



**UNIVERSITY of the
WESTERN CAPE**

Identification of a Transducin (beta)-like 3 protein as a potential biomarker of prediabetes from rat urine using proteomics

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South African **MEDICAL
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DECLARATION

I declare that the “Identification of a Transducin (beta)-like 3 protein as a potential biomarker of prediabetes from rat urine using proteomics” is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledge by complete references.

Henrietta Refiloe Mofokeng

February 2010

Signed.....

A handwritten signature in black ink, appearing to read 'H. Mofokeng', written over a dotted line.

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ABSTRACT

Obesity is a globally increasing disease particularly in developing countries and among children. It is mainly caused by intake of diets high in fat and the lack of physical activity. Obesity is a risk factor for diseases such as type II diabetes, high blood pressure, high cholesterol and certain cancers. Prediabetes is a condition where blood glucose levels are above normal but have not reached those of diabetes. It is difficult to diagnose, as there are no signs or symptoms. Some type II diabetes patients bear no symptoms at all and the disease is discovered late. Proteomics is a field that can provide opportunities for early diagnosis of diseases through biomarker discovery. The early diagnosis of diabetes can assist in the prevention and treatment of diabetes. Therefore there is a need for the early diagnosis of diabetes.

Twenty Wistar rats were used. The rats were initially fed a CHOW diet, which is the standard balanced diet for rats, for 4 weeks. The rats were then divided into 2 groups of 10 where 1 group was fed CHOW and another was fed a high fat (HF) diet in order to induce obesity. The two groups were fed their respective diets for 18 weeks. Rats were weighed. Rats were placed in metabolic chambers and 24 hour urine samples were collected. Ketone levels were measured by Ketostix. Urine proteins were precipitated by acetone, quantified and separated on both the 1D SDS-PAGE and the 2D SDS-PAGE. Protein expression changes between CHOW and HF fed rats were determined and identified using MALDI-TOF mass spectrometry. Protein spots intensities increased and decreased between the CHOW and HF fed rats. Transducin (beta)-like 3 was identified as the only differentially expressed protein, which might serve as a potential biomarker for prediabetes.

Key words: Obesity, type II diabetes, prediabetes, biomarker discovery, proteomics, high fat diet, 1D SDS-PAGE, 2D SDS-PAGE, urine, mass spectrometry

TABLE OF CONTENTS

DECLARATION	i
ACKNOWLEDGEMENTS.....	ii
ABSTRACT.....	iii
TABLE OF CONTENTS.....	iv
LIST OF FIGURES.....	vii
LIST OF TABLES.....	x
LIST OF ABBREVIATIONS.....	xi
CHAPTER 1: LITERATURE REVIEW.....	1
1.1. INTRODUCTION.....	2
1.2. OBESITY	4
1.2.1 Defining overweight and obesity	4
1.2.2 Body Mass Index (BMI).....	4
1.2.3 Causes of obesity.....	6
1.3. OBESITY AS A RISK FACTOR.....	10
1.3.1 Type II Diabetes	11
1.3.2 Hypertension	11
1.3.3 Renal Diseases	12
1.4. DIABETES MELLITUS.....	13
1.4.1 Types of diabetes.....	15
1.4.2 Diabetes diagnosis.....	16
1.5. PROTEOMICS	18

1.5.1	Defining Proteomics.....	18
1.5.2	Urinary proteomics in the discovery of diabetes biomarkers	22
1.5.3	Aims and objectives of the study	26
1.5.4	Hypothesis.....	26
CHAPTER 2: MATERIALS AND METHODS.....		27
2.1	Materials and suppliers.....	28
2.2	Buffers and solutions.....	30
2.3	Animal models.....	33
2.4	Urine collection.....	33
2.5	Urine (sample) preparation.....	33
2.5.1	Protein determination	33
2.5.2	SDS PAGE Staining.....	34
2.5.3	Protein precipitation	35
2.6	One dimensional (1D) SDS-PAGE Analysis	37
2.7	Protein quantification	38
2.7.1	Bradford protein assay	38
2.8	Two dimension (2D) SDS page analysis.....	39
2.8.1	Sample solubilization	39
2.8.2	In-gel rehydration.....	39
2.8.3	Isoelectric focusing (IEF)	39
2.8.4	Equilibration of the strips	40
2.8.5	Second dimension.....	40
2.9	In-gel digestion	41
2.10	Protein identification with MALDI-TOF-MS	41
2.11	Determination of the secondary structure.....	42

CHAPTER 3: RESULTS.....	43
3.1 ANIMAL STUDIES	44
3.1.1 Body weights.....	44
3.1.2 Ketone body levels	45
3.2 OPTIMIZATION OF SAMPLE PREPARATION PROTOCOLS AND SDS PAGES	46
3.2.1 Urine protein determination.....	46
3.3 PROTEIN PRECIPITATION.....	47
3.3.1 Protein Quantification.....	49
3.3.2 2D SDS-PAGE.....	50
3.4 PROTEIN IDENTIFICATION.....	57
3.4.1 Mass spectrometry analysis	57
3.4.2 Transducin (beta)-like 3 protein.....	78
3.5 PROTEIN MODELLING	81
CHAPTER 4: DISCUSSION AND CONCLUSION	87
CHAPTER 5: REFERENCES	93
CHAPTER 6: APPENDIX	102
6.1 ADDRESSES OF SUPPLIERS.....	103

LIST OF FIGURES

Figure 1. 1 Chromosomal location of obesity genes.	7
Figure 1. 2 World map of people with diabetes in developed and developing countries	14
Figure 1. 3 Central dogma indicating the relationship between the genome, transcriptome and the proteome (Lam <i>et al.</i> , 2006).....	18
Figure 1. 4 A typical mass spectrometry-based proteomics experiment	21
Figure 3. 1 Average final body weights of CHOW and HF fed rats at 18 weeks.	44
Figure 3. 2 Average ketone body levels in CHOW and HF fed rats.	45
Figure 3. 3 1D protein profiles of untreated CHOW and HF urine.....	46
Figure 3. 4 A 12 % SDS PAGE of protein profiles obtained from different precipitation methods.	48
Figure 3. 5 1D SDS PAGE urine protein profiles of the pellets from HF and CHOW fed rats following acetone precipitation.	49
Figure 3. 6 2D SDS PAGE urine protein profiles of HF and CHOW fed rats.....	52
Figure 3. 7 A 15 % 2D SDS-PAGE urinary protein profile of CHOW fed rats.	53
Figure 3. 8 A 15 % 2D SDS-PAGE urinary protein profile of HF fed rats.	54
Figure 3. 9 Differentially expressed CHOW and HF urinary proteins.....	56
Figure 3. 10 MALDI-TOF mass spectra of a trypsin digested CH1 and HF1 protein spots.....	62
Figure 3. 11 Mass spectrum of C2 trypsin digested protein spot with the matched protein sequence.....	63
Figure 3. 12 Mass spectrum of C3 and HF3 trypsin digested protein spot with their matched Serotonin 5-HT _{2C} protein sequence.	64
Figure 3. 13 Mass spectra of the digested C4 and HF5 protein spots.	65

Figure 3. 14 Similar CHOW mass spectra of C6, C7 and C11 trypsin digested protein spots.....	66
Figure 3. 15 Similar HF mass spectra of HF7, HF8 and HF12 trypsin digested protein spots.....	67
Figure 3. 16 Mass spectrum of the trypsin digested C8 with the alpha-2u globulin precursor sequence.....	69
Figure 3. 17 Mass spectra of the digested C13 and C15 protein spots and identified sequence of C15.....	70
Figure 3. 18 Mass spectra of the digested C18 and C19 protein spots and their respective identified sequences.....	71
Figure 3. 19 MALDI-TOF mass spectrum of the trypsin digested HF13 and HF 14 with their identified alpha-2u globulin precursor sequence.	72
Figure 3. 20 Mass spectra of the digested HF15 and HF16 protein spots and their respective identified sequences.....	73
Figure 3. 21 MALDI-TOF mass spectra of the digested HF17 and HF18 protein spots and their respective identified alpha-2u globulin precursor sequence.	74
Figure 3. 22 Mass spectrum of HF19 trypsin digested protein spot with the matched protein sequence.....	75
Figure 3. 23 MALDI-TOF mass spectrum of trypsin digested HF21 protein spot with the matched protein alpha-2u globulin precursor sequence.	76
Figure 3. 24 Mass spectrum of trypsin digested HF20 protein spot.....	77
Figure 3. 25 MALDI-TOF mass spectrum of trypsin digested HF2 protein spot.....	79
Figure 3. 26 A transducin (beta)-like 3 amino acid sequence, the matched peptides in bold red indicating 11 % sequence coverage.....	80

Figure 3. 27 The predicted secondary structure of Tbl3 generated using PSIPRED (McGuffin <i>et al.</i> , 2000).	84
Figure 3. 28 A cartoon presentation of the Tbl3 model.....	85
Figure 3. 29 A Ramachandran plot of the Tbl3 model generated using RAMPAGE (Lovell <i>et al.</i> , 2003).....	86

LIST OF TABLES

Table 1. 1 Cut-off points proposed by WHO expert committee for the classification of overweight and obesity.....	5
Table 1. 2 A summary of the diabetes diagnostic criteria according to WHO.	17
Table 3. 1. The identified proteins from the CHOW fed rats analysed by MALDI-TOF MS based on the MASCOT database.....	58
Table 3. 2. The identified proteins from the HF fed rats analysed by MALDI-TOF MS based on the MASCOT database	60

LIST OF ABBREVIATIONS

1D PAGE	One dimensional polyacrylamide gel electrophoresis
2D PAGE	Two dimensional polyacrylamide gel electrophoresis
ACTH	Adrenocorticotropic hormone
APS	Ammonium Persulphate
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
CHAPS	3-[(3-Cholamidopropyl) dimethylammonio]- 1-propanesulfonate
CHCA	α -cyna-hydroxy-cinnamic
cm	Centimetre
DTT	Dithiothreitol
<i>et al</i>	<i>et alia</i>
FPG	Fasting Plasma Glucose
IEF	Isoelectric focusing
IPG	Immobilized pH gradient
kDa	Kilo Daltons
kVh	Kilo Volt-hours
LMW	Low Molecular Weight
M	Molar
<i>m/z</i>	Mass per charge
MALDI-TOF	Matrix assisted laser desorption ionization time of flight
ml	Millilitre

mm	Millimetre
mM	Millimolar
MOWSE	Molecular weight search
MSDB	Mass Spectrometry DataBase
NCBI	National Center for Biotechnology Information
ng	Nano gram
OGTT	Oral glucose tolerance test
pI	Isoelectric point
PDB	Protein Data Bank
PMF	Peptide Mass Fingerprint
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Tb13	Transducin (beta)-like 3
TCA	Trichloroacetic acid
TEMED	N, N, N1,N1- Tetra methylethelene-diamine
µg	Microgram
µg/µl	Microgram per microlitre
µl	Microlitre
V	Volts
v/v	Volume per volume
w/v	Weight per volume
WD	Tryptophan and Aspartate
WHO	World Health Organisation

CHAPTER 1: LITERATURE REVIEW

1.1. INTRODUCTION

Obesity has reached epidemic proportions in developed countries. The prevalence of obesity in developing continues to increase at an alarming rate worldwide (Hill and Peter, 1998). The World Health Organisation (WHO) has classified approximately 1.6 billion people worldwide as overweight and over 400 million people as obese (WHO, 2006b), and South Africa is not spared from this epidemic. A report released in 1998 showed that 57 % of women and 29 % of men across all ethnic groups being regarded as overweight and obese (Puoane *et al.*, 2002).

Obesity can result from mutations at genetic level. However, in recent times the environment has influenced the increase in obesity development with increased consumption of diets high in fat, lack of exercise, increased appetite and to some extent eating disorders, in particular binge eating (Hill, 2006). The increased trend in obesity has been associated with increased consumption of high-fat foods (fast foods) (Jeffery and French, 1998).

Obesity is a risk factor for hypertension, atherosclerosis and type II diabetes among other diseases (Rao *et al.*, 2007; Teasdale *et al.*, 2007). In some cases, death results. The increase in obesity has consequently led to an increase in the prevalence of type II diabetes (WHO, 2006b). According to WHO, approximately 85% of people with diabetes are type II, and of these, 90% are obese or overweight (WHO, 2003). Approximately 3.2 million deaths every year are due to complications of diabetes (Unwin and Marlin, 2004).

Some type II diabetes patients bear no symptoms at all and the disease is discovered late, which makes diabetes is a public health problem that if left untreated and result in complications. More often patients present with end stage complications, such as diabetes nephropathy, kidney disorders, heart disorders and retinopathy. At this point, most of the damages caused are irreversible.

Current methods for diagnosis of disease include diagnosis from symptoms, blood tests that measure antibodies and glucose levels, and urine dipsticks that detect nitrite, ketones, glucose and proteins produced during diseases. Although these methods are reliable for diagnosing late stages of diseases, they produce false negative diagnoses in patients with early stages of diseases. The late discovery or diagnosis of diseases leads to late delivery of treatment, resulting in end stage complications and death.

The increase in obesity-associated diseases and mortalities has increased the urgency for the development of technologies that are quick, simple and can diagnose the early stages of diseases. The early diagnosis of diseases would allow for the proper monitoring of diseases and early efficient delivery of treatment for diseases.

1.2. OBESITY

1.2.1 Defining overweight and obesity

Obesity, also known as adiposity, is an excess accumulation of fat mass in the body. Obesity is principally described as the imbalance that results when energy intake exceeds the energy expenditure (Trayhurn, 2004). That is, when the energy intake equals the energy expenditure then energy is balanced and the body fat is stable (Hill, 2000). The accumulation of high-energy intake favours lipid storage leading to an increase in the number and the size of the fat-storage cells known as adipocytes which are found in the adipose tissue (Loos and Bouchard, 2003). Thus increasing the fat mass and ultimately leading to weight gain. Obesity has also been defined as the expansion of white fat mass (Trayhurn, 2004). At the same time, the adipose tissue is regarded as the source of endocrine signals particularly in the regulation of energy balance (Trayhurn, 2004).

1.2.2 Body Mass Index (BMI)

Adiposity is difficult to characterize and as such there are several measures used to determine adiposity. Some of the measurements used are the waist-to-hip ratio, the body mass index (BMI), skinfold thickness ratio and waist circumference. However, BMI is a common assessment method used in determining the prevalence of overweight and obesity.

Table 1. 1 Cut-off points proposed by WHO expert committee for the classification of overweight and obesity

Adapted from (Kopelman, 2000)

BMI (kg.m ⁻²)	WHO classification	Popular description
<18.5	Underweight	Thin
18.5 – 24.9	-	'Healthy', 'normal'
25.0 – 29.9	Grade 1 overweight	Overweight
30.0 – 39.9	Grade 2 overweight	Obesity
≥40.0	Grade 3 overweight	Morbid obesity

BMI is a common and convenient formula that is used to measure body fatness as it uses height and weight. BMI is the quotient of body weight in kilograms and the square of the height in meters (Gallagher *et al.*, 1996). BMI scores as proposed by the World Health Organisation (WHO) are outlined in table 1.1 above. WHO has categorised BMI scores into 5 groups. The first group has a BMI less than 18.5 kg/m² and is classified as underweight. The second group has BMI scores between 18.5 and 24.9 kg/m² and is classified as the 'normal' acceptable range or a healthy BMI. The third group being the overweight group with BMI scores between 25 and 29.9 kg/m². The fourth group is defined as the obesity group, with BMI scores 30 kg/m² and above. The fifth group with BMI scores 40 kg/m² and higher is defined as the morbid obesity group (Kopelman, 2000; WHO, 2006b).

However, BMI has been reported to inaccurately reflect body fatness since it does not take into account muscularity and body density (Spataro *et al.*, 1996). Also, BMI is considered to be less accurate than the skinfold thickness ratio method that measures the skin thickness (Spataro *et al.*, 1996). BMI is not a recommended method in the

assessment of body fatness in children as BMI scores in children change with age and sex (Wildhalm *et al.*, 2001).

High BMI scores are not only associated with obesity, but they have been correlated with a number of human cancers. A 2006 report by the WHO showed that high BMI scores increase the risk of the different forms of cancers including breast, colon, prostate, kidney, gall bladder and endometrium (Renahan *et al.*, 2008).

1.2.3 Causes of obesity

1.2.3.1 Genetics

Several studies have shown that mutations, genetic disorders and genetic make-up or heredity play a role in the development of obesity. For instance, Prader-Willi syndrome, a genetic disorder caused by imprinting of genes on chromosome 15 increases appetite (Loos and Bouchard, 2003). This is caused by the hyper-secretion of a hormone, ghrelin, which stimulates hunger.

In some instances, obesity is influenced by the interplay between genetics and a slew of other factors such as the social and cultural environment (Barsh *et al.*, 2000; Hill and Peter, 1998; Naggert *et al.*, 1997). This has been illustrated through studies on animal models, and monozygotic and dizygotic twins and adoption studies, where differences in the body weight can be noted among individuals (or animals) living in the same environment (Barsh *et al.*, 2000; Eikelis and Esler, 2005; Hill and Peter, 1998; Naggert *et al.*, 1997).

Spontaneous mutations in the leptin and leptin receptor genes, have been found to play a role in the regulation of energy balance thereby causing obesity (Loos and Bouchard, 2003). Zhang and co-workers isolated the obesity (*ob*) genes using positional cloning technologies and found that *ob* genes appeared to encode a signalling factor produced by the adipose tissue and released into the bloodstream for the regulation of body weight (Zhang *et al.*, 1994). The location of the obesity genes on chromosomes can be seen in figure 1.1.

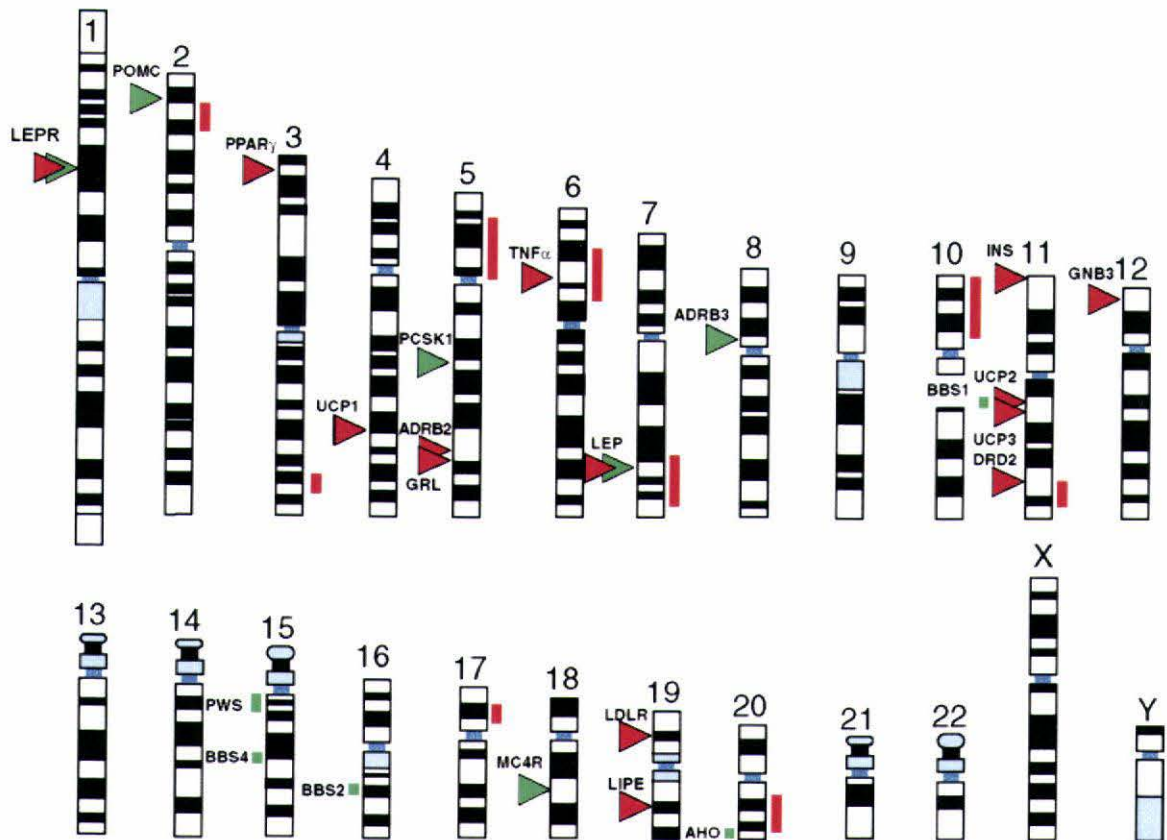


Figure 1. 1 Chromosomal location of obesity genes.

Ideogram of human karyotype with loci for Prader–Willi Syndrome (PWS), Albright hereditary osteodystrophy (AHO), Bardet–Biedl Syndrome (BBS) (green line), monogenic mutations (green triangle), selected obesity candidate genes (red triangle) and QTLs identified by genome-wide linkage scans (red line). Adapted from (Loos and Bouchard, 2003).

1.2.3.2 Dietary intake

Dietary intake is central in the development of obesity. Macronutrients that provide energy are the carbohydrates, fats and proteins. These are balanced by oxidation or storage of the different nutrient (Jéquier and Tappy, 1999). Alcohol, ethanol in particular, has been implicated as another macronutrient that provides energy (Suter, 2000). Moderate alcohol intake leads to ethanol metabolism whereas excessive intake leads to the decrease in lipid oxidation (Suter, 2000). Thus alcohol ingestion can be considered as a risk factor for obesity development.

Changes or shifts in dietary patterns have also been observed, with an increase in diets high in fat. These shift in dietary patterns are evident through the preference and high consumption of fast foods (Stender *et al.*, 2007). An American population study on Coronary Artery Risk Development In young Adults (CARDIA), indicated that fast foods have a high fat content and are made available in bigger portions (Stender *et al.*, 2007).

A high carbohydrate diet has been shown to promote carbohydrate oxidation and a high fat diet to stimulate fat storage (Jéquier and Tappy, 1999). These diets high in fat therefore promote low fat oxidation causing the accumulation of fat in the adipose tissue leading to the development of obesity.

1.2.3.3 Appetite and eating disorders

Appetite can be felt as hunger, leaving individuals yearning for food. Appetite is regulated by the digestive tract, adipose tissue and the brain (Neary *et al.*, 2004). This has been demonstrated through animal model studies (rodents in particular) in the discovery of leptin, an adipose tissue hormone that decreases food intake and loss of weight (Wren *et al.*, 2001).

In addition through animal studies, ghrelin, a digestive tract hormone, has been shown to increase food intake, weight gain and ultimately cause obesity (Tschop *et al.*, 2000; Wren *et al.*, 2001). Ghrelin levels have been shown to be elevated during fasting (Neary *et al.*, 2004).

In the regulation of body weight, Wren and coworkers have suggested ghrelin and leptin to possibly be part of the vital feedback system (Wren *et al.*, 2001). The study revealed that ghrelin levels decreased in lean individuals following food intake. However, following food intake in obese individuals, there was no decrease in the ghrelin levels (English *et al.*, 2002). The study further suggested that the absence or decreased ghrelin levels indicated impaired suppression of food intake, which could result in increased food consumption and weight gain. Thus indicated that food intake failed to suppress ghrelin levels in obese individuals, thereby increasing obesity and its associated diseases.

Eating disorders are defined as the disturbance of eating habits or weight-control behaviours that can result in the impairment of physical health and psychological functioning (Fairburn and Harrison, 2003). Three types of eating disorders have to

date been identified, namely, anorexia nervosa, bulimia nervosa and binge eating disorders. Individuals with anorexia and bulimia nervosa have a tendency to over-assess their shape, weight and are on the contrary usually used by obese individuals to 'control' their weight (Fairburn and Harrison, 2003).

However, binge eating disorders are characterised by uncontrolled overeating without extreme weight-control behaviours, such as vomiting or excessive use of laxatives (Hill, 2006). These over eating episodes may results in increased energy intake. This will thus lead to the development of obesity.

1.2.3.4 Lack of activity

Change is not only observed in dietary patterns but also in the social environment of an individual. The advent of technology, especially in the developed world has promoted a sedentary lifestyle in many people. The environment is enriched by the improvements in technology such as, televisions, computers, electronic games and transportation (Hill and Peter, 1998). These require a great time in an inactive (sitting) position. A sedentary lifestyle is characterised by low levels of physical activity and thus associated with increased risk of obesity development (Hill and Peter, 1998).

1.3. OBESITY AS A RISK FACTOR

Obesity may lead to the impairment of health by increasing the risk of type II diabetes, sleep apnea, atherosclerosis, osteoarthritis, hypertension, coronary artery diseases, osteoarthritis, stroke, respiratory problems and other forms of cancer (Rao *et*

al., 2007; Teasdale *et al.*, 2007). Obesity has also been shown to be the risk factor for the development of eating disorders such as bulimia nervosa, where overweight children or adolescents are tempted to disturb their eating habits in order to lose weight (Hill, 2006). In some cases these obesity-related diseases are fatal.

Described below are the common diseases associated with overweight and obesity.

1.3.1 Type II Diabetes

Insulin is important in the adipocyte biology as it detects glucose in the bloodstream. It causes the absorption of glucose by the skeletal muscle cells, hepatic cells and adipocytes, and storing it in a form of glycogen in the liver and muscle (Khan and Flier, 2000). Hence, insulin promotes adipocyte triglyceride stores and results in the discontinuation of fat as an energy source. The resistance to the effects of insulin on the metabolism of glucose, the uptake of glucose and the storage of glucose is termed insulin resistance (Khan and Flier, 2000). Excess glucose in the bloodstream due to insulin resistance is characteristic of Type II diabetes (Scott *et al.*, 2005).

1.3.2 Hypertension

Hypertension is closely related to renal dysfunction and obesity as hypertensive patients are either overweight or obese (Ribstein *et al.*, 1995). Hypertension is associated with the excretion of sodium salts in the urine caused by natriuretics or the arterial pressure on sodium excretion known as pressure natriuresis (Hall, 2003). The

study further showed that impaired pressure natriuresis accompanies high blood pressure as a result of obesity.

Furthermore, the study revealed that obesity is a predictor for hypertension development through weight gain and raising blood pressure. Hence, essential hypertension develops as a result of renal function alteration and blood pressure is increased when weight is gained (Hall, 2003). The exact mechanism that causes obesity to raise blood pressure is not understood. A relationship has thus been established between hypertension, insulin resistance and abnormal lipid profile (Steinberger and Daniels, 2003).

1.3.3 Renal Diseases

The long-term effects of obesity on the kidneys have been shown through glomerular hyperfiltration in diabetes studies. Glomerular hyperfiltration rate is a process where the glomerular filtration rate increases as a result of increased glomerular capillary pressure and is increased in type II diabetes patients (Jong *et al.*, 2002). It has also been noticed that obesity and diabetes have similar effects on the kidney causing renal damage. Glomerular hyperfiltration is a characteristic of diabetic nephropathy. The increase in glomerular filtration rate leads to increased albumin excretion in the urine resulting in microalbuminuria (Jong *et al.*, 2002). The glomerular filtration rate then falls as a result of excessive albumin excretion leading to noticeable proteinuria and ultimately end-stage renal failure known as diabetic nephropathy.

However, microalbuminuria is not the only predictor of renal failure, obesity, hypertension and complications of diabetes also result in renal damage and ultimately renal failure.

1.4. DIABETES MELLITUS

Diabetes is a disease characterised by hyperglycaemia (high glucose levels) as a result of impaired insulin production or impaired tolerance to its effects ("Report of the expert committee on the diagnosis and classification of diabetes mellitus," 2002). Diabetes is a public health problem that is growing subsequent to the increasing obesity prevalence. In 2000, diabetes mellitus affected 171 million people worldwide. WHO estimates that currently more than 180 million people worldwide are living with diabetes while it is estimated that in 2030, 366 million people will be diabetic. Figure 1.2 demonstrates the world map of diabetics in developed and developing countries. The map also highlights countries with the most diabetics.

Prevalence of diabetes

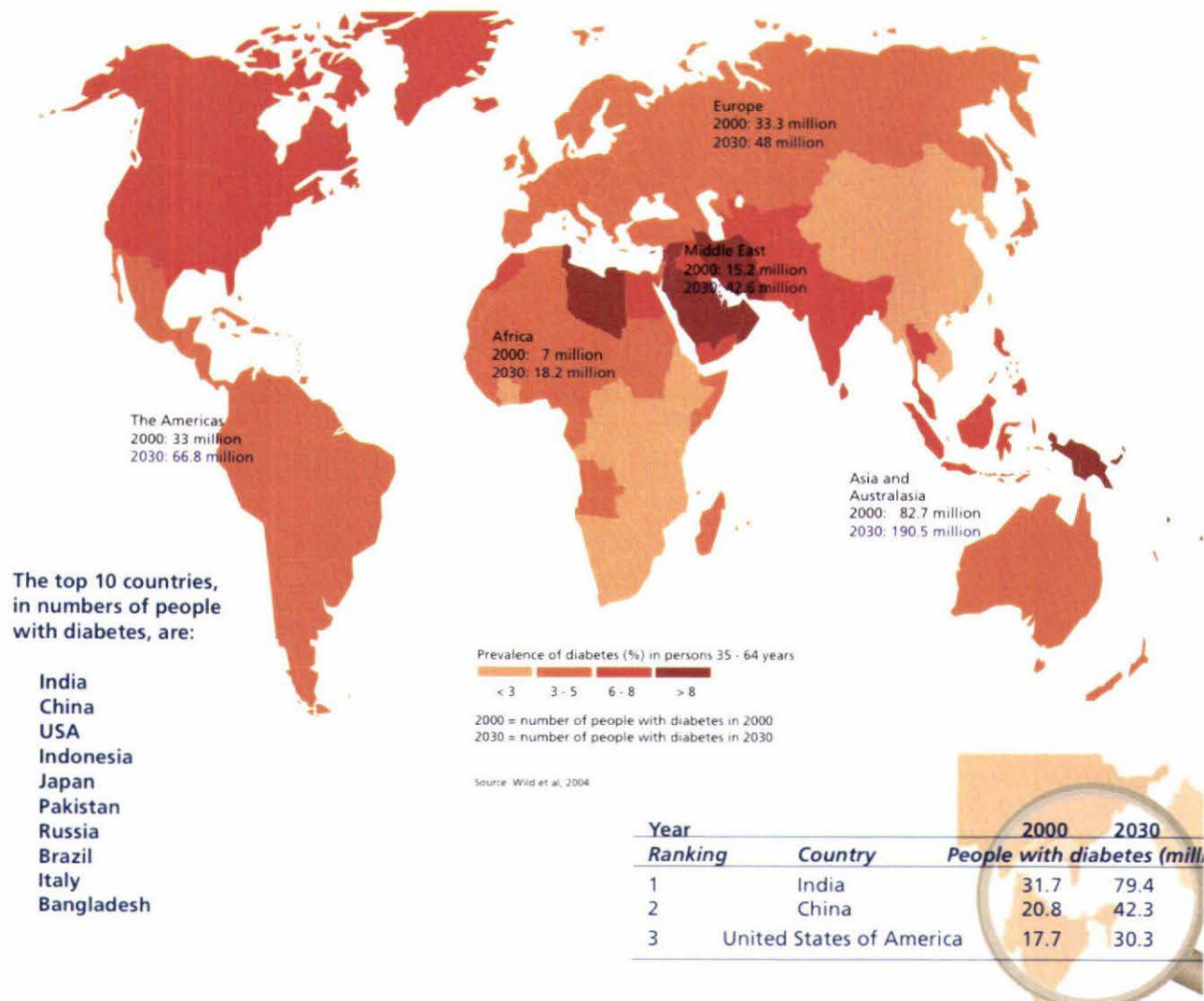


Figure 1. 2 World map of people with diabetes in developed and developing countries

(WHO, 2006a)

1.4.1 Types of diabetes

Diabetes is categorised into 4 groups namely, type I diabetes mellitus, pre-diabetes, type II diabetes mellitus and gestational diabetes. The overall symptoms include excessive excretion of urine, hunger, thirst, fatigue and weight loss (WHO, 2006a). In its advanced stages, diabetes can lead to limb amputations, stroke, blindness and kidney failure.

Type I diabetes results from immune-mediated destruction on insulin-producing islet beta cells.

Prediabetes is usually characterized by the elevation in blood glucose levels, often when the blood glucose levels are above normal but have not reached those of diabetes. It's difficult to diagnose, as there are no signs or symptoms. However people with prediabetes have the same complications with type II diabetic people (WHO, 2006a). People with prediabetes are at high risk of developing type II diabetes.

Type II diabetes is associated with lack of activity and family history of the disease. Type II diabetes is characterised by excess glucose in the bloodstream due to insulin resistance (Scott *et al.*, 2005). Initially the pancreatic beta cells increase the insulin production, resulting in the B-cells been worn out. The worn out beta cells together with insulin resistance and impaired insulin secretion lead to the reduction of insulin-mediated glucose uptake in muscle and adipocytes (Bailey, 2000). Type II diabetes is a disease that typically affected adults over the ages of 40 years but with the increase in obesity, people of younger ages are also affected by the disease (Nolan, 2006; Steinberger and Daniels, 2003).

1.4.2 Diabetes diagnosis

Determining or measuring the amount of glucose in the blood diagnoses diabetes. This is done through the determination of the fasting plasma glucose (FPG) and an oral glucose tolerance test. However, urine properties can also be determined.

1.4.2.1 Oral Glucose Tolerance Test (OGTT)

This test is often used in the diagnosis of prediabetes and diabetes. Blood glucose levels are measured after an 8-10 hour fast. Following this, a person is given 75 g of glucose in a form of a sweet drink. The blood glucose levels are measured at baseline (FPG) and two hours following the intake of glucose. Results are uncertain for diabetes and impaired glucose tolerance if the 2 hour plasma glucose is not measured (WHO, 2006a). Table 1.2 outlines the WHO criteria for diagnosing diabetes and prediabetes.

The impaired glucose tolerance and the impaired fasting glucose are an intermediate stage of diabetes mellitus. The glucose levels resulting are above normal and yet below the diagnostic level of diabetes, a stage often described as prediabetes.

There is however insufficient data to accurately define normal glucose levels (Puavilai *et al.*, 1999). The prediabetes glucose levels differ between WHO and the American Diabetic Association (ADA). In 2003, ADA decided to reduce the prediabetes glucose levels from 6.1 mmol/L to 5.6 mmol/L (WHO, 2006a).

Table 1. 2 A summary of the diabetes diagnostic criteria according to WHO.

Adapted from (WHO, 2006a)

Diabetes	
Fasting plasma glucose	≥ 7.0 mmol/L (126mg/dL)
2 hour plasma glucose*	≥ 11.1 mmol/L (200mg/dL)
Impaired Glucose Tolerance (IGT)	
Fasting plasma glucose	< 7.0 mmol/L (126mg/dL)
2 hour plasma glucose*	≥ 7.8 and < 11.1 mmol/L (140 mg/dL and 200mg/dL)
Impaired Fasting Glucose (IFG)	
Fasting plasma glucose	6.1 to 6.9 mmol/L (110 mg/dL to 125 mg/dL)
2 hour plasma glucose*	< 7.8 mmol/dL (140 mg/dL)

* Venous plasma glucose 2 hour after ingestion of 75g oral glucose load

1.4.2.2 Urine dipsticks

These dipsticks are plastic strips attached with several reagent areas to provide tests for various analytes including pH of the urine, protein, glucose, ketones, leukocytes, nitrites, urobilinogen, specific gravity and bilirubin.

1.5. PROTEOMICS

1.5.1 Defining Proteomics

Proteomics is the study of the proteome, and the proteome is defined as the protein complement of the genome and often referred to as functional genomics (Scott *et al.*, 2005). Figure 1.3 illustrates the central dogma of biology, which is the transcription of DNA to RNA, and the translation of RNA to proteins. The proteome is reported to change over time in response to different situations (Fliser *et al.*, 2007). The complex dynamic nature of the proteome due to post-translational modifications (PTM) has created the field of proteomics to be challenging (Scott *et al.*, 2005).

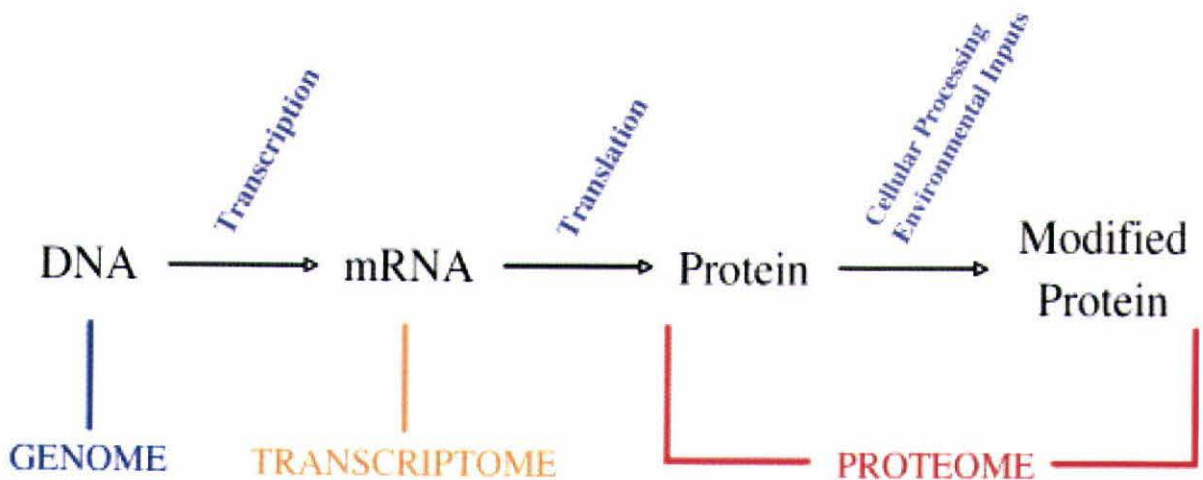


Figure 1. 3 Central dogma indicating the relationship between the genome, transcriptome and the proteome (Lam *et al.*, 2006)

Proteomic techniques date back to the mid 1970s with the introduction of two-dimensional (2D) gel electrophoresis technique by O'Farrell and Klose (Sperling, 2001). However, since the completion of the human genome project, proteomics has become a field of interest (Khan and Packer, 2006). It is believed by scientists that proteomics can reveal proteome changes in biological fluids. Hence, proteomics has a

potential of providing strategies for early diagnosis (through diagnostic marker discoveries) and new therapeutic targets for the treatment of disease (Hanash, 2003; Lam *et al.*, 2006; Scott *et al.*, 2005).

There are many techniques used in the analysis of the proteome. Among them being the techniques for structural proteomics (nuclear magnetic resonance and X-ray crystallography) and for interaction proteomics (yeast two-hybrid and ligand chips). This particular review focuses on expression proteomics. The expression proteomics can help with the identification of expressed proteins in relative samples. Technologies used in expression of proteomics include 2D sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE), protein chips and mass spectrometry (Klein and Thongbookerd, 2004; Roelofsen *et al.*, 2007; Thongboonkerd *et al.*, 2003).

The principles of the expression proteomics are based firstly on the separation of (extracted) proteins by gel electrophoresis on 2D SDS PAGE. The 2D electrophoresis-based technology has been reported to be the most powerful tool when resolving complex protein mixtures from biological samples (Alaiya *et al.*, 2003). Furthermore, its ability to differentiate post-translationally modified proteins have also been reported (Janech *et al.*, 2007).

An important component for proteome analysis is sample preparation. The principles entail inhibition of interfering compounds such as lipids, ionic detergents and nucleic acids that could interfere with 2D electrophoresis, and proper protein solubilization (Alaiya *et al.*, 2003; Lam *et al.*, 2006; Merrell *et al.*, 2004). Another important aspect

of proteome analysis is reproducibility as it is useful in diagnostic applications (Fliser *et al.*, 2007; Merrell *et al.*, 2004).

The first dimension is concerned with separation based on charge according to the isoelectric (IE) point, whereas the second dimension is concerned with separation based on the molecular mass (Scott *et al.*, 2005).

The 2D gel is then stained with an appropriate dye in order to visualise and analyse 'spots' which indicate protein patterns. Relatively high intensity stained 'spots' are selected, excised from the gel and digested by trypsin (Khan and Packer, 2006). This is done in preparation for mass spectrometric analysis which then determines the mass list of the peptides.

According to Aebersold and Mann, a mass spectrometer consists of an ion source, a mass analyzer that measures the mass-to-charge (m/z) ratio and a detector to register the number of ions at each m/z value (Aebersold and Mann, 2003). The most commonly used techniques to volatilize and ionize proteins or peptides for mass spectrometric analyses are the electrospray ionization (ESI) and the matrix-assisted laser desorption ionisation (MALDI). The peaks from the mass list are then matched on the database for protein sequences in the identification of the proteins. Figure 1.4 illustrates the mass spectrometry-based proteomics, as it would be carried out in a typical experiment.

It can thus be said that the use of 2-D SDS PAGE in combination with the mass spectrometry are used to identify and characterise proteins (Thongboonkerd *et al.*, 2003).

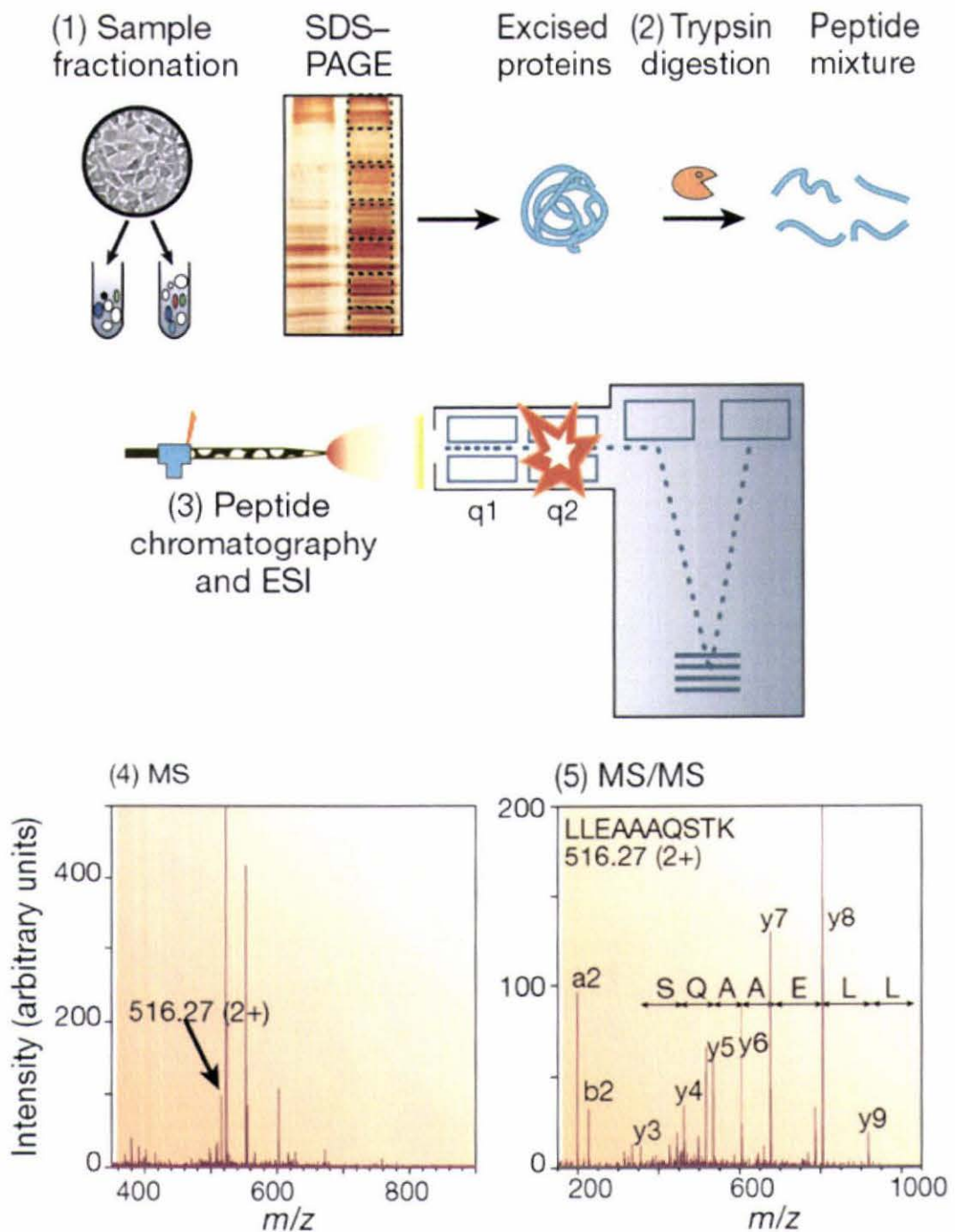


Figure 1. 4 A typical mass spectrometry-based proteomics experiment

In 1, proteins are fractionated and then separated on an SD-PAGE. In 2, spots of interest are then excised and digested by trypsin in preparation of mass spectrometric analysis in 3. The peaks from peptide masses are generated in 4 and then matched on the database for protein sequence generation as in 5. Adapted from (Aebersold and Mann, 2003).

1.5.2 Urinary proteomics in the discovery of diabetes biomarkers

With the completion of the Human Genome Project, the focus has since shifted towards understanding the function and structure of the human genome through the identification of post-translational modifications (PTM), differential expression, protein structure and protein-protein interactions (Sinha *et al.*, 2007). Proteomics has been reported to play a significant role in clinically useful applications through the study of changes in protein expression level or PTM (Vidal *et al.*, 2005). These protein changes highlight the role of peptides as potential biomarkers.

Biomarkers are biological indicators that are used to measure a particular state or progression of diseases (Rifai *et al.*, 2006). The highly employed strategy in the discovery of biomarkers is the top-down proteomics strategy. This strategy involves the separation of proteins by 2D electrophoresis, multidimensional chromatography or capillary electrophoresis followed by tryptic digestion of isolated proteins and the analysis of the resulting peptides by mass spectrometry. Biomarkers are used in the understanding of disease pathogenesis, the early detection of diseases through diagnostic tools, potential drug targets in the treatment of diseases and monitoring the response to therapy (Hanash, 2003; Lam *et al.*, 2006; Scott *et al.*, 2005; Vidal *et al.*, 2005).

Polypeptides in body fluids are relatively accessible for example saliva, cerebrospinal fluid, serum, blood and urine (Miller *et al.*, 2006; Theodorescu and Mischak, 2007; Wait *et al.*, 2001). Thus body fluids are considered interesting biomarker sources for diagnostic purposes. Urine is an easily accessible body fluid that contains complex mixtures of proteins (Roelofsen *et al.*, 2007; Theodorescu and Mischak, 2007). Owing

to the latter factor, urine is thus considered as an interesting source of biomarkers. Urine can also be obtained non-invasively as compared with other body fluids such as blood and cerebrospinal fluids, and that it is plentiful (Hortin and Sviridov, 2007). Urinary proteins are also reported to be stable and to undergo insignificant proteolysis within several hours after collection (Theodorescu and Mischak, 2007). This stability of the proteins also favours urine as an interesting source of biomarkers as it can be stored for several years at -20 °C without significantly altering the proteome.

Urinary proteins have been studied since the 1970s. A study by Anderson and colleagues reported that the human urine contains trace amounts of proteins and some of them diagnostically useful (Anderson *et al.*, 1979). The study further identified types of proteins found in urine. The types included normal plasma proteins, proteins released into the urine from the kidney, proteins reaching the urine after its formation in the kidney, proteins leaked from tissues outside the urogenital tract, hormones or other signal substances, substances released by the products of conception, tumour-associated substances and products of bacterial or viral infections.

Urine proteins originate from ultrafiltration of plasma and from the urinary tract itself (González-Buitrago *et al.*, 2007). As a result, urine contains protein components similar to those found in the blood. Urine is also considered to contain dilute protein concentrations, high salt content and proteins of low molecular weight (LMW) (< 30 kDa) (González-Buitrago *et al.*, 2007; Khan and Packer, 2006; Theodorescu and Mischak, 2007). This high salt content can interfere with the isoelectric focusing of proteins during 2D electrophoresis as it tends to bind to protein surfaces to make them less isoelectric.

Salt is however not the only concern in urine protein analysis. A major concern is the removal of albumin and other abundant proteins. However the removal of albumin and other abundant proteins has been associated with the loss or removal of LMW proteins. The removal of salt and the high abundant proteins from urine through precipitation prior to the separation of urine proteins by electrophoresis is essential.

Another disadvantage of urine is that there are wide variations in protein concentration owing to fluid intake in individuals (Theodorescu and Mischak, 2007). Theodorescu and Mischak further reported on the inconsistency of pH in urine, which may alter the activity of proteases. The latter factor plays a crucial role in polypeptide digestion in preparation for mass spectrometry as the alteration of protease activity may lead to variations in the composition of peptide fragments.

As diabetes continues to be an important clinical problem, proteinuria has been classified as an emerging major cause of morbidity in humans (Candiano *et al.*, 2008). Furthermore it is considered a major factor in renal failure and renal fibrosis and can also potentially trigger kidney inflammation.

Another proteomics study assisted in the identification of urinary protein markers in the prediction of diabetic kidney disorders (Jain *et al.*, 2005). The study revealed four main proteins involved in diabetic kidney disorders. The proteins were identified as Alpha-1 microglobulin which is related to the severity and control of type II diabetes; Alpha-1 acid glycoprotein which is a marker for inflammation and also predicts mortality; Zinc alpha-2 glycoprotein (ZAG) which is expressed in the liver, kidney, breast, prostate, pancreas and various tumours. It is suggested that ZAG is a possible

candidate gene for regulation of body weight. The last protein identified was albumin, which is an early predictor of diabetes, indicating the first signs of the deteriorating kidney function (Jain *et al.*, 2005; Savage *et al.*, 1996). In addition, urinary protein changes in type 2 diabetic patients have been evaluated where transthyretin, a low molecular weight protein, was found to be abundant in type 2 diabetic nephropathy patients (Bellei *et al.*, 2008).

1.5.3 Aims and objectives of the study

Aims:

The study was aimed at using proteomics to identify the differential expression of urinary proteins in lean and obese rats. Also to determine the utility of urine as an alternative source to blood in the diagnosis of prediabetes as urine can be obtained non-invasively.

Objectives:

The specific objectives of the study were to:

- Collect urine from lean and obese Wistar rats and establish a method for the enrichment of low abundance and low molecular weight proteins.
- To generate 2D urine proteome profile maps of the lean and obese Wistar rats.
- To visualize and to identify differentially expressed urinary proteins between the lean and obese Wistar rats through mass spectrometry analysis.

1.5.4 Hypothesis

It is hypothesised that the differentially expressed proteins involved during obesity development will act as potential diagnostic markers and therapeutic targets.

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials and suppliers

Materials (Chemical, reagents)	Suppliers (company details in Appendix)
Acetone	Merck
Acetonitrile	Merck
Acrylamide (40 % 37.5:1 acylamide:bisacrylamide)	Bio-Rad
Agarose	Whitehead Scientific
Ampholytes	Bio-Rad
APS (Ammonium persulfate)	Sigma
Bio Rad dye reagent	Bio-Rad
Bromophenol blue sodium salt	Sigma – Aldrich
BSA (Bovine Serum Albumin)	Seravac
Bovine insulin	Applied Biosystems
CHAPS	Sigma
CHOW diet	Epil
Coomassie Brilliant Blue R-250	Sigma
DTT	Fermentas
Glacial Acetic acid	Merck
Glycerol	Merck
Glycine	Bio-Rad
Hydrochloric acid (HCl)	Merck
Iodoacetamide	Bio-Rad
IPG Strips	Bio-Rad
Ketostix Reagent Strips	Bayer

PlusOne Drystrip cover fluid	Amersham Biosciences
Propan-2-ol	Merck
SDS (Sodium dodecyl sulfate)	Bio-Rad
TCA	Merck
TEMED	Bio-Rad
Tris	Merck
Thiourea	Sigma
Urea	Sigma

2.2 Buffers and solutions

1 % Agarose	1 % agarose dissolved in 1 X SDS buffer
2 X Sample buffer	0.5 M Tris pH 6.8, 20 % glycerol, 10 % SDS and 0.1 % (w/v) bromophenol blue
5 % Stacking gel	0.063 M Tris pH 6.8, 5 % acrylamide, 0.1 % SDS, 0.1 % APS and 0.1 % TEMED
10 % APS	0.1 g ammonium persulfate dissolved in 1 ml distilled water
10 % SDS	10 % SDS dissolved in distilled water
10 X SDS buffer	25 mM Tris, 192 mM glycine and 0.1 % SDS dissolved in distilled water. Adjusted to pH 7.5
1 X SDS buffer	100 ml of mixed 10 X SDS buffer with 900 ml of distilled water
12 % Separating gel	0.375 M Tris pH 8.8, 12 % acrylamide, 0.1 % SDS, 0.1 % APS and 0.1 % TEMED
50 % DTT	0.05 % of DTT and a tinge of bromophenol blue dissolved in lysis buffer

Bio-Rad dye reagent	1 part Bio-Rad dye reagent with 4 parts distilled Water
Coomassie I Staining Solution	10 % glacial acetic acid, 0.025 % (w/v) Coomassie Brilliant blue R-250 and 25 % propan-2-ol dissolved in distilled water
Coomassie II Staining Solution	10 % glacial acetic acid, 0.003125 % (w/v) Coomassie Brilliant blue R-250 and 10 % propan-2-ol dissolved in distilled water
Coomassie III Staining Solution	10 % glacial acetic acid and 0.003125 % (w/v) Coomassie Brilliant blue R-250 dissolved in distilled water
Destaining Solution	10 % glacial acetic acid and 1 % glycerol dissolved in distilled water
Equilibration base buffer	6 M Urea, 2 % SDS, 0.05 M Tris-HCl and 20 % glycerol dissolved in distilled water, pH 8.8
Equilibration buffer 1	6 M Urea, 2 % SDS, 0.05 M Tris-HCl, 20 % glycerol and 2 % DTT dissolved in distilled water, pH 8.8

Equilibration buffer 2	6 M Urea, 2 % SDS, 0.05 M Tris-HCl, 20 % glycerol and 2.5 % iodoacetamide dissolved in distilled water, pH 8.8
High fat diet	5 480 g CHOW (rat pellets), 3 200 g lard, 160 ml oil, 640 g sugar, 80 g vitamin mix, 40 g minerals, 20 g ascorbic acid and 35 ml vitamin D
Solubilization buffer	7 M urea, 2 M thiourea, 4 % CHAPS and a tinge of bromophenol blue dissolved in water
Tris pH 6.8	0.5 M Tris dissolved in distilled water. Adjusted pH with HCl
Tris pH 8.8	1.5 M Tris dissolved in distilled water. Adjusted pH with HCl

2.3 Animal models

For the study, 20 male Wistar rats were used as animal models. The animals were maintained at the MRC Primate and treated according to the rules of the MRC Ethics Committee. The rats were weaned on a balanced standard CHOW diet for four weeks. At four weeks, the rats were equally divided into two groups (10 rats per group). One group was fed CHOW diet and the other was fed a HF *ad libitum*. The two groups were kept on their respective diets for 18 weeks. The body weights were recorded before urine collection.

2.4 Urine collection

Urine was collected from all rats. Rats were placed in metabolic chambers and acclimated for 24 hours. Ketostix were used to measure the ketone levels of each urine sample. To protect the urine from proteases 1 mM PMSF was added to the urine samples. Urine samples were then centrifuged and stored in 2 ml aliquots at -20 °C until required for further use.

2.5 Urine (sample) preparation

2.5.1 Protein determination

This step was taken in order to examine the presence of proteins in urine. Each 2 ml untreated urine sample was thawed and briefly spun. Urinary proteins were resolved on 12 % SDS PAGE gel according to Laemmli's method (Laemmli UK, 1970), which

utilizes a stacking gel to concentrate the proteins into a line before they enter the separating gel, which then separates them according to their molecular weight. Ten μl from each urine sample was mixed with equal volume of 2 X sample buffer and boiled in the heating block (Block Heater, Stuart Scientifica) at 95 °C for 5 minutes. The samples were cooled at room temperature then pulse spun in the microcentrifuge (Eppendorf Microcentrifuge Model 5417, Sigma-Aldrich). The samples were then loaded on the gels. Electrophoresis was carried out at 100 V for 90 minutes.

2.5.2 SDS PAGE Staining

Following electrophoresis, the gels were stained using the three-step coomassie staining. The gels were first placed in the coomassie I stain solution then heated in a microwave for 1 minute at 900 watt and incubated overnight at room temperature with agitation. The stain was decanted, replaced with coomassie II stain solution and heated in a microwave for 1 minute at 900 watt. The gels were incubated with the stain for 30 minutes with agitation. Following staining with coomassie II staining solution, the stain was decanted, replaced with coomassie III staining solution then heated in a microwave for about 1 minute at 900 watt. The gels were incubated with the stain for 30 minutes with agitation. The stain was decanted and the gels were destained with the destaining solution until the desired protein bands or spots were achieved. The images were captured with a digital camera (Olympus Optical).

2.5.3 Protein precipitation

After confirming the presence of proteins in urine, the urinary proteins were precipitated using three different precipitation methods namely; acetone, acetonitrile and TCA-acetone. The acetone, acetonitrile and TCA-acetone were pre-chilled at -20 °C.

2.5.3.1 Acetone precipitation

Precipitation of urinary proteins with acetone was carried out as described by Khan and Packer (Khan and Packer, 2006).

To prepare the samples for acetone precipitation, a 1:5 urine-to-acetone ratio was used to precipitate urine proteins (Khan and Packer, 2006). The urinary proteins were precipitated overnight at -20 °C. Following overnight precipitation, the samples were centrifuged at 14 000 rpm for 15 minutes at 4 °C. The supernatants were stored at -20 °C for later use and the pellets were resuspended in 200 µl of solubilization buffer and stored at -20 °C for later use.

2.5.3.2 Acetonitrile precipitation

Precipitation of urinary proteins with acetonitrile was carried out as described by Khan and Packer (Khan and Packer, 2006).

A 1:5 urine-to-acetonitrile ratio was used to precipitate urinary proteins with acetonitrile (Khan and Packer, 2006). The urinary proteins were precipitated

overnight at -20 °C. Following overnight precipitation, the samples were centrifuged at 14 000 rpm for 15 minutes at 4 °C. The supernatants, middle supernatants and upper supernatants were stored at -20 °C for later use and the pellets were resuspended in 200 µl solubilization buffer and stored at -20 °C for later use.

2.5.3.3 TCA-acetone precipitation

Precipitation of urinary proteins with TCA-acetone was carried out as described by Chen and coworkers (Chen *et al.*, 2005).

Urinary proteins were also precipitated using the TCA-acetone precipitation method (Chen *et al.*, 2005). To prepare the samples for TCA-acetone precipitation, 100 µl of the urine sample was added to 100 µl of distilled water. To the mixture, 4 volumes of ice-cold 10 % TCA-acetone (v/v) was added and immediately mixed by gentle vortexing (Vortex, VELP Scientifica) for 10 minutes. The samples were incubated at -20 °C for 90 minutes. Following incubation, the samples were centrifuged at 16 000 x g (12 274 rpm) for 15 minutes at 4 °C. The supernatants were carefully removed and transferred into new tubes and labeled accordingly. To wash the pellets, 1 ml of ice-cold acetone was added to the pellet and incubated on ice for 15 minutes, followed by centrifugation at 16 000 x g (12 274 rpm) for 15 minutes at 4 °C. The supernatants were removed and mixed with the first supernatants. The pellets were briefly air-dried to remove excess acetone. The tubes were immediately capped. The pellets were then resuspended in 100 µl solubilization buffer and stored at -20 °C for later use.

To the TCA-acetone containing supernatants, 1 ml of ice-cold acetone was added. The tubes were then incubated on ice for 15 minutes, followed by centrifugation at 16 000 x g (12 274 rpm) for 15 minutes at 4 °C. The supernatants were removed and discarded. The pellets were briefly air-dried, to remove excess acetone. The tubes were immediately capped. The pellets were then resuspended in 100 µl of solubilization buffer and stored at -20 °C for later use.

2.6 One dimensional (1D) SDS-PAGE Analysis

From the acetone pellet, the acetone supernatant, the acetonitrile pellet, acetonitrile upper supernatant, acetonitrile middle supernatant, TCA-acetone supernatant and TCA-acetone pellet, 10 µl respectively was mixed with equal volume of 2 X sample buffer and boiled in the Block Heater (Stuart Scientifica) at 95 °C for 5 minutes.

Once the proteins were denatured, the samples were cooled to room temperature, pulse spun in the microcentrifuge, then loaded on the gels by first stacking on the 5 % gels and resolving the proteins on the 12 % gels. The gels were assembled using the Mini-Protean III[®] cell (Bio-Rad). The gels were run at 100 V for 90 minutes. The gels were stained as described in section 2.5.2. The image was captured with a digital camera (Olympus Optical).

2.7 Protein quantification

2.7.1 Bradford protein assay

The acetone pellets were thawed in preparation for the quantification of proteins. The protein concentrations were determined using the Bio-Rad assay. Protein quantification was done in duplicates. Bovine Serum Albumin (BSA) was used as a standard. The standard BSA concentrations were 25 µg/ml, 125 µg/ml, 250 µg/ml, 500 µg/ml, 750 µg/ml and 1000 µg/ml. In each cuvette, 25 µl of the different concentrations was added to 1 ml of the BIO RAD dye reagent.

From the respective acetone precipitated urine samples, 1:50 dilutions were prepared following the manufacturer's directions. In each cuvette, 25 µl from the respective 1:50 dilutions was mixed with 1 ml of the Bio-Rad dye working solution and incubated at room temperature for 30 minutes. Absorbance was read at the wavelength of 595 nm.

2.8 Two dimension (2D) SDS page analysis

2.8.1 Sample solubilization

100 µg of the acetone precipitated HF and CHOW urine samples were added to 1.25 µl of ampholytes, 2 µl of 50 % DTT and solubilization buffer to the final volume of 125 µl.

2.8.2 In-gel rehydration

The samples prepared from respective diet groups in section 2.7.1 were loaded in the grooves of the re-swelling tray (Amersham Biosciences). The gel sides of the 7 cm long IPG strips pH 4-7 (Bio-Rad) were immersed in the prepared samples in the re-swelling tray and the IPG strips were covered with mineral oil (Amersham Biosciences). The respective IPG strips were re-hydrated overnight at room temperature.

2.8.3 Isoelectric focusing (IEF)

Following the overnight re-hydration of IPG strips, the strips were loaded on the EttanTM IPGphorIITM IEF machine (Amersham Biosciences), aligned and overlaid with mineral oil. The IEF machine was programmed for a three-step long run. For the first step, 250 V were applied for 10 minutes. For the second step, 4 000 V were applied for an hour. For the final step, 4 000 V were applied until 12 000 V-hours were reached. The IEF was carried out at 20 °C.

2.8.4 Equilibration of the strips

Equilibration buffers 1 and 2 were prepared fresh by adding 2 % (w/v) DTT (buffer 1) and 2.5 % (w/v) iodoacetamide (buffer 2) to equilibration base buffer respectively to make the final volume of 5 ml. Buffer 1 containing DTT reduces disulfide bonds and maintains monothiols in the reduced state. Buffer 2 contains iodoacetamide which covalently binds cysteines so that the protein cannot form disulphide bonds.

Following the IEF, the strips were rinsed in water and placed in each channel of the rehydration/equilibration tray. Each channel was filled with 2.5ml of equilibration buffer 1 and incubated with gentle agitation for 20 minutes. After 20 minutes, the equilibration buffer 1 was decanted and each channel was refilled with 2.5 ml of equilibration buffer 2. The strips were then incubated with gentle agitation for 20 minutes. The strips were rinsed with 1 X SDS buffer in preparation for the second dimension.

2.8.5 Second dimension

Following equilibration, the strips were placed on top of 15 % SDS PAGE gels. The gels were assembled using the Mini-Protean III[®] cell (Bio-Rad). To secure the strips, 1 % low melting agarose gel was overlaid on top of the strips. The gels were run at 100 V for 90 minutes. The gels were then stained using the three-step coomassie stain as described in section 2.5.2. The images were captured with a digital camera (Olympus Optical).

2.9 In-gel digestion

All the spots with high intensity were excised manually and transferred into sterile microcentrifuge tubes. The gel pieces were washed twice with 50 mM ammonium bicarbonate for 5 minutes and a third time for 30 minutes with occasional vortexing. The gel pieces were then destained twice with 50 % (v/v) 50 mM ammonium bicarbonate and 50 % (v/v) acetonitrile for 30 minutes with occasional vortexing. The gel pieces were dehydrated with 100 μ l of 100 % (v/v) acetonitrile for 5 minutes, and then completely desiccated under vacuum using a Speed Vac SC100 (ThermoSavant). Proteins were in-gel digested with approximately 120 ng sequencing grade modified trypsin dissolved in 25 mM ammonium bicarbonate for 6 hours at 37 °C. The protein digestion was stopped by adding 50 μ l of 1 % (v/v) trifluoroacetic acid (TFA) and incubating 2-4 hours at room temperature before storage at 4 °C until further analysis.

2.10 Protein identification with MALDI-TOF-MS

Protein identification was carried out by Dr. Ludivine Thomas from the Proteomics Research Group (PRG) at the University of the Western Cape.

Respective analytes consisting of digested proteins (1 μ l) were mixed separately with the 1 μ l of α -cyano-hydroxy-cinnamic (CHCA) matrix and spotted onto a MALDI target plate for analysis by MALDI-TOF mass spectrometry using a Voyager DE Pro Biospectrometry workstation (Applied Biosystems) to generate a peptide mass fingerprint (PMF). The MALDI-TOF was operated in the positive ion delayed extraction reflector mode for highest resolution and mass accuracy. Peptides were ionized with a 337 nm laser and spectra were acquired at 20 kV acceleration potential

with optimized parameters. Close external calibration was employed using the Sequazyme calibrationTM mixture II containing angiotensin I, ACTH (1-17 clip), ACTH (18-39 clip) and bovine insulin. This calibration method typically provided mass accuracy of 100 to 200 ppm across the mass range 900 to 5,000 Da. Peptide spectra of accumulated 1,200 shots each were automatically processed for baseline correction, noise removal, and peak deisotoping. Threshold was manually adjusted between 2 and 8 % base peak intensity.

All searches were performed against the National Center for Biotechnology Information (NCBI) and Mass Spectrometry DataBase (MSDB) peptide mass databases using MASCOT (http://www.matrixscience.com/search_form_select.html). Candidate identifications with the highest Molecular weight search (MOWSE) scores were automatically considered as positive assignments. If more than one protein satisfied mentioned threshold criteria, the entry with the highest MOWSE score was assigned.

2.11 Determination of the secondary structure

Protein sequence of Transducin (beta)-like 3 was threaded through the PDB using GenTHREADER (Jones, 1999) and PSIPRED (McGuffin *et al.*, 2000) for the prediction of the secondary structure. Subsequently a model was built using swiss-modeller. The accuracy of the model was assessed using RAMPAGE (Lovell *et al.*, 2003).

CHAPTER 3: RESULTS

3.1 ANIMAL STUDIES

3.1.1 Body weights

The animals (male rats) were fed a high fat in order to induce obesity. The body weights of the rats in the HF group steadily increased over a period of 18 weeks. The rats fed CHOW were labelled lean while the rats fed HF were obese based on the increase in body weight compared to lean rats. Averages of the final body weights from the two groups are illustrated in figure 4.1. The CHOW group had mean body weight of 466.54 ± 4.16 g while the HF group had a mean of 647.54 ± 7.83 g.

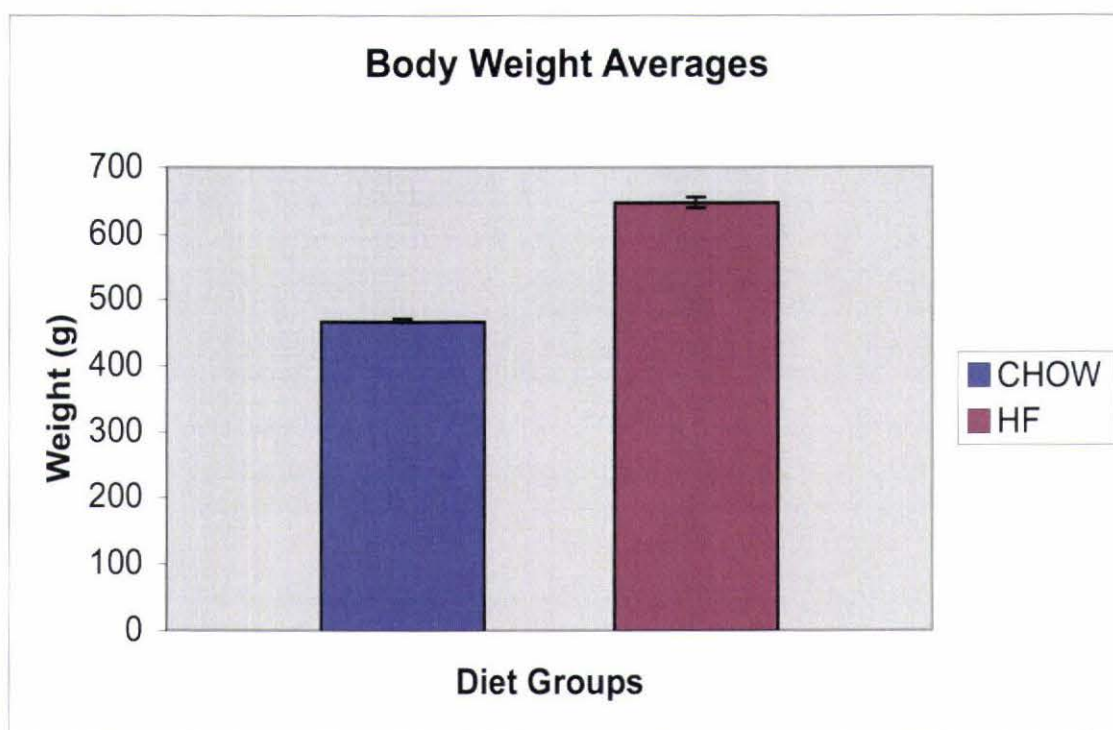


Figure 3. 1 Average final body weights of CHOW and HF fed rats at 18 weeks.

3.1.2 Ketone body levels

Ketone bodies are produced in the blood when fat, instead of glucose, is broken down and used for energy source. The Ketostix Reagent Strip results are expressed as negative, trace, +, ++, and +++ ("Package Insert, Bayer Ketostix Reagent Strips, Bayer Corp., Diagnostics Division, Elkhart, Indiana. ,"). Figure 3.2 demonstrates average ketone body levels in CHOW and HF fed rats. The average ketone body levels in CHOW and HF fed rats were 0.5 ± 0 mmol/L and 1.5 ± 0 mmol/L respectively. A trace of ketone bodies present in the urine of CHOW fed rats is equivalent to 0.5 mmol/L. Ketone bodies of 1.5 mmol/L are equivalent to a +. This indicates the formation of ketone bodies, which should be a caution for diabetes. Individuals with urinary ketone bodies above 1.5 mmol/L, which are ++, and +++, are classified as diabetic.

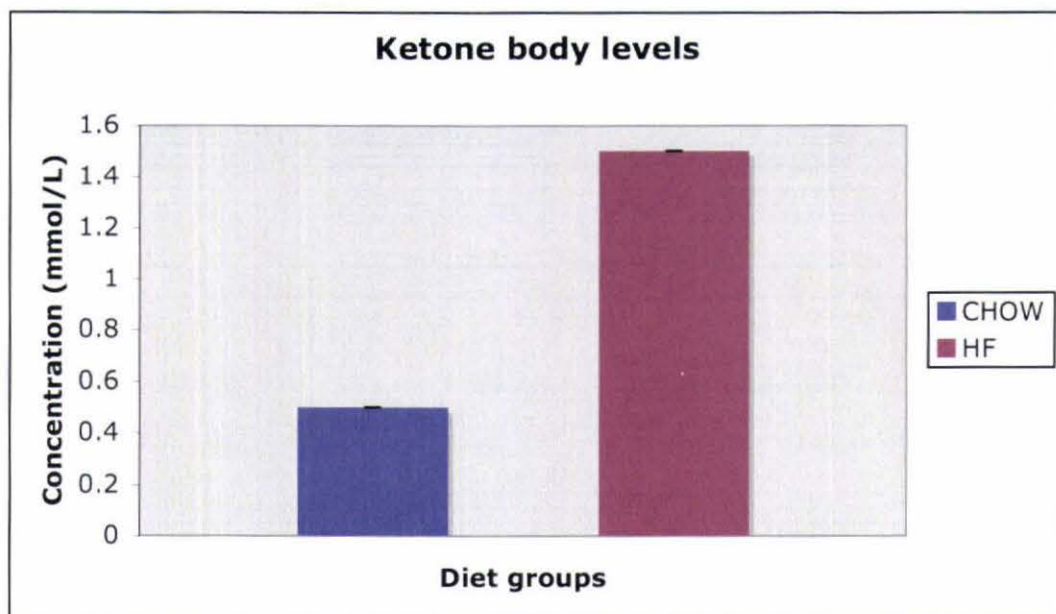


Figure 3. 2 Average ketone body levels in CHOW and HF fed rats.

The Ketostix Reagent Strips were immersed in CHOW and HF urine respectively and the results were read after 40 seconds.

3.2 OPTIMIZATION OF SAMPLE PREPARATION PROTOCOLS AND SDS PAGES

3.2.1 Urine protein determination

To determine whether proteins were present in the urine samples, 10 μ l of urine from the lean (CHOW fed) and obese (HF fed) rats was separated on a 12 % SDS PAGE. Figure 3.3 illustrates untreated urine protein profiles. Three bands were visually identified. The 1D PAGE showed that low molecular weight (LMW) proteins indicated as 'b' were highly expressed in both groups. These proteins were however highly expressed in obese rats than lean rats.

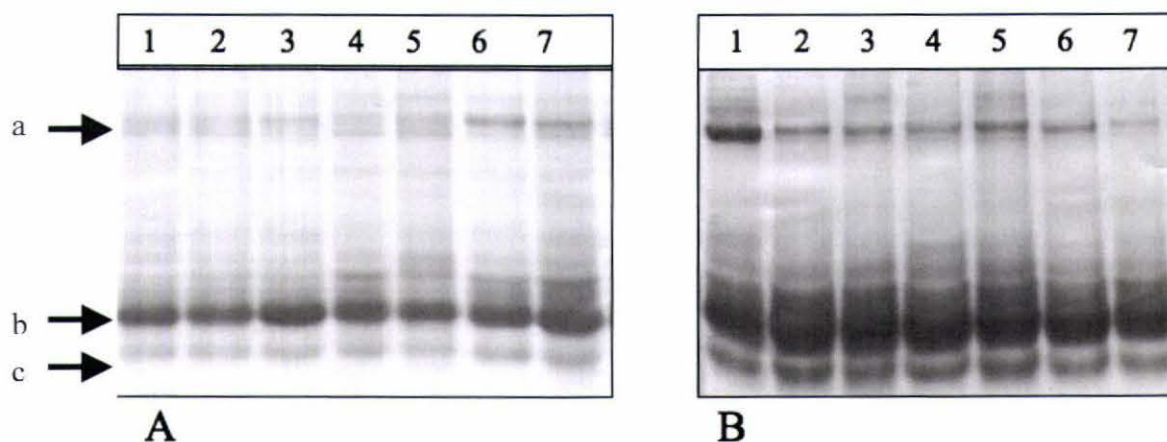


Figure 3. 3 1D protein profiles of untreated CHOW and HF urine.

Untreated urine from CHOW fed (A), and HF fed (B) separated on a 12 % SDS PAGE with protein bands a, b and c indicating highly expressed proteins.

3.3 PROTEIN PRECIPITATION

To remove the salt and to enhance LMW proteins, urinary proteins were precipitated using three different methods, TCA-acetone, acetone and acetonitrile and resolved on a 12 % SDS PAGE. Figure 3.4 shows the protein patterns obtained using different precipitation methods. Using the TCA-acetone method, one band at approximately 17 kDa was observed from the pellet. Faint protein bands were seen in the supernatant. Both the supernatant and the pellet from the TCA-acetone method showed loss or removal of the proteins from the samples.

Using the acetone method, protein bands from the pellet were intense and resembled a similar protein pattern to the untreated urine protein expression pattern. Therefore urinary proteins were enhanced in the pellet when using the acetone method whilst supernatant showed fewer proteins compared to the untreated urine. With the acetonitrile method, no proteins were recovered in the supernatant. The middle supernatant showed the removal of the high molecular weight proteins, which were seen around 55 kDa. However, proteins between 55 kDa and 17 kDa had disappeared and were not recovered in the pellet. Proteins disappeared when using the acetonitrile method. Therefore the acetone precipitation was a desired method as it showed better protein recovery and also enhanced LMW proteins. Urinary proteins were thus precipitated using the acetone precipitation protocol and the pellets were kept for further analysis. Supernatants were discarded, as LMW proteins were not enhanced.

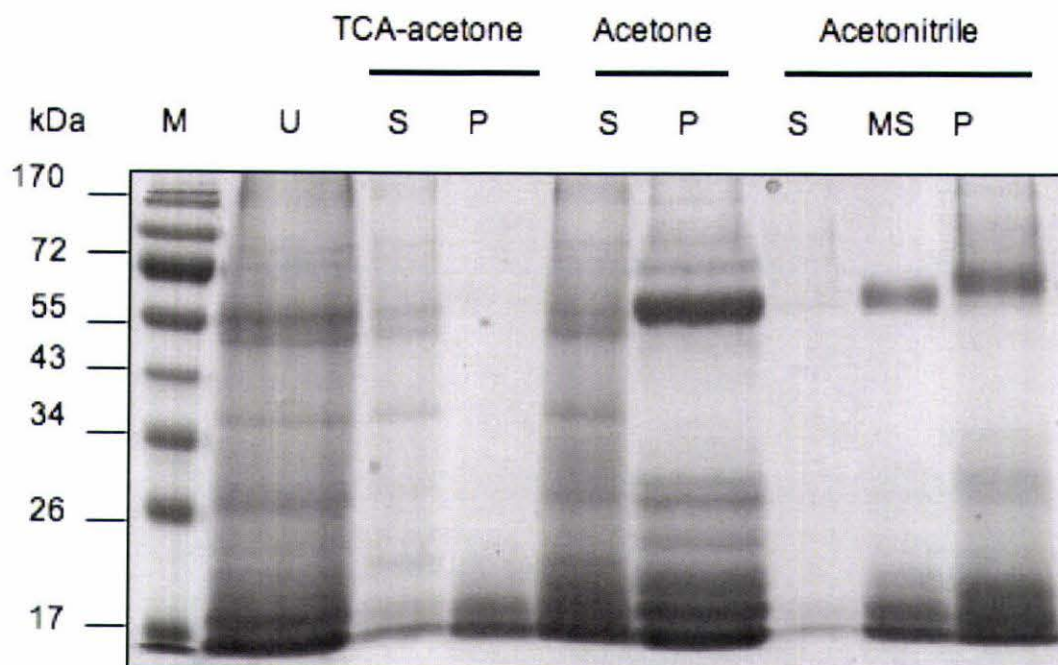


Figure 3. 4 A 12 % SDS PAGE of protein profiles obtained from different precipitation methods.

Lane 1 = molecular weight marker; lane 2 = untreated HF fed rat urine; lane 3 = TCA-Acetone supernatant; lane 4 = TCA-Acetone pellet; lane 5 = acetone supernatant; lane 6 = acetone pellet; lane 7 = acetonitrile supernatant, lane 8 = acetonitrile middle supernatant; and lane 9 = acetonitrile pellet. Where M = molecular weight marker; U = untreated HF urine; S = supernatant; MS = middle supernatant; P = pellet.

3.3.1 Protein Quantification

Following acetone precipitation, the pellet proteins were quantified using the Bio-Rad protocol. Urine protein concentrations varied between the CHOW and the HF group with average concentrations of 0.91 $\mu\text{g}/\mu\text{l}$ and 0.78 $\mu\text{g}/\mu\text{l}$ respectively. Once the protein concentrations were determined, 20 μg of acetone precipitated proteins from CHOW and HF were separated on a 12 % SDS PAGE to determine the 1D urinary protein profiles as illustrated in figure 3.5. The PAGE showed distinct differences between the two groups. CHOW fed rats had 3 proteins at approximately 50 kDa whereas one band was observed from the HF fed rats (indicated as 'a'). Three protein bands were observed below 26 kDa in both groups. This is indicated as 'b'. Although these three proteins were observed in both groups, their intensity was higher in the HF group. The protein profile showed that LMW proteins were abundant in both the CHOW and HF fed rats.

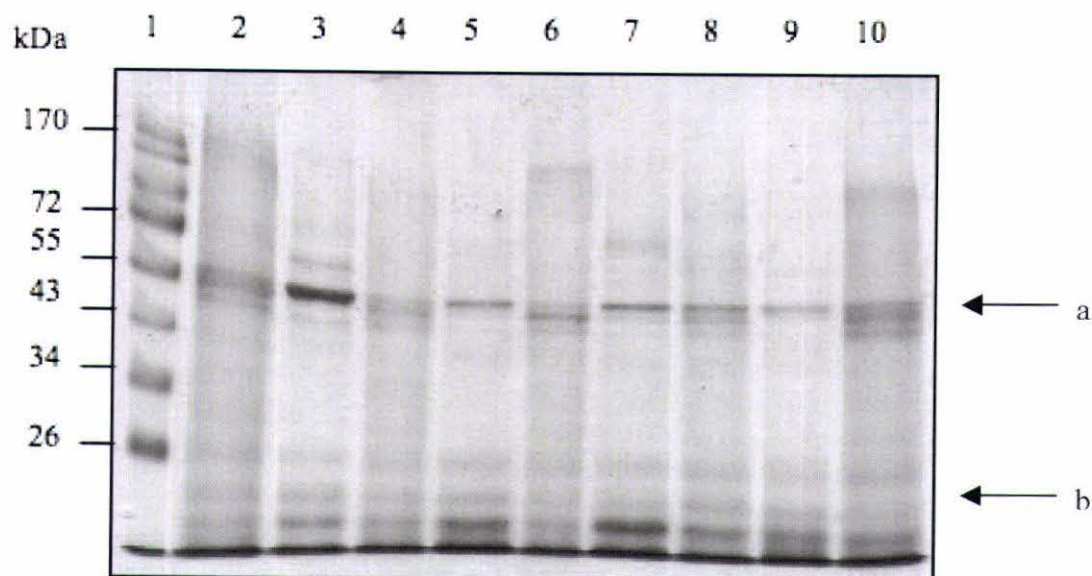


Figure 3. 5 1D SDS PAGE urine protein profiles of the pellets from HF and CHOW fed rats following acetone precipitation.

Lane 1 shows the molecular weight marker. Lanes 2, 4, 6, 8 and 10 show urine proteins from CHOW fed rats and lanes 3, 5, 7 and 9 show urine proteins from HF fed rats.

3.3.2 2D SDS-PAGE

Subsequent to the determination of the 1D SDS PAGE urine profiles, urinary proteins from the CHOW fed and High Fat (HF) fed rats were analysed on 2D SDS PAGE gels to determine their respective protein profiles. Prior to the development of 2D urine profiles certain parameters, such as IPG strips and the focusing conditions were optimised. Different pH range strips namely, pH 3-10 and pH 4-7 were used.

Initially broad range, pH 3-10, IPG strips were used to separate the proteins. 100 µg of urine proteins from the five rats per group were analysed. The proteins were initially focused at 250 V for 10 minutes, then at 4 000 V for an hour and finally at 4000 V until 10 000 V hours were reached. The strips were then equilibrated for 10 minutes and the proteins resolved on a 12 % SDS PAGE. The resulting gel was stained as in section 2.5.2. Upon viewing the gel, the gel was of poor resolution showing clustered protein spots with vertical streaks. The vertical streaks might have been as a result of incomplete reduction or alkylation. Though the gel was of poor resolution, proteins of interest appeared in the narrow range. The protein spots of interest were also observed to be of molecular weight below 30 kDa.

To improve the gel quality for detailed analysis, 7 cm long IPG strips pH 4-7 were used to separate the proteins. To optimize the running conditions, the duration of the third step in the focusing of proteins was increased from 10 000 V-hrs to 12 000 V-hrs at a current of 4 000 V at a constant temperature of 20 °C. To decrease the vertical streaks, the equilibration time was increased from 10 minutes in each buffer to 20 minutes in the respective equilibration buffers.

The resolving gel percentage was increased from 12 % to 15 % SDS PAGE to improve the resolution of LMW proteins. Five SDS PAGE gels in figure 3.6 show the resulting protein profiles. Similar protein expression profiles were seen between the rats in the respective groups. However only two gels, one from each diet group, were chosen to represent the protein profiles from each diet group. The proteomic profiles showed LMW proteins ranging between 10 kDa and 30 kDa. The 2D SDS PAGE gels were analysed visually.

From the CHOW 2D SDS PAGE protein profile, 20 spots were identified. The spots were labelled with C, denoting CHOW followed by the spot number. From the HF 2D SDS PAGE protein profile, 21 spots were identified. The spots were labelled with HF, denoting High Fat followed by the spot number. The CHOW and HF protein profiles are illustrated in figure 3.7 and 3.8, respectively.

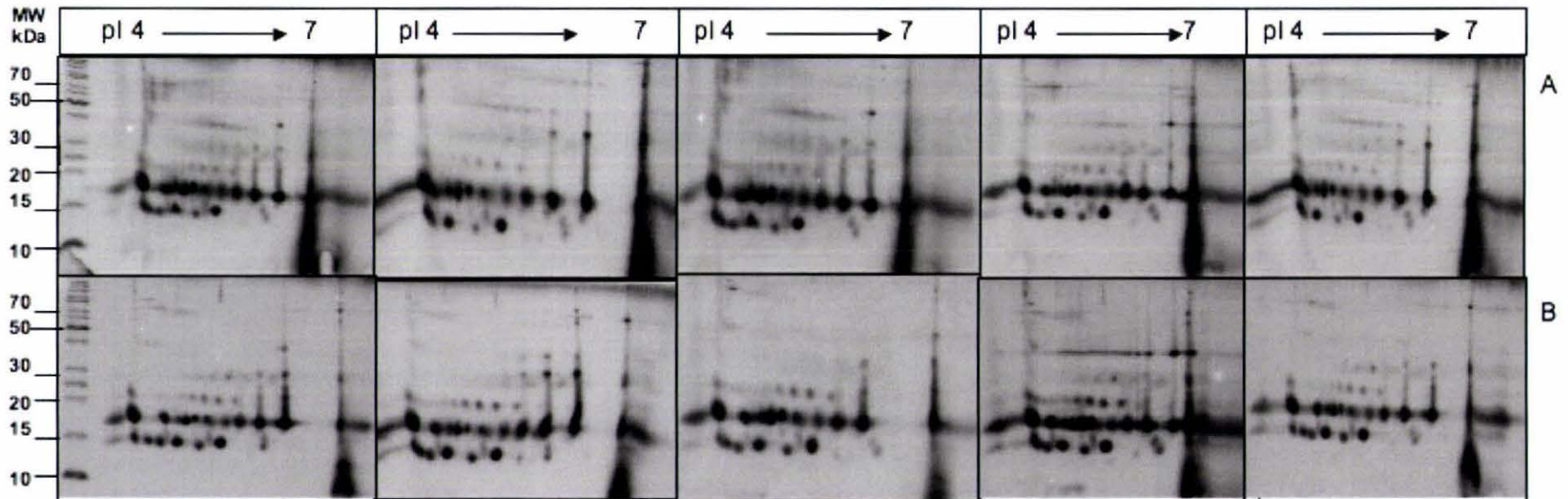


Figure 3.6 2D SDS PAGE urine protein profiles of HF and CHOW fed rats.

Urine proteins of CHOW and HF fed rats were first separated on charge according to their isoelectric point and then separated according to their mass on 15 % SDS PAGE gels. The CHOW 2D profiles are shown in A and the HF 2D profiles are shown in B.

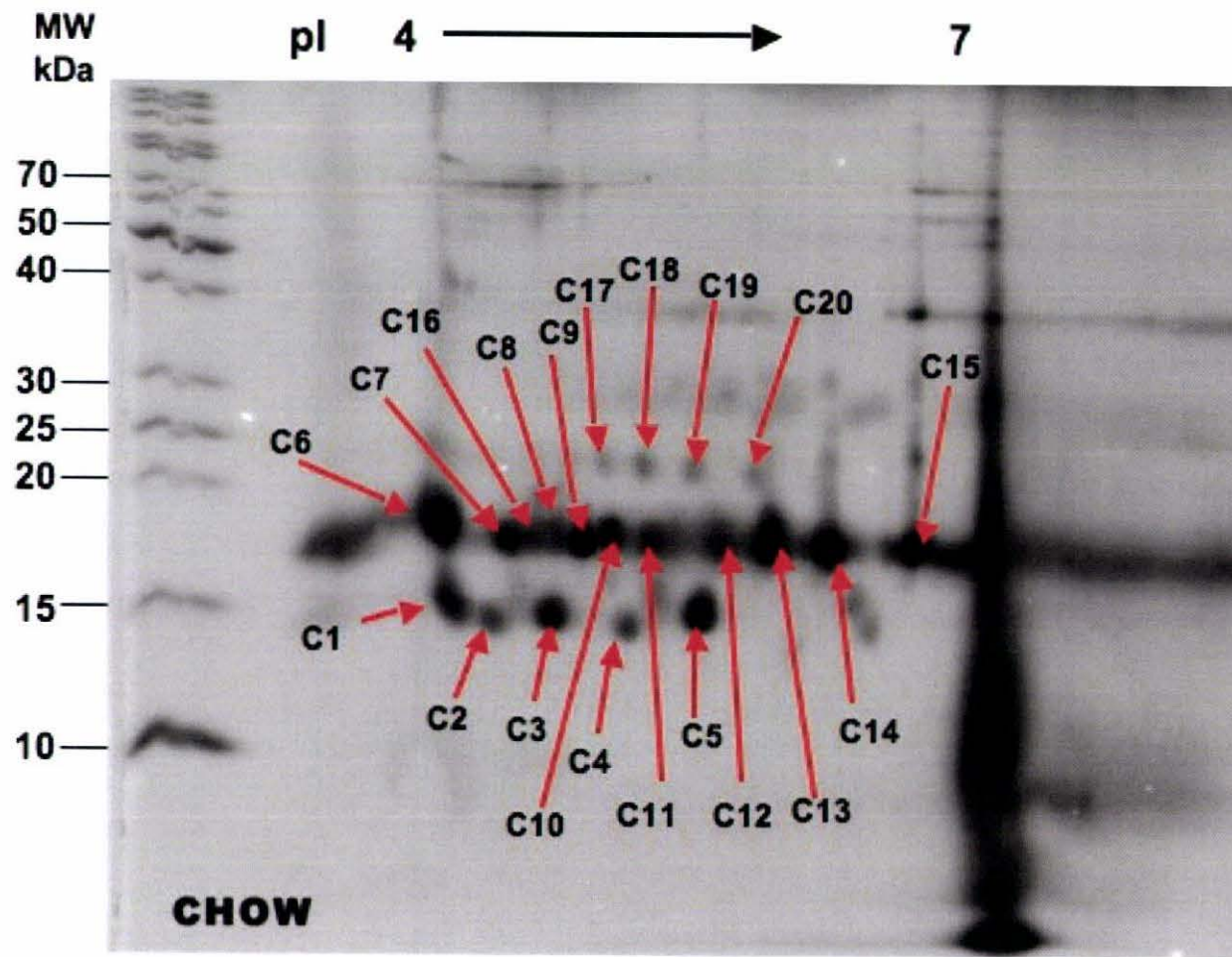


Figure 3. 7 A 15 % 2D SDS-PAGE urinary protein profile of CHOW fed rats.

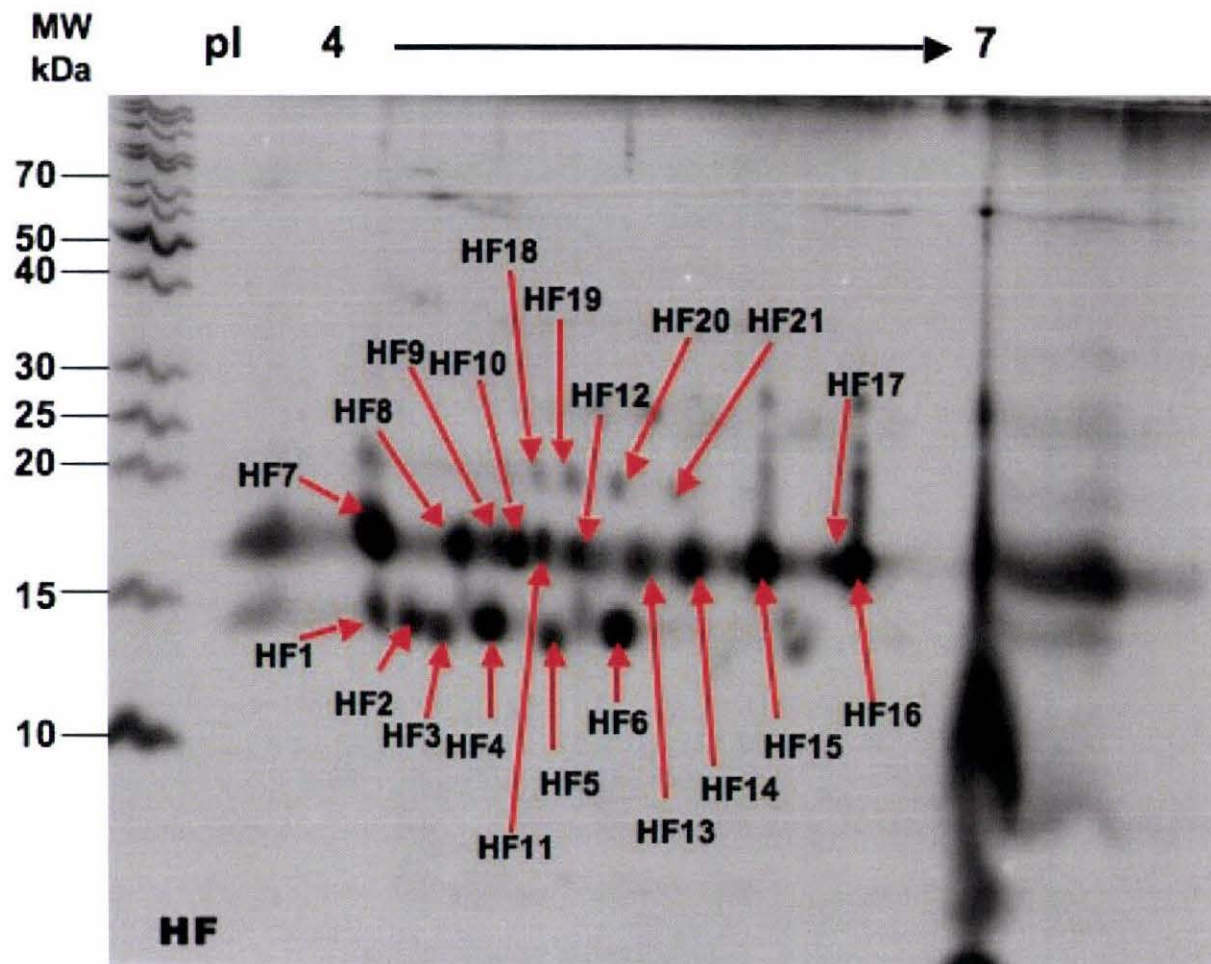


Figure 3. 8 A 15 % 2D SDS-PAGE urinary protein profile of HF fed rats.

The CHOW and HF protein profiles had similar protein expression pattern. However, the HF protein profile had one protein, HF2, which was not expressed by the CHOW fed rats. When comparing the CHOW and HF protein profiles it was noticed that there were increased urinary proteins in the HF group than in the CHOW group. There is however a noticeable decrease in some of the urinary proteins excreted from the HF group when compared to the CHOW group. The increase and decrease in CHOW and HF urinary proteins were measured visually either by the increase, decrease or both the increase and decrease in protein spots intensity and size. When comparing the two groups, there were 3 increased and 4 decreased urinary proteins from the HF protein profile when compared to the CHOW protein profile. Magnified increased and decreased urinary spots from the CHOW and HF 2D protein profiles are provided in figure 3.9.

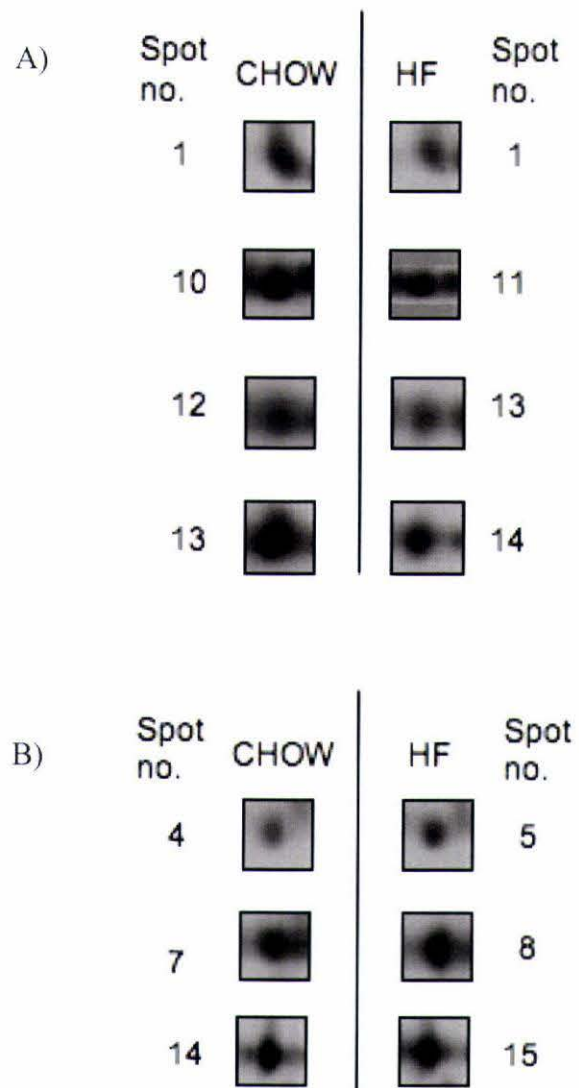


Figure 3. 9 Differentially expressed CHOW and HF urinary proteins

A) Magnified decreased HF urinary proteins in comparison to CHOW urinary proteins. B) Magnified increased HF urinary proteins in comparison to CHOW urinary proteins.

3.4 PROTEIN IDENTIFICATION

Following the successful separation of the proteins, the labelled 41 protein spots (20 CHOW and 21 HF) were excised from the 2D gels and digested with trypsin. This was done in order to generate peptides. The respective analytes were then mixed with the matrix and a laser was applied to the source plate. The matrix absorbed the energy from the laser and transferred it to the analytes. The ions were then analysed according to their mass/charge ratio. A mass list was then created from which peaks were matched on the database to identify the protein.

3.4.1 Mass spectrometry analysis

Significant proteins were identified according to the probability-based Mowse score and were matched on the Mascot database. The protein Mowse score was calculated as $-10\log P$, where P is the absolute probability of matching a number of peaks by random chance at a significance threshold of $p < 0.05$. Tables 3.1 and 3.2 illustrate the summary of the identified proteins from CHOW and HF respectively using the MALDI-TOF-MS.

The Mascot database showed that spots CH1 and HF1 have similar ion spectra although there was no positive identification on the protein. The spectra are shown in figure 3.10. Three similar peaks were identified from the spectra at approximately 2211, 1297 and 2809. This suggests that there is possibility of CH1 and HF1 being the same protein. Spots C2, C3 and HF3 had similar ion spectra, shown in figures 3.11 and 3.12, and were identified as Serotonin 5-HT_{2C} also referred to as 5-hydroxytryptamine (serotonin) receptor 2c.

Table 3. 1. The identified proteins from the CHOW fed rats analysed by MALDI-TOF MS based on the MASCOT database

Spot No.	Protein Name	Accession No.	Estimated MW (kDa)	MW (kDa)	pI	MOWSE Score
1	No positive identification		15			
2	Serotonin 5-HT2c	Q62842	14	33.63	9.52	59
3	Serotonin 5-HT2c	Q62842	14	33.63	9.52	62
4	No positive identification		14			
5	No positive identification		14			
6	No positive identification		18			
7	No positive identification		17			
8	Alpha-2u Globulin precursor	S05440	17	20.33	4.86	86
9	Alpha-2u Globulin PGCL4	gi 76880469	17	20.33	4.86	82
10	Alpha-2u Globulin PGCL4	gi 76880469	17	20.33	4.86	80
11	No positive identification		17			

Spot No.	Protein Name	Accession No.	Estimated MW (kDa)	MW (kDa)	pI	MOWSE Score
12	Alpha-2u Globulin, Chain A	2A2GA	17	18.30	5.30	100
13	Alpha-2u Globulin precursor	gi 204261	17	20.16	5.43	79
14	No spectrum found		17			
15	Alpha-2u Globulin precursor	gi 204261	17	20.16	5.43	82
16	Hypothetical protein LOC683313	gi 155369696	17	59.21	8.06	80
17	No spectrum found		17			
18	Alpha-2u Globulin precursor	gi 204261	17	20.16	5.43	111
19	Alpha-2u Globulin precursor	gi 204261	17	20.16	5.43	107
20	Alpha-2u Globulin precursor	gi 204261	17	20.16	5.43	94

Table 3. 2. The identified proteins from the HF fed rats analysed by MALDI-TOF MS based on the MASCOT database

Spot No.	Protein Name	Accession No.	Estimated MW (kDa)	MW (kDa)	pI	MOWSE Score
1	No positive identification		15			
2	Transducin (Beta)-like 3	Q5U2W5_RAT	14	88.31	6.26	60
3	Serotonin 5-HT2c	Q62842	14	33.63	9.52	61
4	No positive identification		14			
5	No positive identification		14			
6	No positive identification		14			
7	No positive identification		18			
8	No positive identification		17			
9	Alpha-2u Globulin PGCL4	gi 76880469	17	20.33	4.86	73
10	Alpha-2u Globulin PGCL4	gi 76880469	17	20.33	4.86	108
11	No positive identification		17			

Spot No.	Protein Name	Accession No.	Estimated MW (kDa)	MW (kDa)	pI	MOWSE Score
12	No positive identification		17			
13	Alpha-2u Globulin precursor	gi 204261	17	20.16	5.43	72
14	Alpha-2u Globulin precursor	gi 204261	17	20.16	5.43	168
15	Alpha-2u Globulin precursor	gi 204261	17	20.16	5.43	176
16	Alpha-2u Globulin precursor	gi 204261	17	20.16	5.43	146
17	Alpha-2u Globulin precursor	gi 204261	17	20.16	5.43	152
18	Alpha-2u Globulin precursor	gi 204261	17	20.16	5.43	76
19	Alpha-2u Globulin precursor	gi 204261	17	20.16	5.43	68
20	No positive identification					
21	Alpha-2u Globulin precursor	gi 204261	17	20.16	5.43	119

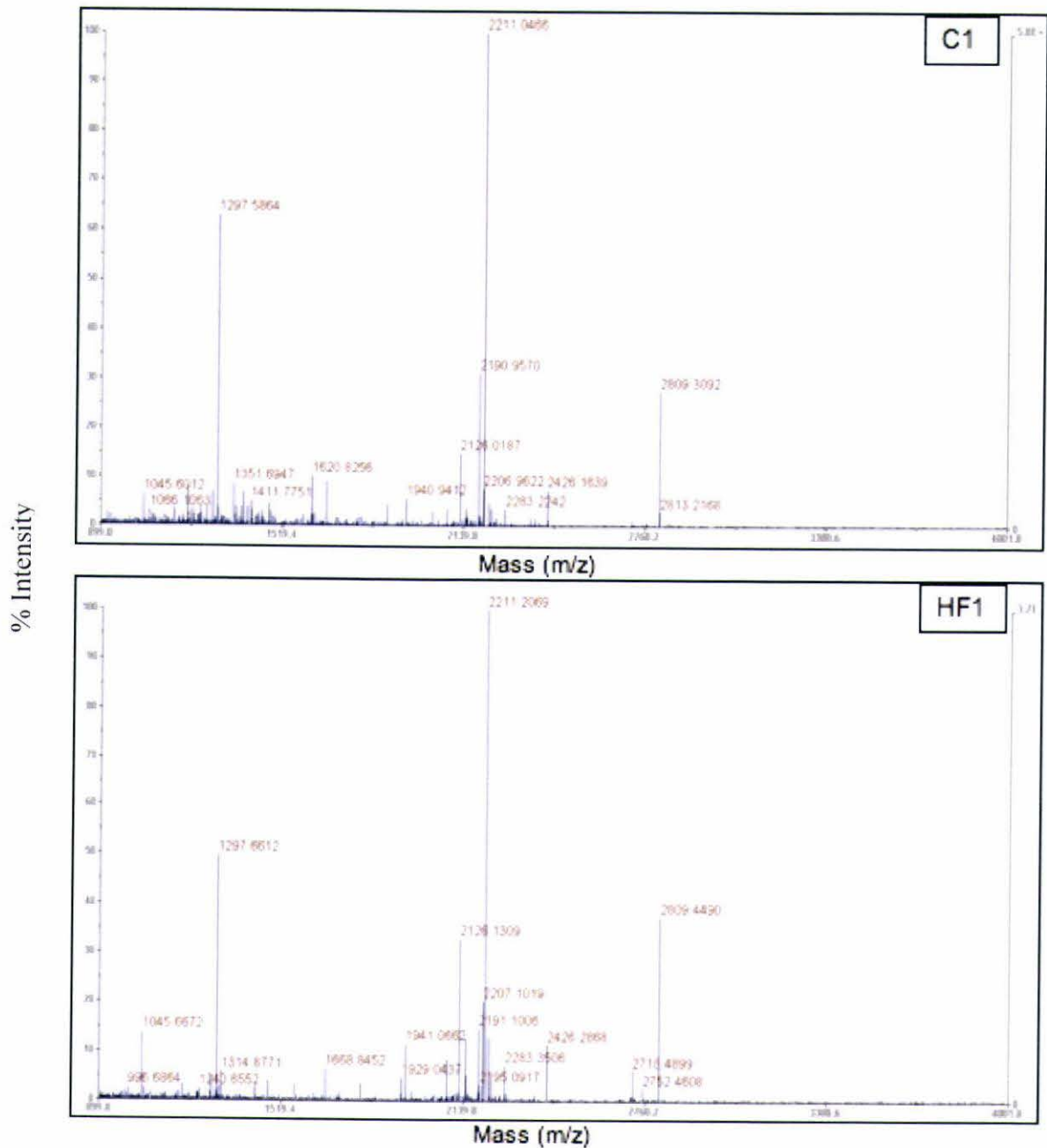


Figure 3. 10 MALDI-TOF mass spectra of a trypsin digested CH1 and HF1 protein spots.

Spots C1 and HF1 were cut from the gel and digested with trypsin. The eluates were mixed 1:1 with μ -CHCA matrix solution, 1 μ l was spotted onto a MALDI plate and allowed to air dry before MALDI-TOF MS analysis.

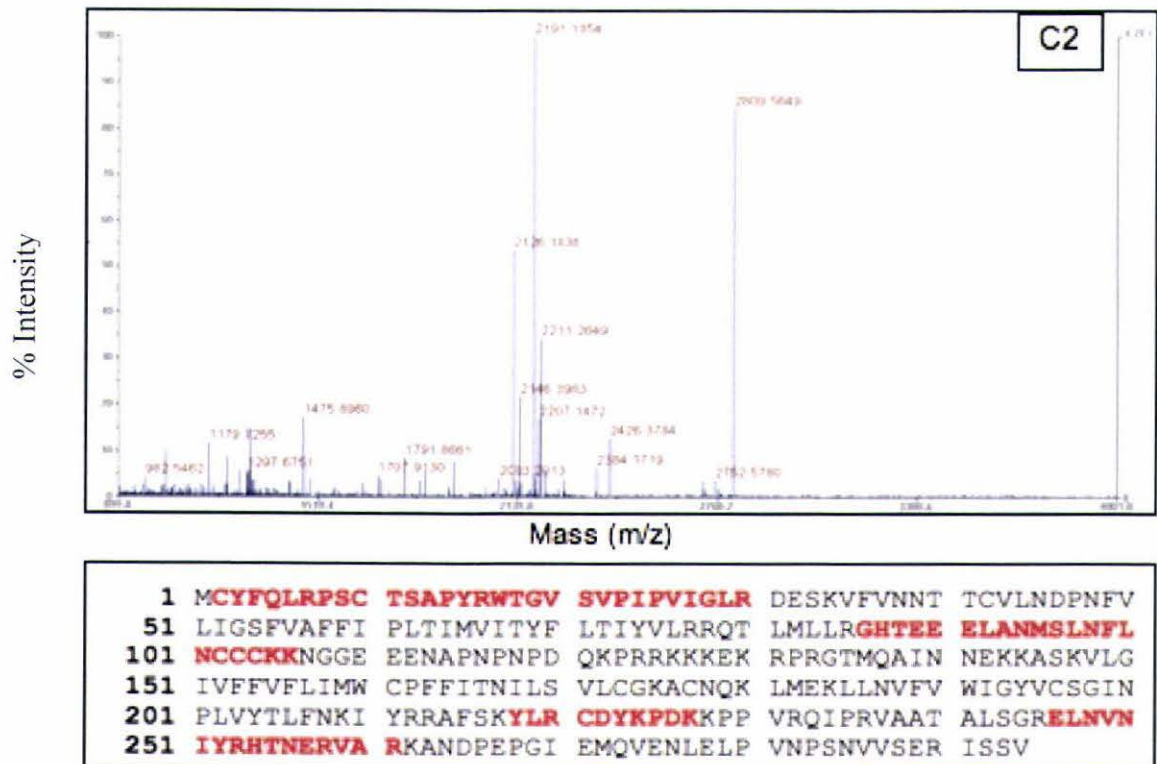
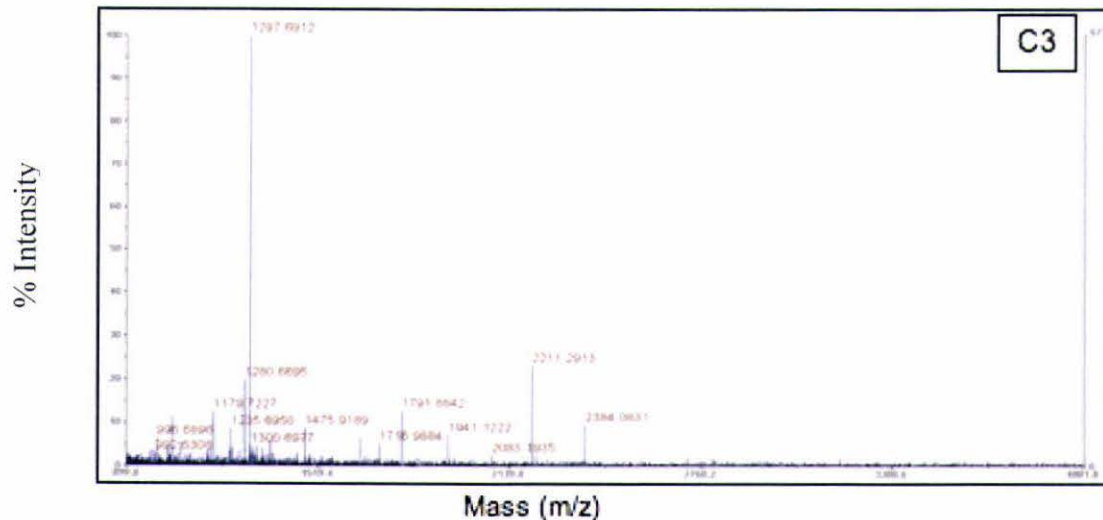
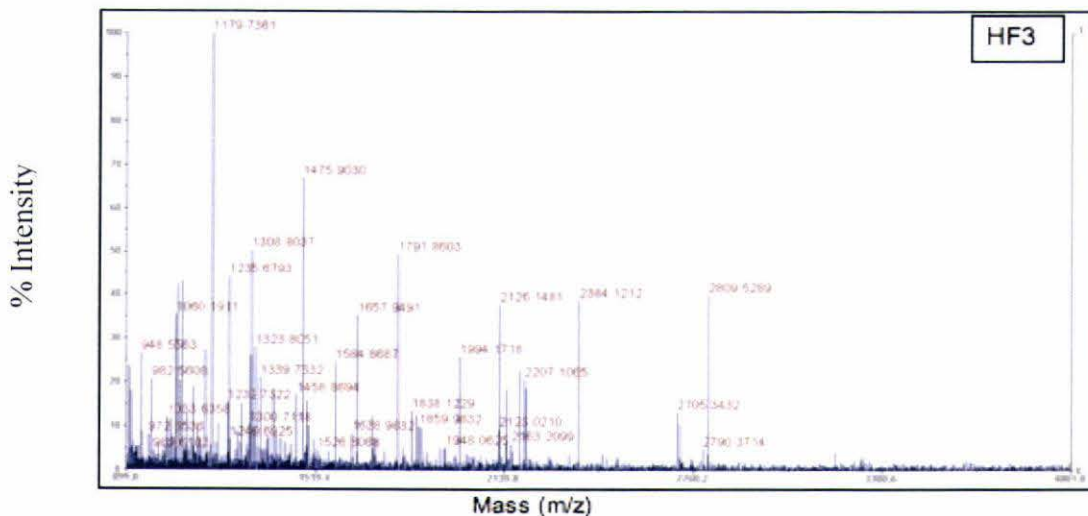


Figure 3. 11 Mass spectrum of C2 trypsin digested protein spot with the matched protein sequence.

Spot C2 was cut from the gel and digested with trypsin. The eluate were mixed 1:1 with μ -CHCA matrix solution, 1 μ l was spotted onto a MALDI plate and allowed to air dry before MALDI-TOF MS analysis. Sequence was identified using the Mascot search that showed the matched peptides in red.



1	MCYFQLR PSC	TSAPYR WTGV	SVPIPVIGLR	DESKVFNNT	TCVLNDPNFV
51	LIGSFVAFFI	PLTIMVITYF	LTIVVLRRT	LMLLRGHT EE	ELANMSLN FL
101	NCCCKK NGGE	EENAPNPDP	QKPRRKKKEK	RPRGTMQAIN	NEKKASKVLG
151	IVFFVFLIMW	CPFFITNILS	VLCGKACNQK	LMEKLLNVFV	WIGYVCSGIN
201	PLVYTLFNKI	YRRAFSKYLR	CDYKPD KKPP	VR QIPRVA AT	ALSGRE LVN
251	IYRHTNER VA	RKANDPE PGI	EMQVENLELP	VNPSNVVSR	ISSV



1	MCYFQLR PSC	TSAPYR WTGV	SVPIPVIGLR	DESKVFNNT	TCVLNDPNFV
51	LIGSFVAFFI	PLTIMVITYF	LTIVVLRRT	LMLLRGHT EE	ELANMSLN FL
101	NCCCKK NGGE	EENAPNPDP	QKPRRKKKEK	RPRGTMQAIN	NEKKASKVLG
151	IVFFVFLIMW	CPFFITNILS	VLCGKACNQK	LMEKLLNVFV	WIGYVCSGIN
201	PLVYTLFNKI	YRRAFSKYLR	CDYKPD KKPP	VR QIPRVA AT	ALSGRE LVN
251	IYRHTNER VA	RKANDPE PGI	EMQVENLELP	VNPSNVVSR	ISSV

Figure 3. 12 Mass spectrum of C3 and HF3 trypsin digested protein spot with their matched Serotonin 5-HT_{2C} protein sequence.

Spots C3 and HF3 were cut from the gel and digested with trypsin. The spectra were generated using the MALDI-TOF MS. Sequence was identified as Serotonin 5-HT_{2C} using the Mascot search that showed the matched peptides in red with a sequence coverage of 25 % for spot C13 and 22 % for protein spot HF3.

Protein spots C4 and HF5 had similar ion spectra with no positive identification on the protein. The spectra are shown in figure 3.13. Three similar peaks were identified from the spectra at approximately 2125, 2146 and 2190.

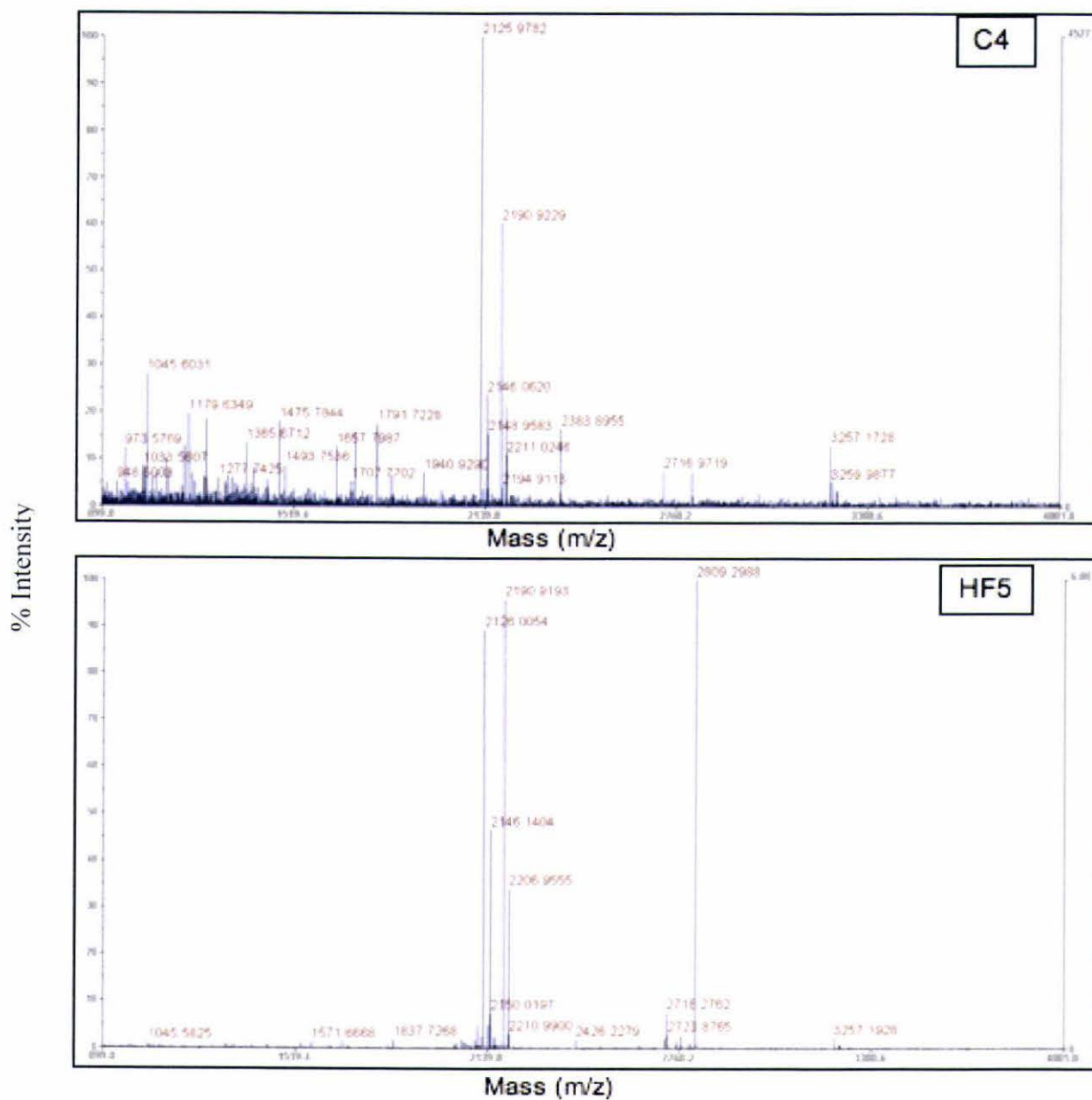


Figure 3. 13 Mass spectra of the digested C4 and HF5 protein spots.

Spots C4 and HF5 were cut from the gel and digested with trypsin. The spectra were generated using the MALDI-TOF MS.

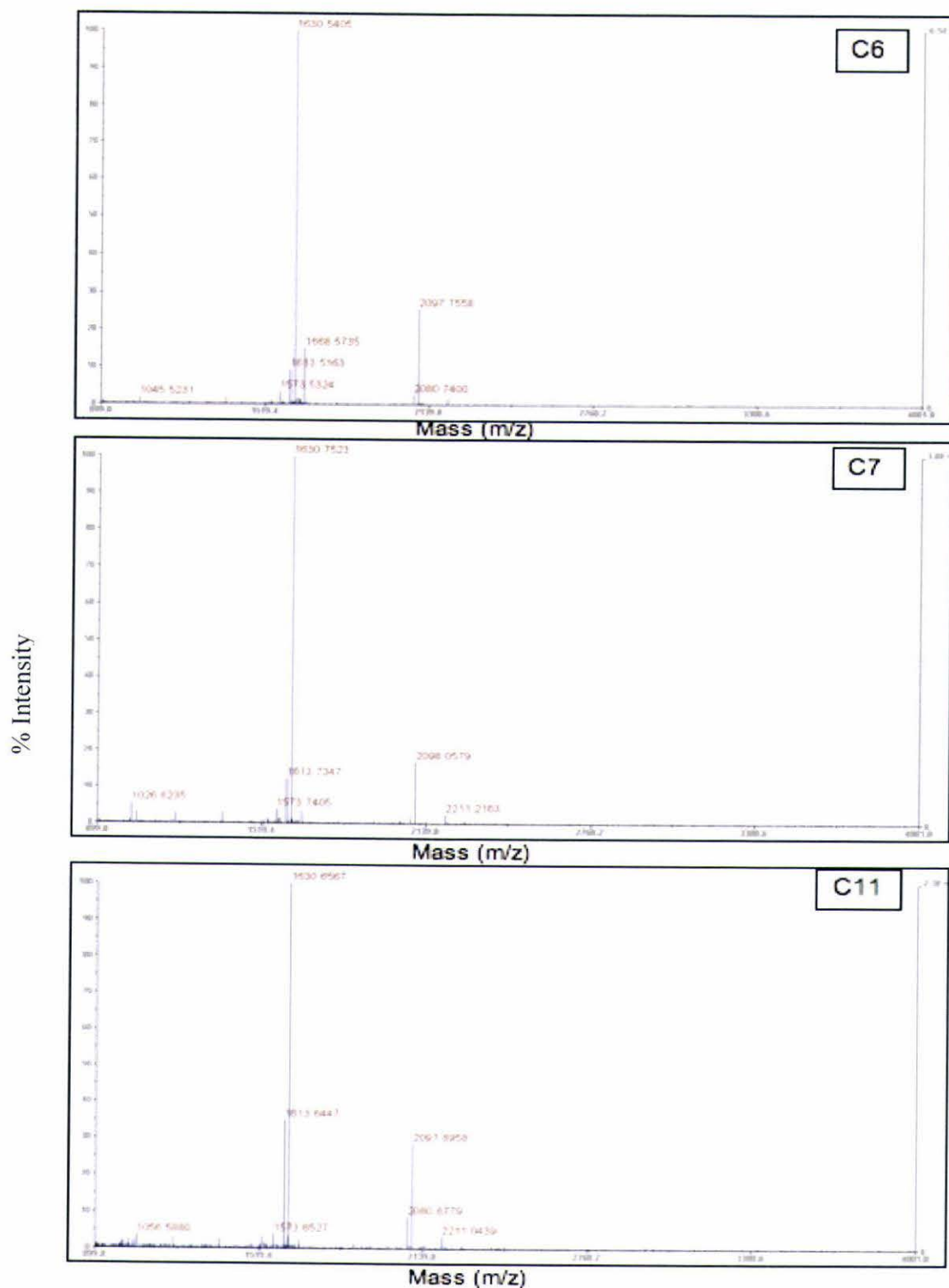


Figure 3. 14 Similar CHOW mass spectra of C6, C7 and C11 trypsin digested protein spots.

Spots C6, C7 and C11 were cut from the gel and digested with trypsin. The eluates were mixed 1:1 with μ -CHCA matrix solution, 1 μ l was spotted onto a MALDI plate and allowed to air dry before MALDI-TOF MS analysis.

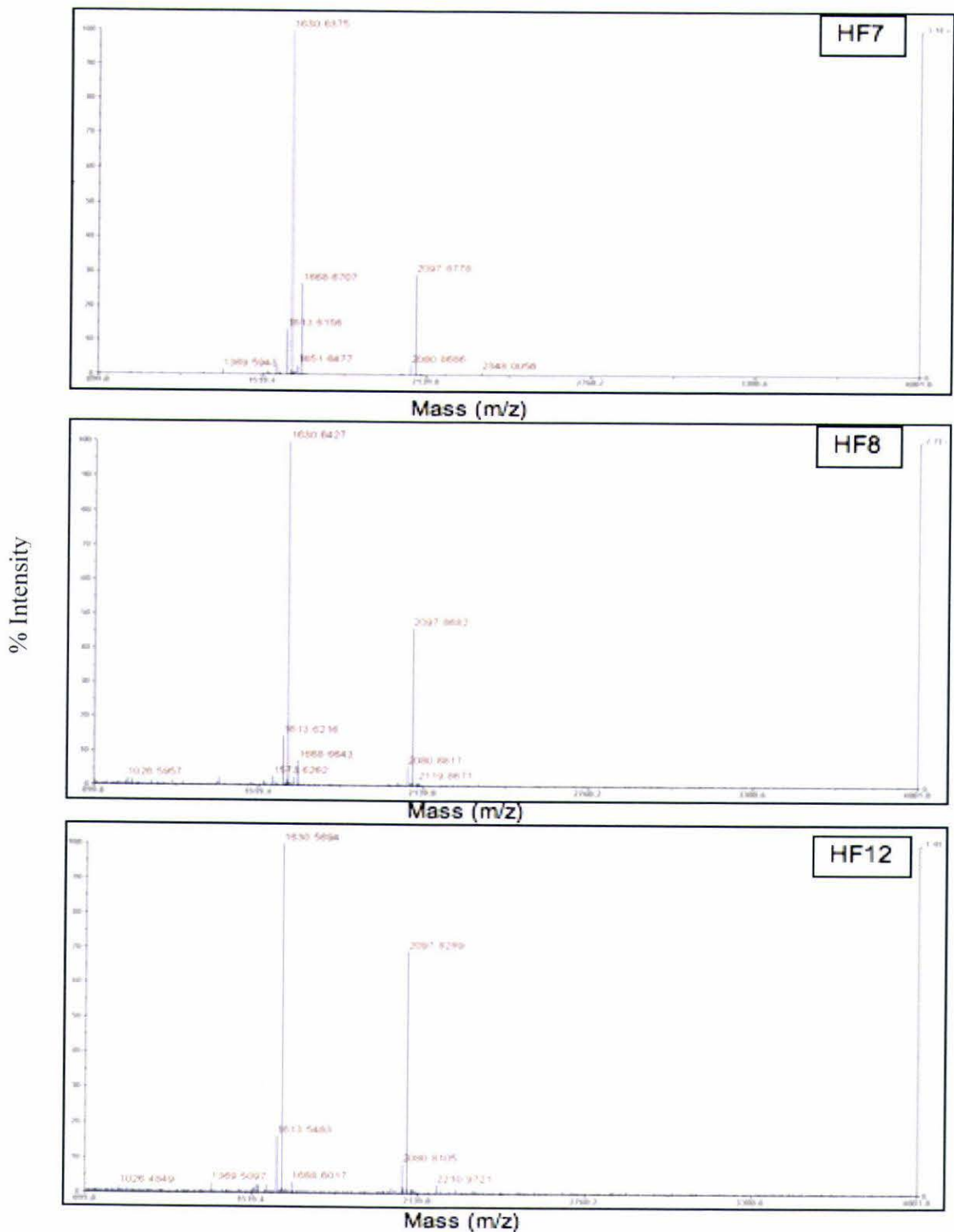


Figure 3. 15 Similar HF mass spectra of HF7, HF8 and HF12 trypsin digested protein spots

Spots HF7, HF8 and HF12 were cut from the gel and digested with trypsin. The spectra were generated using the MALDI-TOF MS.

Protein spots C6, C7, C11, HF7, HF8 and HF12 also had similar ion spectra with no positive protein identity. The spectra are shown in figures 3.14 and 3.15. Three similar peaks were identified from the spectra at approximately 1613, 1630 and 2097. The spectra show that the peptides have similar masses but differ in abundance. The peptides are probable isoforms of the non-identified protein.

Protein spot C12 was identified as alpha-2u globulin, chain A. is also known as the Major Urinary Protein (MUP). Alpha-2u globulins are encoded by a highly homologous multigene family. Moreover, protein spots C9, C10, HF9 and HF10 were identified as alpha-2u globulin isoform, alpha-2u globulin PGCL4. Protein spots C8, C13, C15, C18, C19, C20, HF13, HF14, HF15, HF16, HF17, HF18, HF19 and HF21 were identified as another alpha-2u globulin isoform named alpha-2u globulin precursor. The spectra of the latter protein spots have similar masses but differ in abundance (figures 3.16 – 3.24). Although no identity was found for the HF20 protein spot, it possible that it is also an alpha-2u globulin precursor because its spectrum is similar to that of protein spots C8, C13, C15, C18, C19, C20, HF13, HF14, HF15, HF16, HF17, HF18, HF19 and HF21. It is possible that it could not be identified because of the low abundance of peptides.

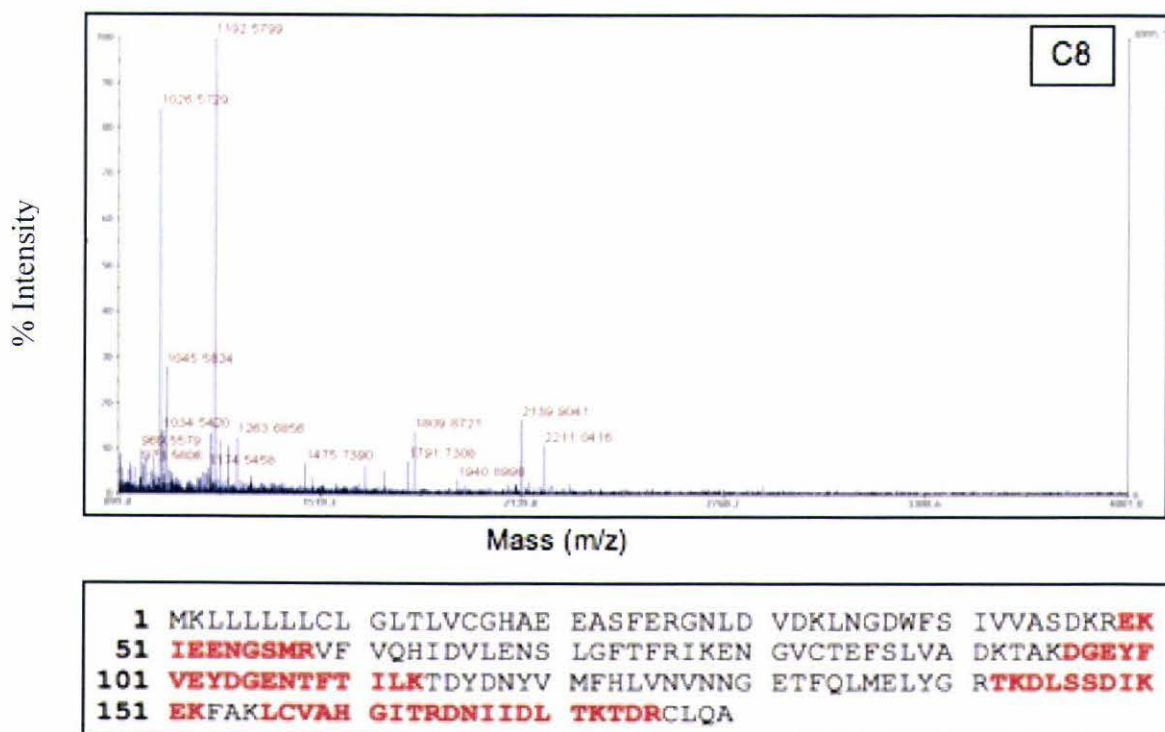


Figure 3. 16 Mass spectrum of the trypsin digested C8 with the alpha-2u globulin precursor sequence.

The spectrum was generated using the MALDI-TOF MS. From the Mascot search 32 % of the sequence was covered with matched peptides indicated in red.

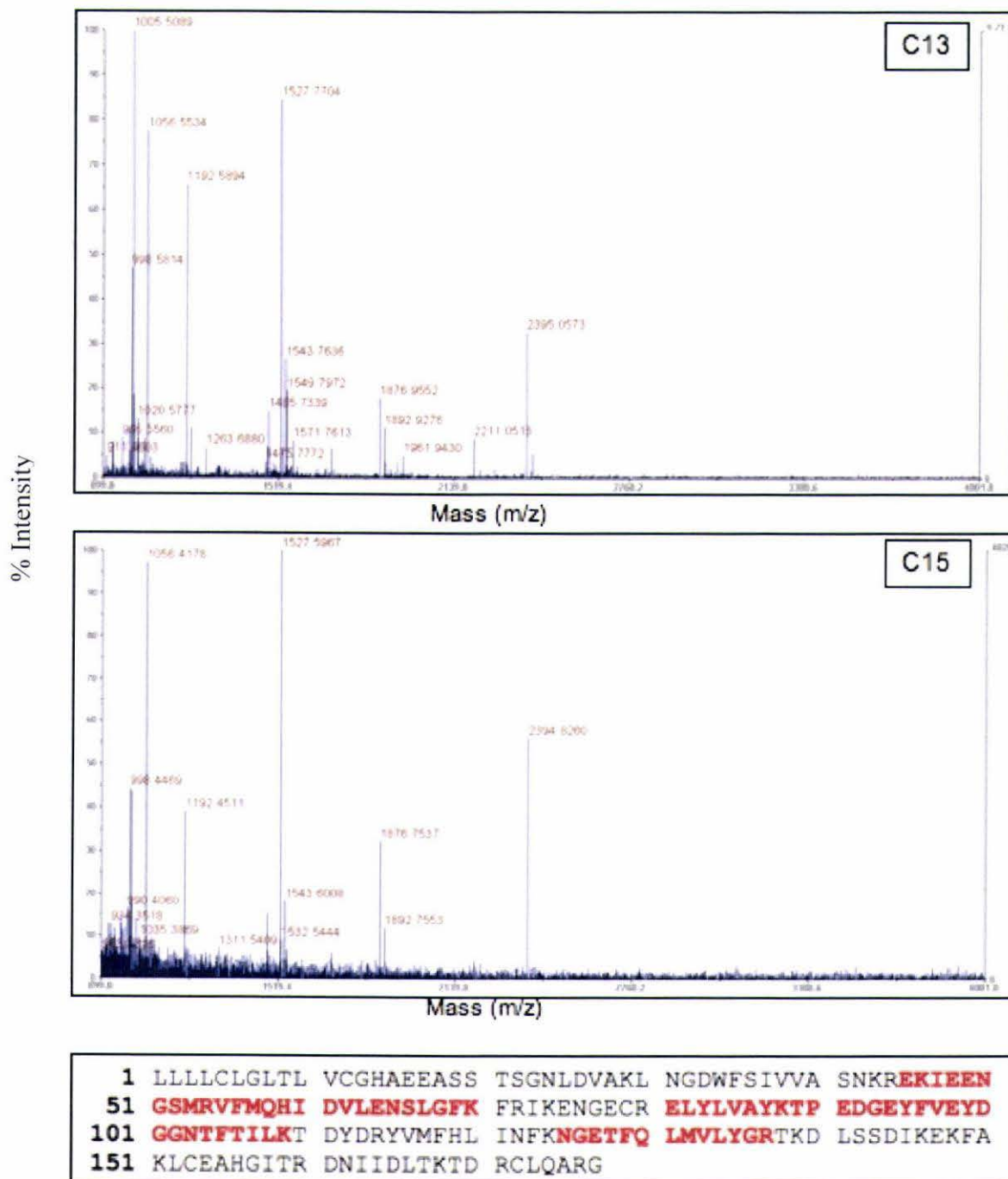


Figure 3. 17 Mass spectra of the digested C13 and C15 protein spots and identified sequence of C15.

Spots C13 and C15 were cut from the gel and digested with trypsin. The eluates were mixed 1:1 with μ -CHCA matrix solution and 1 μ l was spotted onto a MALDI source plate. MALDI-TOF MS was used in the generation of the mass spectra. The Mascot search showed sequence coverage of 38 % from C15 protein spot.

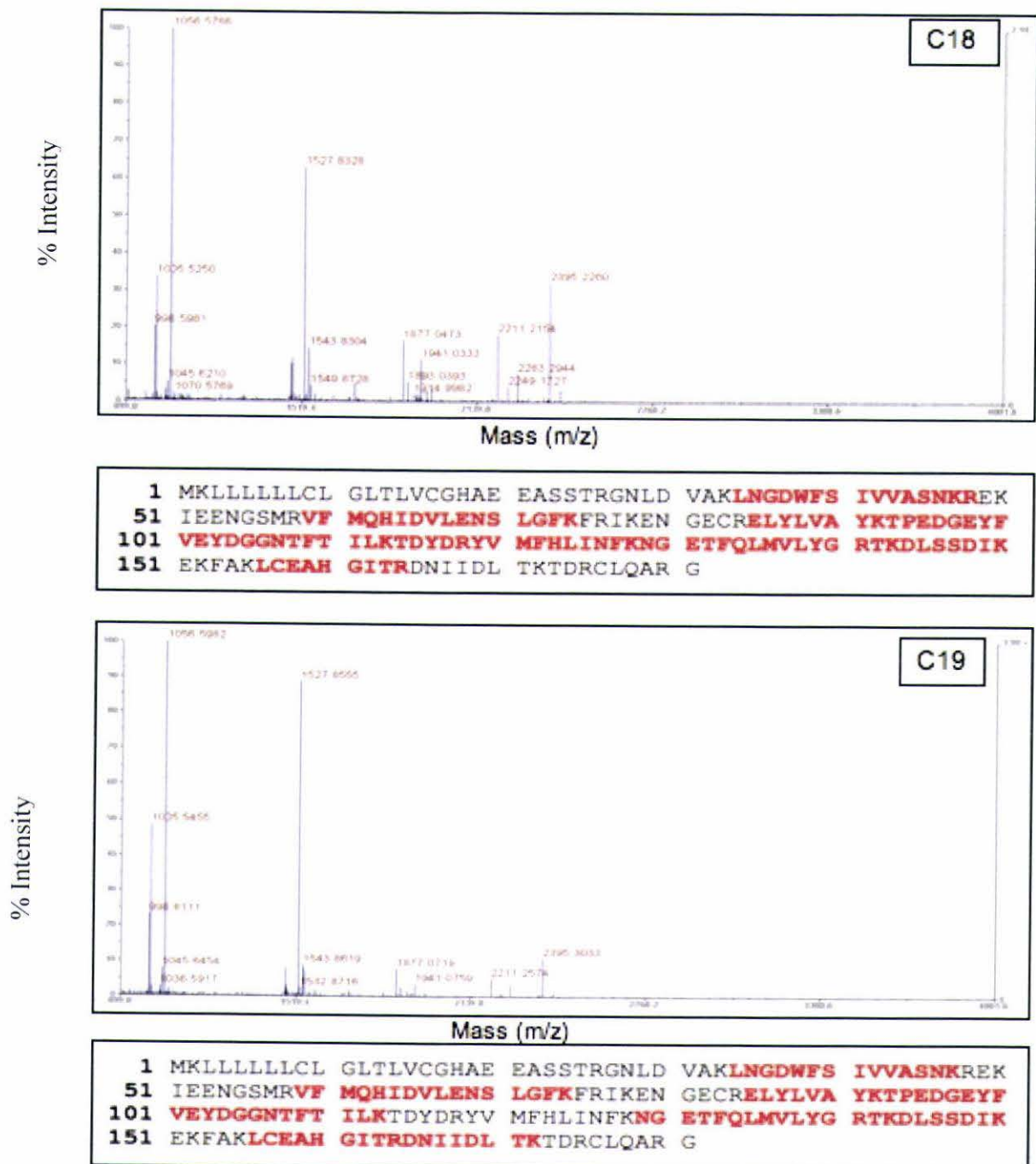
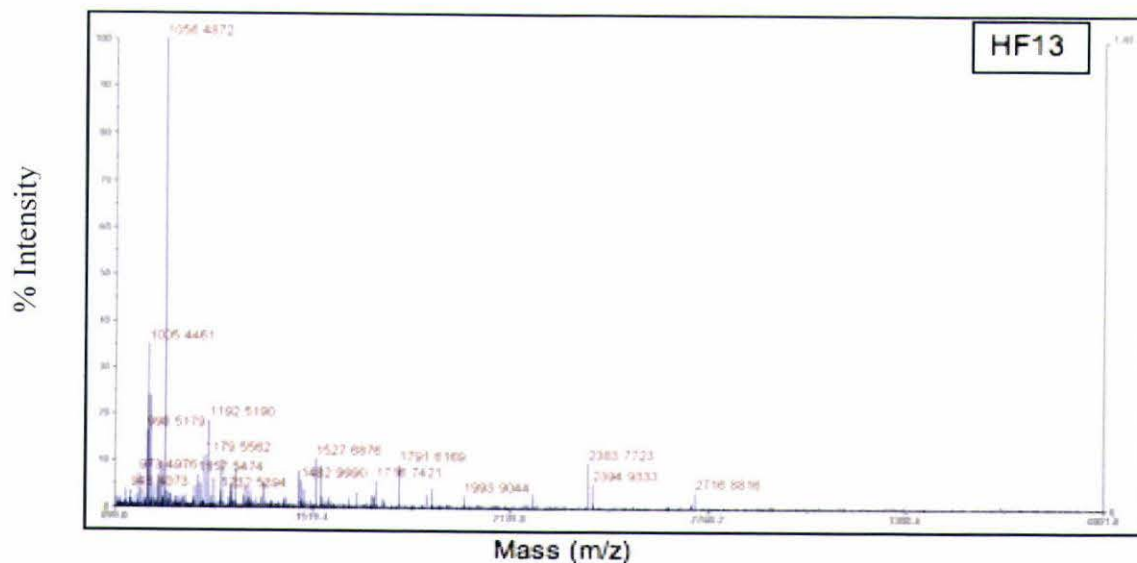
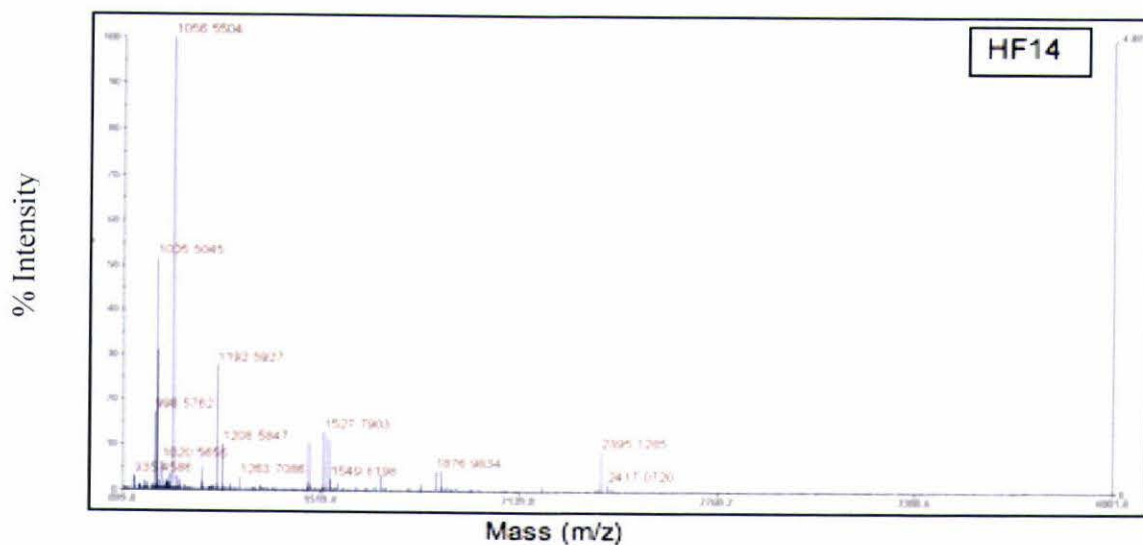


Figure 3. 6 Mass spectra of the digested C18 and C19 protein spots and their respective identified sequences.

Spots C18 and C19 were cut from the gel and digested with trypsin. MALDI-TOF MS was used in the generation of the mass spectra. The Mascot search showed sequence coverage of 58 % from C18 and sequence coverage of 54 % from C19 with these matched peptides indicated in red.



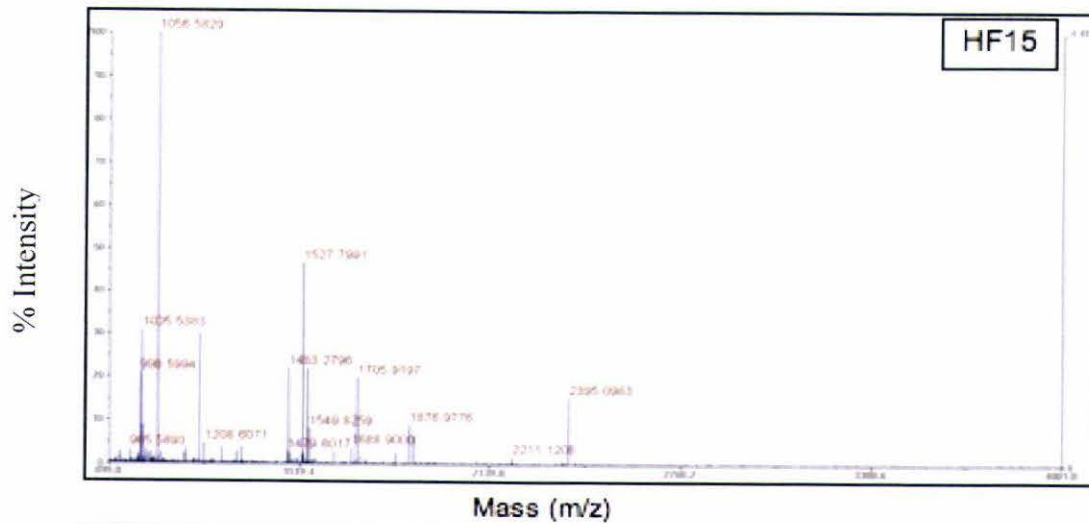
1 LLLLCLGLTL VCGHAE EASS TSGNLDVAKL NGDWFSIVVA **SNKREKIEEN**
51 **GSMRVFMQHI** **DVLENSLGFK** FRIKENGECR **ELYLVAYKTP** **EDGEYFVEYD**
101 **GGNTFTILKT** DYDRYVMFHL INFKNGETFQ **LMVLYGR TKD** LSSDIKEKFA
151 KLCEAHGITR **DNIIDLTKTD** RCLQARG



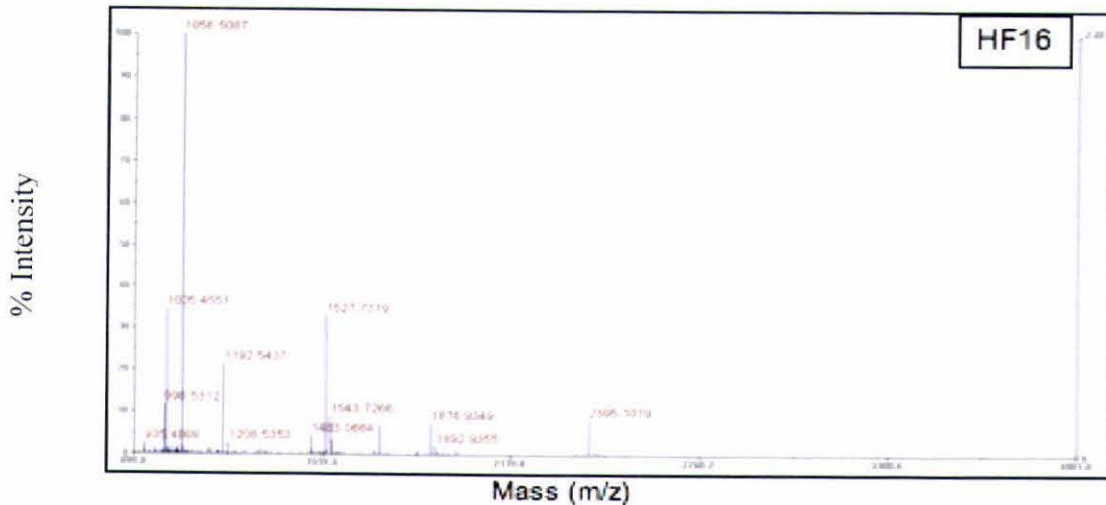
1 LLLLCLGLTL VCGHAE EASS TSGNLDVAKL **NGDWFSIVVA** **SNKREKIEEN**
51 **GSMRVFMQHI** **DVLENSLGFK** FRIKENGECR **ELYLVAYKTP** **EDGEYFVEYD**
101 **GGNTFTILKT** DYDRYVMFHL INFKNGETFQ **LMVLYGR TKD** LSSDIKEKFA
151 KLCEAHGITR **DNIIDLTKTD** RCLQARG

Figure 3. 7 MALDI-TOF mass spectrum of the trypsin digested HF13 and HF 14 with their identified alpha-2u globulin precursor sequence.

Spots HF13 and HF14 were cut from the gel and digested with trypsin. MALDI-TOF MS was used in the generation of the mass spectra. The Mascot search showed sequence coverage of 47 % from HF13 and 67 % for HF14 with matched peptides indicated in red.



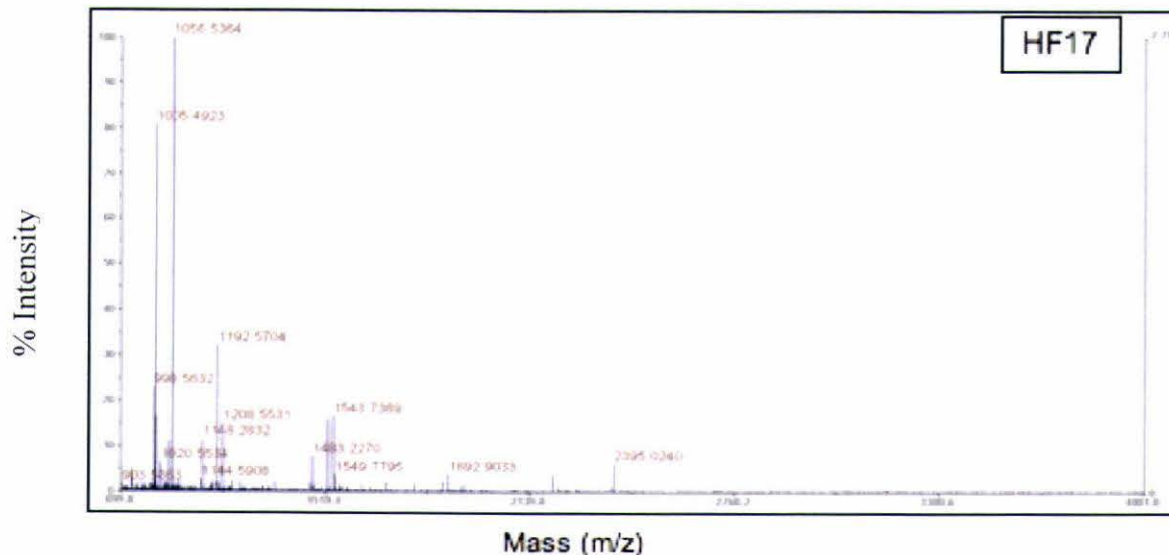
1	LLLLCLGLTL	VCGHAEAEASS	TSGNLDVAKL	NGDWFSIVVA	SNKREKIEEN
51	GSMRVFMQHI	DVLENSLGFK	FRIKENGECR	ELYLVAYKTP	EDGEYFVEYD
101	GGNTFTILKT	DYDRYVMFHL	INFKNGETPQ	LMVLYGRTKD	LSSDIKEKFA
151	KLCEAHGITR	DNIIDLTKTD	RCLQARG		



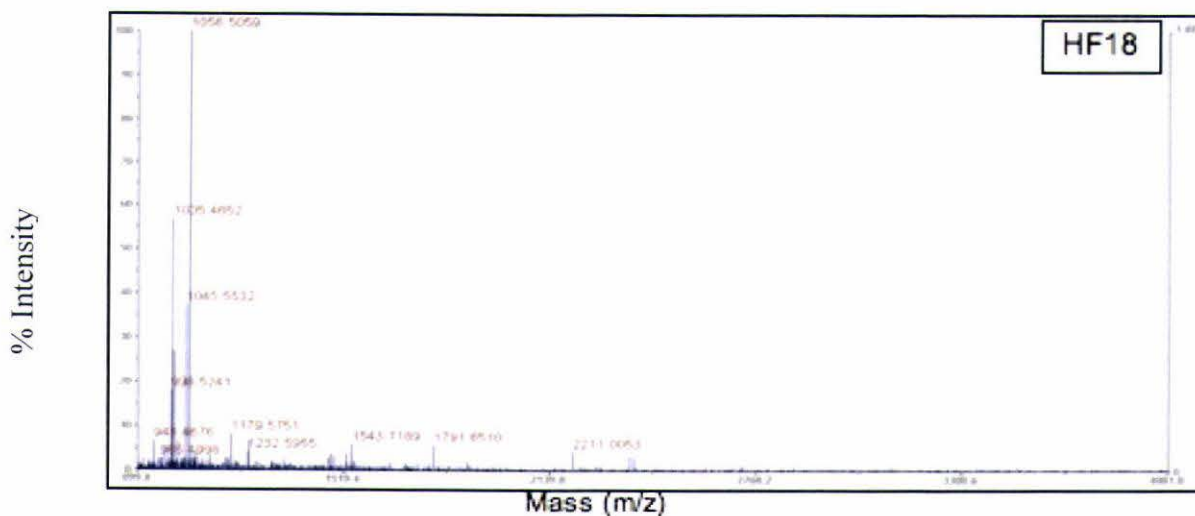
1	LLLLCLGLTL	VCGHAEAEASS	TSGNLDVAKL	NGDWFSIVVA	SNKREKIEEN
51	GSMRVFMQHI	DVLENSLGFK	FRIKENGECR	ELYLVAYKTP	EDGEYFVEYD
101	GGNTFTILKT	DYDRYVMFHL	INFKNGETPQ	LMVLYGRTKD	LSSDIKEKFA
151	KLCEAHGITR	DNIIDLTKTD	RCLQARG		

Figure 3. 80 Mass spectra of the digested HF15 and HF16 protein spots and their respective identified sequences.

Spots HF15 and HF16 were cut from the gel and digested with trypsin. MALDI-TOF MS was used in the generation of the mass spectra. The Mascot search showed sequence coverage of 68 % from HF15 and 57 % for HF16 with matched peptides indicated in red.



1	LLLLCLGLTL	VCGHAEAEASS	TSGNLDVAKL	NGDWFSIVVA	SNKREKIEEN
51	GSMRVFMQHI	DVLENSLGFK	FRIKENGECR	ELYLVAYKTP	EDGEYFVEYD
101	GGNTFTILKT	DYDRYVMFHL	INFKNGETFQ	LMVLYGRTKD	LSSDIKEKFA
151	KLCEAHGITR	DNIIDLTKTD	RCLQARG		



1	LLLLCLGLTL	VCGHAEAEASS	TSGNLDVAKL	NGDWFSIVVA	SNKREKIEEN
51	GSMRVFMQHI	DVLENSLGFK	FRIKENGECR	ELYLVAYKTP	EDGEYFVEYD
101	GGNTFTILKT	DYDRYVMFHL	INFKNGETFQ	LMVLYGRTKD	LSSDIKEKFA
151	KLCEAHGITR	DNIIDLTKTD	RCLQARG		

Figure 3. 21 MALDI-TOF mass spectra of the digested HF17 and HF18 protein spots and their respective identified alpha-2u globulin precursor sequence.

Spots HF15 and HF16 were cut from the gel and digested with trypsin. MALDI-TOF MS was used in the generation of the mass spectra.

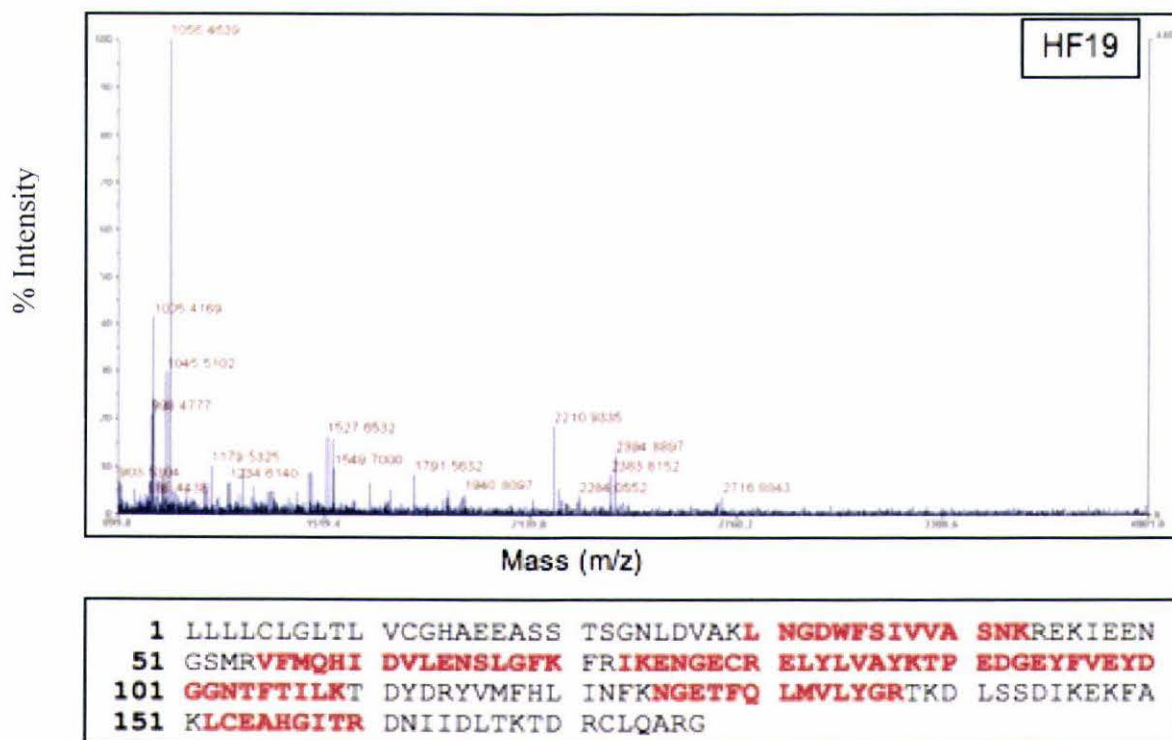


Figure 3. 9 Mass spectrum of HF19 trypsin digested protein spot with the matched protein sequence.

Spot HF19 was cut from the gel and digested with trypsin. The eluate were mixed 1:1 with α -CHCA matrix solution, 1 μ l was spotted onto a MALDI plate and air-dried before MALDI-TOF MS analysis. Sequence was identified using the Mascot search that showed the matched peptides in red with 50 % sequence coverage.

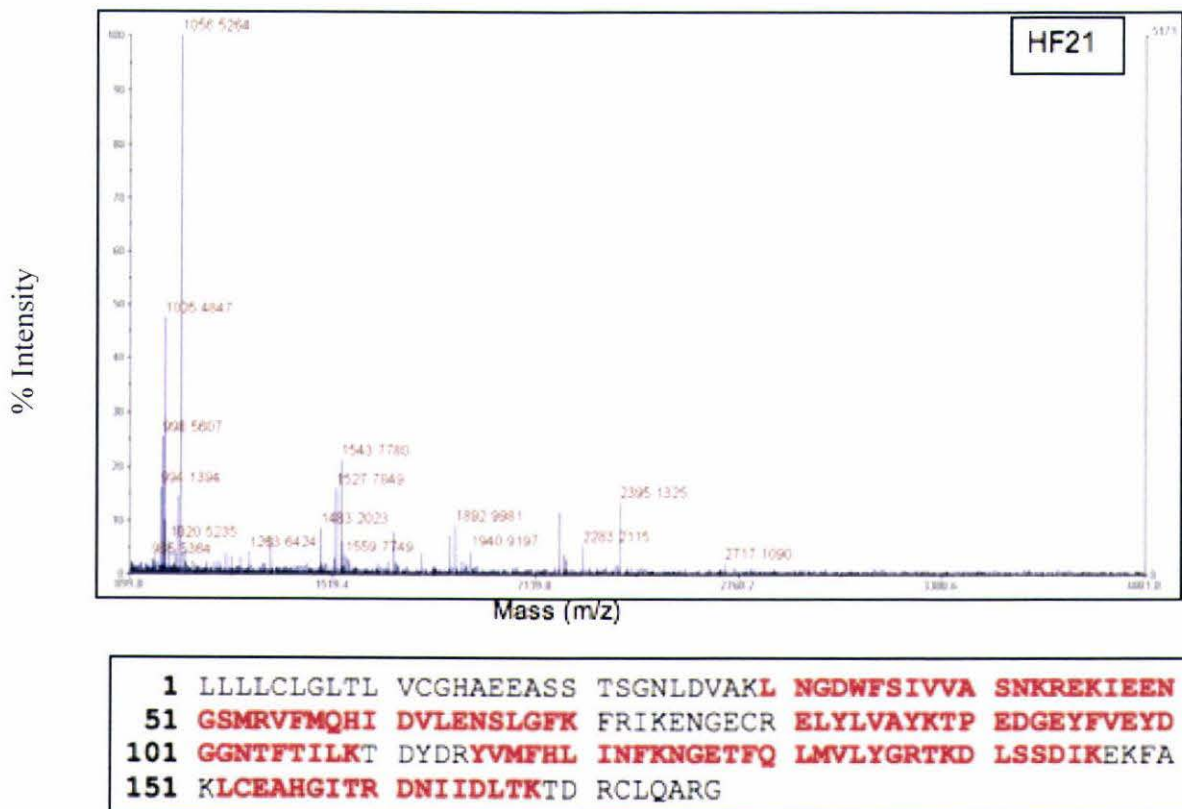


Figure 3. 23 MALDI-TOF mass spectrum of trypsin digested HF21 protein spot with the matched protein alpha-2u globulin precursor sequence.

Spot HF19 was cut from the gel and digested with trypsin. MALDI-TOF MS was used in the generation of the mass spectra. Sequence was identified using the Mascot search that showed the matched peptides in red with 67 % sequence coverage.

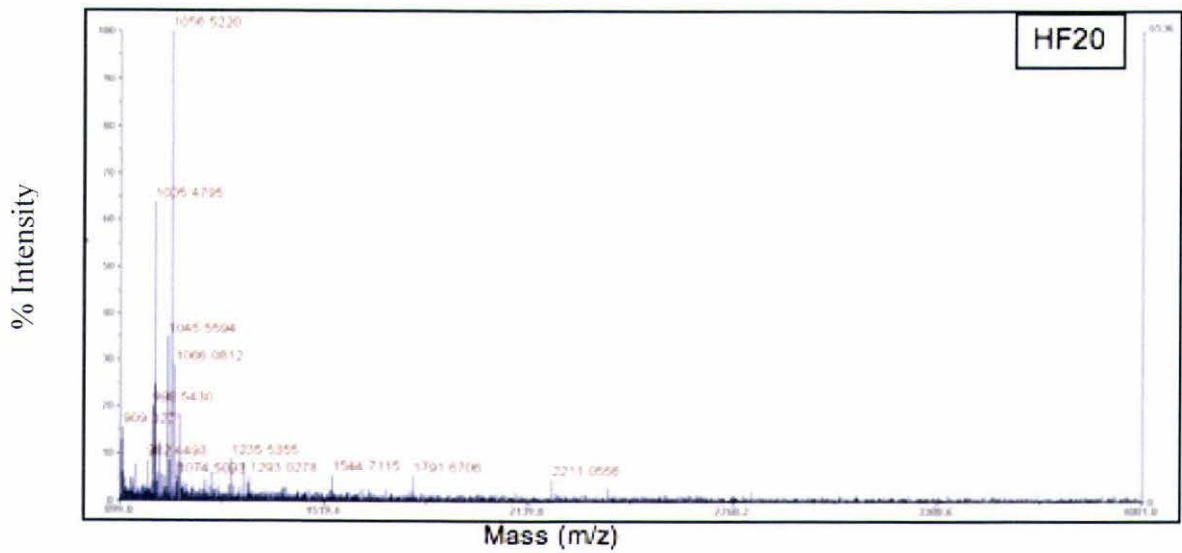


Figure 3. 24 Mass spectrum of trypsin digested HF20 protein spot.

Spots HF20 was cut from the gel and digested with trypsin. MALDI-TOF MS was used in the generation of the mass spectra.

3.4.2 Transducin (beta)-like 3 protein

The only protein spot observed from the obese rats and not from the lean rats was identified as the Transducin (beta)-like 3 protein that is abbreviated as Tbl3. The spectrum obtained using the MALDI-TOF MS is shown in figure 3.25. The Mascot results showed sequence coverage of only 11 %. The matched peptides are indicated in bold red in figure 3.26. The calculated mass of Tbl3 is 88.24 kDa and the protein was observed at approximately 14 kDa. The protein appears to be truncated or to be a proteolytic fragment of a larger protein.

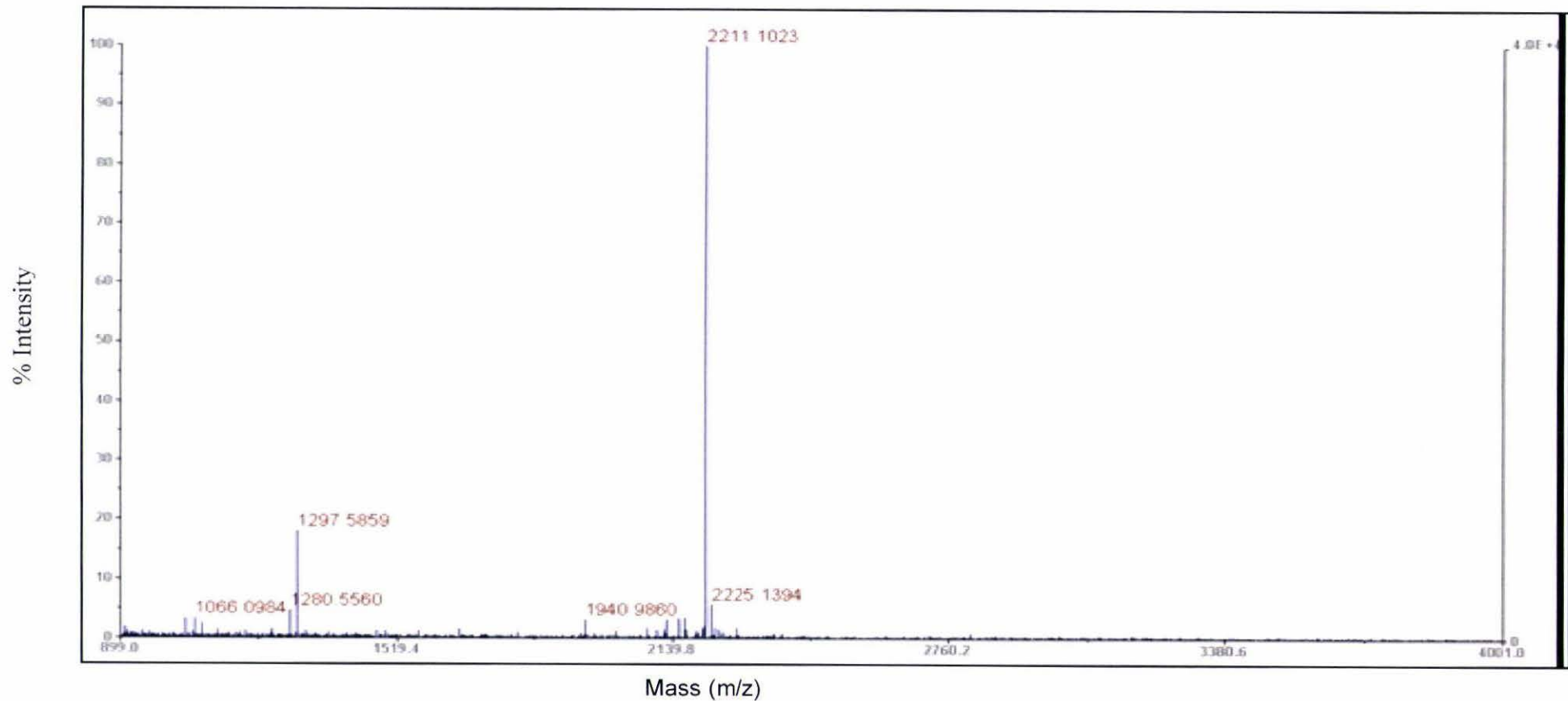


Figure 3. 25 MALDI-TOF mass spectrum of trypsin digested HF2 protein spot.

Spot HF19 was cut from the gel and digested with trypsin. MALDI-TOF MS was used in the generation of the mass spectra. Sequence was identified using the Mascot search that showed the matched peptides in red with 67 % sequence coverage.

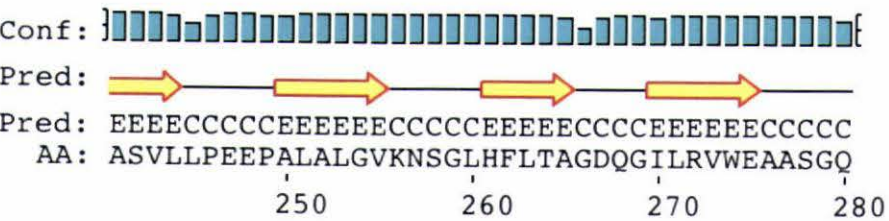
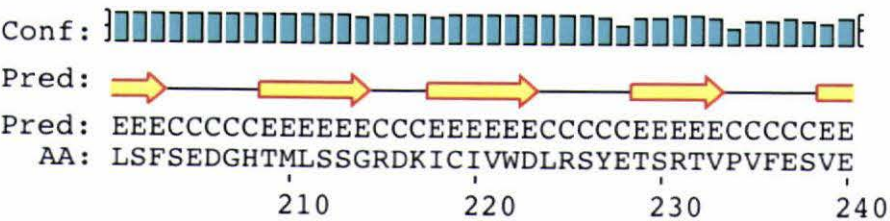
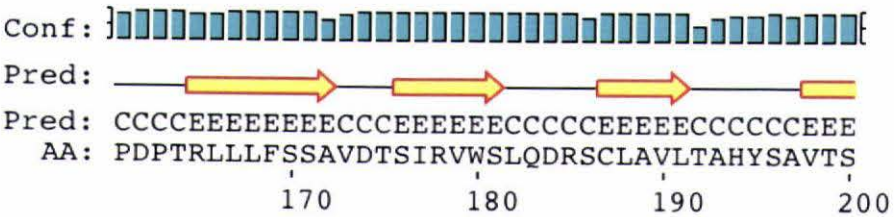
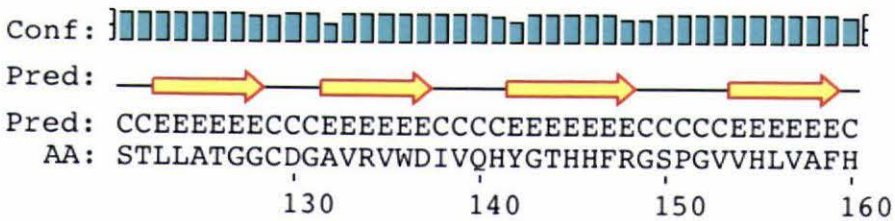
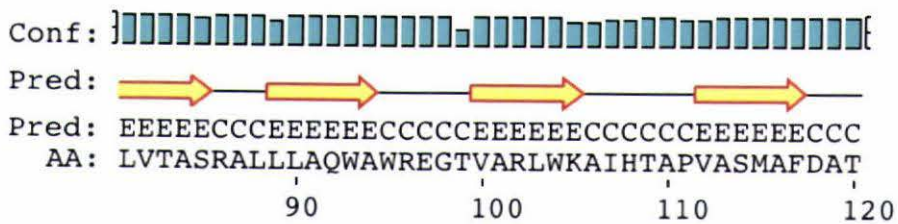
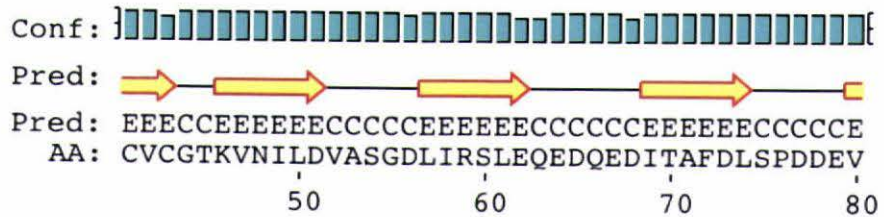
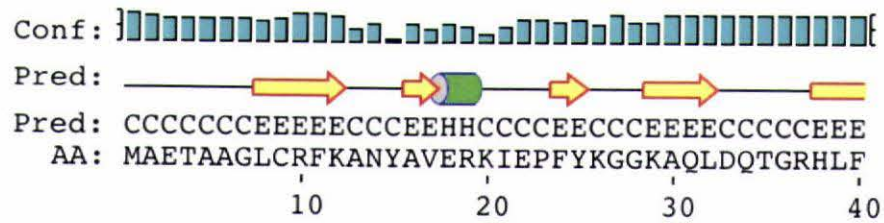
1	MAETAAGLCR	FKANYAVERK	IEPFYKGGKA	QLDQTGRHLF	CVCGTKVNIL
51	DVASGDLIRS	LEQEDQEDIT	AFDLSPDDEV	LVTASRALLL	AQWAWREGTV
101	ARLWKAIHTA	PVASMAFDAT	STLLATGGCD	GAVRVWDIVQ	HYGTHHFRGS
151	PGVVHLVAFH	PDPTRLLES	SAVDTSIRVW	SLQDRSCLAV	LTAHYSAVTS
201	LSFSEDGHTM	LSSGRDKICI	VWDLRSYETS	RTVPVFESVE	ASVLLPEEPA
251	LALGVKNSGL	HFLTAGDQGI	LRVWEAASGQ	CVYTQPQMSG	LRQELTHCTL
301	ARAAGLLLV	TADHNLLLYE	ARSLQLQKQF	AGYSEEVLVDV	RFLGPNDSHI
351	IVASNSPCLK	VFELQTLACQ	ILHGHTDIVL	ALDVERKGWL	FASCAKDQSI
401	RIWRMKNKAGQ	VACVAQGSQH	THSVGTICCS	RLKESFLVTG	SQDCTVKLWP
451	LPEALPSKNT	ASDGDLIPLQ	AQSTQRCHDK	DINSLAVSPN	DKLLATGSQD
501	RTAKLWALPQ	CQLLGVFSGH	RRGLWNVQFS	PTDQVLATAS	ADGTIKLWAL
551	QDFSCCLKTFE	GHDASVLKVA	FVSRGAQLLS	SGSDGLLKLW	TIKSNECVRT
601	LDAHEDKVWG	LHCSRLDDHA	ITGGSDSRII	LWKDVTEAEQ	AEEQAKREEQ
651	VIKQQEELDNL	LHEKRYLRAL	GLAISLDRPH	TVLTVIQAIR	RDPEACEKLE
701	ATVLRRLRRDQ	KEALLRFCIT	WNTNSRHCHE	AQAVLGVLLR	HEAPEELLY
751	DGVRGALEAL	LPYTERHFQR	LSRTLQAATF	LDFLWHNMKL	SPLPAAPPAL
801					

Figure 3. 26 A transducin (beta)-like 3 amino acid sequence, the matched peptides in bold red indicating 11 % sequence coverage.

3.5 PROTEIN MODELLING

In order to gain insight of the differential protein, a model of Tbl3 was generated based on its homology to the WD family. A secondary structure was designed guided by the principles and predictions from GenTHREADER (Jones, 1999) and PSIPRED (McGuffin *et al.*, 2000). PSIPRED (McGuffin *et al.*, 2000) revealed a high prediction confidence secondary structure with 9-helical regions, 59 beta strands and 67 loop regions (shown in figure 3.27). The search for structural alignment using the Tbl3 protein sequence against the known structures in the PDB using GenTHREADER (Jones, 1999) showed 2 candidates, 1pguA and 1nroA. The identified candidates are actin-interacting protein 1 (Aip1p) which is a WD repeat protein reported to regulate cofilin-mediated actin filaments depolymerization.

The model was further evaluated with RAMPAGE (Lovell *et al.*, 2003), to check for possible errors in the stereochemistry. A Ramachandran plot obtained from the model showed that 86.4 % of the residues were in the sterically favoured region where 98 % of the residues in the sterically favoured region were expected. The plot is shown in figure 3.29. The remaining 7.6 % fell within allowed regions whereas approximately 2 % was expected. The model showed that 6 % was in the disallowed regions, this might be owing to the high number of loop regions. A cartoon presentation of the model acquired using PyMOL v0.97 (DeLano, 2002) is shown in figure 3.28.



Conf: }|||||...|||||{
 Pred: → → → →
 Pred: EEEEECCCCCCCCCEEEEECCCCCEEEEECCCEEEEE
 AA: CVYTQPQMSGLRQELTHCTLARAAGLLLTVTADHNLLLYE
 290 300 310 320

Conf: }|||||...|||||{
 Pred: → → → →
 Pred: CCCCCEEEEECCCCCEEEEECCCCCEEEEECCCEEE
 AA: ARSLQLQKQFAGYSEEVLVDVRF LGPNDSHIIVASNSPCLK
 330 340 350 360

Conf: }|||||...|||||{
 Pred: → → → →
 Pred: EEECCCCCEEEEECCCCCEEEEECCCCCEEEEECCCE
 AA: VFELQTLACQILHGHTDIVLALDVFRKGLWFASCAKDQSI
 370 380 390 400

Conf: }|||||...|||||{
 Pred: → → → →
 Pred: EEECCCCCEEEEECCCCCEEEEECCCCCEEEEECCCE
 AA: RIWRMKNKAGQVACVAQGS GHTHSVGTICCSRLKESFLVTG
 410 420 430 440

Conf: }|||||...|||||{
 Pred: → → → →
 Pred: CCCCCEEEEECCCCCCCCCCCCCEEEEEEEEEEEEECC
 AA: SQDCTVKLWPLPEALPSKNTASDGD LIPLQAQSTQRCHDK
 450 460 470 480

Conf: }|||||...|||||{
 Pred: → → → →
 Pred: CEEEEEECCCCCEEEEECCCEEEEECCCEEEEEEECC
 AA: DINSLAVSPNDKLLATGSQDR TAKLWALPQCQLLGVFSGH
 490 500 510 520

Conf: }|||||...|||||{
 Pred: → → → →
 Pred: CCCCCEEEEECCCCCEEEEECCCEEEEECCCEEEEEEE
 AA: RRGLWNVQFSPTDQVLATASADGT IKLWALQDFSCCLKTFE
 530 540 550 560

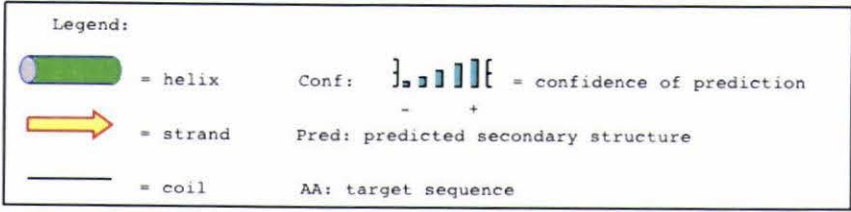
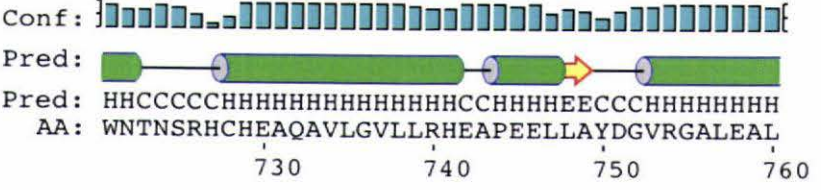
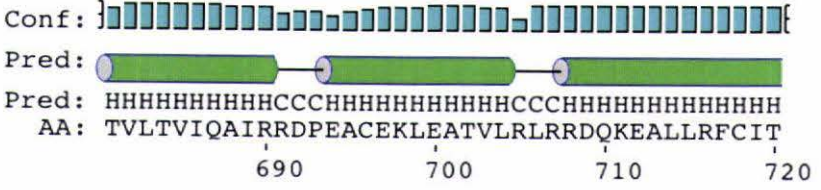
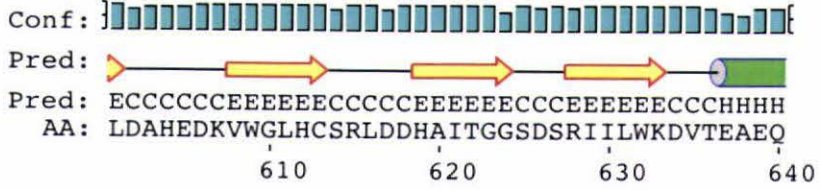
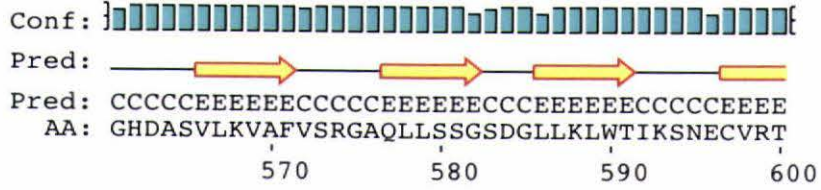


Figure 3. 27 The predicted secondary structure of Tbl3 generated using PSIPRED (McGuffin et al., 2000).

The image suggests that Tbl3 is expected to contain mostly loop regions and beta strands.

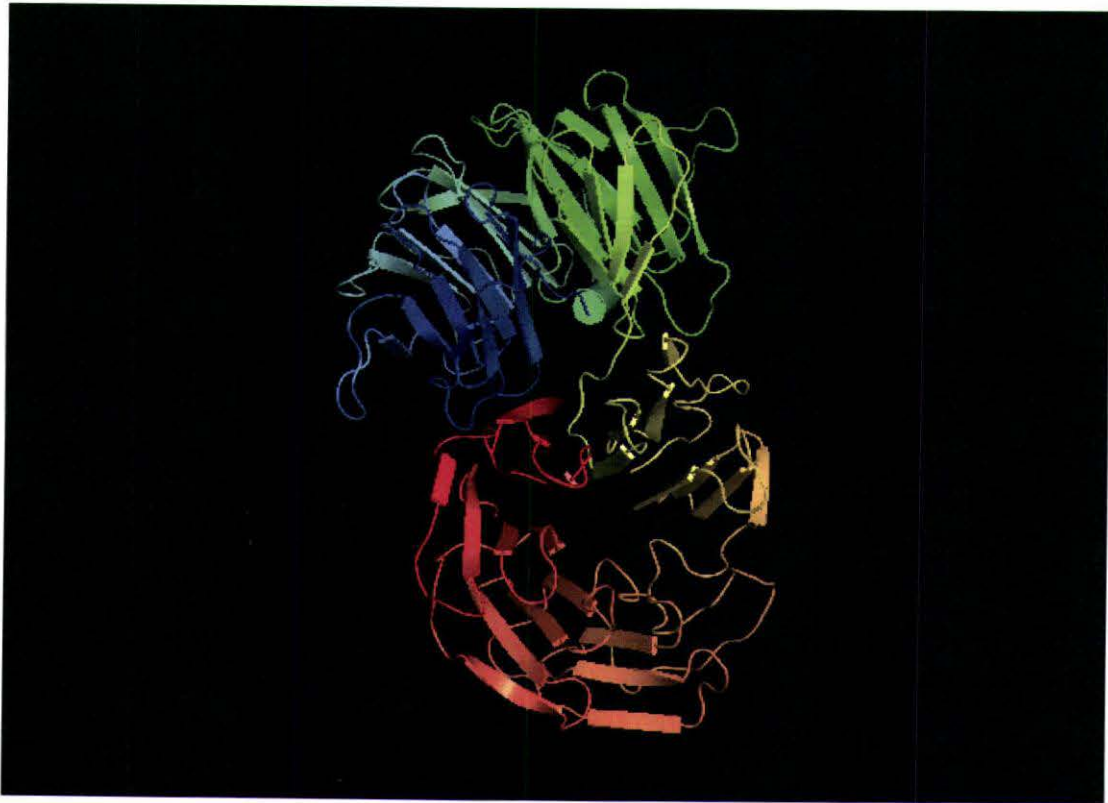


Figure 3. 28 A cartoon presentation of the Tbl3 model.

The figure was generated using PyMOL (DeLano, 2002). The image suggests that Tbl3 model contains mostly loop regions and beta strands.

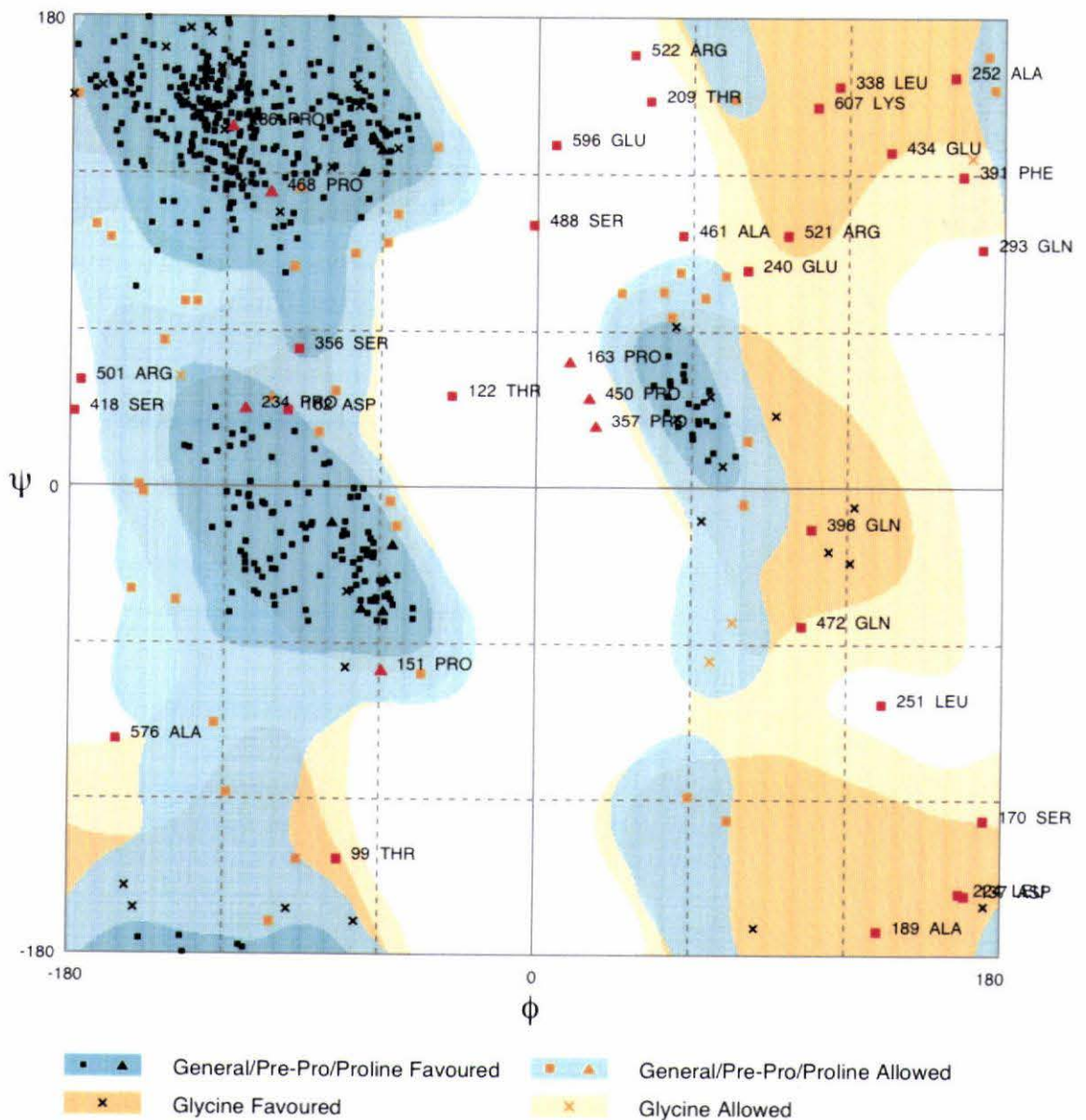


Figure 3. 29 A Ramachandran plot of the Tbl3 model generated using RAMPAGE (Lovell *et al.*, 2003).

The area shaded in blue represents the allowed and favoured regions on the plot. The glycines have been represented by asterisks while the prolines have been represented by triangles. 86.4 % of the residues were predicted within the favoured region, 7.6 % were predicted within the allowed regions while 6 % was in the disallowed regions.

CHAPTER 4: DISCUSSION AND CONCLUSION

The analysis of human body fluids is one of the most important approaches to the diagnosis of diseases or the development of therapeutic interventions in diseases. Sample preparation is said to be the most important in downstream steps, in obtaining reproducible and high-resolution gels, as this will affect the ultimate analytical results. However, sample preparation methods varied from one publication to another. Therefore it was of utmost importance to establish a method for the enrichment of low molecular weight proteins in urine of CHOW and HF fed rats. It is worth noting that urinary protein concentrations are much lower than in serum mainly because the glomerular filtration takes place in the kidneys (González-Buitrago *et al.*, 2007; Theodorescu and Mischak, 2007). This explains the low molecular weight proteins that were observed from the gels. In comparison with other body fluids such as serum, albumin is still a major protein or a highly abundant protein that interferes with the detection of low abundant proteins and low molecular weight proteins. The study established an approach or method for the depletion of high abundant proteins and the enrichment of low molecular weight proteins through acetone precipitation of urine samples.

From the 2D urine proteome profile between the CHOW and HF fed rats only one protein was different. This protein was observed on the HF proteome profile but not on the CHOW proteome profile. Also observed from the HF proteome profile were four decreased protein spot intensities and three increased protein spot intensities. According to Bellei and co-workers, increased excretion of urinary proteins may be owing to various sources such as increased glomerular filtration of plasma proteins; impaired tubular reabsorption of filtered proteins; and proteins that originate from injured glomeruli, tubules, infiltrating inflammatory cells as well as those that enter

the urine in the urinary tract below the kidney (Bellei *et al.*, 2008). It is therefore possible that the rats fed a high fat diet may have developed an injury to the kidney as a result of injured glomeruli. The mechanism behind the increase and decrease of protein spot intensities from the CHOW fed rats is not yet known.

Despite the increase and decrease in protein spot intensities, all the identified spots were identified using MALDI-TOF-MS, their ion spectra were generated, the peaks from the mass list were then matched on the database. All searches were performed against the NCBI and MSDB peptide mass databases using MASCOT. From both the lean and obese rats, Serotonin 5-HT_{2C}, Alpha-2u Globulin PGCL4, Alpha-2u Globulin precursor and the Chain A of Alpha-2u Globulin, which was only observed in the lean rats, were identified.

Even though the proteins were identified, only a percentage of the identified protein sequence was recovered and their observed molecular weight was below the theoretical molecular weight. This suggests that the proteins might have been cleaved or truncated. Fliser and co-workers have also noted that the identified biomarkers from urine of patients with renal diseases are proteolytic fragments of larger proteins (Fliser *et al.*, 2007). In addition, nephropathy and kidney disease studies have indicated that there are specific proteases in urine, which cleave the excreted proteins as a result of several glomerular diseases (Fliser *et al.*, 2007).

The Serotonin 5- HT_{2C} receptor is one of the 14 subtypes of 5-HT serotonin receptors. Serotonin 5- HT_{2C} is a biogenic hormone according to the database, that function as a mitogen, a hormone and neurotransmitter. The 5- HT_{2C} receptor is a seven trans-

membrane G-protein coupled receptor associated with the activation of a phosphatidylinositol-calcium second messenger system. It is also reported that altered patterns of RNA editing in response to drug treatment and stressful situations have been observed in rodent studies such those by (Zhang *et al.*, 2009).

The other identified protein, Alpha-2u globulin, is a tissue and sex-specific protein. It is expressed in male rats and has been shown to be highest in young male rats (Swenberger, 1993). In addition, Alpha-2u globulin is reported to be suppressed by oestrogen. It is synthesized and secreted by parenchymal cells. It is a LMW protein of approximately 19 kDa. It is first synthesized as a 21 kDa precursor that is then processed to be a mature protein (Saito *et al.*, 2000; Unterman *et al.*, 1981).

The only protein spot observed from the obese rats and not from the lean rats was identified as Transducin (beta)-like 3 protein. Transducin (beta)-like 3 protein is abbreviated Tbl3. According to NCBI and MSDB, Tbl3 has conserved protein repeats. These beta-transducin repeats are also known as WD40 repeats. Tbl3 has 13 conserved WD repeat proteins.

WD40 repeats were originally identified in the beta subunit of the heterotrimeric G-protein (G_{β}) (van der Voon and Ploegh, 1992). The repeats are usually 4-8 repeat sequences. The WD40 repeat motifs are divided into two conserved elements A and B (Saeki *et al.*, 2006). The A elements are said to be poorly conserved with a pair of glycine and histidine (GH) whilst the B elements are well conserved with a pair of tryptophan and aspartate (WD). The repeats are thus called WD40, as they are usually approximately 40 amino acid motifs terminating in a WD dipeptide.

The WD40 repeat proteins have been found in a number of eukaryotes. According to van der Voon and Ploegh, the WD40 family members include the β -subunit of guanine nucleotide regulatory proteins of the G-protein; STE4, a functional G-protein in yeast (*Saccharomyces cerevisiae*); CD4, a component of the yeast nuclear cytoskeleton; CDC20; Enhancer of Split, the product of one of the neurogenic genes in *Drosophila*; PRP17, a protein involved in pre-mRNA splicing and AET2/TUP1 a transcriptional repressor (van der Voon and Ploegh, 1992).

STE4 is reported to be involved in a signal pathway that controls the response to mating pheromone. Moreover, CDC4 and CDC20 are required at the late G1/S phase boundary of the cell cycle and several microtubule-dependent processes at multi-stages in the cell cycle respectively.

Taking into consideration the involvement of the WD40 repeats in wide variety of cellular processes in eukaryotes its functions were deduced to be implicated in signal transduction, transcription, RNA processing, regulation in cell cycle and apoptosis (Saeki *et al.*, 2006). The gene that encodes the G protein β 3 subunit (*GNB3*) has also been reported to have a polymorphism (C825T) which has been associated with obesity and the variations of blood pressure causing hypertension (Roskopf *et al.*, 2000). However the association or relationship between obesity, hypertension and the G-protein was not reported.

To gain insight and knowledge into the structure of Tbl3, using SWISS-MODELLER, a 3-dimensional model of Tbl3 was generated based on its homology to IpguA from *Caenorhabditis elegans* and *Saccharomyces cerevisiae*. The modelled structure was a

nine-helix bundle. Evaluation of the model was carried out using RAMPAGE to check for possible errors in protein stereochemistry. The structure was confirmed to have a high number of loop regions indicated by the 6 % that fell within disallowed regions.

The study has a number of implications. The identified differential protein, Tbl3, can serve as a possible indicator of obesity or early stage diabetes, thus making it a potential biomarker. The Tbl3 can also be used in the development of lateral flow devices. This can assist in the design of diagnostic kits that would provide a point of care testing which promises the delivery of early and rapid diagnosis of prediabetes. The early diagnosis of diseases would allow for the monitoring of diseases and early efficient delivery of treatment for diseases. The knowledge of the structure might assist with the design and development of new drugs that may target obesity and possibly prediabetes.

In conclusion, the identification of protein changes between the lean and obese rats using 2D gel electrophoresis and mass spectrometry has been possible. The applications of these techniques on urine have led to the identification of Tbl3 as a potential biomarker although much work still has to be done in order to validate this.

CHAPTER 5: REFERENCES

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CHAPTER 6: APPENDIX

6.1 ADDRESSES OF SUPPLIERS

Applied Biosystems Inc.

850 Lincoln Centre Drive

Foster City, CA 94404

USA

Bayer AG

51368 Leverkusen, Germany

Phone: ++49 214 30-1

Bio-Rad Laboratories Ltd.

Life Science (Research, Education, Process Separations, Food/Animal/Environment Testing)

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E-mail: safrika_helpdesk@bio-rad.com

Epol

www.epol.co.za

Olympus digital camera

Digital Cameras, Binoculars, Professional Imaging

Tudor

4th Floor, Park on Long, 66 Long Street

8001 Cape Town

Tel. +27 21 4242978

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Email: info@tudortech.co.za

Sigma-Aldrich / Fluka / Riedel-de Haën

Box 14508, St. Louis

Missouri 63178, USA

www.sigma-aldrich.com

Stuart scientific

Bibby Scientific Limited, Beacon Road, Stone, Staffordshire ST15 0SA, UK

Telephone: +44 (0) 1785 812121 Fax: +44(0) 1785 810405

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Whitehead scientific

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