with cyclinA/CDK2 which regulates entry into the S-phase of the cell cycle. If cyclin A/CDK2 are not degraded by the proteasome the cell cycle cannot proceed to the next phase. The up regulation of this protein by the high dose tea group could play a role in cancer chemoprevention as it is able to exert some control on the cell cycle by interaction with p53 and cyclin A/CDK2 as well as induce the E3 enzyme in the proteasome that will enhance protein degradation. Up regulation of this protein will slow the cell cycle. This will probably have greater inhibition on fast growing cancer cells than slow growing normal cells. Slowing down of the pre-cancerous cells could allow more time for DNA repair. Even if it does not cause remission of tumors it could slow down the development of tumors, especially very aggressive tumors.

Adenylyl cyclase-associated protein 2 (CAP 2) (Q9CYT6) was up regulated by the high dose group. Adenylate cyclase converts ATP into cAMP. cAMP is a second messenger that is usually increased by the activation of adenylate cyclase. Epinephrine and glucagon stimulates adenylate cyclase. At high cAMP levels epinephrine is able to bind to β-

adrenergic receptors. Thus by up regulating adenylate cyclase with tea more cAMP can be produced for G-protein coupled receptors. Glucagon is secreted by the pancreas to increase blood glucose levels, and also increase the amount of cAMP that is produced by adenylate cyclase. cAMP has many functions in the cells. It is difficult to predict what effect an up-regulation of CAP2 in the overall function may have. It will be interesting to investigate the modulation of this protein by tea in the pancreas.

Developmental control protein (Q9EQN6) is a 304 amino acid residue. This protein was down regulated by the high dose group relative to the control. The function of this protein is unknown. This may be a novel protein whose down regulation may be important in cancer prevention. It may form a novel anti-cancer drug target for intestinal cancers.

Cytochrome P450, family 2, subfamily d, polypeptide 13 (CYP2D13), (AAH18344.1) was up regulated by the high dose group. The liver is the major site for cytochrome P450 (CYP) activity, and thus has more abundant amounts of CYP than any of the other organ such as the lung, kidney or intestine (Lewis, 2001). The effect of green tea has been examined on various cytochrome P450 subfamilies. These families play an important role in hepatic as well as extra-hepatic metabolism and biotransformation of xenobiotics. Most chemicals require chemical activation, as they are not reactive themselves. Cytochrome P450 is one of the major drug metabolizing enzymes involved in activating pro-carcinogens. Studies with green tea and black tea have shown that total cytochrome P450 levels stay constant while some isozymes are up and others are down regulated (Sohn, et al., 1994; Bu-Abbas, et al., 1999 (a)). This indicates that the cytochrome P450 isozymes are indeed affected by green tea and black tea but that this modulation of enzyme activity is at the expense of one another. Up regulation of CYP2D13 could be of benefit to the small intestine.

The identification of the small intestinal proteins will need to be performed by *de novo* sequencing, to ensure the positive identification of the proteins already identified and the spots that were regulated for which no identification was obtained. The problem associated with the identification of the small intestine proteins is due to the fact that at present no complete 2DE map exists for small intestinal proteins of mice. In the literature a need for a 2DE map of the small intestine proteins does exist for comparisons to be made. The protein concentration or peptide recovery of the small intestine proteins was also very

low. This was further illustrated in the MS spectra of Figure 4.5, which had a very low intensity level. Possible a better approach to identifying the proteins of the small intestine would be by the use of MudPIT LC MS. This would be an interesting future prospect as the small intestine does show promise in protein modulation by tea.

The small intestine proteome map presented in Figure 4.1 is a small contribution to the literature. Even if only seven proteins could be tentatively identified it shows that the small intestine can be visualized on the 2DE gels. Further work needs to be done to improve the quality so that better identification can be obtained.

For the liver the following proteins were identified:

Lactoylglutathione lyase (Q9CPU0, EC 4.4.1.5) was used as a control as it was a high abundance spot that could be easily identified with high confidence and can be compared to other 2DE maps in literature. This enzyme is also known as methylglyoxalase, aldoketomutase, glyoxalase I. Lactoylglutathione lyase catalyzes the conversion of hemimercaptal, formed from methylglyoxal and glutathione, to S-lactoylglutathione. It therefore plays a role in the glyoxal pathway. The catalytic activity can be derived from the following equation:

(R)-S-lactoylglutathione → glutathione + methylglyoxal

Lactoylglutathione lyase binds 1 zinc ion per subunit as a cofactor for activity. It has been determined that lactoylglutathione lyase is expressed at higher levels in CD-1 mice that have been bred for low-anxiety-related behavior than in those which have been bred for high-anxiety-related behavior.

Thioether S-methyltransferase (S52102, EC 2.1.1.96) or indolethlamine N-methyltransferase was used as a control as this spot could be correlated to databases and reference 2DE maps in literature. This enzyme catalyzes the N-methylation of tryptamine and structurally related compounds.

S-adenosyl-L-methionine + an amine →

S-adenosyl-L-homocysteine + a methylated amine.

Cytochrome b5 (P56395) was up regulated by the low dose group. Cytochrome b5 was also used as a control for the MS analysis as it was easy to compare the position of cytochrome b5 to other 2DE maps in the literature (Sanchez, et al., 2001). Cytochrome b5

is a membrane bound hemoprotein whose function as an electron carrier for several membrane bound oxygenases. It is also involved in several steps of the sterol biosynthesis pathway. Cytochrome b5 is bound to the cytoplasmic side of the endoplasmic reticulum. It also plays a role in the cytochrome P450 pathway. It was expected that cytochrome b5 would be up regulated by tea, because it plays a role during the CYP450 pathway. Cytochrome b5 supplies electrons to the CYP's during this pathway. Thus if phase I drug metabolism is increased, the activity and expression of cytochrome b5 would also be increased. Our results are similar to previous results that determined that tea is able to increase the activity of cytochrome b5 (Liu, et al., 2003). This thus indicates that tea is able to increase the activity and the expression of cytochrome b5 to the benefit of the cell.

All superoxide dismutase enzymes (SOD) (P08228) are multimeric metalloproteins that are very efficient at scavenging the superoxide radical. SOD plays an important role in protecting living cells against toxicity and mutagenicity of reactive O_2 species by scavenging O^-_2. It is important for living cells to maintain the balance between O^-_2 generated and removed (Scandalios, 1993). Oxidative stress can cause lipid peroxidation and reactive oxygen species will also cause DNA damage and damage to proteins. It was found that EGCg increased both the activity and gene expression of super oxide dismutase (Kim, et al., 2002). Because super oxide dismutase is known to eliminate excess oxygen radicals that are produced, an increase in SOD by EGCg would exert a protective effect on the cell. An increase in SOD in the low dose group was seen in our proteomics study. To counteract oxidative stress cells have several antioxidant enzymes including SOD, glutathione peroxidase (GPx) and catalase. SOD is able to keep the intracellular steady state concentration of O^-_2 low, while catalase and glutathione peroxidase are able to remove H_2O_2 generated from the elimination of O^-_2 (Polavarapu R. et al., 1998). Liver damage like necrosis or inflammation may cause a decrease in the activity and protein expression of GPx, catalase and CuZnSOD. Thus the up regulation of SOD by tea protects the cell from oxidative damage. Similar results were found in the literature (Lin, et al., 1998).

Glutathione peroxidase 1 (GSHPx-1 or GPx-1), (P11352, EC 1.11.1.9) was down regulated by the low dose group, and up regulated in the high dose group. GPx is a selenium containing enzyme located in the cytoplasm and is able to protect the hemoglobin in

erythrocytes from oxidative breakdown. GPx requires GSH for reduction to catalyze the following reaction:

$$2 \text{ glutathione} + H_2O_2 \rightarrow \text{glutathione disulfide} + 2\ H_2O$$

GPx is found in the hepatocytes which, represent about 80% of the cells in the liver by volume and are the major source of metabolic activity. GSH plays a central role in preventing lipid peroxidation because it enables GPx to scavenge inorganic and organic peroxides, thus helping the cell to cope with oxidative stress. Our results showed that GPx expression was decreased in the low dose group but increased in the high dose group. EGCg was able to decrease GPx activity (Ahmed, et al., 2002). This is similar to the results found for the low dose group. Once again the concentration effect seems to play an important role in tea metabolism as the high dose tea group showed an increase in GPx expression. An increase in expression of GPx would probably be of benefit to the cell as GPx is able to relieve the cell from oxidative stress that might cause damage that may result in the onset of cancer.

Glutathione S-transferase mu-1 (P10649, EC 2.5.1.18) was up regulated in the high dose group. GST mu-1 is part of phase II drug metabolism that follow phase I drug metabolism. It follows CYP by conjugation of the xenobiotic to glutathione (GSH). This creates an even more hydrophilic substance, which is easily excreted from the body. Inducers of GST are considered as protective agents. Up regulation of GST mu-1 by tea exerts a protective effect on the cell. Our results are similar to other groups that have also determined that tea is able to up regulate GST activity (Bu-Abbas, et al., 1998).

Glutathione S-transferase theta-1 (Q64471, EC 2.5.1.18) belongs to the GST super family. GST class theta-1 was up regulated in the low dose group. It plays a role in the conjugation of reduced glutathione to a wide number of exogenous and endogenous hydrophobic electrophiles. It is also able to bind to steroids, bilirubin, carcinogens and to numerous organic anions. GST theta-1 has dichloromethane dehalogenase activity and catalyses the following reaction:

$$RX + \text{glutathione} \rightarrow HX + \text{R-S-glutathione}$$

GST theta-1 is present within the liver. The highest expression of GST theta-1 was found in the central vein of hepatocytes. GST theta-1 is also expressed in the interlobular bile duct of epithelial cells. In the lung GST theta-1 is expressed in Clara cells and ciliated cells of the bronchiolar epithelium and in type II alveolar cells of the lung parenchyma. GST

theta-1 has a wide tissue distribution and can exert its protective effect in all of these tissues. The up regulation of both GST mu-1, and GST theta-1 by tea demonstrated the protective role of tea on the cell. Clearly phase II drug metabolism was activated by the consumption of tea. Phase II drug metabolism will then enable the cells to excrete toxic metabolites, and will also exert a cancer chemo preventative effect on the cell. This is the first report to demonstrate the up regulation of hepatic GST theta-1. This may also explain how tea is able to prevent lung cancers due to smoking and the NNK chemical carcinogens from cigarette smoke. Up regulation of GST theta-1 may become an anti-cancer drug target for lung cancer.

Annexin A4 (P97429) was up regulated in the high dose group. Annexin A4 is a calcium/phospholipid-binding protein, which promotes membrane fusion and is involved in exocytosis. The word annexin was derived from the Greek word, *annex*, meaning "bring or hold together". This word was chosen to describe the principle property of nearly all annexins that are able to bind and possibly hold together certain biological structures in particular membranes. By definition an annexin has to fulfill two important criteria: first it must be capable of binding in a Ca^{2+} dependent manner to negatively charged phospholipids, and secondly annexins have to contain a conserved structural element the so-called annexin repeat segment of 70 amino acids. The binding of annexin to calcium is a reversible process. Annexin A4 is able to associate with plasma proteins, and can interact with cytokeratin proteins to participate in the regulation of cytoskeleton dynamics and can also interact with carbohydrates (Gerke and Moss, 2002). The up regulation of annexin by tea is of benefit to the cell. It will thus help stabilize plasma proteins and will also be able to stabilize the cytoskeleton. This is the first time that up regulation of annexin A4 by tea has been reported. This up-regulation of annexin A4 may also help with cell-cell recognition and in this way slow the growth of tumors.

Keratin, type II cytoskeletal 8 (P11679) was up regulated in the low dose group. Keratin polypeptides assemble into 10-14nm filaments. This forms extensive cytoplasmic networks that are associated with nuclear and cytoplasmic membranes. Keratins belong to the intermediate filament family. Keratins are classified as type I and type II based on their charge and their immunological relatedness and sequence similarity. Type I is smaller in molecular weight (40-56 Da, pI 4-6) and more acidic. Type II is larger in molecular weight (53-67 Da, pI 6-8) and has a neutral to basic pI. Keratins assemble into high affinity

filament complexes that form stable resistant networks that impart strength to epidermis and facilitate a role as a protective organ (Albers, et al., 1995). Keratin 8 associates with keratin 18. K8/k18 is involved in modulating and attenuating cellular response to the pro-apoptotic stimuli. They are able to bind to the TNF-α receptor and can thus inhibit it. K8/k18 also plays a role in cellular reinforcement (Owens and Lane, 2003). The up regulation of keratin 8 in the low dose group will help with network formation and reinforcement of cellular membranes. Keratin 8 is also able to bind to TNF- α receptor and inhibit it. Usually TNF-α signalling results in inhibition of IκBα, which will enable NF-κB to translocate into the nucleus to activate transcription and produce even more TNF–α. By inhibiting TNF-α, NF-κB will be unable to translocate into the nucleus. This will play a protective role for the cell and may also play a role in cancer chemoprevention. It has been determined previously that EGCg is able to inhibit TNF-α and thus prevent NF-κB from translocating into the nucleus (Lin, et al., 1999; Ahmad, et al., 2000 (b)). It has also been determined that black tea and TFdg were able to inhibit NF-κB (Lin, et al., 1999). Our results may be the first that can indicate the mechanism of inhibition of TNF-α and NF-κB by keratin 8.

Malate dehydrogenase (P14152, EC 1.1.1.37) was up regulated in the low dose group. Malate dehydrogenase is situated in the cytoplasm and is able to catalyze the following reaction:

$$\text{Oxaloacetate} + \text{NADH} \rightarrow \text{Malate} + \text{NAD}^+$$

The malate shuttle is the most widespread shuttle and is found in the mammalian kidney, liver and heart. First cytosolic malate dehydrogenase reduces oxaloacetate to malate. An antiporter protein in the mitochondrial inner membrane then transports the malate across the membrane. Mitochondrial malate dehydrogenase then converts the malate back to oxaloacetate, which is then further broken down to aspartate. The aspartate is then transported back into the cytosol were oxaloacetate is formed and then again converted to malate. The NADH produced in the mitochondrion passes their electrons directly to the electron transport chain producing 3ATP per mole NADPH. The malate shuttle is able to produce 2.5 moles of ATP for each mole of cytosolic NADH (Campbell, 1999). Therefore by up regulation of malate dehydrogenase the cell is able to produce more aspartate that can be used in protein synthesis as well as the production of more energy. This may be the first report on the regulation of malate dehydrogenase by tea drinking.

Inosine triphosphate pyrophosphatase (Q9D892) occurs in the cytoplasm and is also known as ITPase, and inosine triphosphatase. This 198 amino acid residue enzyme was up regulated in the high dose group. Inosine triphosphate pyrophosphatase hydrolyzes ITP and dITP to their respective monophosphate derivatives. Xanthosine 5'-triphosphate (XTP) is also a potential substrate. ITPase may be the major enzyme responsible for regulating ITP concentration within cells. The reaction catalyzed by ITPase is:

Nucleoside triphosphate + $H_2O \rightarrow$ nucleotide + diphosphate

The up regulation of inosine triphosphate pyrophosphatase by tea will help with the production of nucleotides. This may be of the first results that show that tea drinking is able to regulate inosine triphosphate pyrophosphatase.

Triosephosphate isomerase (TIM) (P17751, EC 5.3.1.1) is also known as triose-phosphate isomerase and plays a role in several metabolic pathways, especially in fatty acid biosynthesis, gluconeogenesis, glycolysis and the pentose phosphate pathway. In glycolysis, triosephosphate isomerase catalyses the following reaction:

D-glyceraldehyde 3-phosphate $\leftrightarrow$ dihydroxyacetone phosphate

This enzyme helps with the breakdown of glucose to form pyruvate that will produce ATP that can be used by the cell. Up regulation will thus increase the rate of glycolysis and increase the rate of ATP production. This may be of the first results that show that tea drinking is able to regulate triosephosphate isomerase.

Ketohexokinase (Hepatic fructokinase) (P97328, EC 2.7.1.3) was up regulated in the high dose group and may be the first reported regulation of ketohexokinase by tea drinking. This enzyme plays a role in carbohydrate metabolism especially the primary metabolism of dietary fructose in mammals. Ketohexokinase is subject to allosteric control and calayses the following reaction:

ATP + D-fructose $\rightarrow$ ADP + D-fructose 1-phosphate

This is similar to the reaction catalyzed by hexokinase in glycolysis, except that hexokinase has specificity for glucose rather than fructose. In mammalian metabolism sucrose is degraded to glucose and fructose. Glucose and fructose will then be further metabolized to produce ATP. In chapter 2 it was determined that the pancreas of the high dose (0.08 g) group weighed significantly less than that of the control (0.10 g) and low dose (0.11 g) groups. This trend was confirmed when expressed as a percentage of the

body weight. The pancreas of the low dose (0.41 %) group was significantly larger than that of the control (0.35 %), while the high dose (0.29 %) group weighed less. The pancreas functions to control glucose levels by secreting insulin to lower glucose levels and secreting glucagon to increase glucose levels. Thus it may seem that tea may play a role in glucose regulation and may therefore play a role in diabetes. It has been reported by (Vinson and Zhang, 2005) and (Tsuneki, et al., 2004) that tea has an effect on diabetes mellitus. It was also reported by (Suganuma, et al., 1998) that the pancreas absorb EGCg and therefore the concentration effect seem to be very important. In another study on diabetes it was reported by (Fajas, et al., 2004) that E2F1 has a role in diabetes and pancreatic function. E2F1 is critical in the cell cycle and regulates pancreas cell proliferation and therefore also regulates insulin secretion and production. Reduction in the pancreatic size may be due to a reduction in islet numbers within the pancreas, but this may also bring about a decrease in insulin secretion. It was reported that EGCg is able to decrease total pRb protein levels in a dose and time dependent manner as well as increase the number of hypophosphorylated Rb, which will ultimately cause cell cycle arrest (Ahmad, et al., 2002). Insulin is secreted from the pancreas β-cells of the islets of Langerhans. It may thus be that this cellular proliferation is controlled by the high dose of tea. This effect may be significant for patients suffering from hypoglycemia. Hypoglycemia is when excess insulin is produced resulting in low blood sugar. Ketohexokinase can be of benefit to the cell by the up regulation by tea. If hypoglycemic patients are able to increase fructose levels their glucose levels could stay constant. Fructose can thus be used as a replacement for glucose especially in patients with hypoglycemia. It also seems that insulin levels can be controlled by the consumption of tea. The effect of an increase in of TIM in the liver on blood glucose is difficult to predict. However if this protein is also increased in the pancreas it may help explain the beneficial effects of tea consumption in diabetes mellitus type II.

Ubiquitin conjugating enzyme E2 (P61089, EC 6.3.2.19) was up regulated in the high dose group. This enzyme consists of 152 amino acids, and is found in various mammalian tissues. It plays a role in the second step of the ubiquitination pathway prior to protein degradation by the proteasome. The ubiquitin pathway makes use of three enzymes and ATP during the ubiquitination of proteins and is thus the rate limiting step in protein degradation. Ubiquitination is catalyzed by the successive action of ubiquitin activating (E1), ubiquitin conjugating (E2) and ubiquitin ligase (E3) enzymes. Ubiquitin becomes

attached to proteins via isopeptide bonds, between the extreme C-terminal glycine residue of ubiquitin and the amino groups of the lysine residues of target proteins. This is demonstrated in the following equation:

ATP + ubiquitin + protein lysine → AMP + diphosphate + protein N-ubiquityllysine

The minimum signal for degradation of protein by the 26 S proteasome is four ubiquitin moieties (Layfeld, et al., 2001). The process repeats until a polyubiquitin chain is attached to the protein marked for degradation. This then draws the protein to the proteasome (Goldberg, et al., 2001). Protein degradation is a very important process, as it may be needed to stop some biochemical reaction by degrading the induced active enzyme. It may play a role in activating some pathway needed by the cell. It also has a key role in maintaining the correct balance within the cell cycle as well as transcriptional activation of various transcription factors like NF-κB. Diseases associated with the inhibition of the proteasome are the development of Parkinson's, Alzheimer's and cancer. Thus the up regulation of ubiquitin conjugating enzyme (E2) plays a protective role within the cell. By up regulation of E2 the protein that needs to be degraded will be tagged faster with ubiquitin and can thus proceed faster to the 26S proteasome for degradation. This is similar to results found by (Nam, et al., 2001). They found that tea and its constituents inhibit only the chymotrypsin activity of the proteasome and not the trypsin activity of the proteasome. The up-regulation of E2 by tea may be an indication of chymotrypsin inhibition by tea. The cells may be over compensating the chymotrypsin inhibition by producing more E2 enzyme.

Myoglobin (P04247) is a 153 amino acid residue with a prosthetic heme group that was up regulated by the high dose group. Myoglobin has eight α-helical regions and no β-plated sheet regions. Myoglobin is a molecule that binds oxygen tightly and has only a single protein chain. Hemoglobin on the other hand is a protein with four myoglobin like sub-units that are fitted together. Myoglobin serves as a reserve supply of oxygen and facilitates the movement of oxygen within muscles. An increase in expression was determined for myoglobin by the high dose group. This increase in expression is probably due to the cell that has produced more myoglobin molecules that will be able to bind more oxygen molecules for storage. Thus by up regulation of myoglobin by tea the cells are able

to store more oxygen for use by the muscles later on. This may also be of an advantage for high altitude climbers and divers.

For the liver various spots were identified by MALDI TOF MS. The identification of liver proteins was easier and more conclusive than that of the small intestine. This could be due to the fact that the concentration of the liver proteins was higher than that of the small intestine. Better resolution was also obtained for the liver proteins as a larger gel system was used during electrophoresis. The proteins identified for the liver also seem to play important roles in metabolism, detoxification and protein degradation. Some of the proteins identified by our MS data are similar to previously identified proteins that are regulated by tea. These regulated proteins were discussed in chapter 1. This is the first report of the identification of modulated liver proteins by tea with 2DE gel electrophoresis approach. By separating liver proteins by two dimensional gel electrophoresis a total of twenty-two modulated spots could be identified that were statistically significant between the gels. Twenty-seven two spots were detected that indicate a regulation of at least two times between the various experimental groups. Only fourteen of the most abundant and easily visible liver proteins were identified. Thus a future prospective would be to identify the rest of the modulated spots either by two-dimensional electrophoresis or by MudPIT. If the two dimensional gel electrophoresis approach is followed the gels will need to be scaled up to at least 18 cm gels, but ideally 24 cm gels would be best to obtain maximum resolution of the proteins. The pancreas should be analyzed next to determine if any proteins are modulated that might be helpful to type II diabetes mellitus.

CHAPTER 5

Concluding Discussion

Tea *(Camellia sinensis)* is one of the most popular beverages consumed worldwide. Tea was first discovered for human use 2737 BC when Emperor Sh'eng Nung took the first sip of tea. Since then tea has been used for its medicinal properties for almost 5000 years. Recently the significance of daily tea consumption and its cancer chemo prevention in humans became an important issue. So much so that the Royal Society of Medicine has launched a "tea4health" campaign to urge Britons to consume at least four cups of tea per day to gain maximum health benefits from tea. It has been demonstrated that the oral intake of tea can protect against stroke (Keli, et al., 1996), obesity (Yang, et al., 2003), inflammation (Varilek, et al., 2001; Sang, et al., 2004), arthritis (Haqqi, et al., 1999), coronary heart disease (Hakim, et al., 2003), oxidative damage (Ishige, et al., 2001; Saffari and Sadrzadeh, 2004), DNA damage (Zhang, et al., 2002), inhibit the development of cancer and tumor progression (Sazuka, et al., 1997; Chen, et al., 1998) and can even interfere with HIV-1 infection (Kawai, et al., 2003). The health effects of tea against disease and tumor growth have been attributed to the unique anti-oxidant activities of the tea polyphenols. The most significant properties of tea polyphenols that may affect health, disease and carcinogenesis are the anti-oxidant activities (Ragione, et al., 2002), modulation of carcinogen metabolizing enzymes (Bu-Abbas, et al., 1999 (a)), trapping of ultimate carcinogens (Suganuma, et al., 1999), induction of cell apoptosis and cell cycle arrest (Ahmad, et al., 2000 (a)). Green tea, black tea, and some tea components all show protective effects on the well being of the cell. Tea is able to regulate gene expression, regulate enzymes, regulate the cell cycle, cause cell cycle arrest, induce apoptosis, and inhibit the proteasome. Even though a considerable amount of research has been done on especially the green tea polyphenol EGCg that has shown many beneficial effects on the cell, there is probably still more merit in research of the whole green tea and black tea extracts.

The aim of this study was to find proteins that may be modulated in mice drinking tea in contrast to control animals that receive water. These proteins would be visualized by

using a proteomics approach. Ultimately it is hoped that these modulated proteins may become new targets for cancer therapy.

Forty-eight, six week old, male C57BL/6 mice were used in the experimental procedure over the six-week period of treatment. Mice have shown similar metabolism and bioavailability of tea to that found in humans. Three groups; control (water only), low dose (0.5% w/v), and a high dose (2% w/v) group were chosen to determine the effect of tea on protein modulation. Liquid consumption was measured daily for each group of mice. The high dose group consumed less liquid than the control group, however this difference did not reach statistical significance. The high dose group started with 3.13 mg tea/mouse/day and ended at 4.38 mg tea/mouse/day. This was higher than that of the low dose group even though the low dose group consumed more liquid. The low dose group started with 1.56 mg tea/mouse/day and ended at 2.97 mg tea/mouse/day. Weight gain was measured for all the groups. The body weight data did not show any significant differences due to the treatment over the six-week period. However longer periods of treatment have produced decreased body weights due to high tea intake in the literature (Kao, 2000), (Klaus, et al., 2005). The liver of the low dose (1.58 g) group was significantly larger than that of both the control (1.34 g) and high dose (1.28 g) group. The same was seen when the liver was expressed as a percentage of the body weight. These results are similar to that of (Yang M. et al., 2003 and Raederstorff, et al., 2003). The increase in liver weight of the low dose group could be due to enhanced enzymatic activity within the liver and expansion of the hepatocytes, while the decreased liver weight of the high dose group could be due the anti-obesity effect of tea that have led to a reduction in hepatic fat levels.

The pancreas of the high dose (0.08g) group weighed significantly less than that of the control (0.10 g) and low dose (0.11 g) groups, this was also seen when the pancreas was expressed as a percentage of the body weight. Tea may have an effect on the pancreas while it functions to control glucose levels by secreting insulin to lower glucose levels and secreting glucagon to increase glucose levels. Reduction in the pancreatic size may be due to a reduction in islet numbers within the pancreas, but this may also bring about a decrease in insulin secretion. The high dose group could have been able to activate the cell cycle by allowing Rb to bind to E2F1 within the pancreas hence causing transcriptional arrest. This may be a reason why the pancreas of the high dose group was so small. For the low dose group it may be possible that the tea concentration is not high enough to affect

the Rb protein phosphorylation and therefore do not have an effect on cellular proliferation within the pancreas. The high dose group increased the weight of the small intestine and the colon. Both the small intestine and the colon play a central role in metabolism. The small intestine is well adapted for absorption and is the major route of absorption for tea compounds in the body.

After the harvesting and sample preparation of the organs of the mice the liver and small intestine were subjected to further proteomic analysis. The aim of the proteomic study was to identify proteins that may be modulated by tea when compared to control groups. The small intestinal and liver proteins were first separated by SDS PAGE and also by tricine SDS PAGE. For the tricine gels no statistically significant differences were detected for protein extracts from the small intestine or the liver. For both the small intestine and the liver three bands were detected by SDS PAGE that showed significant differences between the various groups as determined by Quantity One™. The first band that was detected for the small intestine was a 66 kDa band. This band had decreased expression for the low dose group when compared to the control. The second band that was regulated was a 45 kDa band that had significantly lower expression in the low dose and high dose groups when compared to the control. The third band detected to have significant regulation was a 10 kDa band. This band was up regulated in the low dose group but not the high dose group.

Three bands were also detected for the liver. The first band was a 110 kDa band. This large protein was down regulated in the low dose group. The second significant band was a 66 kDa band that had increased expression of the protein for the low dose group when compared to the control and high dose groups. It is not believed that the 66kDa band from the liver and the small intestine are the same proteins as this band was down regulated in the low dose of the small intestine, while in the liver this band was up regulated for the low dose. One protein would probably exhibit the same regulation even in different tissues. The third band was a 14 kDa band that had increased expression in the low dose and decreased expression in the high dose when compared to the control.

HPLC analysis was done on the 66 kDa and 14 kDa bands to determine the number of proteins present within each band. The HPLC analysis of the 66 kDa indicated the presence of only one protein, while for the 14 kDa band two proteins were detected. The

HPLC chromatograms showed similar modulation of the low dose group than that found for both bands on the SDS PAGE gels. After the separation of the 14 kDa band by HPLC and SDS PAGE the band was subjected to MALDI TOF MS. The protein mixture was identified by Protein Prospector as hypothetical protein XP_358319 (Accession number XP_358319) with a molecular weight of 15 190 Da and immunoglobulin α chain (Accession number AAA38305) with a molecular weight of 13 140 Da. This was not expected, even though the molecular weights of the two identified proteins match that of the 14 kDa band the identification of these proteins will need to be confirmed by LC MS. The possibility does exist that this band is rather a combination of cytochrome b5 (15 110 Da), superoxide dismutase (14 537 Da) or perhaps myoglobin (16 938 Da) that were identified in the 2DE gels that were up regulated.

To increase separating power, two dimensional gel electrophoresis (2DE) was done for the small intestine and the liver. The proteins were hence separated according to their iso-electric point (pI), and according to size. For the small intestine sixteen 2DE gels were done in total and significant spots were determined by PD Quest. Twenty-two spots were regarded as statistically significant between the groups for the small intestine, while twenty-seven spots showed at least two times regulation between the groups. These spots were cut from the gels and subjected to MALDI-TOF MS. The peptide mass fingerprints were used for database searching, but unfortunately none of the scores obtained for the small intestinal proteins were significant according to Mascot. Aldente were also used for identification and thus the protein with the highest score was taken as a positive identification. The molecular weight and pI of the proteins was then matched to the spot position for further identification. Lysosomal acid phosphatase (CAA40485, X57199.1, EC 3.1.3.2) and S-phase kinase-associated protein 1A (Q9WTX5) were high abundance spots that were cut from the small intestinal gels to use as controls. Adenylyl cyclase-associated protein 2 (CAP 2) (Q9CYT6) was up regulated by the high dose group. Adenylate cyclase is stimulated by epinephrine and glucagon and is able to convert ATP into cAMP. Up regulation of adenylate cyclase would thus be important to the cell as it plays an important role for G-protein receptors and may also play a role in insulin regulation.

Developmental control protein (Q9EQN6) has an unknown function and was down regulated by tea. CYP2D13 (AAH18344.1) is mainly involved in phase I drug metabolism and was up regulated by the high dose group. This indicates that the cytochrome P450

isozymes are indeed affected by green tea and black tea but that this modulation of enzyme activity is at the expense of one another. Up regulation of CYP2D13 could be of benefit to the cell in detoxification.

Even though some proteins were identified for the small intestine, the identity of all these proteins will need to confirmed by *de novo* sequencing, to ensure the positive identification of the proteins. At present no 2DE map does exist for the small intestine of mice which increased the difficulty of identifying the proteins. This and the low concentration of the proteins is another reason why these proteins will need to be identified by LC MS/MS. Precipitation of the proteins will only identify a specific group of proteins and thus the MudPIT route of identification would be best.

The sample preparation method for the liver was optimized by increasing solubilization by changing the urea, thiourea and CHAPS concentrations as well as increasing the reducing power by adding more DTT. The IEF procedure was also optimized. Once the sample preparation procedure was optimized for the liver the gels could be run for reproducible results. In total seventeen liver gels were run and from these gels it was determined that a total of 35 spots were statistically different and eleven spots were modulated at least two times between the three groups. For the liver a 2DE map does exit and from this reference 2DE map certain spots were matched to our own gels and these proteins were then identified by MALDI TOF MS and compared to that of the 2DE reference map. The liver proteins were identified by Mascot. The first control for the liver was cytochrome b5 (P56395) that was up regulated by tea. The position of cytochrome b5 could also be confirmed by matching it to other 2DE reference maps. Lactoylglutathione lyase (Q9CPU0, EC 4.4.1.5) and thioether S-methyltransferase (S52102, EC 2.1.1.96) were also controls that were used for the liver. Superoxide dismutase enzyme (SOD) was up regulated by the low dose group and again the position of SOD in our 2DE gels was similar to that of (Sanchez, et al., 2001). SOD protects the cell by scavenging superoxide radicals and in this way protects living cells against toxicity and mutagenicity of reactive O_2 species. Oxidative stress can cause lipid peroxidation and reactive oxygen species will also cause DNA damage and damage to proteins. To counteract oxidative stress cells have several antioxidant enzymes including SOD, glutathione peroxidase (GPx) and catalase. SOD is able to keep the intracellular steady state concentration of O^-_2 low, while catalase and glutathione peroxidase are able to remove H_2O_2 generated from the elimination of O^-_2.

Glutathione peroxidase 1 (GSHPx-1 or GPx-1), (P11352, EC 1.11.1.9) was down regulated by the low dose group, and up regulated in the high dose group. GPx, together with SOD protects the cell from oxidative stress. GPx in particular protect hemoglobin in erythrocytes from oxidative breakdown. An increase in expression of GPx would probably be of benefit to the cell as GPx is able to protect the cell from oxidative stress that might cause damage that may result in the onset of cancer.

Glutathione S-transferase mu-1 (P10649, EC 2.5.1.18) was up regulated in the high dose group. GST mu-1 is part of phase II drug metabolism that follow phase I drug metabolism. Glutathione S-transferase theta-1 (Q64471) plays a role in the conjugation of reduced glutathione to a wide number of exogenous and endogenous hydrophobic electrophiles. It is also able to bind to steroids, bilirubin, carcinogens and numerous organic anions. The up regulation of both GST mu-1, and GST theta-1 by tea demonstrated the protective role of tea on the cell. Clearly phase II drug metabolism was activated by the consumption of tea. Phase II drug metabolism will then enable the cells to excrete toxic metabolites, and will also exert a cancer chemo preventative effect on the cell.

Annexin A4 (P97429) is a calcium/phospholipid-binding protein which promotes membrane fusion and is involved in exocytosis. The up regulation of annexin by tea will help to stabilize plasma proteins and will also be able to stabilize the cytoskeleton. Up regulation of keratin 8 (P11679) in the low dose group will help with network formation and reinforcement of cellular membranes. Keratin 8 is also able to bind to TNF-α receptor and inhibit it. By inhibiting TNF-α, NF-κB will be unable to translocate into the nucleus. This will play a protective role for the cell and may also play a role in cancer chemoprevention. Our results may be the first that can indicate the mechanism of inhibition of TNF-α and NF-κB by keratin 8.

Malate dehydrogenase (P14152, EC 1.1.1.37) up regulation by tea may cause the up regulation of glycolysis and the Krebs cycle that may enable the production of more energy. The up regulation of inosine triphosphate pyrophosphatase (Q9D892) by tea will help with the production of nucleotides. Triosephosphate isomerase (TIM) (P17751, EC) is also known as triose-phosphate isomerase and plays a role in several metabolic pathways, especially in fatty acid biosynthesis, gluconeogenesis, glycolysis and the pentose phosphate pathway. This enzyme helps with the breakdown of glucose to form pyruvate

that will produce ATP that can be used by the cell. Up regulation will thus increase the rate of glycolysis and increase the rate of ATP production. Ketohexokinase (Hepatic fructokinase) (P97328, EC 2.7.1.3) was up regulated in the high dose group. This enzyme plays a role in carbohydrate metabolism especially the primary metabolism of dietary fructose in mammals. It was determined the pancreas of the high dose (0.08 g) group weighed significantly less than that of the control (0.10 g) and low dose (0.11 g) groups. The pancreas functions to control glucose levels by secreting insulin to lower glucose levels and secreting glucagon to increase glucose levels. Thus tea may play a role in glucose regulation and may therefore play a role in diabetes. Ketohexokinase can be of benefit to the cell by the up regulation by tea. If hypoglycemic patients are able to increase fructose levels their glucose levels could stay constant. Fructose can thus be used as a replacement for glucose especially in patients with hypoglycemia. It also seems that insulin levels can be controlled by the consumption of tea.

Ubiquitin conjugating enzyme E2 (P61089, EC 6.3.2.19) was up regulated in the high dose group. It plays a role in the second step of the ubiquitination pathway prior to protein degradation by the proteasome. Protein degradation is a very important process as it may be needed to stop some biochemical reaction by degrading the active enzyme or it may play a role in activating some pathway needed by the cell. It also has a key role in maintaining the correct balance within the cell cycle as well as transcriptional activation of various transcription factors like NF- κB. Diseases associated with the inhibition of the proteasome are the development of Parkinson's, Alzheimer's and cancer. Thus the up regulation of ubiquitin conjugating enzyme (E2) plays a protective role within the cell. By up regulation of E2 the protein that needs to be degraded will be tagged faster with ubiquitin and can thus proceed faster to the 26S proteasome for degradation.

Myoglobin (P04247) serves as a reserve supply of oxygen and facilitates the movement of oxygen within muscles. By up regulation of myoglobin by tea the cells are able to store more oxygen for use by the muscles later on.

From the PMFs the various regulated proteins of the liver could be identified. It is clear from the functions that were described that tea does have an overall protective effect on the cell and general well being of an organism. Proteins that were modulated include proteins and enzymes involved in detoxification, energy metabolism, and protein turn over. The

results that were obtained by the proteomic approach that was followed are similar to results that were previously obtained for tea as described in chapter 1 with approaches like enzyme assays, substrate probes and antibodies. This is the first study that has taken a proteomic approach to the identification of the overall regulation of proteins by tea. The initial aim of this study was met by identifying the regulated proteins. It was hoped that new and novel proteins would be identified to aid in cancer chemoprevention. Unfortunately no novel proteins could be identified, but some newly regulated proteins were identified for tea drinking. But this study did improve our knowledge of the protective effects of tea. It was illustrated that tea may have an anti-obesity effect on mice, and that tea may also be able to control diabetes. This was seen by the regulation of the pancreas weight by tea, as well as the regulation of ketohexokinase that may play a role in diabetes and energy regulation. Annexin A4 may play a protective role during cancer, as it will induce apoptosis. Tea is able to promote apoptosis of cancerous cell but not normal cells. This chemo-protective effect is also further shown by the up regulation of the ubiquitin conjugating enzyme that will aid in protein turn over. Up regulation of SOD, GPx and the phase II detoxification enzymes is a further illustration of the protective role of tea in cancer chemo-prevention.

The goal of this study was to discover new proteins that may be modulated in mice drinking tea in contrast to control animals that receive only water. It was hoped that some of the known proteins modulated by tea would be detected by our proteomic approach. Figure 5.1 is an extension of Figure 1.12 shown in chapter 1. In Figure 5.1 the proteins that are regulated by tea as known in the literature is marked in green for inhibition (Θ) and activation (⊕). The newly discovered proteins found in this study are marked in red for inhibition (-) and activation (+). The names of the newly discovered proteins are also boxed in purple. The various pathways affected by the regulation of the novel proteins are also linked to each other as indicated in Figure 5.1 (Also see text of chapter 4 and chapter 5).

Hypothesis I: Tea drinking is able to modulate the proteome in normal cells from various organs, was supported by the proteomics study as for the small intestine thirty regulated spots were found and for the liver thirty three regulated spots were found on the respective 2DE gels. Hypothesis II: Some of the modulated proteins will be ones that have already been discovered by other approaches for example by substrate probes for specific CYP

isozymes, was also supported as we did identify glutathione S-transferase mu-1, superoxide dismutase, glutathione peroxidase and cytochrome b5 to be regulated by tea that are similar to the literature. Hypothesis III: Some of the modulated proteins will be new discoveries that have never been shown before to be modulated by tea drinking was also supported as we identified glutathione S-transferase theta-1, annexin A4, keratin 8, malate dehydrogenase, ketohexokinase, ubiquitin conjugating enzyme E2, inosine-triphosphate-pyrophosphatase, triosephosphate-isomerase, and myoglobin to be regulated by tea in the liver. The small intestinal proteins are all new discoveries.

It is thus clear that tea is able to exert a protective effect on the well being of the experimental animals. Further studies are needed to identify possible cancer therapy targets. At the moment it seems like tea is nature's wonder cure for almost all ailments. Skip the apples and drink four cups of tea a day to keep the doctor away!

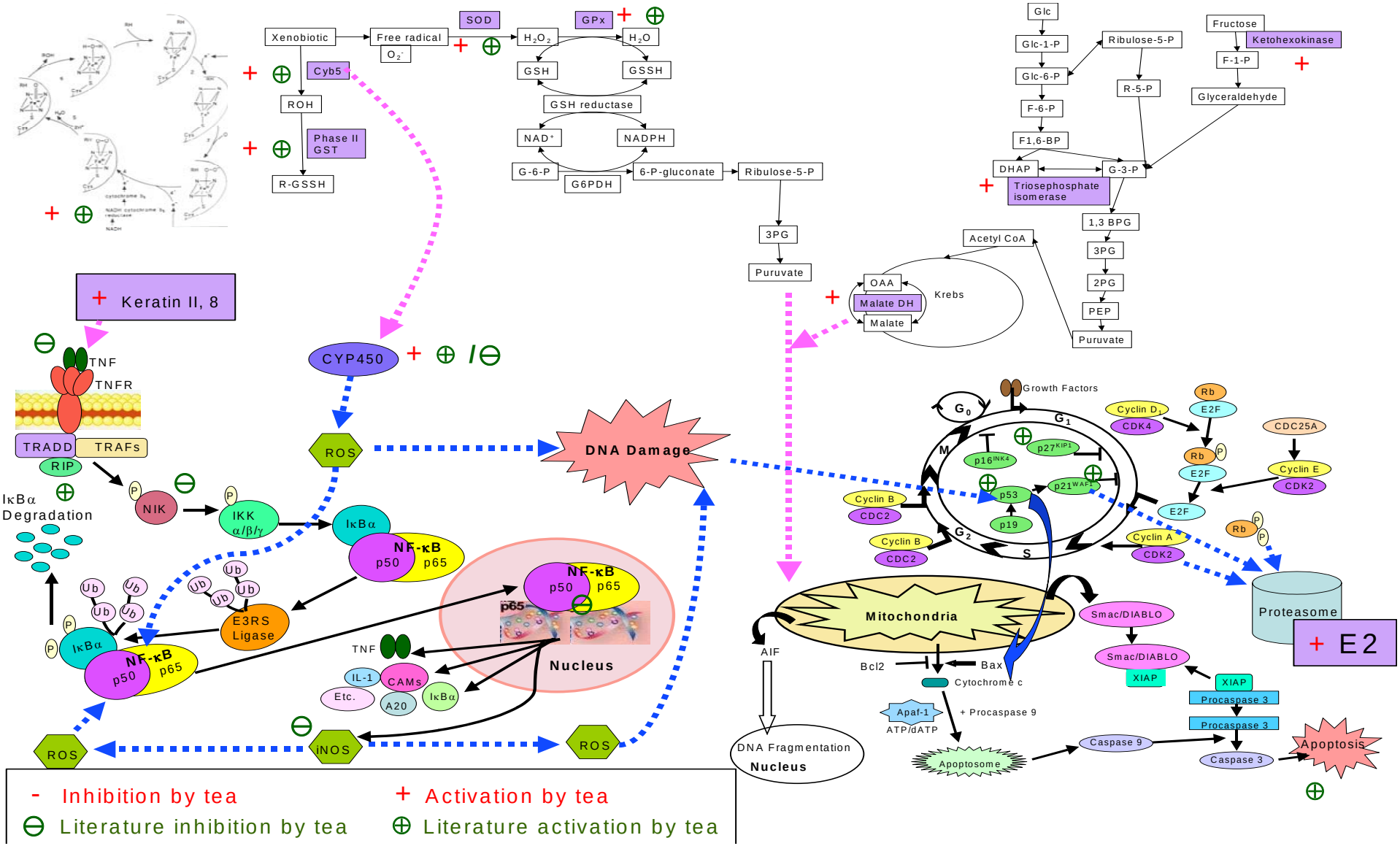


Figure 5.1: Overall regulation of various processes by tea as determined by the experimental study.

Summary

The oral intake of green tea, black tea and some of the tea constituents has been demonstrated to protect against various diseases and show protective effects on the cell. Tea is able to regulate gene expression, regulate enzymes, regulate the cell cycle, cause cell cycle arrest, induce apoptosis, and inhibit the proteasome.

The aim of this study was to find proteins by a proteomic approach that may be modulated in mice drinking tea in contrast to control animals that receive water.

Three groups; control, low dose, and a high dose tea group were chosen to determine the effect of tea on protein regulation of C57BL/6 male mice. Daily liquid consumption was measured, and even though the high dose group consumed less liquid they still ingested more tea than the low dose group at the end of the study. Weight gain was measured for all the groups but no significant differences were found. Some differences were found in organ weights of the low and high dose groups. There was no dose dependent effect for the liver and small intestine, while the colon showed a positive and the pancreas a negative dose dependent effect.

Small intestine and liver proteins were separated by one and two dimensional gel electrophoresis. No significant differences were found for the small intestine and liver when the proteins were separated by tricine SDS PAGE. However some significant differences were found on the glycine SDS PAGE gels of both the small intestine and the liver. The small intestine had three significant bands at 66kDa, 45kDa and 10kDa. The three significant liver bands were at 110kDa, 66kDa and 14kDa. HPLC analysis of the liver 66kDa band showed that the band consisted of only one protein while the 14kDa band consisted of possibly two proteins. MS analysis of the 14kDa band identified the proteins as hypothetical protein XP_358319 (15 190Da) and immunoglobulin α chain (13 140Da). Although the identified proteins match the molecular weight of the 14kDa band these results will need to be confirmed by MudPIT.

Thirty 2DE spots of the small intestine were regulated by tea. Ten of these spots were analyzed by MALDI TOF MS, but only seven of these proteins were identified. These

proteins were S-phase kinase associated protein p19, hypothetical protein XP_903753, unnamed protein product, adenylyl cyclase-associated protein 2, developmental control protein, lysosomal acid phosphatase, and cytochrome P450 (CYP2D13). All seven the small intestinal proteins will need to be confirmed by *de novo* sequencing, to ensure the positive identification of the proteins. Currently there is no 2DE map in literature of the small intestine. This study will provide the first 2DE map of the murine small intestine proteins.

Thirty three 2DE spots of the liver were regulated by tea. Twenty of these were analyzed by MALDI TOF MS, but only fifteen of these proteins were idenitifed. These regulated proteins are: superoxide dismutase, and glutathione peroxidase that are antioxidant enzymes to counteract oxidative stress, detoxification enzymes like glutathione S-transferase mu-1, glutathione peroxidase theta-1 and cytochrome b5. Annexin A4 is able to help stabilize plasma proteins and the cytoskeleton and may induce apoptosis, keratin 8 may help with network formation and reinforcement of cellular membranes, malate dehydrogenase for energy expenditure and ketohexokinase in carbohydrate metabolism, while ubiquitin conjugating enzyme E2 plays a role in protein turn over. Other identified proteins include inosine-triphosphate-pyrophosphatase, triosephosphate-isomerase, and myoglobin. This study provides a novel 2DE map for liver protein regulation by tea.

This was the first study that has taken a proteomic approach to the identification of the overall regulation of proteins by tea. The aim of this study was met by identifying the tea regulated proteins and elaborating on the protective effects and possible cancer chemo preventative effects of tea.

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