ABSTRACT

Diabetes mellitus is a chronic metabolic disorder due to carbohydrate, fat and protein metabolism disturbances, resulting from defects of insulin secretion (Type 1), increased cellular resistance to insulin (Type 2) or both. Diabetes is characterized by an abnormally high level of blood glucose (hyperglycemia) that leads to serious damage of the body organs. World Health Organization estimates almost 220 million people worldwide diagnosed by diabetes and likely to be doubled by the year 2030. Furthermore, untreated diabetes will contribute to complications such as heart disease, stroke, hypertension, nervous system disease and amputation. Currently, researchers approach for an alternative therapeutic for combating the most prevalent diabetes, which is type 2 diabetes, by delaying the absorption of carbohydrates/glucose through inhibition of carbohydrate hydrolysing enzymes; α-amylase and α-glucosidase enzymes. Previous research reported that *Pleurotus pulmonarius* (Fr.) Quél. has a good potential in reduction of hyperglycemia in type 2 diabetes and ameliorates the course of cardiovascular-related complications. Bioactive compounds from *P. pulmonarius* sources are considered to be less toxic and free from side effects compared to synthetic drugs available in market such as sulfonylureas, biguanides, glinides, tolbutamide, troglitazone, rosigitazone and repaglinide. Besides, the production of *P. pulmonarius* has been increasing worldwide at a rapid rate due to its broad adaptability, aggressiveness, ease of cultivation with high yield potential and high nutritional value. According to Icons of Medicinal Fungi from China, the medicinal part of the oyster mushroom is in its basidiocarps. Hence, the present study aims to isolate and to characterize potentially active protein from *P. pulmonarius* basidiocarps. Crude aqueous, methanol, dichloromethane and polysaccharide extracts as well as ammonium sulphate precipitated protein fraction (F10-F100) from *P. pulmonarius* basidiocarps were explored. All samples were tested via *Saccharomyces cerevisiae* α-glucosidase

and porcine α-amylase inhibitory assays *in vitro*. Preliminary results demonstrated that at 25 µg/ml, F30 protein fraction inhibited α-glucosidase with 24.18% and F100 protein fraction inihibited α-amylase activity with 41.80%. Nevertheless, crude aqueous, methanol, dichloromethane, polysaccharide extracts at 25 mg/ml showed meagre inhibitory activities towards both assays. Selected anti-diabetic inhibitors F30 and F100 protein fractions showed a very distinct SDS-PAGE protein bands profile, 10 (5-70 kDa) and 9 protein bands (6-70 kDa) respectively thereby, both samples were further purified by RP-HPLC. Following RP-HPLC, peak 3 at 2.5 µg/ml demonstrated 25% inhibition towards α-glucosidase (F30) but very low inhibition for α-amylase activities at 6.25 µg/ml with only 2.84% (F100). Characterization of peak 3 protein from F30 protein fraction using MALDI-TOF/TOF mass spectrometry identified four different proteins, which could be involved in the regulation of blood glucose level via various mechanisms. This study revealed the presence of several anti-diabetic related proteins such as profilin-like protein (in-solution MALDI-TOF/TOF MS), glyceraldehyde-3-phosphate dehydrogenase-like protein, trehalose phosphorylase-like protein and catalase-like protein (in-gel MALDI-TOF/TOF MS), thus suggests that the basidiocarps of *P. pulmonarius* has high potential in lowering blood glucose level, reduce insulin resistance and the risk of diabetic-related complications.

ABSTRAK

Diabetes mellitus (kencing manis) adalah masalah metabolik kronik yang disebabkan oleh gangguan metabolisme karbohidrat, lemak dan protein, terhasil akibat daripada kecacatan rembesan insulin (Jenis 1), peningkatan rintangan sel terhadap insulin (jenis 2) atau kedua-duanya. Diabetes dicirikan oleh paras tinggi glukosa darah (hiperglisemia) yang luar biasa dan membawa kepada kerosakan yang serius kepada organ badan. 'World Health Organization' menganggarkan hampir 220 juta orang di seluruh dunia disahkan menghidapi kencing manis dan jumlah ini berganda menjelang tahun 2030. Tambahan pula, kencing manis yang tidak dirawat akan menyumbang kepada komplikasi seperti penyakit jantung, strok, darah tinggi, penyakit sistem saraf dan amputasi. Pada masa ini, penyelidik mendekati terapi alternatif untuk memerangi diabetes yang paling lazim, iaitu diabetes jenis 2, dengan melengahkan penyerapan karbohidrat/glukosa melalui perencatan enzim pencernaan karbohidrat; α-amilase dan α-glucosidase. Kajian sebelum ini melaporkan bahawa *Pleurotus pulmonarius* (Fr.) Quel. mempunyai potensi yang besar dalam mengurangkan hiperglisemia dalam diabetes jenis 2 serta mencegah komplikasi yang berkaitan dengan kardiovaskular. Sebatian bioaktif daripada *P. pulmonarius* dianggap kurang toksik dan bebas daripada kesan sampingan berbanding dengan ubat-ubatan sintetik yang terdapat di pasaran seperti sulfonylureas, biguanid, glinides, tolbutamide, troglitazone, rosigitazone dan repaglinide. Selain itu, pengeluaran *P. pulmonarius* telah meningkat di seluruh dunia pada kadar yang pesat disebabkan oleh penyesuaian yang luas, agresif, kemudahan penanaman dengan hasil yang berpotensi tinggi dan nilai pemakanan yang tinggi. Menurut 'Icons of Medicinal Fungi from China', bahagian perubatan cendawan tiram ialah basidiokap. Oleh itu, kajian ini bertujuan untuk mengasingkan, mengenal pasti dan mencirikan protein daripada basidiokap *P. pulmonarius*. Ekstrak air mentah, metanol, diklorometana dan polisakarida serta fraksi protein melalui pemendakan ammonium

sulfat (F10-F100) daripada basidiokap *P. pulmonarius* telah dikaji. Semua sampel telah diuji secara *in vitro* melalui ujian perencatan enzim α-glucosidase dari *Saccharomyces cerevisiae* dan α-amilase dari khinzir. Keputusan awal menunjukkan bahawa pada 25 μg/ml, fraksi protein F30 merencatkan α-glucosidase sebanyak 24.18% dan fraksi protein F100 merencatkan aktiviti α-amilase sebanyak 41.80%. Walau bagaimanapun, ekstrak air mentah, metanol, diklorometana dan polisakarida pada 25 mg/ml menunjukkan aktiviti perencatan yang rendah terhadap kedua-dua ujian. Fraksi protein, (F30 dan F100) menunjukkan profil jalur protein SDS-PAGE yang berbeza masing-masing dengan 10 (5-70 kDa) dan 9 jalur protein (6-70 kDa) dan dipilih untuk analisis RP-HPLC. Melalui penulenan menggunakan RP-HPLC, puncak 3 pada 2.5 μg/ml merencat aktiviti α-glucosidase (F30) dengan 25% peratus sedangkan aktiviti perencatan α-amilase pada 6.25 μg/ml adalah sangat rendah dengan hanya 2.84% (F100). Pencirian puncak 3 protein dari F30 fraksi protein menggunakan MALDI-TOF/TOF MS menunjukkan kehadiran empat protein berbeza yang mungkin terlibat dalam merendahkan tahap glukosa darah melalui pelbagai mekanisma. Kajian ini menunjukkan kehadiran beberapa protein yang berkaitan dengan anti-diabetik seperti ‘profilin-like protein’ (in-solution MALDI-TOF/TOF MS), ‘glyceraldehyde-3-fosfat dehidrogenase-like protein’, ‘trehalose phosphorylase-like protein’ dan ‘catalase-like protein’ (in-gel MALDI-TOF/TOF MS) dan mencadangkan bahawa basidiokap *P. pulmonarius* mempunyai potensi yang tinggi dalam merendahkan tahap glukosa darah, mengurangkan rintangan insulin dan risiko komplikasi yang berkaitan dengan kencing manis.

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Nurul Azwa Abd Wahab, 2013

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LIST OF SYMBOLS AND ABBREVIATIONS

%	percentage
ºC	degree Celsius
±	plus-minus
Da	dalton
cm	centimetre
g	gram
kDa	kiloDalton
L	litre
µl	microlitre
µm	micrometre
mg	miligram
mm	milimetre
ng	nanogram
nm	nanometre
mM	milimolar
v/v	volume per volume
w/v	weight over volume
rpm	revolutions per minute
V	voltan
α-amylase	alpha amylase
α-glucosidase	alpha glucosidase
abs.	absorbance
ACN	acetonitrile
AGEs	advanced glycation end products
ANOVA	analysis of variance

APS	ammonium persulphate
EDTA	ethylenediaminetetraacetic acid
GAP	glyceraldehyde-3-phosphate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
H_2O	water
HCl	hydrochloric acid
IDF	International Diabetes Federation
MALDI-TOF/TOF MS	Matrix Assisted Laser Desorption/Ionization-Time of Flight/Time of Flight Mass Spectrometry
MW	molecular weight
Na_2CO_3	sodium carbonate
Na_2HPO_4	disodium hydrogen phosphate
OD	optical density
PKC	protein kinase C
RPF	reversed-phase HPLC fraction
RP-HPLC	Reversed-phase High Performance Liquid Chromatography
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
TEMED	N, N, N', N'- tetramethylenediamine
TP	trehalose phosphorylase
WHO	World Health Organization

LIST OF APPENDICES

1.0 INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disorder in the endocrine system resulting from defects of insulin secretion (Type 1), increased cellular resistance to insulin (Type 2) or both. A consequence of this is characterized by an abnormally high level of blood glucose or known as hyperglycemia that leads to serious damage to many body organs. Without sufficient insulin available, body tissues, in particular, the liver, muscle and adipose tissues fail to take up and utilize glucose from the blood circulation. Although genetic is a main factor in the development DM, it is possible to modify DM via lifestyle and food diet in combating the disease (Okada, 2011).

DM is rapidly increasing in the whole world and currently become the third "killer" of human health. The prevalence of type 2 DM has been estimated to increase from 150 million, to 225 million by the end of the decade and is expected to increase to 300 million by 2025 (Maggi *et al.*, 2013). Uncontrolled DM disease leads to the development of both acute and long-term chronic complications such as retinopathy, neuropathy, amputation, organ dysfunction involving the eyes, kidneys, nervous, heart and damage of vascular systems thereby increase the risk of cardiovascular disease (Genuth *et al.*, 2003). Untreated complications resulted from this disease can lead to death (Ortiz *et al*., 2010).

Currently, there are many kinds of anti-diabetic medicines that are available in the market such as sulfonylureas, biguanides, glinides, tolbutamide, troglitazone, rosigitazone and repaglinide. However, most of them are too toxic, costly, and they give rise to negative effects to the patient and significantly fail to alter the course of diabetic complications. Furthermore, the major focus of current anti-diabetic research is the development of anti-hyperglycemic agents that are safe and free of negative side effects such as nausea, diarrhoea, liver problems and weight gain (Malviya *et al*., 2010).

Recently, many researchers are working on an alternative therapeutic approach

for combating DM by decreasing postprandial hyperglycemia via delaying the absorption of carbohydrates/glucose through the inhibition of carbohydrate hydrolysing enzymes; α-amylase and α-glucosidase enzymes in the digestive tract using compound from natural sources such as polysaccharides, proteins, steroids terpenoids and alkaloids (Etxeberria *et al*., 2012).

Bioactive proteins can be isolated via proteomics study which is an excellent tool in profiling, discovering, and identifying proteins produced in response to a changing cellular environment (Aebersold & Mann, 2003). Proteome analyses involve a wide variety of methods that include protein purification such as ammonium sulphate precipitation, separation using high performance liquid chromatography (HPLC), SDS-PAGE and identification using MALDI-TOF/TOF mass spectrometry.

Many mushroom species such as *Agaricus bisporus*, *Agaricus subrufescens*, *Grifola frondosa*, *Pleurotus ostreatus*, and *Cordyceps sinensis* are found to be effective for controlling the blood glucose levels and modify the course of diabetic complications (Barra *et al.*, 2012). *Pleurotus pulmonarius* (grey oyster mushroom) is an excellent candidate mushroom claimed to have anti-diabetic properties. Bioactive compound from mushrooms are considered to be less toxic and free from side effects compared to synthetic drugs. Moreover, it contains numerous bioactive compounds including polysaccharides, protein and dietary fibres isolated whether from the whole mushroom or mycelia. These compounds showed potential as antihyperglycemic agents (Khan & Tania, 2012).

In addition, the production of *P. pulmonarius* has been increasing worldwide at a rapid rate due to its broad adaptability, aggressiveness, ease of cultivation with high yield and high nutritional value. Furthermore, it can be cultivated within a wide range of temperatures on different natural resources and agricultural wastes (Tan & Wahab, 1997). Previous research reported that *P. pulmonarius* has a good potential in reduction

of hyperglycemia in type 2 DM and ameliorate the course of cardiovascular-related complications (Kanagasabapathy *et al.*, 2012). Meanwhile, the aqueous extract of *P. pulmonarius* basidiocarp also contains vitamin B1, B2, and C and it can also reduce the blood cholesterol level (Pramanik, 2005).

To date, there are no reports on the active protein(s) with anti-diabetic properties isolated from the *P. pulmonarius* basidiocarps.

Hence, the objectives of this research were focused on:

1. To prepare the organic, aqueous and protein extracts from basidiocarps of *P. pulmonarius*.
2. To evaluate the anti-diabetic activities *in vitro* of the prepared extracts.
3. To identify and characterise the peptides isolated from the bioactive extract by MALDI TOF/TOF mass spectrometer.

2.0 LITERATURE REVIEW

2.1 Diabetes Mellitus

Diabetes mellitus (DM) is a group of metabolic disease characterized by hyperglycemia resulting from defects of insulin secretion by pancreas, increased cellular resistance to insulin or both. In the human normal system, when foods containing starch or simple sugars are digested and absorbed into the blood through the intestines, insulin helps the body to store these nutrients throughout the body for later use as a source of energy. Therefore, it will keep removing extra glucose from the blood, causing the blood sugar level to decrease and always maintained at normal levels (Shobana *et al.*, 2009). DM appears due to improper homeostasis regulation of carbohydrate and lipid metabolism by insulin. As a consequence, abnormally elevated levels of glucose accumulated in the blood occurs which in turn leads to serious damage to many of the body's systems, especially the nerves and blood vessels (Nagappa *et al.*, 2003; Li *et al*., 2012b).

Nowadays, DM is becoming a dangerous threat to human health all over the world. It is a heterogeneous disease which is caused by genetic and environmental factors as well as obesity and lack of exercise (McCue *et al.*, 2005; Smith *et al.*, 2012). Although genetic factors are important in the development of type 2 DM, it is possible to modify them to prevent the disease (Sanz *et al*., 2010). People in high-risk groups are those who are obese, having a history of giving birth to big baby (weighing more than 4 kg), history of gestational diabetes, having a family history of type 2 DM, or those belong to high-risk ethnic groups due to their lifestyle and tradition such African American, native American, Hispanic, Pacific Islanders. Moreover, smoking and alcohol drinking are also potential cause of the disease (Risch *et al.*, 2002).

Besides, diabetes will be the fifth leading cause of death in the world by the year 2030 and becomes the most challenging health problems in the 21st century (Mimino,

2011). According to the statistics of International Diabetes Federation (IDF), two individuals develop diabetes every 10 seconds all over the world, and two individuals die of diabetes-related disease every 10 seconds worldwide (World Diabetes Media Kit: every 10 seconds 1 person dies of diabetes, 2007).

The IDF also estimates at least 336 million people worldwide suffered from diabetes in 2011 and the number has been expected to increase to a staggering 552 million by 2030. That concludes one in ten adult will have diabetes and the number of people with type 2 DM is increasing in every country (Shaw *et al*., 2010; Kwon, 2012). Moreover, the death cases were estimated to be 3.96 million of diabetic people in the age group 20–79 years in 2010 (Roglic & Unwin, 2010). The number of death of diabetic people was increased in the year 2011 for at least 4.6 million deaths worldwide (Colagiuri, 2010; Nickerson & Dutta, 2012). Hence, the statistic was greater than the global mortality for hypertension, AIDS, and tuberculosis (Hu, 2011).

Meanwhile, increasing number of diabetic cases gives a negative impact to low- and middle-income countries because it imposes economic burden on national health care system. People with diabetes indeed have more outpatient visits, need more medications, have a higher probability of being hospitalized, and are more likely to require emergency and long-term care compared to healthy people (Zhang *et al.*, 2010). In 2011, at least US$465 billion dollars have been spent which constitutes about 11% of the total health expenditure in adults (Oputa & Chinenye, 2012).

Nevertheless, untreated hyperglycemia (type 1 and type 2 DM) can also lead to the development of both acute and long term chronic complications such as retinopathy, neuropathy, amputation, renal dysfunction, tissue damage and failure of different organs such as eyes, kidneys, nerve, heart and vascular systems (Weiss & Sumpio, 2006; Kowalska, 2007; Winkley *et al.*, 2012). Besides, chronic hyperglycemia is known to damage almost all cell types in the body and also increase the risk of cardiovascular

disease (Li *et al.,* 2012b). Both experimental and clinical studies also suggest that there are a close link between hyperglycemia and diabetic complications which cause a high mortality and morbidity (Rabbani *et al.,* 2009). Furthermore, without any cure and medical emphasis of this chronic disease will end up with many diabetes-related disease and untreatable complications such as coronary heart disease (Gossain & Aldasouqi, 2010). Therefore, early diagnosis and a healthy lifestyle are pivotal choice to avert risk of DM (Yates *et al.*, 2012).

Up until now, other than insulin treatment, there are many kind of anti-diabetic medicines available to control and treat diabetes and its complications mainly depend on the bioactive compound. However until now, there has never been a report saying that someone had totally recovered from DM (Li *et al.*, 2004). Although currently various oral anti-diabetic agents are available on the market such as phenformin, troglitazone, biguanides, rosigitazone, glinides, tolbutamide, repaglinide and sulfonylureas, which are widely used drugs for diabetic treatment, they produce negative side-effects, fail to alter the course of diabetic complications as well as affects other body metabolisms (Patel *et al.*, 2012).

The definitions and categories of DM used in this document are based on the most recent classifications reported by the American Diabetes Association. There are three main types of DM; type 1 DM, type 2 DM and gestational diabetes. Other forms of diabetes which were caused by a single gene mutation and damaging the quality of insulin were seldom found (Velho & Froguel, 1997).

2.1.1 Type 1 Diabetes Mellitus

Type 1 DM is also known as insulin dependent diabetes mellitus (IDDM) or juvenile diabetes. This type occurs as a result of the body's failure to produce insulin due to destructive lesions of insulin producing β-cells of the islets of Langerhans

located in the pancreas and usually progresses to the stage of absolute insulin deficiency (Kobayashi, 1994; Pozzilli & Buzzetti, 2007).

Commonly, type I diabetes occurs in children or adults subjects with acute onset thus it was termed as "juvenile diabetes" (Imagawa *et al.*, 2000). People with type 1 DM depend on exogenous insulin by daily intake of insulin injections to stay alive and they also have a risk for ketoacidosis. Type 1 DM develops approximately 5–10% of diagnosed diabetes cases in all countries but the incidence is rapidly increasing 40% higher in the year 2010 compared to 1999 (Sierra, 2009). Only half a million people have type 1 DM worldwide, and only 78 000 children develop the disease every year (Oputa & Chinenye, 2012). Until now, there are no specific or identified agent capable to control or prevent this type of diabetes (Atkinson & Eisenbarth, 2001).

2.1.2 Type 2 Diabetes Mellitus

Type 2 DM is also known as non-insulin dependent diabetes mellitus (NIDDM). NIDDM is far more common because it accounts for 90 to 95% of all diagnosed diabetes cases. This type of diabetes is one of the major public health challenges of the 21st century (Hussain *et al.*, 2007; Oputa & Chinenye, 2012).

Type 2 DM occurs when body's cells fail to use insulin properly (insulin resistance) in peripheral tissues such as muscle, liver and adipose. Among individuals suffering type 2 DM, their disease factor can be attributed to environmental exposure and genetics from family member diagnosed of diabetes (Eriksson & Lindstrom, 2001). Typically, type 2 DM develops after middle age but may occur in younger people and it is associated with obesity, age, and physical inactivity (Rosenbloom *et al.*, 1999). Type 2 DM was unheard of in children but recently it makes up 40-50% of cases with the rest being type 1 DM (Davis & Christoffel, 1994).

2.1.3 Gestational Diabetes Mellitus

The third type of diabetes is gestational diabetes (GDM) which develops in pregnant women who have never had diabetes before but have a high blood glucose level during pregnancy (Zhang & Ning, 2011). GDM develops in only a small proportion of pregnant women (3–5%) (Ben-Haroush *et al.*, 2004).

Indeed, women with GDM are at high risk for having or developing diabetes after pregnancy. This was proven as almost 50% cases of GDM may end up as type 2 DM. Thus, GDM requires careful medical supervision throughout the pregnancy (Buchanan & Xiang, 2005; Oputa & Chinenye, 2012).

2.2. Treatment and Management of Type 2 Diabetes Mellitus

Admittedly, type 2 DM is the most prevalent form of the disease, which diagnosed is increasing rapidly and can remain undiagnosed for many years. The number of patients with diabetes disease-related complications such as heart disease, stroke, hypertension, nervous system disease and amputation are also increasing unabated (Zimmet, 2003). Type 2 DM occurs when the body resistance to natural insulin hormone produced from the pancreas gland, essentially ignoring its insulin production. Insulin is continuously being secreted but the body does not respond to the effects of natural hormonal insulin as it should function. Insulin resistance is first demonstrated in skeletal muscle, in which concentrations of insulin are necessary to allow glucose to enter the cells (Weyer *et al.*, 2000).

Nevertheless, onset of type 2 DM is difficult to detect because in the early stages, there are no symptoms shown. It will be discovered only when blood glucose levels exceed the "renal threshold" where glucose appears in the urine by medical tests. At this stage, the symptom of diabetes will nascent which are constant thirst (polydipsia), excessive excretion of urine (polyuria), immediate weight loss, constant

hunger, blurred vision, itchy skin, fatigue and slowly cuts healing (García, 2005).

As a consequences, untreated DM in a long term can damage human health and can increase the risk of diabetes related-disease such as heart disease, stroke, kidney failure, nerve damage and blindness. The risk of diabetic complications usually begins as early as in the pre-diabetic phase. Generally, the injurious effects of hyperglycemia are separated into macrovascular complications (coronary artery disease, peripheral arterial disease, and stroke) and microvascular complications (diabetic nephropathy, neuropathy, and retinopathy) (Fowler, 2008).

Close to 50% of death that due to diabetes resulted from cardiovascular disease as well as heart disease and stroke and approximately 2% of diabetic patients become blind. Diabetic retinopathy can cause blindness among diabetic patient because of a long-term accumulated damage to the small blood vessels in the retina. Therefore, early treatment to avert the disease and its complications is a key issue because of the increasing huge premature morbidity, mortality and economic burdens (Zimmet, 2001).

Currently, medical researchers worldwide emphasize that a combination of insulin treatment, oral anti-hyperglycemic drugs, physical exercise, losing excess weight and healthy diet are effectively used to control and lower blood glucose to a near-normal glucose level (Sanz *et al.*, 2010). Indeed, in the management of type 2 DM, it is very important to achieve blood glucose level as close as possible to normal without resulting in either chronically high (hyperglycemia) or dangerously low blood glucose levels (hypoglycemia). Both can cause severe disease complications (Mooradian & Thurman, 1999).

Despite of being the mainstay treatment to diabetic patient, insulin still has its shortcomings. The main drawback of this treatment is it must be given only by injection. Moreover, insulin treatment can cause up to 4 kg increase in body weight and risk of mild to severe hypoglycemia with increasing durations of the treatment

(Swinnen *et al.*, 2009). Besides, majority of patients need multiple therapies correlated with insulin to attain normal glycemic target levels in the longer term (Turner, 1999). Hence, the search for active oral anti-hyperglycemic drugs was demanded not only because of its effectiveness but also because of each drug has unique mechanism of action (Inzucchi, 2002).

In year 2006, there are five major classes of oral anti-hyperglycemic agents in the market. One of the most commonly used drug is metformin which reduces the amount of glucose production by the liver (hepatic glucose) and improving the way glucose is used by muscles. It is therefore considered as insulin sensitizer. Intake of metformin is associated with weight loss or no weight gain and has a lower risk of hypoglycemia. Meanwhile, sulfonylureas are also commonly used because it helps stimulate pancreas to increase insulin release from pancreatic islets. Second-generation sulfonylureas, such as glyburide, glipizide, and glimepride, are safer, potent and lesser drug interactions than first-generation sulfonylureas such as chlorpropamide, tolazamide, and tolbutamide (Riddle, 2000). Prandial-glucose regulators for example, repaglinide also encourage pancreas to produce more insulin (Shoelson, 1995).

Other mostly consumed oral anti-hyperglycemic agent is α-glucosidase inhibitors such as acarbose and voglibose which were introduced in 1996. It is a complex oligosaccharide which reversibly inhibits α-glucosidase, the final enzymes which cleaves more complex carbohydrates into sugars in the brush border of small intestinal mucosa. This drug lowers blood glucose by slowing the breakdown of some ingested carbohydrates into glucose thus maintain the blood glucose level (Wehmeier & Piepersberg, 2004).

Hence, in order to search for safer new drug in preventing and treatment of type 2 DM and complications, more research is needed in this field. This is due to the existing synthetic drugs have several limitations in term of tolerability and significant

mechanism-based side effect which prompt patients to stop taking these anti-diabetic medications. Major drawbacks of oral anti-diabetic agents are most of them may cause weight gain, abnormal colon function, give metallic taste, nausea, and the severity of gastrointestinal disturbances such as frequent diarrhoea, colon bloating and flatulence (Glucotrol, 2001; Fisman *et al.*, 2004). The most bothersome side effects of synthetic drugs is on postprandial hyperglycemia (DeFronzo, 1999).

For diabetes control, rather than the synthetic drugs, many agents from the plant origin such as proteins and polysaccharide have been explored and used particularly for the treatment of type 2 DM (Arayne *et al.*, 2007). However, a number of herbal and plants origin medicines have already been used since ancient times but yet to be commercially formulated as modern medicines (Grover *et al.*, 2002). Therefore, new or alternative therapies using other natural sources for new drugs as no drug which are definitely considered to be less toxic and free from harmful side effects compared to synthetic drugs need to be further researched (Pari & Umamaheswari, 2000).

2.3 Blood Glucose Homeostasis

Carbohydrate taken by human or mammals from various dietary sources is a primary source of glucose. Since glucose is the main energy for the body, it is required to be continuously supplied to ensure all body cells function properly. The glucose level in bloodstream fluctuates because it depends on the body needs. Blood glucose homeostasis is maintained by a balance between glucose entry into the blood circulation and its removal from the blood circulation (Postic *et al.*, 2004). The diet, type and amount of carbohydrate, the rate of carbohydrates digestion, absorption of carbohydrates, breakdown of glycogen as well as the activity of the hepatic gluconeogenesis and glycogenolysis process influence glucose entry into the circulation. Nevertheless, insulin and glucagon are hormones that have a great

responsibility to precisely regulate the amount of glucose in the bloodstream. These hormones drain directly into the liver.

Insulin is an important hormone, where it regulates the blood glucose level to the normal level. Insulin producing beta cells, clumps together to form the "islets of Langerhans" which are found in pancreas gland (Grover *et al.*, 2002). The pancreatic "islets of Langerhans" are the sites for the production of insulin and glucagon. Hence, the pancreas plays a primary role in the metabolism of glucose either to secrete the insulin or glucagon hormones.

Commonly, insulin will be released from pancreas mostly when the level of postprandial blood glucose is high. When glucose is abundant, the pancreatic beta cells will stimulate secretion of insulin into the bloodstream (Reaven, 1988). In order to maintain blood glucose level, insulin will triggers the cells to use glucose in protein synthesis. Insulin promotes glucose storage and stimulates cells to breakdown glucose (glycolysis) by releasing it as an energy in the form of ATP and citric acid cycle (Yakar *et al.*, 2004). Meanwhile, insulin also ease the entry of glucose into the adipose and muscle tissues and store it as glycogen for short-term energy storage whereas for long-term storage, adipose tissue will store glucose as fat (Bekele, 2008). As a result, the glucose level in the blood decreases. On the other hand, insulin inhibits the production and release of glucose from the liver (gluconeogenesis and glycogenolysis). Likewise, insulin inhibit glucagon production.

Otherwise, glucagon hormone will be released by pancreas when blood glucose level is scarce and insulin levels are low. Generally, glucagon stimulates the liver to produce glucose and releases it into the bloodstream. However, when blood glucose and insulin levels are high after a meal, glucagon levels will immediately drop, and the liver stores excess glucose for later use. The actions of glucagon and insulin work in an opposing manner so as to regulate and maintain a rigid control on glucose

concentrations in the blood (Aguilar-Parada *et al.*, 1969).

When the blood glucose levels are diminished, in order to raise the blood glucose level approximately between 70 to 110 mg/dl, glucagon will be released. Glucagon raises blood sugar levels by stimulating the mobilization of glycogen stores in the liver, release energy and providing a rapid burst of glucose. Furthermore, glucagon stimulates both gluconeogenesis and glycogenolysis thereby releases blood glucose into the blood circulation. Conversely, it inhibits glycogenesis process. Glucagon also helps to stimulate glucose production by favouring the hepatic uptake of amino acids, the carbon skeletons of which are used to make glucose. Moreover, glucagon acts to trigger the body to switch and use resources other than glucose, such as fat and protein (Cummings, 2006).

In patients with diabetes, a blood glucose level remains high regardless of the level of insulin since its function or the production of insulin has been stopped. This differentiates between type 1 and type 2 DM patients. For type 2 DM, the blood glucose concentration remains increased despite the presence of normal to high insulin concentration in the bloodstream and this is referred as insulin resistance.

2.4 Mechanisms to Lower Blood Glucose Level

Carbohydrates are the major constituents of human diet and polysaccharides are one of the main components of carbohydrates that mainly play a role in the energy supply (Nickavar & Yousefian, 2009). In order to be absorbed in the duodenum and upper jejunum as energy, the dietary carbohydrates should first be hydrolysed to monosaccharides by α-glucosidase enzyme in small intestinal brush-border, since only monosaccharides can be absorbed by intestinal lumen (Kwon *et al.*, 2006a).

As well as dietary polysaccharides, it first are hydrolyzed in the gastrointestinal tract by the α-amylase enzyme to produce oligosaccharides and disaccharides. The

resulting oligosaccharides and disaccharides are further hydrolyzed by α-glucosidase enzymes to further produce glucose and other monosaccharides such as fructose and galactose before being absorbed as blood glucose (Bhat *et al.*, 2011). Glucose and other monosaccharides resulting from digestion of carbohydrates and polysaccharide are absorbed through the small intestine into the hepatic portal vein thus result in elevation of the postprandial blood glucose level. This proves that the postprandial hyperglycemia strongly depends on the amount of absorbed monosaccharides and the rate of digestion into small intestines.

Indeed, in the effort to control postprandial hyperglycemia, many researchers have focused on the starting process of carbohydrate metabolism, particularly in glucose releasing and transport across the intestinal brush border membrane down to the blood stream (Tiwari & Madhusudanarao, 2002). They found that the inhibition of carbohydrate hydrolysing enzymes, α-amylase and α-glucosidase delaying the breakdown and absorption of carbohydrates (sucrose, dextrins, maltose, and starch) from the digestive tract can reduce postprandial hyperglycemia. However, the treatment of type 2 DM is complicated because of various factors affecting the disease occurrence and one of it is elevated postprandial hyperglycemia (Gin & Rigalleau, 2000). Good glycemic control can reduce postprandial hyperglycemia and decreases the risk for both type 2 DM and its complication. Thus, these enzyme inhibitor drugs have been one of the major focus nowadays (Jayasri *et al.*, 2009).

2.5 Alpha Glucosidase and Alpha Amylase Enzymes Inhibitors

Alpha glucosidase which is one of the carbohydrate metabolism enzymes, is responsible to degrade complete carbohydrates into monosaccharides. The released non-reducing sugar is easily absorbed through intestines into the bloodstream (Shobana *et al.*, 2009). Alpha glucosidase inhibitors acts as a competitive inhibitors of glucosidases which are located in the brush border of epithelial cells, mainly in the upper half of the

small intestine (Moller, 2001; Bailey, 2004). This process occur when the α-glucosidase inhibitors bind reversibly in a dose-dependent manner to the oligosaccharide binding site of α-glucosidase enzymes thereby delay the degradation of polysaccharides and starch to glucose (Franco *et al.*, 2002). Hence, this enzyme inhibitor helps in slowing down food digestion in the gut thereby reduce peak blood glucose concentrations. Importantly, enzyme inhibitors function without increasing insulin secretion, do not cause hypoglycemia or weight gain and may also act as effective anti-obesity agents (Kotowaroo *et al.*, 2006).

Another inhibitor related to α-glucosidase inhibitor is α-amylase inhibitor that also offer an alternative therapeutic approach in reducing the postprandial hyperglycemia. In order to ultimately slowing glucose breakdown from starch, α-amylase enzyme that cleaves at internal bonds of large polysaccharides is inhibited (Da Silva Pinto *et al.*, 2008). This α-amylase inhibitor inhibits the action of α-amylase enzyme leading to retard the liberation of maltose from starch hydrolysis which shows beneficial effects on glucose level control in diabetic patients (Notkins, 2002).

It has been recently reported that therapeutic approach to delay the absorption of carbohydrates or glucose into gastrointestinal after meal to prevent postprandial hyperglycemia is now increasingly important as alternative to treat type 2 DM (Ademiluyi & Oboh, 2011). Since 90% of the diabetic patients are suffering from type 2 DM, it is important that dietary α-glucosidase and α-amylase inhibitors are featured and be emphasized upon the production of new drug (Ranilla *et al.*, 2010). A safe drug that reduce postprandial hyperglycemia by suppressing the absorption of carbohydrate are effective in type 2 DM prevention and treatment. The purification and characterization of the new inhibitor of carbohydrate hydrolysing enzymes may provide an insight into the reasons of development of alternative anti-diabetic drugs. Besides, a research regarding glucosidase inhibitors is important and become an important biochemical

tools for studying the mechanism of enzyme-catalyzed glycoside, oligosaccharide structure, medicinal chemistry and researchers can understand the cause of diabetes intensively (Chen *et al.*, 2004).

These types of observations led to the hypothesis that drugs act on the gastrointestinal tract to interfere with carbohydrate digestion might be useful agents in the treatment of diabetes, reduced risk of cardiovascular disease and hypertension (Lebovitz, 1997; Van de Laar *et al.*, 2007). Although powerful synthetic α-glucosidase and α-amylase inhibitors such as acarbose, miglitol, voglibose and emiglitate for the treatment of type 2 DM are widely used nowadays, it has been reported that it can cause several harmful side effects such as hepatic disorders, cause abdominal pain, flatulence, diarrhoea and may increase the incidence of renal tumours, hepatic injury and acute hepatitis (Singh *et al.*, 2008). This results from the undigested carbohydrate available in the colon which is then fermented into short-chain fatty acids and releasing gas (Nolte & Karam, 2004). In addition, there is no report found about the long-term effectiveness of commercial inhibitors reducing chronic complication (Cheng & Fantus, 2005). The effect of drugs from natural sources has not yet been reported. Due to this, it is important to identify natural new α-glucosidase and α-amylase inhibitors as alternative to reduce diabetes cases.

Since 2001, several safer natural α-amylase and α-glucosidase inhibitors compounds such as alkaloids, triterpene, acids, phytosterol, myoinositol, flavonoids, anthraquinones, anthrones, xanthones, anthocyanin and others were isolated from plants that contributes to management of hyperglycemia linked to type 2 DM (Matsui *et al.*, 2001; Kim *et al.*, 2005; Da Silva Pinto *et al.*, 2008; Benalla *et al.*, 2010). It has been reported that many medicinal plant and herbal extracts have been found to inhibit the enzymatic activity of α-glucosidase and α-amylase (McCue & Shetty, 2004). Kobayashi *et al.*, (2002) also reported screening of various plants for α-amylase inhibitory activity

and the resultant *in vivo* postprandial hyperglycemia activity. However, Kwon *et al.*, (2006b) had reported that plant origin for both inhibitors with stronger activity attenuates the α-glucosidase enzyme but not significant inhibitor effects on α-amylase enzyme activity.

In the recent years, more researchers have moved on to venture new exciting candidate other than plants and herbs to help control and manage postprandial hyperglycemia. Natural resources such as seaweed and mushroom has now gain an interest of many researchers (Silva *et al.*, 2012). As an example, Mohamed *et al*., (2012) reported that seaweed, *Gymnopilus edulis* showed a potent inhibitory action against enzyme glucosidase. Nowadays, screening of α-amylase and α-glucosidase enzymes inhibitors from mushroom species have received much attention throughout the world (Silva *et al.*, 2012). Edible mushrooms such as *Lentinus edodes*, *Ganoderma lucidum*, *Schizophyllum commune* and *Trametes versicolor* are now considered to be an important source of nutrients and popular as non-toxic medicines (Wasser & Weis, 1999); and therefore worth to be investigated or screen for their potential as inhibitors for the α-amylase and α-glucosidase enzyme.

2.6 Nutritional and Medicinal Properties of Mushrooms for the Prevention of Diabetes Mellitus

Mushrooms are defined as macrofungus with a fleshy and distinctive fruiting body structure, large enough to be picked by hand or to be seen with naked eyes (Chang, 2008; Guillamon *et al.*, 2010). It is a popular delicacy, widely consumed as a human daily food for thousands of years in many countries probably due to their attractive taste and texture (De Silva *et al.*, 2012). In ancient times, only the basidiocarps are consumed but nowadays the mycelia are also being processed and widely commercialized (Smith, *et al.*, 2002; Lindequist *et al.*, 2005; Hyde *et al.*, 2010).

Wild or cultivated mushrooms have vast prospects as natural sources of medicine with a high nutritional, pharmacological effect and economic values. Furthermore, many previous studies have confirmed that mushrooms are an important source of nutritional food as well as income in for many countries (Wong *et al.*, 2001). Amongst the many species used in health prospect are *Claviceps purpurea*, *Cordyceps sinensis*, *Ganoderma lucidum*, *Laricifomes officinalis*, *Lentinula edodes* and *Trametes versicolor* (Molitoris, 2005). However, according to Hawksworth (2001), there are 7000 yet undiscovered species with medicinal potential.

Recently, mushrooms become popular as a functional food and as a remarkable source for development of new drug because it contains a numerous bioactive compounds that have shown therapeutic benefits against the development of many diseases (Carbonero *et al.*, 2008; Cheung, 2008; Lee & Hong, 2011). The most popular edible mushrooms include *Agaricus bisporus*, *Pleurotus* species, *Lentinula edodes*, *Volvariella volvacea*, *Auricularia* species, *Flammulina velutipes*, *Tremella fuciformis*, *Hypsizygus marmoreus*, *Pholiota nameko* and *Grifola frondosa* (Manzi *et al.*, 2001; Sanchez, 2010). Hence, consumption of whole, extracts or concentrates edible medicinal mushroom provides nutraceutical properties and helps in prevention or treatment of many diseases because it contains high molecular weight compounds such as polysaccharides, digestible proteins and lipids as well as a number of low molecular weight metabolites such as lectins, lactones, terpenoids, alkaloids, sterols and phenolic substances (Barros *et al.*, 2008; Chun, 2011). Mushrooms species also contains high amount of functional bioactive compounds, rich in minerals, proteins, fibers, carbohydrates and have high levels of water (Lindequist *et al.*, 2005; Caglarirmak, 2007; Elmastas *et al.*, 2007; Smiderle *et al.*, 2012).

Besides that, mushrooms also contain vitamins, minerals and low fat levels thus is excellent to be included in daily low caloric diets (Agrahar-Murugkar &

Subbulakshmi, 2005). Mushroom has been established as a high source of minerals such as copper, iron, potassium, magnesium, selenium, phosphorus, manganese, sodium, calcium and zinc in the human diet and are very low in sodium (Román *et al.*, 2007; Gençcelep *et al.*, 2009). Furthermore, mushroom proteins is comparable to animal proteins, which is of particular importance to counterbalance a high consumption of protein animal food sources, especially in daily diets of developed countries (Okoro, 2012). Mushroom also acts as a excellent replacement of vegetable in daily diet routine (Sadler, 2003). Moreover, one species can possess a high variety of bioactive compounds, and therefore indicates various pharmacological effects. For example is *Ganoderma lucidum*, which not only contains more than 120 different triterpenes but also polysaccharides, proteins and other bioactive compounds (Lindequist *et al.*, 2005).

Because of the high content of various beneficial compounds, many of edible mushrooms have shown the ability and potential to prevent several chronic diseases such as anti-tumour, cardiovascular, cancer, hypertension, metabolic syndrome, antiviral, antibacterial, anti-parasitic and anti-diabetic (Bobek *et al.*, 1998; De Silva *et al.*, 2012) (Table 2.1). Recently, many studies have focused on immunomodulatory and anti-tumour effects because mushrooms may contain a diverse array of biologically active metabolites (β-D-glucans) with well-known immune enhancing capabilities (Wasser, 2011; Wu *et al.*, 2012). Moreover, mushrooms are important source of strong antioxidant and have potent free radical-scavenging activities (Ajith & Janardhanan, 2007). Besides, mushroom helps to inhibit platelet aggregation, reduce of blood cholesterol concentration, prevent heart disease, and block induced liver peroxidation (Ghaly *et al.*, 2011). Therefore, high production of mushrooms in a larger scale are important in order to meet the high demands of the global market (Guillamon *et al.*, 2010).

Table 2.1: Examples of medicinal mushrooms and their pharmacological effects

Species (active compound identified)	Function	References
Agaricus bisporus, *A. brasiliensis*, *Trametes versicolor*, *Grifola frondosa*, *Inonotus obliquus*, *Lentinus edodes*, *Leucoagaricus americanus*, *Pleurotus ostreatus*, and *Sparassis crispa*	Breast cancer inhibitory activity	(Petrova *et al.*, 2005)
Agaricus blazei (polysaccharide-protein complex)	Treatment of tumour, hypertension, diabetes, obesity and hepatitis	(Mizuno *et al.*, 1999)
Flammulina velutipes (flammulin, a basic simple protein)	Protective against increased serum cholesterol level and inhibit tumour cells	(Ling *et al.*, 1990)
Flammulina velutipes and *Hypsizygus marmoreus*	High anti-tumour activity, treat liver diseases, gastric ulcers and cancer prevention	(Ikekawa, 2001)
Ganoderma lucidum	Enhancement of lymphocyte proliferation and antibody production, producing both anti-genotoxic and anti-tumour promoting activities	(Bao *et al.*, 2001; Wasser, 2002)
Ganoderma lucidum (triterpenes)	Anti-tumour effect	(Bao *et al.*, 2001)
Grifola frondosa	Antiviral properties	(Gu *et al.*, 2007)
Inonotus obliquus (melanin complex)	Antioxidant and genoprotective effects	(Chihara *et al.*, 1969)
Lentinula edodes (shiitake) (lentinan, a β-1,3-glucan)	Anti-tumour, increasing production of T-cells and macrophages	(Ikekawa, 2001; Sadler, 2003)
P. cornucopiae	Antihypertensive activity	(Hagiwara *et al.*, 2005)
P. florida	Inhibited platelet aggregation and anti-inflammatory activities	(Jose *et al.*, 2004).
P. linteus	Anti-inflammatory effect	(Kim *et al.*, 2004)
P. ostreatus and *P. citrinopileatus*	Antihyperlipidaemic	(Hossain *et al.*, 2003; Hu *et al.*, 2006a)
P. ostreatus, *Pleurotus ferulae* and *Clitocybe maxima*	Antioxidant properties	(Tsai *et al.*, 2009)
Phellinus rimosus, *P. florida*, *P. sajor caju* a.k.a *P. pulmonarius* and *Ganoderma lucidum*	Antioxidant activity	(Lakshmi *et al.*, 2004)

Table 2.1, continued

Species (active compound identified)	Function	References
Piptoporus betulinus	Treatment of rectal cancer and stomach diseases	(Lemieszek *et al.*, 2009)
Pleurotus ostreatus	Exert anti-tumour activity against Hela tumour cell, antibacterial and antifungal properties	(HaiBin *et al.*, 2009; Hearst *et al.*, 2009)
Pleurotus tuber-regium	Anti-tumor	(Tao *et al.*, 2006)
Schizophyllum commune (polysaccharide schizophyllan)	Anti-cancer activity	(Ooi & Liu, 2000; Daba & Ezeronye, 2004)
Trametes versicolor (krestin and polysaccharide-peptide)	Liver protective effect, antitumor and powerful immunostimulant and anti-cancer agent	(Ng, 1998; Ooi, 2001)
Tremella fuciformis (glucuronoxylomannan)	Anti-cancer activity and enhance immune functions	(Hobbs, 1995)
Tricholoma giganteum	The blood pressure-lowering action	(Ooi, 2001)

Some mushroom, especially *Pleurotus* genus also possess a number of bioactive compounds such as polysaccharides, mevinolin and other statins with hypocholestero-laemic activities (Gunde-Cimerman & Plemenitas, 2001). There are also excellent producers of lovastatin, a cholesterol-lowering drug that was reported to be the best therapeutic agent for ameliorating hypercholesterolemia (Gunde-Cimerman & Plemenitas, 2001; Mattila *et al.*, 2001; Jayakumar *et al.*, 2006). *Pleurotus* genus have also been proven to be a good source of essential amino acids that have several medicinal properties such as antioxidant activities (Badole *et al.*, 2006). The aqueous extract of *Pleurotus* fruit body also contains vitamin B1, B2, C, D and K which can reduce the cholesterol level in blood (Pramanik *et al.*, 2005). Meanwhile, the fat fraction in mushroom is mainly composed of unsaturated fatty acids (Mattila *et al.*, 2001).

In the future, it is anticipated that mushroom have a good potential and vital in medicine and biotechnology field because of their unique biosynthetic capabilities and contain various metabolic compounds (Román *et al.*, 2007). The ongoing research will explore a new generation of food originated from mushrooms due to great potential for the prevention or cure of disease more than in other plant species. Nevertheless, more research needed to be noteworthy to ensure the mushrooms species are free from poisonous and high heavy-metal concentrations.

2.6.1 Anti-diabetic Properties of Mushrooms

Up to now, many researchers have shown that certain mushrooms may have potential to lower elevated blood sugar levels and have been targeted as potential hypoglycemic and anti-diabetic agents (Table 2.2). Bioactive metabolites including polysaccharides, protein and dietary fibres such as β-glucans have been successfully isolated from basidiocarps and mycelia of mushroom which markedly decrease blood glucose (Xiao *et al.*, 2011). However, in order to identify the active principles for the

Table 2.2: Mushroom species that have medicinal properties to prevent and ameliorate diabetes and their major effective compounds

Species	Major effective compounds	Mechanism	References
Agaricus bisporus	Dehydrated fruiting body extracts, high amount of acidic polysaccharide	Lower blood glucose and cholesterol level	(Fukushima, *et al.*, 2001)
Agrocybe cylindracea	Glucan and heteroglycan	Hypoglycemic activity	(Wang *et al.*, 2002)
Agaricus campestris	Aqueous fruiting body extracts	Anti-hyperglycemic, insulin releasing and insulin-like activity	(Gray & Flatt, 1998)
Agaricus subrufescens	Beta-glucans and oligosaccharides	Anti-hyperglycemic and improves pancreatic beta cells through inhibition of α-glucosidase	(Liu *et al.*, 2008)
Agaricus blazei	Beta-glucans and oligosaccharides	Improved insulin resistance	(Hsu *et al.*, 2007)
Auricularia auricula	Water soluble polysaccharide from fruit body	Lowering plasma glucose, insulin, urinary glucose and food intake	(Yuan *et al.*, 1998)
Coprinus comatus	Vanadium	Maintains a low level of blood glucose and improves glucose tolerance	(Han & Liu, 2009)
Cordyceps sinensis	Polysaccharide CSP-1	CSP-1 may stimulate pancreatic release of insulin and/or reduce insulin metabolism	(Li *et al.*, 2006)
Ganoderma lucidum	Polysaccharides (Ganoderan A and B, glucans)	Strong α-glucosidase inhibition and lowered the serum glucose levels	(Zhang & Lin, 2004)
Grifola frondosa	Alpha-glucan (MT-alpha-glucan) and methanol of fruit bodies	Effect on insulin receptors (increase insulin sensitivity and ameliorating insulin resistance) and α-glucosidase inhibitor	(Matsuura *et al.*, 2002; Lo *et al.*, 2008)
Hericium erinaceus	Methanol extracts	Hypoglycemic effects with lower elevation rate of blood glucose level	(Wang *et al.*, 2005)
Inonotus obliquus	Dry matter of culture broth	Anti-hyperglycemic activity	(Sun *et al.*, 2008)

Table 2.2, continued.

Species	Major effective compounds	Mechanism	References
Lentinus edodes	Exo-polymer	Hypoglycemic effect with lower blood glucose level	(Yamada *et al.*, 2002)
Pleurotus pulmonaris aka *P. sajur caju*	Aqueous extracts of the mushroom	Reduced the serum glucose level in alloxan-treated diabetic mice and increased glucose tolerance	(Li *et al.*, 2010).
T. versicolor Tremella aurantia	β-glucan–protein complex glucuronoxylomannan	exhibited hypoglycemic and ameliorated the symptoms of diabetes	(Kiho *et al.*, 2000)
Tremella fuciformis	Exopolysaccharide (submerged mycelial culture) and Glucuronoxylomannan (fruit bodies)	Reduced blood glucose levels and improve insulin sensitivity	(Cho *et al.*, 2007)
Wolfiporia cocos, Laricifomes officinalis, Fomitopsis officinalis and *Laetiporus sulphureus*	Dehydrotrametenolic acid	Acts as an insulin sensitizer in glucose tolerance tests and reduces hyperglycemia	(Sato *et al.*, 2002)
Wolfiporia extensa (*Poria cocos)*	Dehydrotumulosic acid, dehydrotrametenolic acid, pachymic acid extracts and triterpenes	Reduced postprandial blood glucose levels and enhanced insulin sensitivity	(Sato *et al.*, 2002)

treatment of DM and its complications, further isolation and research is needed (Perera & Li, 2011). Mushroom does not only appear to be effective to control blood glucose, it also has a potential in controlling the modification caused by diabetic complications because it is capable of producing bioactive metabolites that directly act upon glucose metabolism and related biochemical pathways (Silva *et al.*, 2012). As an example, *G. frondosa*, which does not only possess hypoglycemic action in type 2 DM patients but also helps in maintaining optimum cardiovascular functions and healthy circulatory system (Lindequist *et al.*, 2005; Shavit *et al.*, 2009). Matsuura *et al.*, (2002) reported that *G. frondosa* also contains α-glucosidase inhibitors whereas Xiao *et al.*, (2011) reveals that its polysaccharide is a potent anti-diabetic agent with considerably strong hypoglycemic activity and high potency of increasing insulin sensitivity. Previous clinical research has shown that several triterpenes; ganoderans A, B, C and a ganopoly (polysaccharide extract) that are isolated from *G. lucidum* fruit bodies have been shown to have a strong hypoglycemic effect (Lindequist *et al.*, 2005). Meanwhile, the development of bioactive compounds isolation from basidiocarps and mycelia of many other mushrooms including *Agaricus bisporus, Agaricus subrufescens*, *G. frondosa, P. ostreatus*, and *Cordyceps sinensis* as curative drugs for DM and its complications has been a great demand (Barra *et al.*, 2012).

2.6.2 Anti-diabetic Research Related to *Pleurotus* Species

Pleurotus species or also known as oyster mushrooms is the third most popular cultivated edible mushrooms after *Agaricus bisporus* and *Lentinula edodes* (Chang, 2008) and being an important culinary mushroom all over the world (Abdullah *et al.*, 2012). According to Guzman (2000), several species of *Pleurotus* are widely used by traditional practitioner for treating approximately 35 diseases. Thus, due to increasing market demand, the cultivation of the oyster mushroom has also increased greatly

throughout the world in the last few decades (Banik & Nandi, 2004).

In addition, *Pleurotus* species have been discovered to have definite nutritive and medicinal values because it contains a number of biologically active compounds, dietary fibres, essential amino acid with various therapeutic activities including anti-diabetic, antioxidant, antimicrobial, antiviral, anti-tumour, anti-inflammation, lower blood lipid concentrations, prevent high blood pressure, atherosclerosis, and antithrombotic activities (Jayakumar *et al.*, 2006; Chirinang & Intarapichet, 2009; Agrawal *et al.*, 2010; Vamanu *et al.*, 2011). It has been reported that, polysaccharides from *P. tuber-regium* and *P. abalones* showed effective antidiabetic property, antioxidant substances and boost immunity (Badole *et al.*, 2006; Huang *et al.*, 2012). Moreover, novel antioxidant polysaccharide-peptide complex LB-1b from the basidiocarps of *P. abalone* mushroom exhibited a high antioxidant activity with a significant hypoglycemic effect (Li *et al.*, 2012a). *Pleurotus ostreatus* extract was more effective in decreasing the genetic alterations and sperm abnormalities in diabetes conditions and could reduce the high blood glucose level in hyperglycemic rats (Ghaly *et al.*, 2011). Another important species, the *P. eryngii* has been tested for insulin resistance, anti-hyperglycemic and anti-hyperlipidemic effects in mice. Moreover, dietary polysaccharides from this species significantly improved insulin sensitivity (Kim *et al.*, 2010). Water-soluble polysaccharides from *P. citrinopileatus* demonstrated enhanced insulin secretion and inhibited the increased of blood glucose level (Hu *et al.*, 2006b).

2.7 *Pleurotus pulmonarius* (Fr.) Quél. (Grey Oyster Mushroom)

More than 1000 species of *Pleurotus* have been described throughout the world, in more than 25 related and/or confused genera. However, only approximately 50 valid species are recognized in *Pleurotus*. Other popular *Pleurotus* species are *P. ostreatus*, *P.*

cornucopiae, P. florida, P. eryngii, P. cystidiosis, P. flabellatus, P. cornucopie and *P. ostreatoroseus* (de Carvalho *et al.,* 2010) that were majorly produced in China (Chang & Miles, 2004).

Many of the mushroom growers, manufacturers and cultivators often marketed *P. pulmonarius* using the incorrect name *P. sajor caju*. Some other researchers believed that *P. pulmonarius* is a synonym of the species *P. sajor caju*. It was also being called by several other names such as *P. sapidus* (Shnyreva *et al.*, 2012). According to index of fungorum, *P. sajor caju* no longer exist. The scientific classification of *P. pulmonarius* is from fungi, basidiomycota, agaricomycotina, agaricomycetes, agaricomycetidae, agaricales and pleurotaceae. These species are easily confused due to their similar morphology and substrate specificity. However, compare to other *Pleurotus* species, *P. pulmonarius* has a favourable properties such as tolerant to high temperature, fast fruiting and yield efficiencies and also can grown in warmer weather (Akinmusire, 2011).

Pleurotus pulmonarius is called oyster mushroom because of the shell-like, spatulate pileus with eccentric or lateral stipe (Figure 2.1) (Ohga, 2000). Currently, *Pleurotus pulmonarius* a.k.a *P. sajor caju* is cultivated throughout the world at a rapid rate due to high demand as it is popularly consumed (Gregori *et al.*, 2007; Gern *et al.*, 2008). Moreover, this species can be cultivated within a wide range of temperatures on various natural resources and agricultural wastes such as cereal straws, sawdust and banana leaves (Poppe, 2004). Nowadays, this species become the major types of non-agaric mushroom that are cultivated on a large scale (Fu *et al.*, 2006). Previous research, Alam *et al.,* (2008) suggested that there are different nutritional values in the different part of cultivated mushrooms but according to Icons of Medicinal Fungi from China, part with the most medicinal properties of this mushroom is in the basidiocaps which are low in calories, carbohydrate, and calcium (Ying *et al.*, 1987; Chang & Miles,

2004).

Pleurotus pulmonarius and its extracts may have possible medicinal applications for a wide range of conditions. Apart from having potential anti-diabetic property, *P. pulmonarius* was able to reduce the total plasma cholesterol and triglyceride level and thus reduce the chance of atherosclerosis and other cardiovascular and artery related disorders. *P. pulmonarius* also has hypertensive effects through its active ingredients, which affect the renin-angiotensin system. These medicinal properties might be due to the presence of some important substance in certain dietary mushrooms (Chang & Buswell, 1996).

Previously, Kanagasabapathy (2012) has reported that glucan-rich polysaccharide of *P. pulmonarius* can serve as a potential agent for prevention of glucose intolerance, insulin resistance and inflammation. In addition, *P. pulmonarius* extract with acarbose produced a greater synergistic anti-hyperglycemic effect than either acarbose agent alone (Badole *et al.*, 2008). It also has substances in aqueous extract, hot water extracts with antihyperglycemic effect, hypoglycemic activity and increasing glucose tolerance (Wang *et al.*, 2011). Discovery of the great potential of *P. pulmonarius*, its usefulness in the prevention of DM. Other than containing low fat, high soluble fiber, very little lipid and starch, it also contains a good quality proteins and vitamins such as B1, B2 , and C (Kanagasabapathy *et al.*, 2011).

Figure 2.1: Basidiocarps of *P. pulmonarius*

3.0 MATERIALS AND METHODS

3.1 Preparation of *P. pulmonarius* Fruit Bodies

Fruiting bodies of *P. pulmonarius* used in this experiment were purchased from the hypermarket. The sample was washed with distilled water for several times and cleaved into small pieces. Fruit bodies were kept in freeze-drying flasks, -20 °C and later subjected to freeze-drying. Freeze-dried mushroom was then ground using a dry grinder to obtain fine powdered sample. The freeze-dried samples were bottled and kept in dry container at room temperature before extraction.

3.2 Preparation of Solvents and Protein Extracts of *P. pulmonarius*

The bioactive compounds present in the mushroom fruit bodies of *P. pulmonarius* were extracted using four different methods. They were polysaccharide extraction, methanol extraction, dichloromethane extraction, and crude aqueous extraction procedures (Figure 3.1). All the extracts were kept in 4 °C prior to analysis.

3.2.1 Methanol and Dichloromethane Extracts

Ground freeze-dried *P. pulmonarius* powder was weighed and soaked with methanol and dichloromethane solvents separately in Schott Duran bottle at a ratio of 1:10 each. Then each mixture was left on a shaker for 100 rpm overnight at room temperature. The mixture was then filtered using a vacuum pump and the filtrate was collected.

The volume of remaining residue from methanol and dichloromethane extraction was measured and repeatedly soaked again with an equal amount of the solvents at a ratio of 1:1 separately. The mixture was left on shaker for 100 rpm overnight and filtered on the next day. Both filtrates from each extraction were then pooled. Total filtrates from each extraction was then subjected to rotary evaporation and the semi-

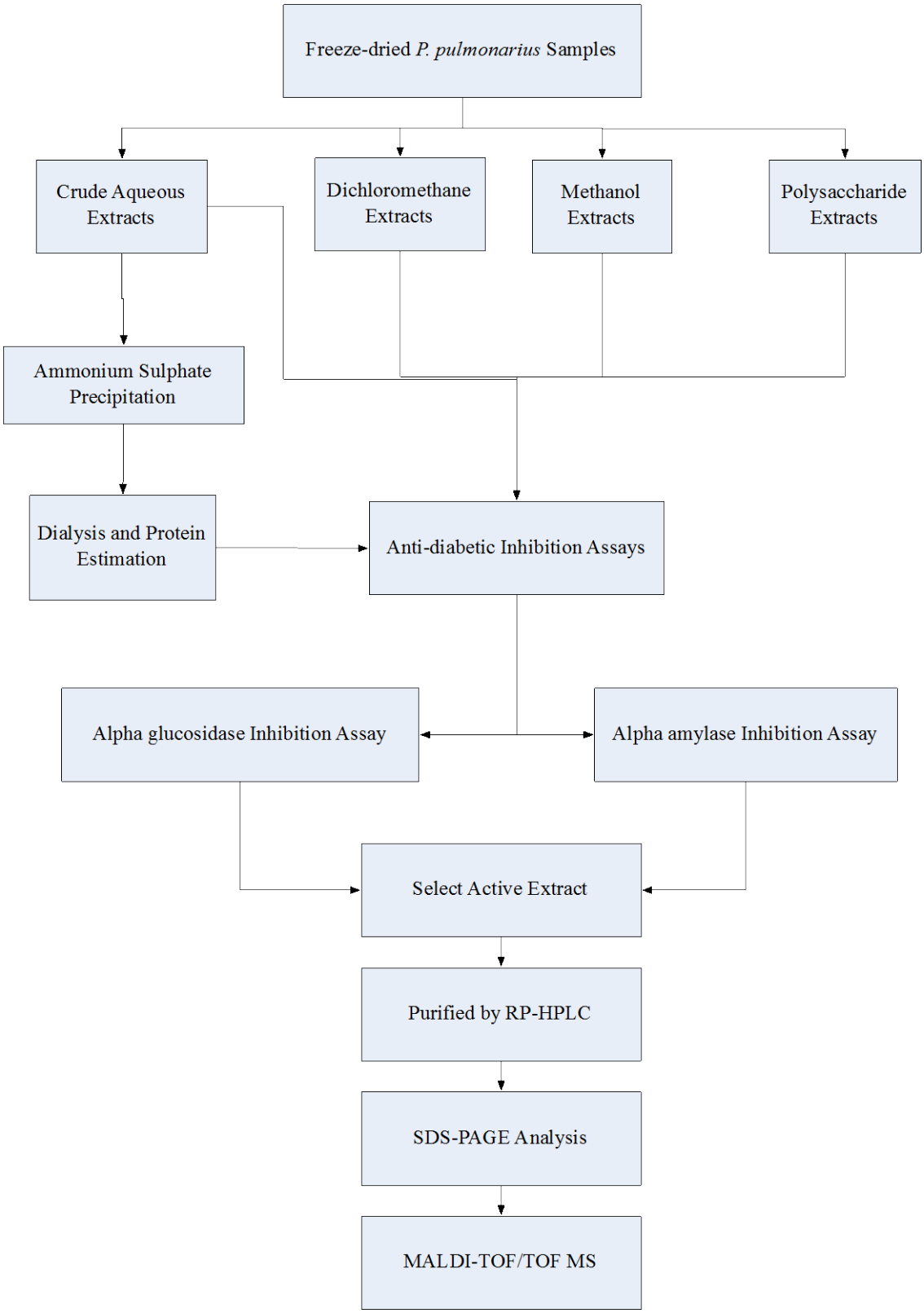

Figure 3.1: Schematic diagram of mushroom extraction procedures, anti-diabetic assays, SDS-PAGE, HPLC and MALDI-TOF/TOF MS analysis

solid sample was bottled and stored at 4 °C. Yield of extract was determined by weighing the semi-solid extract.

3.2.2. Polysaccharide Extracts

Ground freeze-dried fruiting bodies of *P. pulmonarius* (30 g) was weighed and soaked in 900 ml of distilled water at 1:30 ratio. The mixture was then homogenized using a blender for almost 3 minutes and the homogenate was later boiled for 3 hours. Boiled mixture was then left to cool at room temperature and filtered using vacuum pump. Filtrate (200 ml) was added to same amount of 75% ethanol (1:1 ratio) and the mixture was left at 4 °C overnight.

The filtrate-ethanol mixture was centrifuged at 4000 rpm for 15 minutes at 4 °C. Supernatant was discarded and the precipitate was then subjected to rotary evaporation prior to freeze drying and the dried sample was then bottled and stored at 4 °C. Yield of extracts was determined by weighing the dried extract.

3.2.3 Crude Aqueous Extracts

Ground freeze-dried *P. pulmonarius* powder (30 g) was weighed and soaked in 300 ml distilled water (ratio 1:10). The mixture was then homogenized using a blender for almost 3 minutes. The homogenate were separated by filtration using a vacuum pump and the filtrate was collected. To obtain a clear supernatant, it was centrifuged at 5000 rpm for 20 minutes at 4 °C. The pellet formed was discarded while the supernatant referred as aqueous extracts was freeze-dried and kept at -20 °C until further analysis.

3.2.4 Preparation of Protein Fraction Using Ammonium Sulphate Precipitation

One litre of crude aqueous extract obtained above (Section 3. 2. 3) was subjected to ammonium sulphate ($(NH_4)_2SO_4$) precipitation, followed by dialysis and protein estimation. The beaker containing supernatant was continuously stirred at 4 °C and

covered with parafilm. A sufficient amount of solid ammonium sulphate were calculated and weighed to prepare the desired 10 to 100% salt saturation (F10-F100) solutions according to the nomogram (Table 3.1). Ammonium sulfate salt was added slowly and constantly stirring. Salt was allowed to dissolve completely before addition of the remaining salt. Once all have been added, the mixture was left to sit for 45 minutes on an ice bath with continuous stirring.

Then the mixture was centrifuged at 10 000 rpm for 30 minutes (4 °C) to separate the precipitated protein from the solution. Precipitated protein was recovered by re-suspending it gently in 1 ml of distilled water by a quick vortex and transferred to a clean tube and kept at 4 °C, prior to dialysis. Supernatant will be subjected to further gradual addition of ammonium sulphate to pellet out proteins with various hydrophobicity.

The protein suspension was transferred to visking dialysis tubing, Snake Skin™ Pleated Dialysis Tubing, 3 500 molecular weight cut-off (MWCO). The required length of tubing was pulled out from the stick and cut to the appropriate size. The amount of tubing required was calculated by assuming ~3.5 ml sample per cm of dry tubing. The open ends of the tubing were briefly dipped for 5 seconds in distilled water.

A knot was tied and tightly pulled at one of the open end. Precipitated protein fraction was added through the other open end of the tubing, which was then securely tied as well. The dialysis tubing containing protein samples was immersed in distilled water at 4 °C with continuous stirring for 48 hours with four times water change. The dialysate was then freeze-dried and stored at -20 °C and referred as $(NH_4)_2SO_4$ protein fractions.

Table 3.1: Nomogram for determination of ammonium sulphate $(NH_4)_2SO_4$ amount needed for each percentage (%) of saturation.

		10	**20**	**30**	**40**	**50**	**60**	**70**	**80**	**90**	**100**
Original Saturation		Quantity (gram) of ammonium sulphate $(NH_4)_2SO_4$ must be added to 1 liter of distilled water									
	0	56	114	176	243	313	390	472	561	662	767
	10		57	118	183	251	326	406	494	592	694
	20			59	123	189	262	340	424	520	619
	30				62	127	198	273	356	449	546
	40					63	132	205	285	375	469
	50						66	137	214	302	392
	60							69	143	227	314
	70								72	153	237
	80									77	157
	90										79

3.2.5 Measurement of Total Protein in *P. pulmonarius* Crude Aqueous Extracts and Protein Fractions

Following semi-purification by $(NH_4)_2SO_4$ precipitation and dialysis process, protein fractions F10-F100 were subjected to protein estimation using Pierce BCA Protein Assay Kit according to the protocols recommended by the manufacturer. Bicinchoninic acid (BCA) is a calometric procedure for quantification of protein. The BCA assay measures the formation of Cu^{+} from Cu^{2+} by the Biurett complex in alkaline solutions of protein using bicinchonic acid (BCA).

A volume of 25 µl of each standard or unknown sample replicate were pipetted into a microplate well (working range 20-2000 µg/ml). A range of bovine serum albumin (BSA) concentrations (0-2 mg/mL) was used to construct a standard curve. Preparation of standard Diluted Albumin (BSA) Standards can be referred to Appendix 1.1(a).

Working reagent (200 ml) was added to each well of 96 wells microplate and mixed thoroughly on a plate shaker for 30 seconds (Appendix 1.1(b)). The plate was covered and the solution was incubated at 37 °C for 30 minutes. The absorbance was measured at 562 nm on a Tecan Sunrise ELISA Reader. All protein determinations were completed in triplicate. Pierce BCA Protein Assay Kit instructions from product's manufacturer can be referred in Appendix 1.1 (c).

3.3 Preparation of Extracts Stock Concentration

For crude aqueous solution preparation, 10 mg of freeze dried sample were weighed and dissolved in 10 ml of distilled water in order to prepare 10 mg/ml stock solutions. For polysaccharide, methanol and dichloromethane dried extracts, yield obtained was weighed and dissolved in distilled water in order to prepare 5 mg/ml of stock solutions prior use. All the solutions were mixed well by using a vortex mixer.

The solutions were then kept at -20 ^{o}C for further use. To obtain the desired concentration for each assay, the sample stock solutions were subjected to serial dilution using appropriate amount of distilled water.

3.4 *In vitro* Screening of *P. pulmonarius* Extracts for Anti-diabetic Enzyme Inhibitory Activities

Two anti-diabetic inhibition assays were used in this experiment; *S. cerevisea* α-glucosidase inhibitory assay and porcine pancreatic α-amylase inhibitory assay.

3.4.1 *In vitro* Screening of *S. cerevisiae* Alpha Glucosidase Inhibitory Activity Assay

The determination of α-glucosidase inhibitory activity was performed by the spectrophotometric method according to Apostolidis *et al.*, (2006) and Kim *et al.*, (2004) with modification. This assay was carried out in the dark due to *p*-nitrophenyl-α-D-glucopyranoside used is light sensitive. Alpha glucosidase enzyme from *S. cerevisiae* (Type 1) (Sigma Aldrich Co) was chosen in this anti-diabetic inhibitory assay.

Sample from stock solution for each solvent extraction and protein fraction with different concentration were prepared and labelled. For crude aqueous, methanol, dichloromethane and polysaccharide extracts, 25 mg/ml concentration were prepared while 25 µg/ml protein concentration for F10-100 protein fractions.

For α-glucosidase inhibitory assay, a volume of 250 µl of each sample solution (all crude extracts and protein fractions with varying concentrations prepared) and 250 µl of 1.0 M potassium phosphate buffer at pH 6.9 (Appendix 1.2.1(a)) containing α-glucosidase solutions (0.1 U/ml) (Appendix 1.2.1(d)) were premixed in a test tubes. All extracts were carried out in three replications.

For control, 250 µl of distilled water was added to replace the amount of sample

used and contained only distilled water, enzyme and substrate. Furthermore for positive controls, voglibose was used. Preparation of voglibose was described in Appendix 1.2.1(e).

The mixtures were then pre-incubated at 37 °C for 2 minutes. After the pre-incubation, 250 µl of *p*-nitrophenyl-α-D-glucopyranoside in phosphate buffer (Appendix 1.2.1(b)) was added to the mixtures as a substrate at 5 seconds intervals to start the reaction. The reaction mixture was further incubated for another 30 minutes at 37 °C. The catalytic reaction was terminated by addition 2 ml of 0.1 M sodium carbonate (Na_2CO_3) solution to each sample (Appendix 1.2.1(c)).

Alpha glucosidase inhibitory activity was determined by measuring the release of *p*-nitrophenol from *p*-nitrophenyl- α -D-glucopyranoside at 405 nm by using a Shimadzu UV Mini 1240 spectrophotometer. The α-glucosidase inhibitory activity under experimental condition was expressed as percentage of inhibition and was calculated by following formula:

$$\text{Percentage of Inhibition} = \frac{[(\text{A}_{405}\text{Control} - \text{A}_{405}\text{Extract})] \times 100}{\text{A}_{405}\text{Control}}$$

Where,

$\text{A}_{405}\text{Extract}$ = OD 405 value with sample extracts, of *p*-nitrophenyl- α-D-glucopyranoside and α-glucosidase

$\text{A}_{405}\text{Control}$ = OD 405 value with *p*-nitrophenyl-α-D-glucopyranoside and α-glucosidase

Distilled water was served as a blank for correcting the background absorbance. The activity in controls (with an enzyme but without inhibitor) was considered to be 100% activity. The percentage of α-glucosidase inhibition was plotted against different

sample extracts to identify active samples for further study.

3.4.2 *In vitro* Screening of Porcine Pancreatic Alpha Amylase Inhibitory Activity Assay

The α-amylase inhibition assay was adapted from Apostolidis *et al.*, 2007 with some modifications. Porcine pancreatic α-amylase was purchased from Sigma Chemical Co. According to the manufacturer product description, one unit of amylase enzyme will liberate 1.0 mg of maltose from starch per minute at pH 7.0 at 20 °C.

Sample from stock solution of solvent extracts and protein fractions at different concentrations were prepared and labelled. For crude aqueous, methanol, dichloromethane and polysaccharide extracts, 25 mg/ml concentration were prepared while 25 µg/ml protein concentration for F10-100 protein fractions.

Sample extracts and protein fractions (1 ml) were pre-mixed in 1 ml of 0.02 M sodium phosphate buffer pH 6.9 (Appendix 1.2.2(a)) with 0.006 M sodium chloride containing 0.5 mg/ml α-amylase (Appendix 1.2.2(d)). The mixture solution was pre-incubated in a water bath at 37 °C for 5 minutes. For control, 1 ml of distilled water was added to replace the amount of sample used and contained only distilled water, enzyme and substrate. Furthermore for positive control, acarbose was used (Appendix 1.2.2(e)).

After pre-incubation, 1 ml of a starch solution (substrate) in 0.02 M sodium phosphate buffer, pH 6.9 with 0.06 M sodium chloride (Appendix 1.2.2(b)) was added to each tube at predetermined time intervals. The reaction mixtures were then incubated again at 37 °C for 10 minutes. The reaction was stopped by directly transferring the reaction mixture to a boiling water bath (100 °C) and 2 ml of dinitrosalicyclic acid (DNS) colour reagent was quickly added while boiling (Appendix 1.2.2(c)). The test tubes were incubated in boiling water bath for 15 minutes.

The reaction mixture was cooled to room temperature before being diluted with

the addition 5 ml of distilled water. This reaction (corresponding to a colour change from orang-yellow to red) is detectable at 540 nm using Shimadzu UV Mini 1240 spectrophotometer. Experiments were performed in three replicates for each sample. The α-amylase inhibitory activity under experimental condition was expressed as percentage of inhibition and was calculated by following formula:

$$\text{Percentage of Inhibition} = \frac{[(\text{A}_{540}\text{Control} - \text{A}_{540}\text{Extract})] \times 100}{\text{A}_{540}\text{Control}}$$

Where,

$\text{A}_{540}\text{Extract}$ = OD 540 value with sample extracts, starch and α-amylase enzyme

$\text{A}_{540}\text{Control}$ = OD 405 value with starch and α-amylase

Distilled water was served as a blank for correcting the background absorbance. The activity in control (with an α-amylase enzyme but without inhibitor) was considered to be 100% activity. The percentage of α-amylase inhibition was plotted against different sample extracts to identify active samples for further study.

3.5 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis to Profile Proteins in Protein Fractions

Molecular weight pattern for each protein was profiled through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) that was performed based on the continuous buffering system by using method from Laemmli (1970), with some modifications.

Active protein fractions that showed the highest inhibition for the *in vitro* screening assays were subjected to SDS-PAGE analysis. For α-glucosidase assay, F30 protein fraction was selected while for α-amylase assay, F100 protein fraction was

selected. SDS-PAGE was used in this experiment to observe the protein profile of each fraction. Preparation of stock solutions for glycine SDS-PAGE system was attached as Appendix 1.3.

3.5.1 Preparation of Separating and Stacking Gels

The protein fraction with highest enzyme inhibitory activity was loaded on to 16% separating gel SDS-PAGE with 4% stacking gel in vertical gel electrophoresis system. The 2 sets of vertical slab gel units were rinsed with distilled water and assembled vertically. Spacer strips with the thickness of 0.75 mm spacer strips were placed at both edges of the plates. The solutions of 16% separating gels (Appendix 1.4.1) were mixed well and poured into each sandwich using a pipette. TEMED and 10% APS were freshly added to the mixture prior to pouring into gel-casting apparatus.

Then each poured gel was overlaid with distilled water. The separating gel was allowed to polymerize at room temperature for a minimum of 1 hour. Before pouring the stacking gel, the overlaid distilled water was removed by slanting the gel assembly. The gel surface was rinsed with 1 or 2 ml of stacking gel solution, which was then removed.

The stacking gel solution (Appendix 1.4.2) was poured onto the separating gel and a comb was then inserted. It is important to avoid trapping any bubbles below the teeth of the combs to ensure a successful protein transfer. The gel was left at room temperature for at least 2 hours to polymerize. The polymerized gel with the comb in place can be stored overnight in 4 °C refrigerator.

In this experiment, a molecular weight markers (Pre-stained Protein Marker, Broad Range 6-175 kDa) protein ladder was used to ensure the correct molecular weight of desired band was observed. The pre-stained protein marker stock solution was shook to mix the solution and stored as aliquot in eppendorf tubes to reduce contamination and

later kept in -20 °C freezer. Prior to use the marker was heated to 95 °C to 100 °C for 3-5 minutes. A quick microcentrifuge spin was applied before loading it into a gel.

3.5.2 Gel Electrophoresis Running Procedure

Samples with determined concentration and sample buffer were mixed at 1:3 ratios for SDS-PAGE and then boiled for 5 minutes. For the screening purposes 12 µl of each selected protein fraction (F10-F100) was used. The casted sandwiches were placed in the dual tank. The tank buffer was filled into dual vertical mini-gel unit tank. The combs were slowly removed from the gels and overfilled with tank buffer.

Appropriate volume of samples from selected protein fractions and molecular weight markers (pre-stained protein marker, broad range (6-175 kDa) for 16% gel thick was filled into the wells. The wells without sample or standard solution were loaded with sample buffer. Electrophoresis was performed using Bio-Rad Power Pac 300 at constant voltage of 60 V for the first 15 minutes followed by gradual increased to 80-100 V, to avoid excess heat generated. When the blue dye front reached approximately 1 cm from the bottom of the gel, the power supply was turned off and power cable was disconnected. The gels were then stained with Coomassie Brilliant Blue. The gels were be treated immediately to prevent band diffusion and the image was then captured.

3.5.3. Comassie Brilliant Blue Staining Protocols

To visualize the protein bands, the 16% acrylamide gel was stained with Coomassie Brilliant Blue R-250. The stacking gel was removed and discarded from the SDS-PAGE gel. The remaining gel was placed in a transparent container that contained staining solution (Appendix 1.5(a)). Sufficient volume of staining solution was added until the gel freely floated. It was shook slowly for 30 minutes on an orbital shaker. To accelerate staining, the staining solution was heated at 45 °C prior to use.

The staining solution was then replaced with de-staining solution (Appendix 1.5(b)). Addition of Kimwipe tissue was added to the corner of the tray will help remove Coomassie blue from the gel without much changing of the de-staining solution. The de-staining solution was frequently replaced until suitable gel background was achieved and gave a good protein band resolution. For better results, gel with de-staining solution was left overnight with gentle shaking on an orbital shaker. De-staining process was stopped by washing the gel several times with distilled water.

3.6 Purification of Selected Active Protein Fractions by Reversed-Phase High Performance Liquid Chromatography (RP-HPLC) Analysis

After gel image for protein fraction screening was obtained, there were two active protein fractions which showed high percentage of preliminary inhibition assays for α-glucosidase inhibitory activity (F30 protein fraction) and α-amylase inhibitory assays (F100 protein fraction). Both fractions showed a different range of observed bands and protein molecular weight thus either one is responsible to treat diabetes mellitus. The protein fraction that has show active inhibition activity was further purified by reversed-phase high performance liquid chromatography (RP-HPLC).

Two different columns were used in this experiment; analytical column for protein profile analysis (Table 3.2) and a semi-preparative column for protein collection (Table 3.3). Analytical column was used for a protein profile analysis and to optimize solvent system; 1 mg/ml of protein fractions concentration was used by using a 20 µl loop while for collection purpose protein fractions 2 mg/ml was used using a preparative column with 200 µl loop. The freeze-dried samples were diluted with distilled water and transferred to 1.5 ml eppendorf tubes and then centrifuged at 4000 rpm for 3 minutes to precipitate any particulate matter and to get clear solution. The samples were stored at 4 °C until analysis.

Chromatographic optimization of a separation was accomplished using RP-HPLC Shimadzu Model (RP-HPLC) from Japan. An analytical column, which is Waters Atlantis Column (4.6 mm x 250 mm x 5 µm particle size) and a preparative column, Chromolith SemiPrep RP-18 endcapped 100-10 mm for a protein sample collection were used. The mobile phase consisted of two solvent systems that includes pump A; (0.1% trifluoroacetic acid (TFA) in water (v/v)) and pump B; (100% acetonitrile) in a gradient mode (Appendix 1.6). System used to purify was elution buffer 0% to 95% acetonitrile for 40 minutes with the flow rate 1.0 mg/ml.

All solvents were filtered by cellulose membrane filter paper 0.45 µm and nylon membrane filter with Bunchi Filtration system under vacuum, to avoid of air bubbles and contamination by any small particles. Prior to the injection to HPLC system, all the protein samples after preparation was filtered through the filter disc (13 mm nylon, 0.45 um) with syringe filter to avoid blockage of the HPLC column. The syringe filter was rinsed with 2-3 ml aliquots of the methanol (Appendix 1.6(c)) between each use.

Baseline was established prior to the injection of sample. Eluent, which is 100% acetonitrile (Appendix 1.6(a)) and 0.1% TFA (Appendix 1.6(b)), was prepared and run for few minutes to ensure that the baseline is stable. [Note: reversed-phase column should be flush or “conditioned” for first time use, after long terms storage or when changing buffer for a system].

An aliquot of the F30-F100 selected protein fractions extract was analysed by a Waters HPLC system with UV-VIS detection (220 and 254 nm) using a Water Atlantis Column. A volume of protein sample, which is 20 µl with 1 mg/ml concentration, was injected each time into the column with a 20 µl injection loop used, at a flow rate 1 ml/min. Each of the protein samples for both assays was injected 3 times into the column and the corresponding chromatograms were obtained. From these chromatograms, the retention times and the areas under the peaks of the protein samples

were noted.

Peaks detected for particular protein fractions were identified by comparing the samples retention time with the baseline at 220 nm and 254 nm. Based on analytical chromatographic separation, preparative chromatographic separation was carried out for a sample collection. For peak collection, 200 µl of the sample solution with 2 mg/ml concentration was injected each time into the column with a 200 µl injection loop. The eluted peaks were collected for further used in re-evaluation of the sample for both assays.

Based on UV detection, several peaks were collected manually. All individual HPLC peaks were collected, labelled and kept in 4 °C. Acetonitrile was totally removed under a stream of nitrogen gas and the residue (water solution) was freeze-dried. Method development for HPLC can be referred at Appendix 1.7.

Table 3.2: Analytical reversed-phase HPLC parameters for profiling

Parameter	Condition
Column	Waters Atlantis Column (4.6 mm x 250 mm x 5 µm), RP-HPLC
Mobile phase	Acetonitrile: 0.1% TFA with distilled water (Binary gradient)
Injection volume	20 µl
Flow rate	1.0 ml/min
Sample Concentration	1 mg/ml
Elution System	0% to 95% acetonitrile for 40 minutes
Detector Signal (PDA)	220 nm

Table 3.3: Semi-preparative reversed-phase HPLC parameters collection of samples

Parameter	Condition
Column	Chromolith SemiPrep RP-18 endcapped 100-10 mm, RP-HPLC
Mobile phase	Acetonitrile: 0.1% TFA with distilled water
Injection volume	200 µl
Flow rate	3.9 ml/min
Sample Concentration	2 mg/ml
Elution System	0% to 95% acetonitrile for 40 minutes
Detector Signal (PDA)	220 nm

3.7 Anti-Diabetic Enzyme Inhibitory Assays of RP-HPLC Purified Protein

Following fractionation by RP-HPLC, all peaks obtained from the chromatogram were re-evaluated again by α-glucosidase and α-amylase assays. For α-glucosidase inhibitory assay, the protein concentration use (F30 protein fraction) was 2.5 µg/ml which was 10 fold diluted from the previous assay (section 3.4.1). Similarly, 6.25 µg/ml protein (F100 protein fraction) was used for α-amylase assay which was four fold diluted compound to the previous assay 3.4.2. The experiments for anti-diabetic assays were carried out as described in Section 3.4.

3.8 SDS-PAGE Analysis of Active RP-HPLC Separated Protein Fractions

In this experiment, SDS-PAGE analysis was used to characterize the degree of purity and also the preparation for in-gel digestion prior to Matrix Assisted Laser Desorption/Ionization-Time of Flight/Time of Flight Mass Spectrometry (MALDI-TOF/TOF MS) analysis. From the re-evaluation of anti-diabetic assays (Section 3.4), the selected HPLC purified protein that showed the highest activity for each assays were subjected to SDS-PAGE analysis.

In the case of in-gel digestion for MALDI-TOF/TOF MS analysis, 1µg/µl of purified protein fraction was used. All the procedures and reagents preparation for SDS-PAGE was the same as previous, Section 3.5 and stained with Coomassie Brilliant Blue stain (Section 3.5.3). Coomassie staining is easier and more rapid thus Coomassie Brilliant Blue staining was first used to stain the gel. However no band was observed. Thus, the silver staining method, which is more sensitive was used to detect smaller amount of protein. Prior to MALDI-TOF/TOF MS analysis, a compatible silver staining was used to avoid permanent binding of the protein to the gel.

3.8.1 Silver Staining Protocol for Coomassie-stained 16% polyacrylamide gel (MS Compatible)

Mass spectrometry compatible silver staining method used was conducted according to Shevchenko *et al.*, (1996) with modifications.

3.8.1.1 Protocol of Silver Staining for Coomassie-Stained Protein from SDS-PAGE Analysis

The Coomassie-stained gel was de-stained as described in Section 3.5.3 overnight and then rinsed with distilled water. The gel was then soaked in a fixing solution (Appendix 1.8(a)) for 30 minutes to overnight with gentle shaking on an orbital shaker.

Subsequently, it was incubated in the sensitizing solution (Appendix 1.8(b)) for another 30 minutes. The gel was then washed three times for 5 minutes each with distilled water. The gel was then stained in the silver stain solution (Appendix 1.8(c)) for 20 minutes. Prior to developing step, the gel was washed again with distilled water for a few seconds and the water was poured off.

Then small volumes (3-5 ml) of developing solution (Appendix 1.8(d)) was added, swirled briefly, and then discarded. Development took 4-5 minutes or until the protein bands were nicely resolved. The gel was rinsed quickly with distilled water and gels development was blocked by placing it in a stop solution (Appendix 1.8(e)) for 10 minutes. The gels were replaced and stored in distilled water for further use.

3.8.1.2 Preparation of Silver-Stained Protein SDS-PAGE for MALDI-TOF/TOF MS Analysis

De-stained gel with a clear protein band from MS compatible silver staining was

observed and image was captured. The gel was transferred to a clean culture dish and unnecessary parts (top, bottom, MW marker lanes) was cut away with a sterile scalpel and blade, leaving only the lanes with proteins of interest. All protein bands were excised as precise as possible. Any diffused stained edge to the band was removed.

All excess stain was removed otherwise residual stain may interfere with data acquisition. Each gel slice was placed into a plain 1.5 ml eppendorf tube. Fresh scalpel was used to cut up each slice and the same scalpel used to transfer the cut up slices to the labelled tube. The gel slice was washed with distilled water at least 2 times for 10 minutes with occasional vortex mixing. Wash solution was discarded. Small plastic pipette tips were used (10 µl -100 µl) to remove the solution. Work was quickly as possible because the gel becomes stickier as it dries out.

3.9 Protein Identification by MALDI-TOF/TOF Mass Spectrometry Analysis

Two digestion protocols were applied for the MALDI-TOF/TOF MS analysis; the in-solution and in-gel digestion. The in-solution digestion involved the protein samples which were collected from HPLC protein fractions and could directly be characterized by MS meanwhile for in-gel digestion, it was necessary to efficiently recover the proteins from the SDS-PAGE gel. In this experiment, both protocols of MALDI-TOF/TOF MS were used and compared.

3.9.1 In-Solution Digestion Protocol of MALDI-TOF/TOF Mass Spectrometry for Protein

MALDI-TOF/TOF MS analysis was performed at the Proteomic Facilities in Medical Biotechnology Laboratory, Faculty of Medicine, University of Malaya. Procedure used in this experiment was modified from Pierce In-solution Tryptic

Digestion Kit.

Following re-evaluation bioassay of several HPLC fractions, the peak that showed the highest inhibition of α-glucosidase inhibitory assay was furthered to MALDI-TOF/TOF MS analysis. This is for identification of the purified proteins and measuring the mass more accurately. Protein samples were prepared as previously described in Section 3.2.4.2.

The in-solution MALDI-TOF/TOF MS analysis worked well with protein concentrations in the range 0.1-1.0 mg/ml (Appendix 1.9(e)). The protocol of in-solution tryptic digestion was divided to three steps; reduction and alkylation, followed by digestion and extraction.

A. REDUCTION & ALKYLATION:

Fifteen µl of 50 mM ammonium bicarbonate (digestion buffer) was prepared (Appendix 1.9(a)) and 1.5 µl of 100 mM DTT (reducing buffer) (Appendix 1.9(b)) were added to a clean 500 µl microcentrifuge tube. Ten-µl protein sample containing 10 µg of protein was added to the tube and the final volume adjusted to 27 µl with ddH_2O. The sample was incubated at 95°C for 5 minutes. The sample was then left to cool at room temperature. Alkylation buffer (100 mM iodoacetamide) (Appendix 1.9(c)) was freshly prepared and 3 µl of the buffer was added to the tube. The mixture was then incubated in the dark at room temperature for 20 minutes.

B. DIGESTION:

Stock solution, 0.1 µg/µl of trypsin (Appendix 1.9(d)) was prepared separately. Trypsin (2 µl) was added to the reaction tube and incubated at 37 °C for overnight. The mixture was then vortexed briefly and spun down at 1000 rpm for 1 minute.

C. EXTRACTION:

The mixture was shaken with 50 µl of 50% acetonitrile for 15 minutes. All liquid was transferred to fresh tubes. The mixture was then mixed with another 50 µl of 100% acetonitrile for 15 minutes. The liquid then transferred to the previous tube. The digested sample was then completely dried by using vacuum at low speed usually for 2 hours. Unused sample may be frozen at -20 °C in case further analysis is required. The samples were furthered to ziptip desalting and spotting.

3.9.1.1 Zip tip protocol (desalting and spotting)

a. Material and Preparation of solutions

- Material: C_{18} ZipTip. Preparation of solutions:

I. Wetting solution = 100% acetonitrile

II. Sample preparation = 0.1% Formic acid

III. Equilibrium solution = 0.1% Formic acid

IV. Washing solution = 0.1% TFA

V. Elution solution = 0.1% TFA in 50% acetonitrile

Matrix solutions:

VI. Buffer A: 2% Acetonitrile; 0.1% TFA; 98% MiliQ water

VII. Buffer B: 98% Acetonitrile; 0.1% TFA; 2% MiliQ water

VIII. 10 mg of α-Cyano-4-hydroxycinnamic acid (CHCA) dissolved in (0.4 ml buffer A and 0.6 ml buffer B mixture)

b. Desalting sample protocol:

Protein samples were reconstituted in 10 µl of 0.1% TFA. Elution buffer (1.5 µl) was added into a new tube.

Pre-wetting: 10 µl of 100% acetonitrile was aspirated and dispensed for three cycles

Equilibrate: To equilibrate tips, 10 µl of 0.1% Formic acid was aspirated and dispensed for five cycles

Binding: 10 µl of protein samples was passed through the zip-tips repeatedly by pipetting in and out for 10 cycles without completely dry

Washing: 10 µl of 0.1% TFA was aspirated and dispensed for five cycles

Elution: 1.5 µl of elution buffer was aspirated and dispensed for three cycles

Then, the sample was fully dispensed in the sample solution.

Note: The zip-tip resin should not be completely dry (except for final elution step) after aspiration of solution to avoid resin damage and alteration of binding capacity.

c. Spotting sample:

Matrix solution (1.5 µl) was added to 1.5 µl elution buffer (with sample). The mixture was mixed well and 3 µl of each sample was spotted on MALDI plate twice. Samples were allowed to completely dry before the analysis.

The peptides resulted from the in-gel or in-solution solution digestion were anaylzed by MALDI-TOF/TOF spectra to obtain the peptides' masses. The results were matched with the translated open frames in database of the National Centre for Biotechnology Information (NCBI) and Masscot, Swissprot database.

3.9.2 In-Gel Digestion Protocol for MALDI-TOF/TOF MS Analysis of an SDS-PAGE Gel Separated, Silver-Stained Protein

MALDI-TOF/TOF MS analysis for In-gel digestion was sent to Genome Research Centre, The University of Hong Kong (HKU). ABSciex MALDI 4800 TOF/TOF Analyser was used and the scanning range for general protein identification was 900-4000 *m/z*, reflector mode, then the five most abundant peptides (precursors) that are not on exclusion list was selected for further fragmentation (MALDI-TOF/TOF) analysis to generate the full scan mass spectrum. Trypsin enzyme was used to digest

proteins. Overall schematic diagram for In-gel digestion protocol was simplified in Figure 3.2 while sample preparation for zip-tip desalting and detail procedure were attached in Appendix 1.10.

MASCOT (ver. 2.1) & NCBI search analysis settings were as follows:

Fixed modification: carbamidomethyl (C);

Variable modification: oxidation (M);

MSMS Fragment tolerance: 0.2 Da;

Precursor tolerance: 75 ppm;

Peptide charge: +1; Monoisotopic

3.10 Statistical Analysis

All assays were performed in triplicates and the values were averaged. Each data expressed represent the mean $\pm$ standard deviation (SD) and was analyzed by using the Microsoft Excel 2011. Data significance was performed by one-way analysis of variance (ANOVA) by Minitab Release 14 Statistical Software program version (14.12.0). Differences of 95% ($p<0.05$) were considered statistically significant.

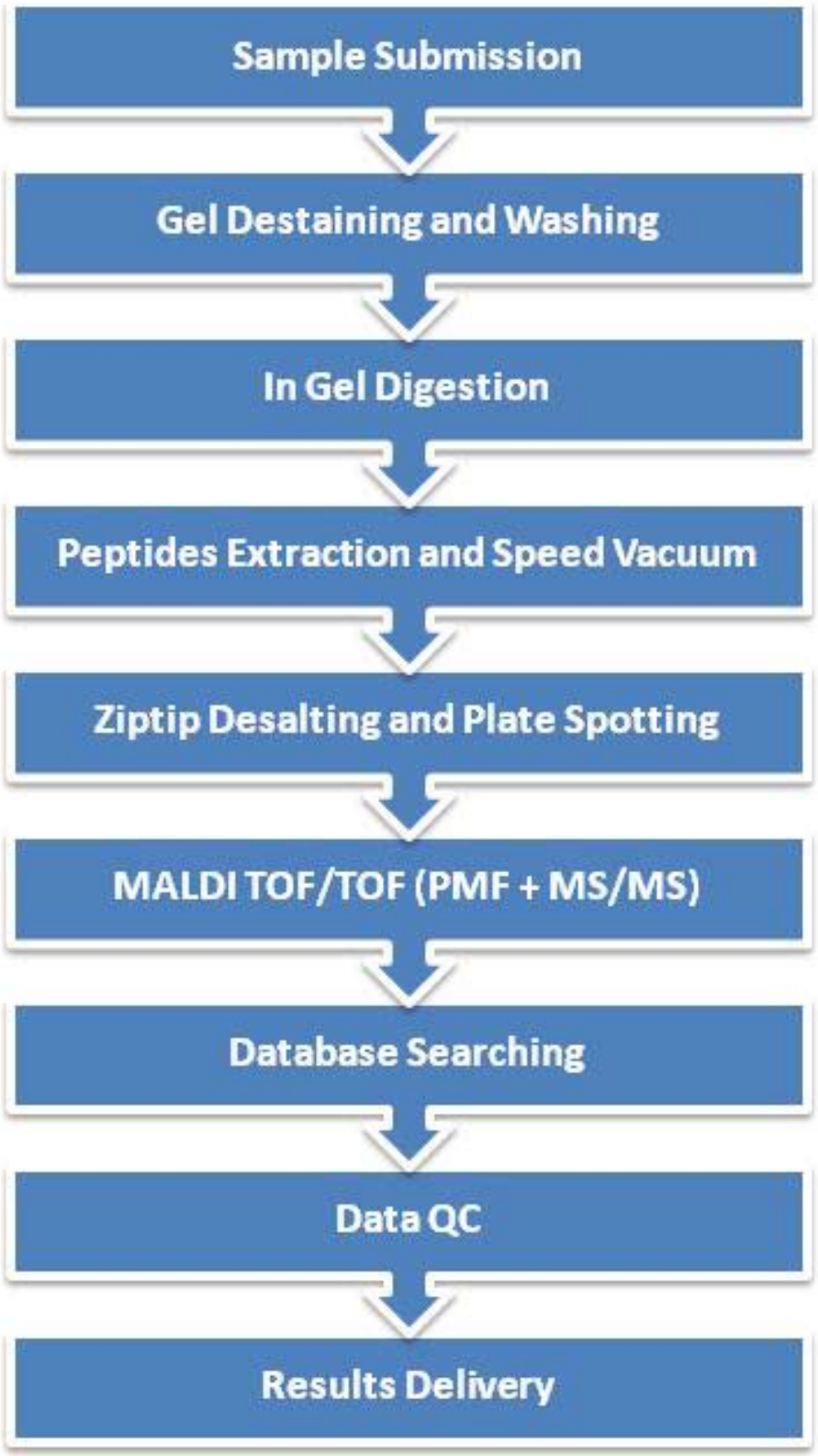

Figure 3.2: Overall schematic for in-gel digestion protocol done by Genome Research Centre, The University of Hong Kong (HKU)

4.0 RESULTS

4.1 Yield of *P. pulmonarius* Extractable Solutes Using Different Extraction Solvents

Ground freeze-dried *P. pulmonarius* basidiocarps were used in this study for the preparation of extracts. Freeze drying technique applied to the extract was found to remove 83.65% water from the basidiocarps. Powdered samples were soaked in water, methanol and dichloromethane to yield aqueous extracts (AE), methanol extracts (ME) and dichloromethane extracts (DCME), respectively. Polysaccharide extract (PE) was obtained by ethanol precipitation of hot water extract.

Yield of extractable solutes using different solvents is shown in Table 4.1. The results indicated that highest percentage yield was AE (18.34 %) followed by ME (1%) and DCME (0.525%). PE showed the lowest percentage yield of only 0.27%.

4.2 Quantitation of Protein by Pierce BCA Protein Assay Kit

Quantitation of total proteins was done in triplicates and the average mean of protein concentration was recorded. Protein content was estimated and calculated by referring to BSA Standard Curve of Albumin Standard (BSA). Table 4.2 shows the protein contents from aqueous extract and protein fractions that were derived from ammonium sulphate precipitation.

Total protein content (Table 4.2) showed that AE contained the highest amount of protein content (3000 µg/ml). Among the protein fractions derived from ammonium sulphate precipitation technique, F90 salt saturation solution showed the highest protein content of 1820 µg/ml. This is followed by protein fraction with salt saturation F70, F50 and F60 with 1450, 1550 and 1000 µg/ml protein content, respectively.

Table 4.1: Percentage yield of *P. pulmonarius* extractable solutes using various solvents

Procedure of Extraction	Percentage Yield (g/100g dry weight)
Aqueous extracts (AE)	18.34
Polysaccharide extracts (PE)	0.27
Methanol extracts (ME)	1.0
Dichloromethane extracts (DCME)	0.525

Table 4.2: Protein contentof aqueous extract and protein fractions of *P. pulmonarius* basidiocarps obtained following ammonium sulphate precipitation

Protein fractions and aqueous extracts of *P. pulmonarius* basidiocarps	Protein concentration (µg/ml)
F10	60
F20	90
F30	190
F40	450
F50	1550
F60	1000
F70	1450
F80	790
F90	1820
F100	330
Aqueous extract (AE)	3000

4.3 *In vitro* Screening of Solvents Extracts and Protein Fractions from *P. pulmonarius* for Anti-Diabetic Enzymes Inhibitory Assays

Crude extracts (AE, ME, DCME and PE) as well as the ammonium precipitated protein fractions (F10-F100) were assayed for the inhibition of α-glucosidase and α-amylase enzymes. For both anti-diabetic assays, the protein fractions were diluted to 25 µg/ml protein content and 25 mg/ml for solvents and crude AE.

4.3.1 *In vitro* Screening for *S. cerevisiae* Alpha Glucosidase Inhibitory Activity Assay

Crude extracts (AE, ME, DCME and PE) and protein fractions (F10-F100) were tested for α-glucosidase inhibitory activity. The activity was expressed as percentage of inhibition (%) shown in Figure 4.1. DCME extract showed the strongest inhibition effect of α-glucosidase activity (91.13%) at 25 mg/ml concentration. This is followed by sample from PE and AE with 85.82% and 73.50% inhibition respectively. ME showed the lowest inhibitory activity against α-glucosidase activity with only 2.61% inhibition. The activity was expressed as percentage of inhibition (%) shown in Figure 4.1.

Inhibition of α-glucosidase activity by F10-F100 is shown in Figure 4.2. Among all the fractions, at 25 µg/ml protein content, F30 protein fraction strongly suppressed the activity of α-glucosidase enzyme (24.18%), followed by F60, F80 and F90 fractions with 19.57%, 16.94% and 15.82% inhibition respectively. There was no inhibitory activity observed for F20 and F100 protein fractions. Majority of the protein samples (F30-F90) appeared to be better inhibitors of α-glucosidase enzymes than a known inhibitor for α-glucosidase, voglibose. Based on the result, F30 protein fraction was selected for further chemical analysis.

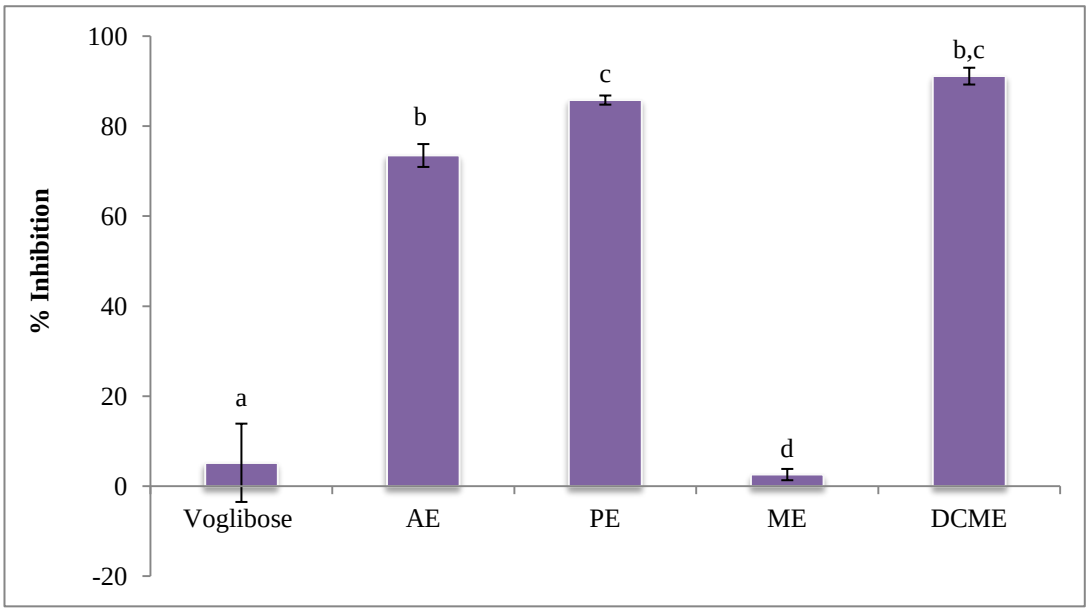

Figure 4.1: Percentage inhibition of α-glucosidase enzyme activity by crude aqueous, polysaccharide, methanol and dichloromethane extracts at 25 mg/ml. (AE= aqueous extract, PE= polysaccharide extract, ME= methanol extract, DCME= dicholoromethane extract)

Values were expressed as mean ± SEM of three replicate determinations. Mean values with different lower case letters (a-d) indicate significant difference at $p < 0.05$. *Voglibose was used as negative control with the concentration at 25 µg/ml.

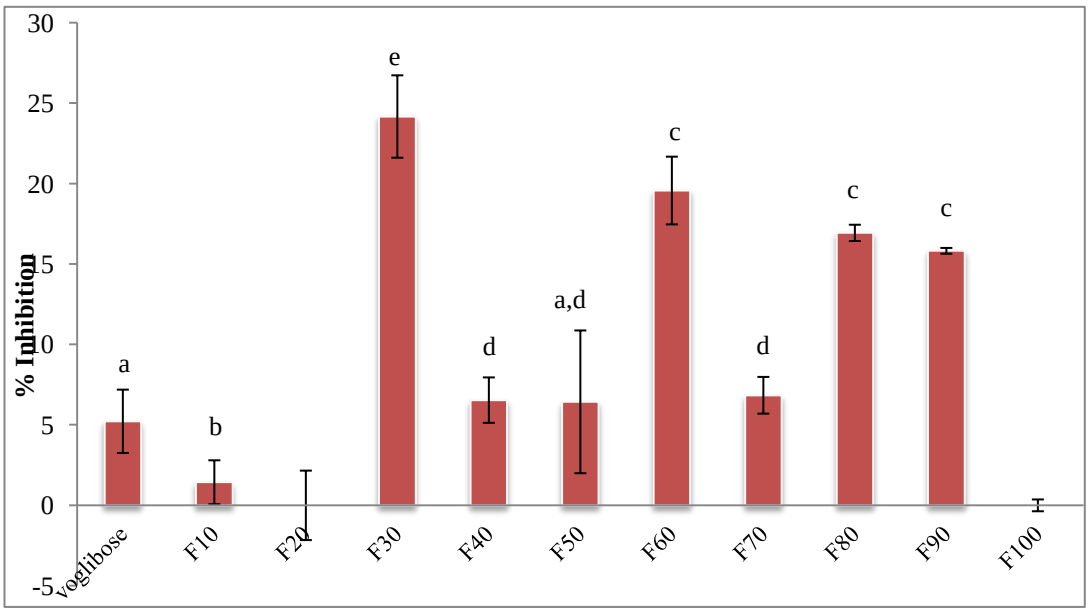

Figure 4.2: Percentage inhibition of α-glucosidase enzyme activity by ammonium percipitated protein fractions (F10-F100) at 25 µg/ml

Values were expressed as mean ± SEM of three replicate determinations. Mean values with different lower case letters (a-e) indicate significant difference at $p < 0.05$. *Voglibose was used as negative control with the concentration at 25 µg/ml.

4.3.2 *In vitro* Screening for Porcine Pancreatic Alpha Amylase Inhibitory Activity Assay

Crude extracts (AE, ME, DCME and PE) and protein fractions (F10-F100 protein fraction) both from *P. pulmonarius* basidiocarps were tested for α-amylase inhibitory activity.

At 25 mg/ml concentration, AE extracts showed a significant inhibitory action against α-amylase activity with 87.74%. This was followed by samples from PE and ME with 48.10% and 42.73% inhibition respectively. DCME yielded the lowest inhibition activity with only 29.82% activity. The activity was expressed as percentage of inhibition (%) (Figure 4.3).

Inhibition of α-amylase activity by F10-100 from *P. pulmonarius* basidiocarps was shown in Figure 4.4. Among all the protein fractions at 25 µg/ml, F100 protein fraction showed the highest inhibition activity of α-amylase which was 41.80% followed by F70 and F90 with 30.98% and 28.99% inhibition, respectively. The inhibitory effect of the F70, F90 and F100 appeared to be better inhibitors than a known inhibitor for α-glucosidase, acarbose. Among all the crude and protein extracts screened for the α-amylase inhibitory assay, F100 protein fraction that showed the best inhibitory activity was selected for further chemical analysis.

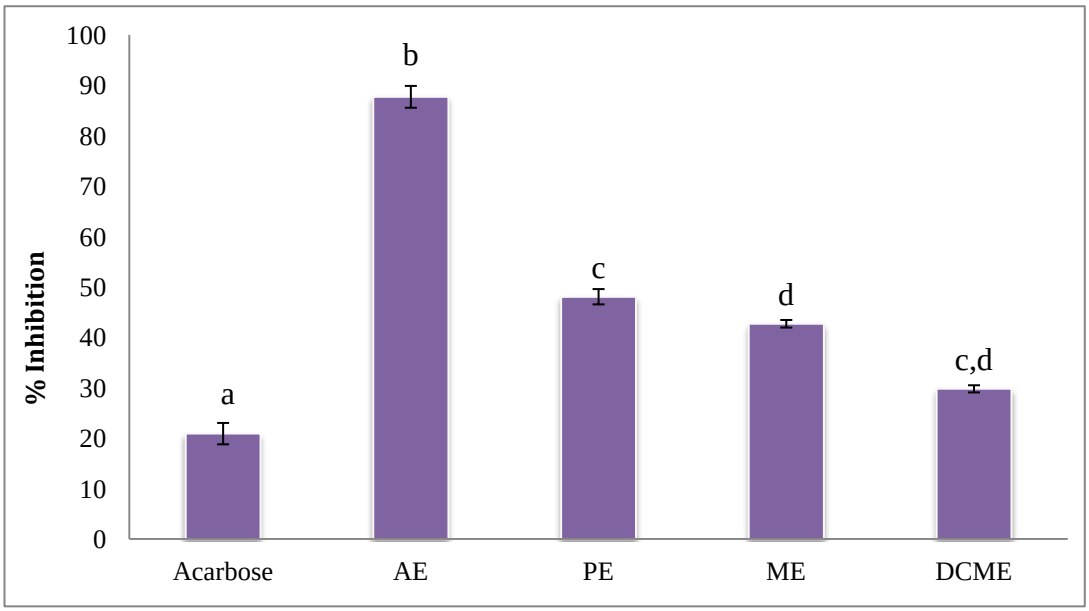

Figure 4.3: Percentage inhibition of α-amylase activity by crude aqueous, polysaccharide, methanol and dichloromethane extracts at 25 mg/ml. (AE= aqueous extract, PE= polysaccharide extract, ME= methanol extract, DCME= dicholoromethane extract)

Values were expressed as mean ± SEM of three replicate determinations. Mean values with different lower case letters (a-d) indicate significant difference at $p < 0.05$. *Acarbose was used as negative control with the concentration at 25 µg/ml.

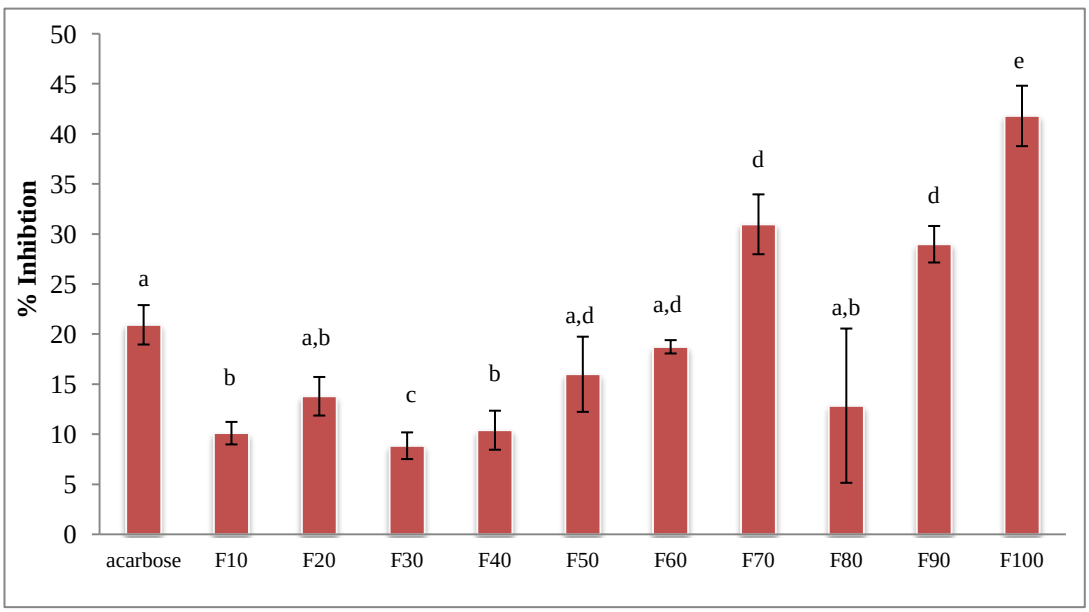

Figure 4.4: Inhibition of α-amylase activity by F10-F100 protein fraction with acarbose at 25 µg/ml.

Values were expressed as mean ± SEM of three replicate determinations. Mean values with different lower case letters (a-e) indicate significant difference at $p < 0.05$.
*Acarbose was used as negative control with the concentration at 25 µg/ml.

4.4 Profiling of Seleted Active Protein Fractions by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis

Protein profile for selected active protein fractions (F30 and F100) that inhibited α-glucosidase and α-amylase respectively were resolved and captured on 16% SDS polyacrylamide gel image as in Figure 4.5. Based on the image, F30 protein fraction (α-glucosidase inhibitor) showed the presence of 10 protein bands (Lane 2) with molecular weight ranged approximately 5-70 kDa. Whereas F100 protein fraction (α-amylase inhibitor) showed the presence of nine protein bands ranged approximately from 6 kDa to 70 kDa.

From this SDS-PAGE analysis, the two selected anti-diabetic inhibitors F30 and F100 protein fractions showed a very distinct band profile, thereby was further fractionated by RP-HPLC.

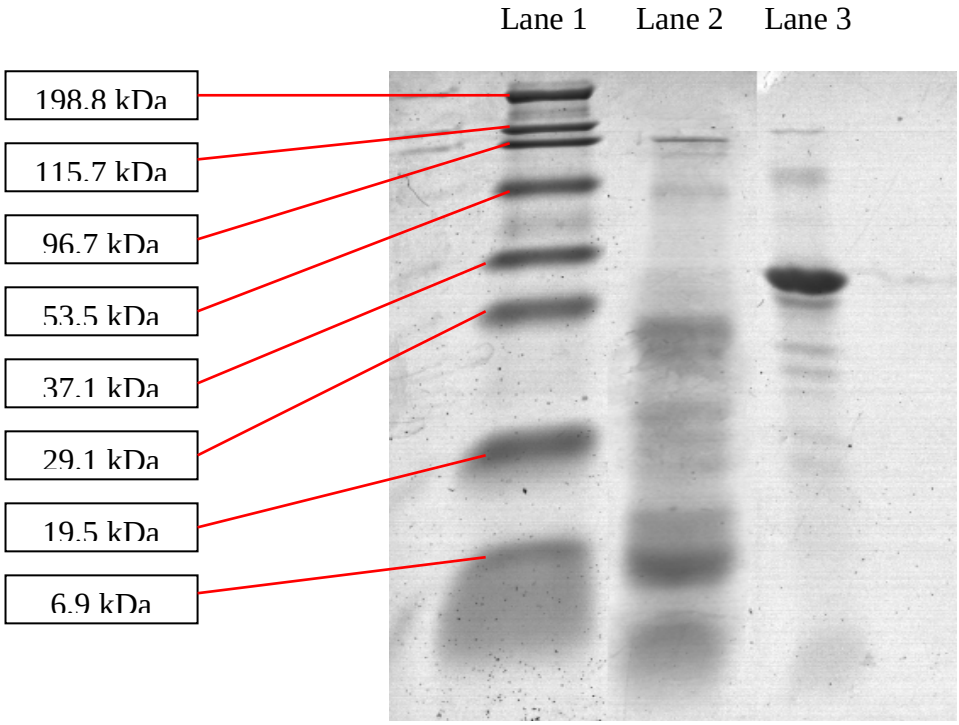

Figure 4.5: Coomassie Brilliant Blue-stained 16% SDS-PAGE protein profile of *P. pulmonarius* F30 and F100 protein fractions

The SDS-PAGE gel was stained by Coomassie Brilliant Blue. Lane 1: Pre-Stained SDS-PAGE standards protein marker. Lane 2: F30 protein fraction in sample buffer with DTT. Lane 3: F100 fraction protein in sample buffer with DTT.

4.5 Purification of Active Protein Fractions by Reversed-phase High Performance Liquid Chromatography (RP-HPLC)

Both active fractions with anti-diabetic potential, which were F30 protein fraction α-glucosidase inhibitor, and F100 protein fraction α-amylase inhibitor, were further fractionated by RP-HPLC.

4.5.1 Purification Profile of F30 fraction separated by RP-HPLC

The selected protein fraction, F30 with α-glucosidase inhibitory activity was injected into HPLC column. The peaks obtained were referred as RP-HPLC fractions (RPF). The peaks and its retention times at 220 nm were shown in the chromatogram in Figure 4.6. Ten major peaks representing RPF identified in the chromatogram with its retention times (Rt) were listed in Table 4.3.

4.5.1.1 Alpha Glucosidase Inhibitory Activity of F30 Fractions Separated by RP-HPLC

Alpha glucosidase inhibitory assay was conducted to evaluate the RPF. All collected peaks evaluated showed α-glucosidase inhibitory activities (Figure 4.7) even though the concentration has been reduced by 10 times lower (2.5 µg/ml) compared to its actual sample concentration before purification by RP-HPLC.

Among all the RPF, at 2.5 µg/ml protein concentration, peak 3-RPF strongly suppressed the activity of α-glucosidase enzymes (25%) followed by peak 4-RPF and peak 8-RPF with same percentage of inhibition of 22% (Table 4.4).

All peaks of RPF appeared to be better inhibitors of α-glucosidase enzymes compared to voglibose. Peak 3-RPF shows a significant anti-diabetic activity and was chosen as the best candidate for further characterization using MALDI-TOF/TOF MS analysis.

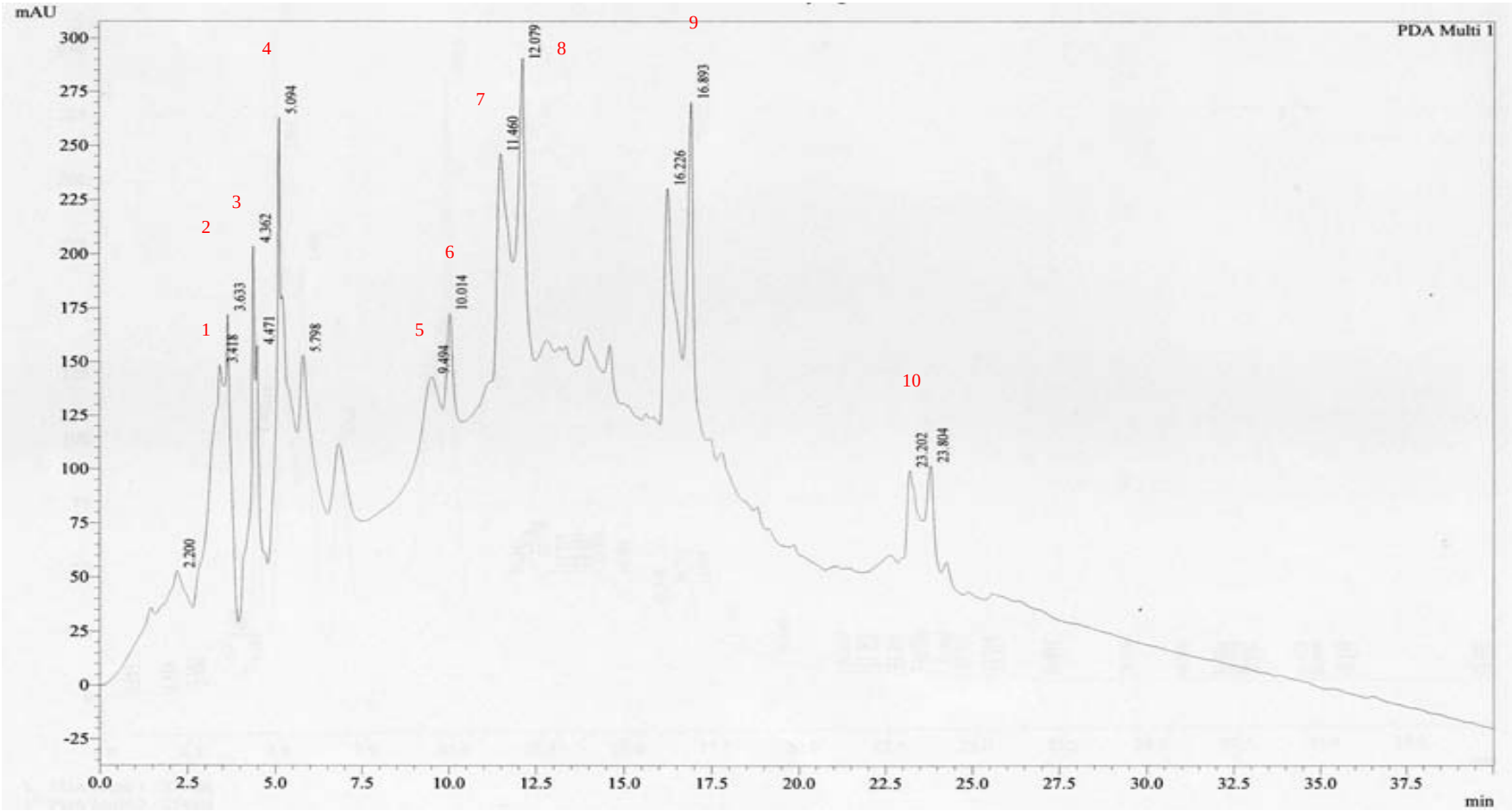

Figure 4.6: HPLC profile of F30 protein fraction

Chromatogram for determination of protein profile for F30 fraction according to optimized method by column Chromolith SemiPrep RP-18 endcapped 100-10 mm; flow rate 1 ml/min, at sample concentration of 2 mg/ml and detection wavelength of 220 nm.

Table 4.3: Retention times (Rt) at 220 nm for all RPF peaks from F30 protein fraction

RPF Peaks	Retention time at 220 nm
Peak 1	3.418
Peak 2	3.633
Peak 3	4.362
Peak 4	5.094
Peak 5	9.494
Peak 6	10.014
Peak 7	11.460
Peak 8	12.079
Peak 9	16.893
Peak 10	23.804

Table 4.4: Percentage of α-glucosidase inhibition of all RPF peaks from F30 protein fraction at 2.5 µg/ml

RPF Peaks	% Inhibition of α-glucosidase inhibitory assay
Voglibose	13±2.03
Peak 1	17±0.70
Peak 2	18±1.11
Peak 3	25±2.57
Peak 4	22±2.99
Peak 5	17±0.98
Peak 6	19±2.37
Peak 7	14±0.72
Peak 8	22±2.07
Peak 9	18±1.20
Peak10	17±1.17

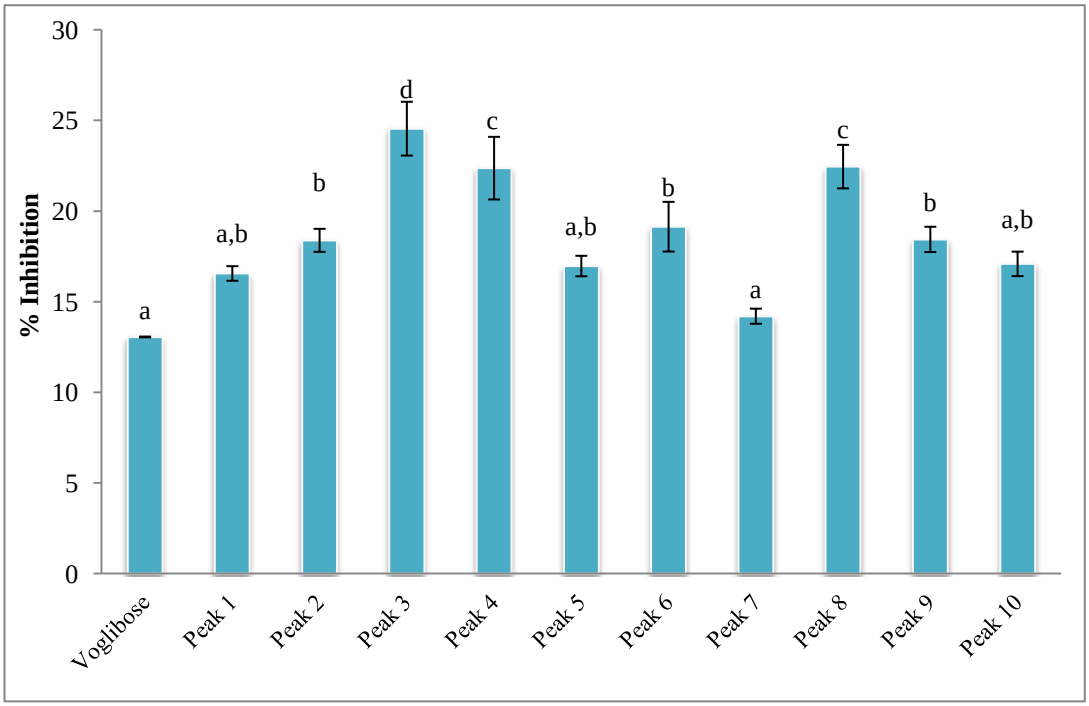

Figure 4.7: Inhibition of α-glucosidase enzyme activity by all protein fractions (RPF) from F30 at 25 µg/ml

Mean values with different lower case letters (a-d) indicate significant difference at $p < 0.05$.
*Voglibose was used as negative control with the concentration 25 µg/ml.

4.5.2 Purification profile of F100 fractions separated by RP-HPLC

The selected protein fraction, F100 with α-amylase inhibitory activity was injected into HPLC column. The peaks obtained were referred as reversed-phase HPLC fractions (RPF). The peaks and its retention times at 220 nm were obtained as shown in the chromatogram in Figure 4.8. Six major peaks representing RPF identified in the chromatogram with its retention times (Rt) were listed in Table 4.5.

4.5.2.1. Alpha Amylase Inhibitory Assay of F100 Fraction Separated by RP-HPLC

Alpha amylase inhibitory assay was conducted to evaluate the active fractions. All peaks collected showed α-amylase inhibitory activities even though the concentration has been reduced 4 times lower (6.25 µg/ml) compared to its actual sample concentration before purification by HPLC.

Among all the RP-HPLC fractions (RPF) (Figure 4.9), at 6.25 µg/ml protein concentration, peak 3-RPF showed the highest activity of α-amylase but with very low percentage of inhibition (2.84%) followed by peak 5-RPF and peak 4-RPF with percentage of inhibition, 1.45% and 1.1% respectively. There was no inhibition activity observed for peak 1-RPF and peak 2-RPF of RP-HPLC fractions (Table 4.6). Moreover, only peak 3-RPF appeared to be better inhibitors of α-amylase enzymes than a known acarbose.

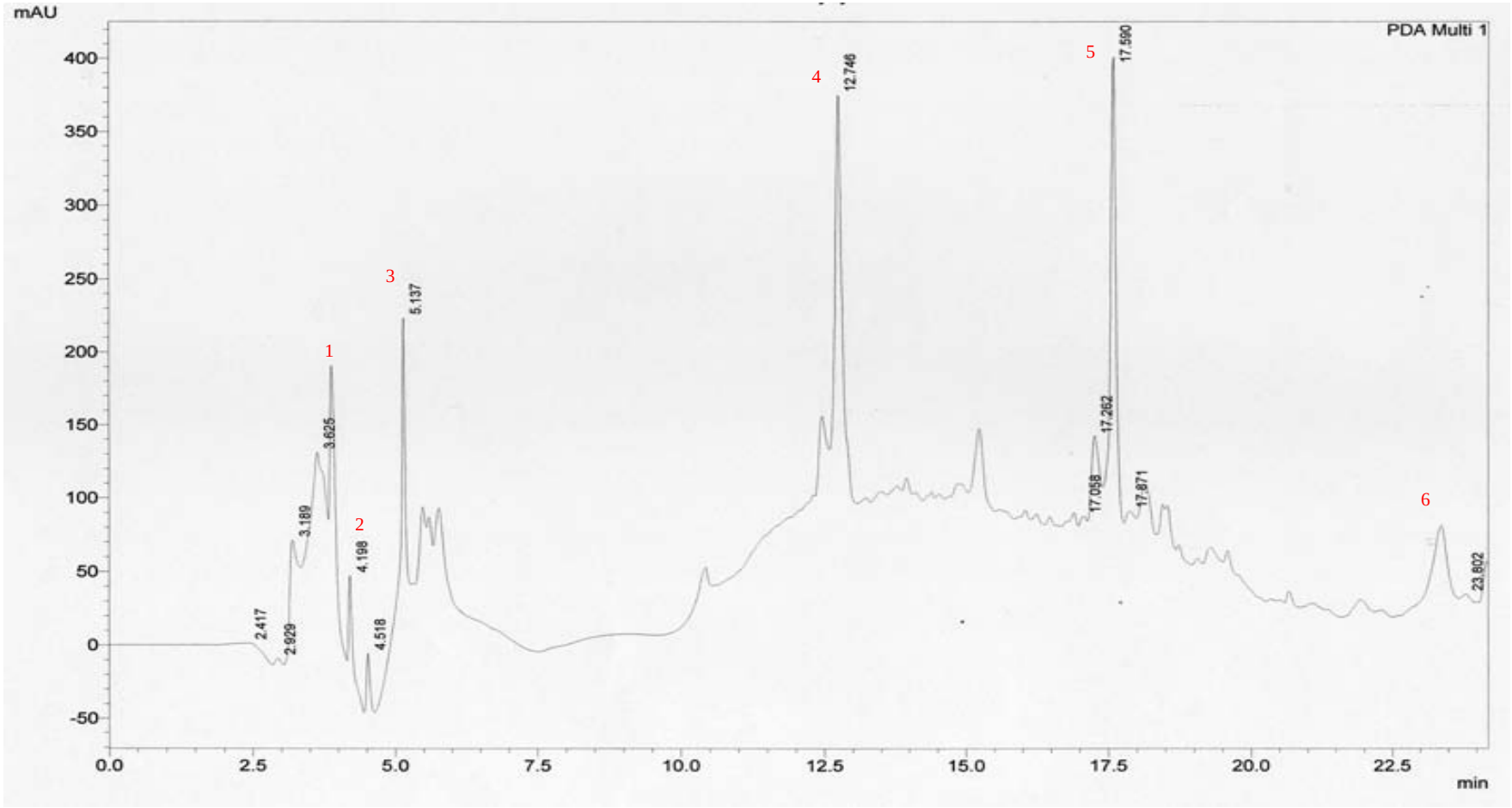

Figure 4.8: HPLC profile of F100 protein fraction

Chromatogram for determination of protein profile for F100 RPF according to optimized method by column Chromolith SemiPrep RP-18 endcapped 100-10 mm; flow rate 1 ml/min, at sample concentration of 2 mg/ml and detection wavelength of 220 nm.

Table 4.5: Retention times (Rt) at 220 nm for all RPF peaks from F100 protein fraction

RPF Peaks	Retention time at 220 nm
Peak 1	3.625
Peak 2	4.198
Peak 3	5.137
Peak 4	12.746
Peak 5	17.590
Peak 6	23.369

Table 4.6: Percentage of α-amylase inhibition of all RPF peaks from F100 protein fraction at 6.25 µg/ml

RPF Peaks	% Inhibition of α-amylase inhibitory assay
Acarbose	2.10±0.41
Peak 1	0
Peak 2	0
Peak 3	2.85±0.36
Peak 4	1.11±0.25
Peak 5	1.44±0.23
Peak 6	0.11±0.25

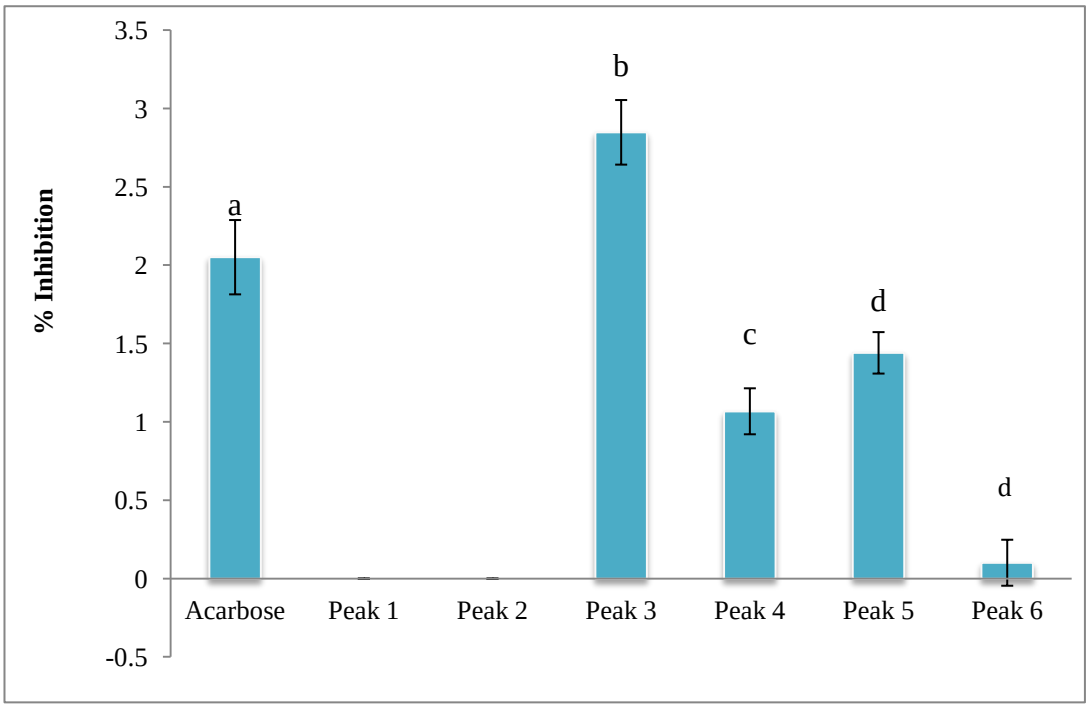

Figure 4.9: Inhibition of α-amylase enzyme activity by all protein fraction (RPF) from F100 at 6.25 µg/ml

Mean values with different lower case letters (a-d) indicate significant difference at $p < 0.05$.
*Acarbose was used as negative control with the concentration 25 µg/ml.

4.5.2.2 Evaluation of Synergism of Peak 3-RPF from F100 Fractions for Alpha Amylase Inhibitor

For α-amylase inhibitor, only peak 3-RPF from F100 protein fraction showed inhibition result. Due to low percentage, the sample was subjected for a synergism mechanism study. Alpha amylase inhibitory assay was run at the 6.25 µg/ml for pre-purified (F100 protein fraction) and post-purified fractions (Peak3-RPF) by HPLC. Percentage of inhibition for F100 protein fraction pre-purified was 12% and 2.85% for sample post-purified RP-HPLC (Peak 3- RPF) shown in Figure 4.10. Hence, because of the very low percentage of inhibition after purified by HPLC, peak 3-RPF from α-amylase inhibitor was not chosen for further characterization using MALDI-TOF/TOF mass spectrometry analysis.

4.6 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) Profile of Peak 3-RPF of Alpha Glucosidase Enzyme Inhibitor

The active peak 3-RPF from F30 protein fraction as α-glucosidase enzyme inhibitor was further resolved using SDS-PAGE thereby silver-stained electrophoregram was obtained (Figure 4.11). Lane 1 was a pre-stained SDS-PAGE standard protein marker and Lane 2 was an active peak 3-RPF.

SDS-PAGE profile of the active peak 3-RPF showed the presence of five distinct protein bands (Table 4.7). From the SDS-PAGE gel, the bands were excised and prepared to further identification by MALDI-TOF/TOF MS analysis.

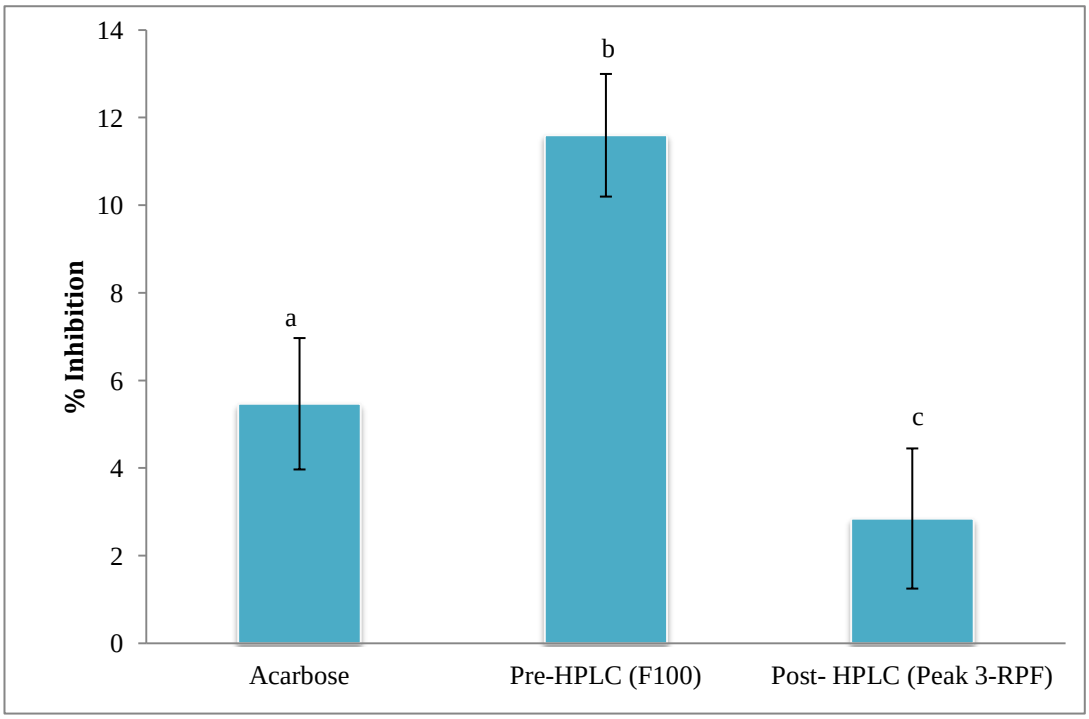

Figure 4.10: Inhibition of α-amylase enzyme activity for pre and post-purified by HPLC and at 6.25 µg/ml

Pre-purified was F100 protein fraction and post-purified was Peak 3-RPF by RP-HPLC purification. Mean values with different lower case letters (a-c) indicate significant difference at $p < 0.05$.
*Acarbose was used as negative control with the concentration 6.25 µg/ml.

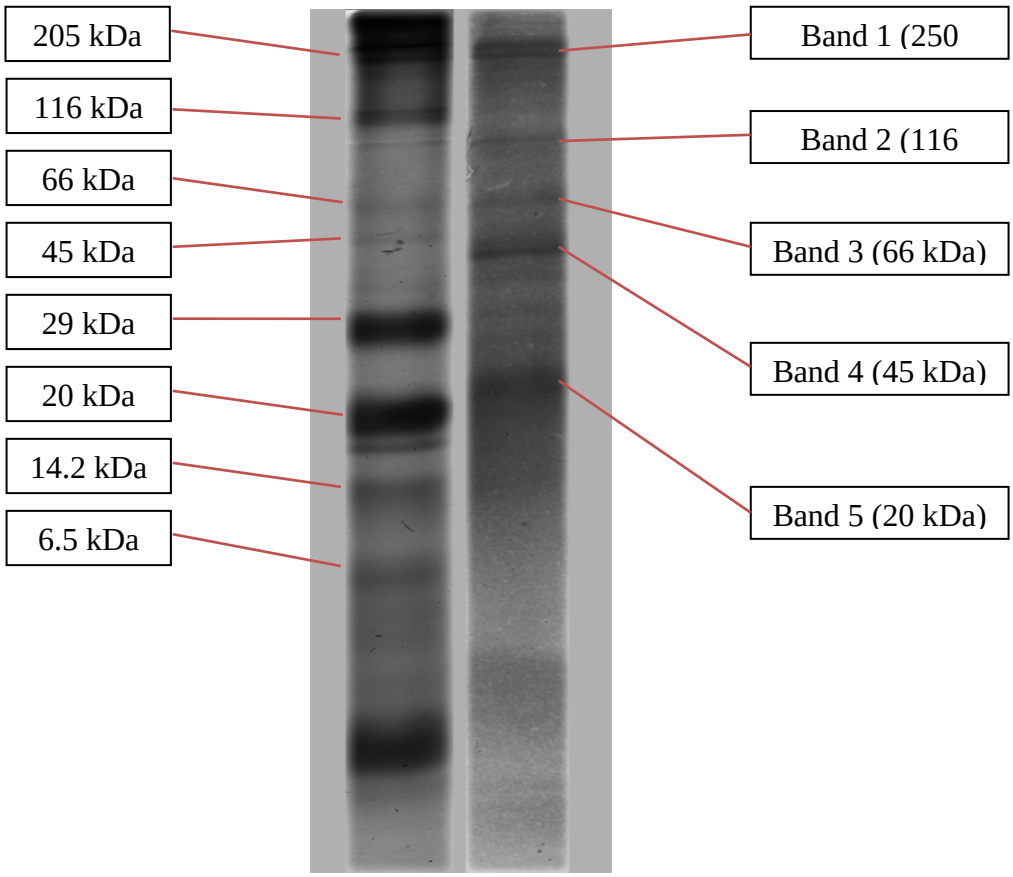

Figure 4.11: Silver-stained electrophoregram of active peak 3-RPF (α-glucosidase inhibitor

Peak 3-RPF showing several different protein bands. The SDS gel was stained with silver staining (MS compatible). Lane 1: Pre-stained SDS-PAGE standards protein marker. Lane 2: Active peak 3 reversed-phase fraction (RPF)

Table 4.7: SDS-PAGE gel protein bands with approximate molecular weight

Band in SDS-PAGE Gel	~Molecular Weight
Band 1	250 kDa
Band 2	116 kDa
Band 3	66 kDa
Band 4	45 kDa
Band 5	20 kDa

4.7 Identification of Anti-diabetic Related Proteins by MALDI-TOF/TOF Mass Spectrometry Analysis

In this experiment, two protocols of MALDI-TOF/TOF MS (In-gel and In-solution digestions) were used to identify protein and the results were compared. For proteins which were collected into fractions and then subjected to protein analysis (in-solution digestion), they could be directly characterized meanwhile SDS-PAGE gel plugs were excised is necessary to recover the protein from the gel (in-gel digestion).

4.7.1 MALDI-TOF/TOF MS Analysis of Five Protein Bands Obtained From Peak 3-RPF/F30 Silver-Stained Electrophoregram by In-Gel Digestion Protocol

From the protein bands observed by SDS-PAGE gel profile from electrophoregram (Figure 4.11), the 5 proteins band indicated with arrows were excised, in-gel digested and analysed by MALDI-TOF/TOF MS analysis.

Several available protein databases were used such as All Swissprot, Fungi National Center for Biotechnology Information (NCBI), Fungi Swissprot, *Lentinus* NCBI and *Lentinus* Swissprot. Among all databases used, only proteins obtained using the *Lentinus* NCBI showed the relationship with diabetes. The name of the protein, molecular weight, protein score and accession number of identified proteins (band 1-5) (Appendix D (1.0)) from the *Lentinus* NCBI database were listed in simplified form in Table 4.8.

According to the protein score from the MALDI-TOF/TOF analysis (Table 4.8), *Lentinus* NCBI database represented 10 identified proteins. Three proteins were suggested to be related to diabetes (based on their function). The proteins are trehalose phosphorylase (84 kDa), followed by glyceraldehyde-3-phosphate dehydrogenase and catalase (43 kDa) (Table 4.9). These three proteins have been reported to have properties that indirectly inhibit diabetes and diabetic-related complication pathways.

However, the anti-diabetic mechanism of action of these proteins may be different from each other.

4.7.2 MALDI-TOF/TOF MS Analysis of Peak 3-RPF by Using In-Solution Digestion Protocol

The peak of RPF fraction that showed the best percentage of α-glucosidase inhibition (Peak 3-RPF) was chosen for further characterization using MALDI-TOF/TOF MS analysis. Ten most significant proteins with its molecular masses were listed in Table 4.10.

The data was obtained by using Mascot searching database. The top score was 42 for PROF1_PHAVU, Profilin-1-*Phaseolus vulgaris* (Kidney Bean) but it is not significant according to Mascot however, from the record and previous research, profilin protein has a function to inhibit anti-diabetic indirectly. Mascot database software (Matrix Science Ltd, London, UK) (Aminudin *et al.*, 2008) reports a match as significant if it has a match with a less than 5% chance of being a random hit (Appendix D (2.0))

Table 4.8: List of matched identified proteins based on *Lentinus* NCBI database

Band ID	Name of the Protein	Accession number NCBI	Protein score	Protein Molecular Weight (Da)	Protein PI	No. Of Peptide Match
Band 1						
1	Trehalose phosphorylase	gi/74626081	18	83999.8	6.38	4
2	Glyceraldehyde-3-phosphate dehydrogenase	gi/30580405	11	36 156.7	6	3
3	HSP 100	gi/62998570	9	99 394	5.71	6
4	WUN [*L. sajor-caju*]	gi/6018116	8	5174.7	10.43	1
5	RNA polymerase II second largest subunit	gi/194306409	7	33 383	8.34	3
6	Antimicrobial ribonuclease	gi/71153693	6	1015.5	6.13	1
7	Heat-induced catalase [*L. sajor-caju*]	gi/13183346	5	60 039	6.77	2
8	laccase 3 [*L. sajor-caju*]	gi/11036960	5	18 143.7	5.23	1
9	Translation elongation factor 1 alpha	gi/170517001	4	20 207.6	7.07	1
Band 2						
1	HSP100 [*L. sajor-caju*]	gi/62998570	14	99 394	5.71	4
2	Glyceraldehyde-3-phosphate dehydrogenase	gi/30580405	13	36 156.7	6	2
3	RNA polymerase II second largest subunit	gi/194306409	6	33 383	8.34	1
4	Catalase [*L. sajor-caju*]	gi/28558774	5	43 768.6	6.19	1
5	Heat-induced catalase [*L. sajor-caju*]	gi/13183346	5	60 039	6.77	1
6	Trehalose phosphorylase	gi/74626081	4	83 999.8	6.38	1
Band 3						
1	RNA polymerase II second largest subunit	gi/194306409	8	33 383	8.34	1
2	Glyceraldehyde-3-phosphate dehydrogenase	gi/30580405	8	36 156.7	6	1
3	Trehalose phosphorylase	gi/74626081	8	83 999.8	6.38	1

Table 4.8, continued.

Band ID	Name of the Protein	Accession number NCBI	Protein score	Protein Molecular Weight (Da)	Protein PI	No. of Peptide Match
4	Catalase [*L. sajor-caju*]	gi/28558774	7	43 768.6	6.19	1
5	HSP100 [*L. sajor-caju*]	gi/62998570	7	99 394	5.71	2
6	Heat-induced catalase [*L. sajor-caju*]	gi/13183346	6	60 039	6.77	3
Band 4						
1	Trehalose phosphorylase	gi/74626081	32	83 999.8	6.38	1
2	Glyceraldehyde-3-phosphate dehydrogenase	gi/30580405	11	36 156.7	6	2
3	HSP100 [*L. sajor-caju*]	gi/62998570	10	99 394	5.71	3
4	RNA polymerase II second largest subunit [*L. sajor caju*]	gi/194306409	8	33 383	8.43	2
5	laccase 4 [*L. sajor-caju]*	gi/11036962	5	18 143.7	4.92	1
6	Catalase [*L. sajor-caju*]	gi/28558774	5	43 768.6	6.19	1
7	Heat-induced catalase [*L. sajor-caju*]	gi/13183346	4	60 039	6.77	1
Band 5						
1	Trehalose phosphorylase	gi/74626081	18	83 999.8	6.38	5
2	RNA polymerase II second largest subunit	gi/194306409	12	33 383	8.34	4
3	Heat-induced catalase [*L. sajor-caju*]	gi/13183346	10	60 039	6.77	4
4	Glyceraldehyde-3-phosphate dehydrogenase	gi/30580405	10	36 156.7	6	3
5	HSP100 [*L. sajor-caju*]	gi/62998570	10	99 394	5.71	7
6	Catalase [*L. sajor-caju*]	gi/28558774	9	43 768.6	6.19	3
7	WUN [*L. sajor-caju*]	gi/6018116	8	5174.7	10.43	1
8	Translation elongation factor 1 alpha [*L. sajor-caju*]	gi/170517001	4	20207.6	7.07	1

Table 4.9: Potential proteins obtained from *Lentinus* NCBI database (In-gel digestion MALDI-TOF/TOF) which are indirectly related to anti-diabetic mechanisms

Anti-diabetic related protein	Protein Molecular Weight (Da)	Protein Band	Protein Score
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	36, 000	2	13
Catalase	43, 000	5	9
Trehalose Phosphorylase	84, 000	4	32

Table 4.10: Ten abundant proteins identified of Peak 3-RPF from Mascot database (In-solution digestion MALDI-TOF/TOF)

	Accession	Mass (Da)	Score	Name of Protein	No. Of Peptide Match
1	PROF1_PHAVU	14 172	42	Profilin-1- *Phaseolus vulgaris* (Kidney Bean) (French bean)	2
2	PROF1_RICCO	14 199	42	Profilin_1- *Ricinus communis* (Castor bean)	2
3	PROF1_SOYBN	14 091	42	Profilin-1- (GmPRO1) (Allergen Gly)- *Glycine max* (Soybean)	2
4	PROF2_HEVBR	14 142	42	(Pollen allergen Hev b)- *Hevea brasiliensis*	2
5	PROF2_MALDO	14 043	42	Pollen allergen Mal d- *Malus domestica* (apple)	2
6	PROF2_SOYBN	14 091	42	Profilin-1- (GmPRO1) (Allergen Gly)- *Glycine max* (Soybean)	2
7	PROF_FRAAN	14 058	42	Profilin (Allergen Fra a 4)- *Fragaria ananassa* (strawberry)	2
8	PROF_PRUPE	13 980	42	Profilin (Allergan Pru p 4.02)- *Prunus persica* (Peach)	2
9	EX7S_GEOSL	8693	38	Exodeoxyribonuclease 7 small subunit	2
10	Y1491_LACJO	18 213	37	UPF0090 protein LJ_1491- *Lactobacillus johnsonii*	2

5.0 DISCUSSION

Pleurotus pulmonarius basidiocarps was extracted by several methods of extractions including distilled water, methanol, dichloromethane and ethanol to yield the aqueous, methanol, dicholoromethane and polysaccharide extracts. Most solvent extracts, crude and protein fractions of *P. pulmonarius* basidiocarps demonstrated the *in vitro* anti-diabetic activity based on two assays involving carbohydrate metabolizing enzymes; α-glucosidase and α-amylase. The inhibition of these enzymes, which are involved in the digestion of carbohydrates, can significantly reduce postprandial elevations of blood glucose and therefore offers an alternative strategy in the management of blood glucose level especially in type 2 DM (Codario, 2011).

Aqueous extracts showed the highest yield percentage (18.34 %) compared to other extraction methods. Extract yield differences might be due to the different availability of extractable components in the extracts resulting from the varied chemical composition (Sultana *et al.,* 2009). Furthermore, different extraction methods were used because *Pleurotus* genus possibly produce various range of bioactive as a pharmacological compounds such as pigments, aromas, organic acids, polysaccharides, enzymes, vitamins and amino acid (Dalonso *et al.,* 2010).

In this study, protein fractions of *P. pulmonarius* basidiocarps were shown to exhibit potent anti-diabetic inhibitory activity *in vitro*. It has been reported that aqueous extracts of *P. pulmonarius* have hypoglycemic effects and when interact with oral anti-diabetic agents such as glyburide, acarbose and rosiglitazone it could monitor on serum glucose level (Badole *et al*., 2006; Badole *et al*., 2007; Badole *et al*., 2008). According to Kanagasabapathy *et al.,* (2013), *P. pulmonarius* contains low fat and high soluble fiber content, with no starch and low sugar. Additionally, it also have gastronomic, nutritional and medicinal properties besides easily cultivated on a large range of substrates.

Salting out method using ammonium sulphate to precipitate water soluble protein from aqueous extracts was used as early step purification resulted with the formation of 10 protein fractions (F10-F100). Proteins are soluble in water due to their interaction with hydrophilic amino acid side-chains facing outwards. Ammonium sulphate interferes these interactions between amino acids side-chains and water by reducing the available water thus reduce the solubility of protein, and hence, the protein will precipitate out from the solution. Low temperature is required throughout the experiment (4 ^{o}C) to ensure protein is not denatured. Different proteins precipitate at different concentrations of ammonium sulphate (Komsta *et al.*, 2011).

Twenty-five mg/ml of four crude organic extracts with 25 µg/ml protein content of F10-F100 protein fraction were assayed for anti-diabetic inhibitory assays. A higher concentration of solvent extract were used because there are severeal compounds mix together and the active compound could only composed a minor protein. Therefore, a higher amount are needed compared to the protein since there are considered to be purified protein from crude extract. Among all the solvent extracts, aqueous extract and protein fractions, F30 strongly suppressed the activity of α-glucosidase enzyme (24.18%). Meanwhile F100 protein fraction for inhibitory activity of α-amylase enzyme (41.80%). Methanol, dichloromethane and polysaccharide extracts showed very low inhibition percentage although the concentration used was 10-fold higher. According to Jarald *et al.*, (2008), aqueous extract and non-polysaccharide fraction of the plant reported can decrease the serum glucose level and other complications in alloxan diabetic animals.

Based on the results, it could be hypothesized that these proteins have different hydrophobic strength with F30 protein fraction being more hydrophobic meanwhile F100 protein fraction is very much hydrophilic (Scopes, 1994). In order to demonstrate synergism between the protein fractions, a lower concentration of each fractions were

mixed and re-evaluated. If the mixture gave an effect higher than individual protein fraction, this suggests than both fractions are required and thus prove synergustic relationship. Hydrophobicity (and hydrophilicity) had been shown to have a major role in the binding behaviour of specific drugs and proteins. This can be explained by the enzymes work at the different places in human intestinal tract and with different mechanisms of action therefore, the hydrophobicity which explaining the binding behaviour of certain drugs also may differ (Seedher & Kanojia, 2008). According to Melander, (2004) the hydrophobicity characteristic explains the differences in clinical efficacy or safety therapeutic drugs even though it shows identical mechanisms of action. As an example for anti-diabetic drugs, sulfonylureas, phenformin and adenosine monophosphate-activated protein kinase (AMPK) are hydrophobic (Gruzman *et al.*, 2009) meanwhile metformin is hydrophilic (Viollet *et al.*, 2012).

As displayed in Figure 4.5, SDS-PAGE analysis of the selected F30 protein fraction for α-glucosidase inhibitor (10 bands) and F100 protein fraction α-amylase inhibitor (9 bands) demonstrated a very distinct band profile; hence, was further fractionated by RP-HPLC. Distinct RPF peaks from the fraction were then collected and furthered re-evaluated with the same assays. Based on the assays, peak 3-RPF from F30 protein fraction, exerted strong inhibitory activity (25%) towards α-glucosidase enzyme meanwhile peak 3-RPF from F100 protein fraction only demonstrated 2.84% inhibition towards α-amylase enzyme. The result indicated a possible synergistic interaction between proteins in F100 protein fraction since they effectively inhibited α-amylase inhibitory activity when present together but the inhibition effect became insignificant once they were separated through RP-HPLC (Oikonomakos *et al.*, 2002; Thompson & Orvig, 2006).

Semi pure fraction represented by peak 3-RPF from F30 protein fraction was subjected to SDS-PAGE and visualised using compatible silver staining prior to in-gel

digestion for MALDI-TOF/TOF-MS analysis. Mass spectrometry analysis against *Lentinus* NCBI database revealed three possible proteins that have a direct/indirect relation with the prevention of DM which are glyceraldehyde-3-phosphate dehydrogenase (GAPDH), catalase and trehalose phosphorylase. *Lentinus* NCBI was selected in detecting proteins because it is the nearest genus. Since many fungi database are still largely incomplete, many proteins present in *P. pulmonarius* are absent in those databases. This also explain the low score for all proteins obtained.

On the other hand, for in-solution MALDI-TOF/TOF-MS, profilin is the most abundant proteins detected via Mascot database search. It may have a potential mechanism in inhibiting diabetic. We hypothesized that profilin was present in high abundance in the fraction and thus, easily identified. Due to its small molecular weight, it could be possible that it diffuses out from the SDS-PAGE gel, and therefore, not identified via in-gel digestion MALDI-TOF/TOF-MS.

All four proteins which were profilin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), catalase and trehalose phosphorylase involved in the regulation of blood glucose level through various mechanisms based on its ability to inhibit antidiabetic activity *in vitro*. Profilin (14 kDa) is ubiquitous small proteins that is commonly found in mammals, animal cells, plants, and viruses (Witke *et al.*, 2001). Profilin is also essential for sustainability of eukaryotes ranging from fungi to animals (Vidali *et al.*, 2007). Profilin-2 is predominantly expressed at lower levels in neuronal cells, skeletal muscle, uterus and kidney (Geese *et al.*, 2000).

Profilin binds to actin and affects the structure of the cytoskeleton. At high concentration, profilin prevents the polymerization of actin, whereas it enhances it at low concentration (Saarikangas *et al.*, 2010). Furthermore, profilin also acts as a tumour suppressor molecule for breast cancer cells and has a potential to inhibit metastasis (Krishnan & Moens, 2009). Deletion of profilin genes will give rise to Miller-Dieker

syndrome, a genetic disorder (Kullmann *et al.*, 2012). On another account, profilin is a signalling molecule involved in the binding of phosphatidylinositol 4,5-bisphosphate (PIP_2), which means it also indirectly involve in the regulation of diabetes metabolism or biomolecules.

In human, activation of protein kinase C (PKC) contributes to hyperglycemia induced cell damage. Activation of the PKC pathway through the de novo synthesis of diacylglycerol (DAG) from glycolytic intermediates could results with complication of DM. It is also associated with neurovascular changes, insulin resistance and many vascular abnormalities in the retinal, renal, neural and cardiovascular tissue. Therefore, PKC may have vital role in the cardiovascular diseases (Geraldes & King, 2010).

PKC requires its activator, DAG and calcium for activation (Chu & Silverstein, 2012). In DAG-PKC mechanism, PIP_2 is hydrolysed by phospholipase C (PLC) into two intracellular signalling molecules, inositol trisphosphate (IP_3) and DAG. This will increase intracellular calcium available (Pintérová *et al.*, 2011). PKC activation occurs with binding of DAG, in the presence of calcium ions (Ca^{2+}) resulting in translocation of the PKC-DAG complex to the cell membrane (Zuzek *et al.*, 2013). IP_3 opens calcium channel to allow calcium entry from the endoplasmic reticulum and extracellular fluid. PIP_2 is the precursor of two-second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), which regulate a variety of cellular processes meanwhile DAG major function is to activate the PKC (Hille, 2012).

Activation of PKC induced by glucose increases production of extracellular matrix and cytokines which leads to enhance contractility, permeability and vascular cell proliferation. Oxidants such as hydrogen peroxide (HP) can also contribute to PKC activation either directly or by increasing DAG production (Nishikawa *et al.*, 2000).

In diabetic, the augmented availability of glucose caused an augmented availability of DAG through *de novo* synthesis and consequent activation of PKC.

Effect of this extra activation is different and detrimental for vascular functionality because it increase vascular permeability (Piconi *et al.*, 2007). In addition, the secondary metabolic products such as reactive oxygen intermediates, oxidative stress, fatty acid and advanced glycation end products (AGEs) can also activate PKC independently of DAG (Rains & Jain, 2011).

From the previous research, profilin has been discussed as a negative regulator of the PLC activity in DAG-PKC pathway (Goldschmidt-Clermont *et al.*, 1990). Profilin binds with high affinity to small clusters of PIP_2 molecules in micelles and also in bilayers with other phospholipids. In the case of profilin and actin, it has been assumed that PIP_2 binds to the profilin. By binding to PIP_2, it interferes and lowers rate of PIP_2 hydrolysis by soluble PLC and consequently inhibits the formation of IP_3 and DAG. PIP_2-profilin complex is a poor substrate for PLC (Elnakish & Hassanain, 2012).

Profilin competes efficiently with platelet cytosolic phosphoinositide-specific PLC for interaction with the PIP_2 substrate and thereby inhibits PIP_2 hydrolysis. Profilin is an effective inhibitor of PIP_2 hydrolysis by PLC, even when the concentration of calcium ion (Ca^{2+}) is optimal for PLC activity. The low Ca^{2+} concentration together with profilin should reduce the further activity of PLC (Kang & Othmer, 2007).

Thus, it could be possible to suggest that profilin might play a critical role and may represent a new therapeutic target to prevent various disease pathogenesis and as a mediator in diabetic vascular injury (Caglayan *et al.*, 2010). However, more studies need to be done in order to fully understand the profilin-signalling pathway and its mechanism of regulation. Drug development in this area is necessary as it may offer therapeutic options for diabetic complications, cancer, autoimmune disease and inflammation (Skvortsova, 2013).

The second protein identified is GAPDH enzyme (~37 kDa), responsible to catalyzes the reaction of glyceraldehyde-3-phosphate (GAP) conversion to 1,3

diphosphoglycerate (Tatton *et al.*, 2000). GAPDH provides a common link between hyperglycemia-induced, oxidative stress and activation of some major pathways associated with the pathogenesis of diabetic complications. Madsen-Bouters *et al.*, (2010) reported that GAPDH acts as a mediator in the activation of pathways implicated in the pathogenesis of diabetic retinopathy.

GAPDH functions actively to regulate the insulin pathway. The segregation of GAPDH releases the suppressor of insulin signaling from the cell membrane, and thus activates the insulin signaling (Devitt, 2006). Min *et al.*, (2006) also suggested that GAPDH is an active regulator in the phosphoinositide-mediated signaling pathway and a potential new target for insulin resistance treatment which eventually help to treat diabetes.

However, GAPDH activity and abundancy are significantly perturbed in DM because it is exquisitely sensitive to modification by cellular processes. It occurs because the enzyme contains a highly reactive thiol at its active site which is sensitive to modification by a variety of compounds such as reactive aldehydes, glycating agents and oxidative radicals (Schuppe *et al.*, 1994). Recently, this perturbations in GAPDH have been connected to cellular dysfunction in several neurodegenerative diseases (Chuang *et al.*, 2005).

Inhibition of GAPDH involves the formation of methylglyxol (MG), AGEs, activation of PKC, induction of the hexosamine and polyol pathways; which eventually contribute to the development of diabetic complications (Beisswenger *et al.*, 2003; Brownlee, 2005). GAPDH increases AGEs formation by increasing MG and activates PKC by the formation of DAG. Furthermore, toxic potential of MG has effects on insulin secretion from pancreatic beta cells (Ulrich & Cerami, 2001). Decrease GAPDH activity may also play a role in the high rates of artherosclerosis associated with diabetes and the insulin resistance syndrome (Leyva, 1998).

Structural mutations of the GAPDH gene and altered gene expression could lead to significantly altered function and increased triose phosphate and MG production in response to hyperglycemia (Nemet *et al.,* 2006). Thus, GAPDH is suggested to control a crucial metabolic step that determines the levels of MG precursor and can therefore regulate its production.

In hyperglycemia, AGEs glycate with protein, making it saturated and results in loss of function and poor gene expression. AGEs modulate the initiating steps in atherogenesis involving blood-vessel wall interactions, cause plaque formation and block the blood vessel. This will leads to high blood pressure, atherosclerosis, amputation, cataracts, and attract bacterial attachment to unavoidable injuries or cuts (Aronson & Rayfield, 2002). It also could lead to neuropathological changes in the brain and the subsequent behavioural deficits; characteristics of autism (Maher, 2012).

Both MG and AGEs are able to induce oxidative stress, inflammation and mitochondrial dysfunction, which implicated in diabetic complications and multiple neurological diseases. Therefore, researchers are trying to develop various pharmacological approaches in order to avoid deleterious effects of AGEs reactions. It has been postulated that environmental factors or genetic dysregulation can modify GAPDH production consequent effects in MG production and AGEs production (Beisswenger *et al.,* 2003).

Besides, GAPDH gene expression can be regulated by hormonal, nutritional and metabolic factors including insulin, which can increase GAPDH mRNA and activity up to 10 fold in liver and fat cells. Thus when GAPDH is higher, none of MG and AGEs will be produced. The discovery of GAPDH adds another target that can be addressed in combating the disease (Jagt, 2008).

The third possible protein is trehalose phosphorylase (TP), which is widely distributed in many microorganisms, plants, and animals such as in bacteria, fungi,

yeast, insects, nematodes, shrimp and invertebrate animals (Elbein *et al.*, 2003). This enzyme also has been found in mushrooms such as *Flammulina velutipes*, *Pichia fermentans*, *Euglena gracilis*, cyanobacterial *Scytonema* species (Inoue *et al.*, 2002), *Grifola frondosa* (Saito *et al.*, 1998), *Agaricus bisporus* (Wannet *et al.*, 1998), *Catellatospora ferruginea* (Aisaka *et al.*, 1998) and *Pleurotus ostreatus* (Schwarz *et al.*, 2007).

TP participates in starch, sucrose and glycogen metabolism and also plays a vital role especially in trehalose metabolism (Ng, 2004). Its function is to catalyzes the phosphorylase of trehalose into α-glucose-1-phosphate (α-Glc-1-P) and glucose and the reaction is reversibly. According to Eis & Nidetzky (1999), the equilibrium of the reaction lies in the direction of trehalose synthesis. However, since the reaction was ditermined *in vitro*, there is uncertainty about the participation of TP in the synthesis or degradation of trehalose *in vitro* (Avonce *et al.*, 2006).

Trehalose (α, α-1, 1-glucosyl-glucose) is a non-reducing disaccharide of d-glucose in which the two glucoses are linked in a α, α-1, 1-glycosidic linkage. It is a common sugar, concentrations that can exceed significantly those of other storage carbohydrate such as glycogen and found in vegetative cells, spores of fungi and mushrooms which contain up to 10–25% by dry weight (Elbein *et al.*, 2003). Trehalose can play a number of different roles in biological systems, such as serving as a reservoir of glucose for energy or carbon, functioning as a stabilizer or protectant of proteins and membranes during times of stress, acting as a regulatory molecule in the control of glucose metabolism, serving as a transcriptional regulator and playing a structural and functional role as a component of various cell wall glycolipids in mycobacteria and related organisms (Avonce *et al.*, 2006). It may also act as a signalling or regulatory molecule in some cells and link trehalose metabolism to glucose transport and glycolysis (Elbein *et al.*, 2003).

Because of its ubiquitous role, trehalose is currently known as a component in food especially for DM patient because it can help to inhibit progression of type 2 DM. In 1995, the development of a large-scale biological procedure for commercial production of trehalose, enable the community to use trehalose as a component in foods and cosmetics (Arai *et al.*, 2001). It is a natural sugar found in nature, easy to process, has a pleasant taste and have a potential to protect against metabolic syndrome. Trehalose will not trigger a rise in blood glucose levels, unlike other foods and drinks. Intake of trehalose has been shown to evoke lower insulin secretion and mitigates insulin resistance (Arai *et al.*, 2010). Moreover, trehalose has a suppressive effects on osteoporosis development (Higashiyama, 2002).

There are several mechanisms that can increase the trehalose production and one of them involves a single enzyme TP that catalyses the interconversion of maltose and trehalose (Pan *et al.*, 2004). This happens because TP control intracellular levels of trehalose by converting excess trehalose to maltose, which can then be converted by a-glucosidases to glucose (Wolf *et al.*, 2003).

Trehalose and TP are both involved in the production and degradation of glycogen, making both metabolisms of trehalose and glycogen interconnected. TP affects the levels of glycogen and utilization of trehalose in cells. For example, TP accumulates large amounts of glycogen when grown in high concentrations of trehalose, but mutants missing TP activity do not accumulate glycogen, regardless of the amount of trehalose in the media (Pan *et al.*, 2008). TP acts as a sensor or regulator of trehalose levels in these cells by catalysing the conversion of glycogen to trehalose when cytoplasmic trehalose levels are low. TP enzyme also can expedite the conversion of trehalose to glycogen when cytoplasmic trehalose levels become high. Thus, TP represents another pathway for the production of trehalose from glycogen, involving maltose as an intermediate (Pan *et al.*, 2008).

TP is not only involved in the production of trehalose from glycogen, but also appears to play an essential role in the formation and accumulation of glycogen (Elbein *et al.,* 2003). Thus it is good for diabetic patients to replace the intake of sugar (sucrose) with trehalose, since TP manage to control the level of trehalose in the body by changing it to glycogen and trehalose has been proven better than sucrose. Removal of excessive trehalose from the body is necessary because high levels of trehalose will cause toxic effect. Thus, it could be suggested that TP proteins found in by *P. pulmonarius* extracts can help to maintain the level of trehalose in diabetic patients and ensure a balanced glucose-glycogen metabolism.

The fourth possible protein is catalase enzyme (43 kDa), commonly found in nearly all organisms. In human, catalase could be found in high concentration in the human liver, kidney and erythrocytes but much lower in connective tissue, pancreas, brain and serum (Scandalios *et al.,* 1997). Almost 98% of blood catalase activity is localized in the erythrocytes (Lai, 2008). Catalase catalyses the breakdown of hydrogen peroxide (HP) into oxygen and water (Góth *et al.,* 2001). An absence of catalase (acatalasemia) in human body resulted with the homozygous mutations of the gene prone to have links with DM, Alzheimer's disease and tumours (Góth & Nagy, 2012).

Catalase acts as antioxidant, inhibits the oxidation of other molecules, helps enhance the insulin secretion and insulin sensitization (Lai, 2008). High free radicals and low antioxidant defence mechanism will damage of high lipid peroxidation, cellular organelles, enzyme and results insulin resistance (Prentki & Nolan, 2006).

Free radicals involved in oxidative stress and lead to the damage of pancreas (Xu *et al.,* 1999). It also leads to retinopathy, neuropathy, nephropathy atherosclerosis, cataract and cardiopathy. In human tissues, catalase plays a primary role in controlling HP concentration, increase in response to oxidative stress and protect pancreatic beta cells from damage. Therefore catalase is important to inhibit the activity of free radicals

and avoid diabetic long term complications (Giacco & Brownlee, 2010).

Sartoretto *et al*., (2011) demonstrated deficiency of catalase may cause elevated HP concentration with both toxic and physiological effects. Prolonged high HP concentration leads to long-term oxidative stress and contribute to DM and its complication. Factors that decrease catalase level include catalase gene mutations, catalase enzyme inactivation, or downregulation (Hamada *et al.*, 2004).

HP is a byproduct of normal respiration and also formed from superoxide anion by action of superoxide dismutase. Low level concentration of HP acts as a cellular messenger in insulin signalling while in an excessive concentration it may cause toxic to protein, RNA, DNA, lipids and usually damages pancreatic cells (which are catalase poor) and also inhibits insulin signalling (Barbosa *et al.*, 2013). It can be postulated that deficiency of this catalase might leads to cumulative oxidant damage and ultimately, failure of pancreatic beta cells and diabetes (Styskal *et al.*, 2012).

The risk may be due to peroxide damage of normally catalase-poor pancreatic beta cells, which are sensitive to oxidation (Chen *et al.*, 2001). This is because, one of the unique features of beta cells is their relative low expression of many oxidant enzymes but it is further lowered by acatalasemia mutations (Vitai & Góth, 1997). Beta cell susceptibility could increase to oxidative damage, cell death induced by HP. The damage by peroxide should depend on whether the low catalase effect is chronic or acute (Rains & Jain, 2011).

Diabetic patients are usually accompanied by increased production of free radicals or impaired antioxidant defences. In type 2 diabetic patient, the downregulation of catalase synthesis may cause decreased blood catalase activity which consequently increase the level of HP (Baynes & Thorpe, 1999).

In summary, catalase is highly beneficial to the organs and body processes because it has a substantial capacity for removing intra- and extracellular HP. Catalase

provide protection for tissues with low catalase activity because it has high turnover numbers; one molecule of catalase can decompose more than one million molecules of HP per second (Góth & Vitai, 2003).

It is necessary to take the right amount of minerals and proteins to sustain the sources of catalase in the body. For example the natural catalase sources are barley grass, fruits, vegetable and *Aspergillus niger* (Marsili *et al.*, 2004; Bankar *et al.*, 2009). Currently, treatment of antioxidant-based drug formulations which is due to catalase gene alterations by captopril, aminoguanidine, melatonin and acetylsalicylic acid are available but as discussed, drugs have negative side effects (Messner & Imlay, 2002).

6.0 CONCLUSION

Protein fractions from *P. pulmonarius* basidiocarps have significant potential inhibition towards α-glucosidase and α-amylase enzymes higher than crude aqueous, dichloromethane, methanol and polysaccharide extracts. The protein fractions were further separated via RP-HPLC and all the eluted peaks were collected and assayed for the antidiabetic activities. Based on SDS-PAGE analysis, the two selected fractions with anti-diabetic potentials (F30 and F100 protein fractions) showed a very distinct banding profile and thereby was further purified by second round of RP-HPLC. Among all the eluted sub-fraction peaks, peak 3 RPF from F30 showed an inhibition towards α-glucosidase enzyme. F100 fraction showed low inhibition percentage probably because of synergistic interaction since they effectively inhibited α-amylase activity as a group but the inhibition effect became insignificant once they were separated through RP-HPLC. Identification by MALDI-TOF/TOF-MS revealed the presence of promising anti-diabetic related proteins in these sub-fractions.

As a conclusion, we have characterized four potential inhibitors from *Pleurotus pulmonarius* basidiocarps, which could be useful in combating diabetic disease with reference to their ability to inhibit *in vitro* α-glucosidase activity. However, there is no significant activity of α-amylase *in vitro* inhibition observed. We claimed that the profilin-like, trehalose phosphorylase-like, gylceraldehyde-3-phosphate dehydrogenase-like and catalase-like proteins might promote the lowering of glucose level in blood, reduce insulin resistance and prohibit the risk of cardiovascular diseases.

Based on this research, the consumption of *P. pulmonarius* should be promoted as it contains 4 types of proteins with very close relationship to diabetic and its complication issues. These proteins from *P. pulmonarius* could be further developed as alternative therapeutics for the treatment of DM as well as its complication and other carbohydrate mediated diseases. Any therapeutic peptide derived from an edible

mushrooms that can be eaten daily are potentially safer and may have lesser possibility to exert adverse side effects.

However, in order to determine the identity of the α-glucosidase inhibitor, further protein isolation is required. It is necessary to fully characterize this active principle, both structurally and functionally, to validate its role as potential anti-diabetic molecule. *In vivo* study using animal could be proposed to add assurance regarding the safety and efficacy of the new drug.

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APPENDIX A: PREPARATION OF EXTRACTS AND REAGENTS

1.1 Preparation of Standard and Working Reagent for Pierce BCA Protein Assay Kit

a. Preparation of Diluted Albumin (BSA) Standards

Table 1 was used as a guide to prepare a set of protein standards. The contents of the one Albumin Standard (BSA) ampule were diluted into several clean vials, preferably using the same diluent as the sample(s). Each 1 ml ampule of 2.0 mg/ml Albumin Standard is sufficient to prepare a set of diluted standard for either working range suggested in Table 1. There will be sufficient volume for three replications of each dilute standard.

Table 1. Preparation of Diluted Albumin (BSA) Standards; Dilution Scheme for Standard Test Tube Protocol and Microplate Procedure (working range=20-2000 μg/ml)

Vial	Volume of Diluent	Volume and Source of BSA	Final BSA Concentration
A	0	300 μl of Stock	2000 μg/ml
B	125 μl	375 μl of Stock	1500 μg/ml
C	325 μl	325 μl of Stock	1000 μg/ml
D	175 μl	175 μl of vial B dilution	750 μg/ml
E	325 μl	325 μl of vial C dilution	500 μg/ml
F	325 μl	325 μl of vial E dilution	250 μg/ml
G	325 μl	325 μl of vial F dilution	125 μg/ml
H	400 μl	100 μl of vial G dilution	25 μg/ml
I	400 μl	0	0

b. Preparation of the BCA Working Reagent (WR)

i. The following formula was used to determine the total volume of WR required:

(# standards + # unknowns) x (# replicates) x (volume of WR per sample) = Total volume WR required

Note: There is only 200 μl of the WR reagent required for each sample in the microplate procedure.

ii. WR was prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A: B). For the above example, 50 ml of Reagent A was combined with 1 ml of Reagent B.

Note: When Reagent B is first added to Reagent A, turbidity is observed that quickly disappears upon mixing to yield a clear, green WR. The sufficient volume of WR was prepared based on the number of samples to be assayed. The WR is stable for several days when stored in a closed container at room temperature (RT).

c. Microplate Procedure (Sample to WR ratio = 1.8)

i. 25 µl of each standard or unknown sample replicate was pippete out into a microplate well (working range 20-2000 µg/ml).

Note: If sample size is limited, 10 µl of each unknown sample and standard can be used (sample to WR ratio 1: 20). However, the working range of the assay in this case will be limited to 125-200 µg/ml.

ii. 200 µl of the WR was added to each well and a plate mixed thoroughly on a plate shaker for 30 seconds.

iii. The plate was covered and incubated at 37°C for 30 minutes.

iv. The plate was cooled to RT.

v. The absorbance was measured at 562 nm on a plate reader.

Notes:

Wavelengths from 540-590 nm have been used successfully with this method. Because plate readers use a shorter light path length than cuvette spectrophotometers, the Microplate Procedure requires a greater sample to WR ration to obtain the same sensitivity as the standard Test Tube Procedure. If higher 562 nm measurements are desired, increase time to 2 hours.

Increasing the incubation time or ratio of sample volume to WR increases the net 562 nm measurement for each well and lowers both the minimum detection level of the reagent and the working range of the assay. As long as all standards and unknowns are treated identically, such modifications may be useful.

vi. The average 562 nm absorbance measurement of the Blank standard replicates was subtracted from the 562 nm measurements of all other individual standard and unknown sample replicates.

vii. A standard curve was prepared by plotting the average Blank-corrected 562 nm measurement for each BSA standard versus its concentration in μg/ml. The standard curve was used to determine the protein concentration of each unknown sample.

Note: If using curve-fitting algorithms associated with a microplate reader, a four-parameter (quadratic) or best-fit curve will provide more accurate results than a purely linear fit. If plotting results by hand, a point-to-point curve is preferable to a linear fit to the standard points.

1.2 Preparation of Reagents for Anti-diabetic Inhibitory Assays

1.2.1 Preparation of Reagents for Alpha Glucosidase Inhibitory Assay

a. Preparation of 1.0 M Potassium Phosphate Buffer, pH 6.9

3.722 g of potassium dihydrogen phosphate (KH_2PO_4) and 3.713 g of dipotassium hydrogen phosphate (K_2HPO_4) were weighed and dissolved in 400 ml of distilled water. The solution was mixed well in a beaker using a magnetic stirrer. The solution was adjusted to pH 6.9 by adding either K_2HPO_4 as base or KH_2PO_4 as acid. Then the sodium phosphate buffer as topped up to 500 ml with distilled water in a volumetric flask. The solution was kept at RT.

b. Preparation of 3mM *p*-nitrophenyl-α-D-glucopyranoside, pH 6.9

0.045 g of *p*-nitrophenyl-α-D-glucopyranoside (*p*NPG) (Sigma Aldrich) was weighed and dissolved in 50 ml of 1.0 M phosphate buffer, pH 6.9. Since the chemical

is sensitive to light, the solution preparation was carried out in the dark condition and stored in an aluminium foil wrapped bottle. The solution was prepared freshly upon usage and kept in an ice bath.

c. Preparation of 0.1 M Sodium Carbonate (Na_2CO_3) Solution

5.30 g of sodium carbonate (Na_2CO_3) was weighed and dissolved in 500 ml distilled water and kept at room temperature.

d. Preparation of *Saccharomyces cerevisiae* Alpha Glucosidase Enzyme

Alpha glucosidase enzyme from *Saccharomyces cerevisiae* (Type I) was purchased from Sigma Aldrich Co., which in lyophilized powder. For a stock solution (1 U/ml), *Saccharomyces cerevisiae* alpha glucosidase (100 U) was dissolved in 100 ml of pre-chilled sodium phosphate buffer, pH 6.9. The enzyme was then aliquot into 1 ml in each in eppendorf tubes that has been fully covered with aluminium foils. The enzyme solution was stored at -20°C freezer for further use. According to the manufacturer's product description, one unit will liberate 1.0 μmole of D-glucose from p-nitrophenyl-α -D-glucoside per min at pH 6.8 at 37°C.

For *Saccharomyces cerevisiae* alpha glucosidase inhibitory assay, 1 ml from stock solution of alpha glucosidase enzyme was dissolved in 9 ml of 1.0 M sodium phosphate buffer, pH 6.9. The solution was placed in an ice bath during the experiment and prepared freshly prior to assay. For storage, the *Saccharomyces cerevisiae* alpha glucosidase enzyme solution was stored at -80°C prior to assay. [Note: The *S. cerevisiae* alpha glucosidase enzyme was sensitive and unstable, therefore, freeze and re-thaw is not preferable].

e. Preparation of Voglibose

Voglibose was used as a positive control for alpha glucosidase inhibitory assay. It was purchased from Sigma Aldrich Co. in lyophilized form. According to manufacturer's product description, it was a modified tetrasaccharide that acts as a

reversible alpha glucosidase inhibitor. Stock solution (1 mg/ml) was prepared by dissolving 10 mg of enzyme powder in 10 ml in distilled water.

The stock solution was then diluted to a concentration that was required for *Saccharomyces cerevisiae* alpha glucosidase inhibitory assay which was 25 μg/ml for voglibose and protein fraction, while for crude sample extracts was 25 mg/ml. The stock solution was stored at -20°C refrigerator for further use.

1.2.2 Preparation or Reagent for Amylase inhibitory Assay

a. 0.02 M Sodium Phosphate buffer, 0.006 M sodium chloride, pH 6.9

For sodium phosphate buffer, the following two solutions were prepared separately:

- Solution of 0.2 M Sodium dihydrogen phosphate dehydrate ($NaH_2PO_4.2H_2O$) was prepared by dissolving 15.6 g $NaH_2PO_4.2H_2O$ with 500 ml dH_2O.
- Solution of 0.2 M Disodium hydrogen phosphate heptahydrate, ($Na_2HPO_4.7H_2O$) was prepared by dissolving 53.65 g $Na_2HPO_4.7H_2O$ with 1 L dH_2O.

Firstly 0.1 M sodium phosphate buffer was prepared by adding 135 ml of 0.2 M $NaH_2PO_4.2H_2O$ solution and 165 ml of 0.2 M $Na_2HPO_4.7H_2O$ solution. The mixture was mixed well using a stirrer followed by addition of distilled water until reached 600 ml. The solution was adjusted to pH 6.9 by adding either Na_2HPO_4 as base and NaH_2PO_4 as acid.

For final concentration 0.02 M sodium phosphate buffer 0.006 M sodium chloride pH 6.9, a dilution has been made by pipetted out 200 ml from stock of 0.1 M sodium phosphate buffer and topped up until 1000 ml with distilled water in volumetric flask. Finally 0.3506 g of sodium chloride (NaCl) was added and pH was adjusted to pH 6.9.

b. Starch (0.5 %)

Soluble starch from potato (0.5 g), which purchased from Sigma Aldrich Co. was dissolved in 100 ml of 0.02 M Sodium Phosphate buffer, 0.006 M sodium chloride,

pH 6.9. Constant stirring at 90°C during the assay period helped the dissolution of starch in buffer. The starch solution was then cooled at room temperature. The starch solution prepared freshly prior to assay.

c. Preparation of Dinitrosalicylic Acid (DNS) solution. (1 % (w/v) DNS, 12 % (w/v) sodium potassium tartrate in 0.4 M sodium hydroxide).

300 ml of distilled water was filled in aluminium foil covered bottle and placed on a hot plate with a stirrer. Lyophilized 3-5 dinitrosalicylic acid (DNS) (3 g) were added with continuous stirring. Then, 4.8 g of sodium hydroxide was added gradually until completely dissolve. 36 g of sodium potassium tartrate were the added very slowly over 20-30 minutes period, with constant stirring. The mixture was left at room temperature. This preparation was done in a dark room.

d. Preparation of Alpha Amylase Enzyme

Alpha amylase from porcine pancreas was purchased from Sigma Aldrich Co. in lyophilized form (Type VI-B). According to the manufacturer's product description, one unit of enzyme will liberate 1.0 mg of maltose from starch in 3 minutes at pH 6.9, 20°C. Stock solution (1 mg/ml) was prepared by dissolving 100 mg of enzyme powder in 100 ml pre-chilled sodium phosphate buffer pH 6.9. The enzyme was then aliquot into 10 ml each in plastic centrifuge tubes, which has been fully covered with aluminium foil. For alpha amylase inhibitory assay, the stock solution was diluted to 0.5 mg/ml using the same buffer solution. The solution was placed in the ice bath during the experiment and prepared freshly prior to assay. For storage, the enzyme solution was stored at temperature -20°C prior to assay. [Note: The enzyme was sensitive and unstable furthermore need re-thaw is not preferable].

e. Preparation of Acarbose

Acarbose (G5003) was used as a positive control for alpha amylase inhibitory assay (Sigma Aldrich Co.), which in lyophilized form. For stock solution, 10 mg/ml of

acarbose was prepared. The stock solution was then diluted to a concentration that was required for alpha amylase inhibitory assay for acarbose and protein fraction, which were 25 μg/ml, while for crude sample extracts was 25 mg/ml. The stock solution was stored at -20°C refrigerator for further use.

1.3 Preparation of stock solutions for glycine SDS-PAGE system

a. Solution A: Monomer acrylamide and bisacrylamide (30.8 % T, 2.7 % Cbis)

30 g of acrylamide and 0.8 g of N, N'-Methylenebisacrylamide were weighted and dissolved in distilled water. The mixture left with stirring and after dissolved, topped up with distilled water to 100 ml. The solution was deionised using a small quantity of Amberlite IRN-150L and continued stirred for 1 hour. Amberlite IRN-150L is a homogenous mixture of strong cationic and strong anionic resins. It is used to remove trace ionic impurities from non-ionic acrylamide solution. The amberlite IRN-150L was then removed from the mixture by filtered and stored up to 3 months in a dark bottle. The solution was kept at 4°C.

b. Solution B: 4x running gel buffer (1.5 M Tris- HCl pH 8.8)

18.7 g of Tris base was weighted and dissolved then made up to 100 ml with distilled water. The pH was then adjusted to pH 8.8 using concentrated hydrochloric acid (HCl). The solution was kept at 4°C freezer.

c. Solution C: (10 % (w/v) SDS)

10 g of Sodium Dodecyl Sulfate (SDS) was dissolved and made up to 100 ml with distilled water. The solution was stored at room temperature.

d. TEMED (N, N, N, N'- Tetramethyl ethylenediamine)

TEMED was purchased from Sigma Aldrich Chemical Co. The reagent was directly used and needed to be stored at room temperature.

e. 10 % APS (10 % (w/v) ammonium persulphate)

10 g of APS was dissolved and made up to 100 ml with distilled water. The solution was prepared prior to use or prepared earlier and stored in aliquots. The solution was kept at -20°C freezer.

f. Solution D: Stacking gel buffer (0.5 M Tris – HCl, pH 6.8)

6.06 g of Tris base was dissolved and made up to 100 ml with distilled water. The pH was then adjusted to pH 6.8 using concentrated HCl. The solution was kept at 4°C freezer.

g. 4x SDS-PAGE sample buffer (0.1 % (w/v) Bromophenol Blue, 10 % (w/v) Glycerol, 2 % (w/v) SDS, 1 % (w/v) DTT 62.5 mM Tris – HCl pH 6.8)

Bromophenol blue (20 mg), 2.0 ml of Glycerol, 400 mg of SDS and 200 mg of DTT (dithiothreitol) were mixed together. Then 2.5 ml of solution D was added and made up to 20 ml with ddH_2O. The buffer was stored at -20°C in aliquots for further use.

h. Tank buffer or cathode buffer (25 mM Tris, 198 mM glycine, 0.1 % (w/v) SDS, pH 8.3)

3.03 g Tris base, 14.4 g Glycine and 1.0 g SDS were mixed and made up to 1 litre with distilled water. The solution was kept at room temperature. The buffer can be reused but make sure it was filtered and not contaminated.

1.4 Preparation of SDS-PAGE gels

1.4.1 Recipe for the preparation of separating gel with final concentration 16 %

Running gel final concentration	Volume for 16 % acrylamide gels
Solution A	10 680 µl
Solution B	5000 µl
Solution C	200 µl
Distilled water	4020 µl
* TEMED	6.6 µl
* 10 % APS	100 µl
Total Volume	20 ml

This recipe was used to prepare 0.75 mm thick gel. Final volume of (20ml) is enough to prepare 2 gels. *10 % APS and TEMED were freshly prepared and added prior to pouring into gel-casting apparatus.

1.4.2 Recipe for the preparation of stacking gel which final concentration 4 %

Running gel final concentration	Volume for 4 % acrylamide gels
Solution A	650 µl
Solution D	1250 µl
Solution C	50 µl
Distilled water	3050 µl
* TEMED	5 µl
* 10 % APS	25 µl
Total	5.3 ml

This recipe was used to prepare 0.75 mm thick gel. Final volume of (5 ml) is enough to prepare 2 gels.*10 % APS and TEMED were freshly prepared and added prior to pouring into gel-casting apparatus

1.5 Preparation of Stock Solution of Coomassie Staining:

a. Staining solution (0.1 % Coomassie Brilliant Blue R-250 in 10 % acetic acid and 40 % methanol)

1.0 g of Coomassie Brilliant Blue R-250 was added to 400 ml of methanol and stirred until dissolved. Then 100 ml of acetic acid were mixed and made up to 1 liter with distilled water. Filtering was not required. The solution was stored in a dark place.

b. De-staining solution (10 % Acetic acid)

100 ml of acetic acid was added to 900 ml of distilled water. The solution was kept at room temperature.

1.6 Preparation of Mobile Phase for High Performance Liquid Chromatograph (HPLC):

a. Preparation of pump A solution: 0.1 % Trifluoroacetic acid (TFA)

For preparation of 1 L the solution, 1 ml of Trifluoroacetic acid (TFA) HPLC grade was purchased from Fisher Scientific was added to 999 ml of distilled water. The solution mixed using a magnetic stirrer. Before use, the solution was filtered with Whatman cellulose filter membrane by using vacuum pump.

b. Preparation of pump B solution: 100 % Acetonitrile (ACN)

Acetonitrile (ACN) HPLC grade were purchased from Fisher Scientific. 1 L of ACN was measured and filtered using Whatman nylon membrane filter paper.

c. Methanol

Methanol with HPLC grade were purchased from Fisher Scientific was measured to 100 ml, transferred to a Schott Duran bottle and then furthered to a filtration by using Whatman nylon filter.

1.7 Method Development of High Performance Liquid Chromatogram (HPLC)

The mobile phase consisted of two solvent system; pump A- (0.1 % (v/v) trifluoroacetic acid (TFA) in water and pump B- (100 % acetonitrile). Prior to chromatography, the instrument with a column needs to be flushed with 95 % acetonitrile for 30 minutes at flow rate of 0.9 mL/min in isocratic flow. Then the column was flushed with 0 % of acetonitrile in pump B for 10 minutes. The filtered methanol was injected to the injector to rinse the loop for several times for contaminants removal.

For the baseline, the system was run at 0-90 % acetonitrile (pump B) for 40 minutes with binary gradient flow. The mobile phase solution was allowed to pass through the HPLC column until a stable baseline signal was equilibrated. After optimization, the system was set as A: 0.1 % trifluoroacetic acid (TFA) in water (v/v) and B: 100 % acetonitrile in a gradient (40 min, 0-95 % pump B) by binary gradient mobile composition.

For analytical run, the injection volume was 20 µl with 2 mg/ml concentration and the wavelength was monitored at 220 nm and 254 nm. All the systems, temperature and sample ID were set prior to the HPLC run.

The syringe filter was rinsed with 2-3 ml aliquots of methanol between each use. Sample was dissolved in distilled water and filtered with 13 mm, 0.45 µm syringe filter prior to chromatography. The manual injector was switched the to the “load”

position at least three 20 µl volumes of each sample were pushed through the loop, making certain each volume does not contain air bubbles.

With the syringe still in the injector, the knob was turned to the “inject” position, at which the instrument will automatically start data acquisition. The injector was left in the “inject” position, and the syringe was removed after 5 minutes. The column temperature was kept at 40°C and the flow rate were set at 1.0 mL/min.

For sample collection, the column was changed with semi preparative column and the concentration also remains as 2 mg/ml. The HPLC system was maintained. Methanol was used as to rinse a syringe and a loop with at least 3 volumes of the methanol and later dispensed into the waste beaker. At the end of the day of multiple runs, the column was washed for 30 minutes with 100 % acetonitrile to remove unbound components.

1.8 Preparation of Stock solutions for Silver Staining Protocols (MS Compatible)

a. Fixing solution (40 % (v/v) ethanol, 10 % (v/v) glacial acetic acid)

400 ml of ethanol and 100 ml of glacial acetic acid were mixed and made up to 1 litre with distilled water. The solution was kept in room temperature. The solution was freshly prepared before use.

b. Incubation/Sensitizing solution (30 % (v/v) ethanol, 6.8 % (w/v) sodium acetate, 0.2 % sodium thiosulfate)

300 ml of ethanol, 68 g of sodium acetate and 2 g of sodium thiosulphate* were mixed and made up to 1 litre with distilled water. The solution must be freshly prepared before use.

c. Silver solution (0.5 % (w/v) silver nitrate)

1 g of silver nitrate were mixed with 1 litre distilled water. The solution must be freshly prepared before use.

d. Developing solution (0.24 M sodium carbonate, 0.02 % (v/v) formaldehyde)

25 g of sodium carbonate and 100 µl of formaldehyde* were mixed and made up to 1 litre with distilled water. The solution must be freshly prepared before use.

e. Stop solution (400 mM EDTA-Na_2.2H_2O)

14.6 g of EDTA-Na_2.2H_2O were made up to 1 litre with distilled water. The solution was kept at room temperature.

* The material was added freshly prior to use

1.9 Preparation of Reagents Matrix-Assisted Laser Desorption/Ionization (MALDI) for In-Solution Digestion Protocol.

a. Digestion buffer (50 mM Ammonium bicarbonate)

10 mg of ammonium bicarbonate was weighed and dissolved in 2.5 ml of ddH_2O for a final concentration of ~50 mM. This solution can be stored at 4°C for up to two months.

b. Reducing solution (100 mM Dithiothreitol (DTT)

8 mg of DTT as weighed and dissolved with 500 µl of ddH2O for a final concentration ~100 mM. Reducing solution can be stored at -20 °C.

c. Alkylation solution

Alkylation solution was freshly prepared just before use. Iodoacetamide (IAA) (9 mg) was weighed and added to a foil-wrapped tube to avoid exposure of light. DdH_2O (500 µl) was added for a final concentration of ~100 mM. Excess solution cannot be stored.

d. Trypsin stock

Trypsin stock solution was prepared at concentration of 0.1 µg/ml . Two hundred µl of trypsin suspension buffer was added to 20 µg of trypsin. This solution was stored at -20°C for up to two months.

e. Protein concentrations

The procedure described was been found to work well with protein concentrations in the range 0.1-1.0 mg/ml. This concentration range allowed working with protein from 0.1 μg to 10 μg, respectively in 10 μl total volume for the digestions.

1.10 Preparation of Reagents and Procedure for Matrix-Assisted Laser Desorption/Ionization (MALDI) for In-Gel Digestion Protocol

a) Stock materials in the set

Stock materials	**Label**	**Storage**
Potassium ferricyanide $K_3Fe(CN)_6$	PF	Room temperature, in dark
Sodium thiosulfate	ST	Room temperature, in dark
50 mM Ammonium bicarbonate NH_4HCO_3	50 AB	Room temperature
10 mM Ammonium bicarbonate NH_4HCO_3	10 AB	Room temperature
Acetonitrile (HPLC grade)	100 % ACN	Room temperature, in dark
50 % Acetonitrile (HPLC grade)	50 % ACN	Room temperature, in dark
5 % Formic acid/ 50 % Acetonitrile	5 % FA / 50 % ACN	Room temperature, in dark
0.1 % Formic acid/ 50 % Acetonitrile	0.1 % FA / 50 % ACN	Room temperature, in dark
0.1 % Formic acid	0.1 % FA	Room temperature, in dark
Menthol (HPLC grade)	MeOH	Room temperature, in dark
100 mM Dithiothreitol	100mM DTT	-20ºC
Iodoacetamide	IAA	4ºC
Trypsin (Stock)	Trypsin	-20ºC
ZipTip uC-18 (10 μL)		Room temperature

b) Solutions prepared prior use

Chemicals	**Preparation**
30 mM Potassium ferricyanide $K_3Fe(CN)_6$	2 ml MilliQ water was added to the tube (can be stored at 4ºC for a few days)
100 mM Sodium thiosulfate	2 ml MilliQ water was added to the tube (can be stored at 4ºC for a few days)
Silver Destaining solution	"**PF**" and "**ST**" were mixed in 1:1 ratio (Note: This solution must be freshly prepared prior use, and cannot be re-used)

10 mM Dithiothreitol DTT	100 mM DTT was diluted 10 times using 10 mM Ammonium bicarbonate (Note: This solution must be freshly prepared prior use, and cannot be re-used)
55 mM Iodoacetamide IAA	1 ml 10 mM NH_4HCO_3 was added to the tube (Note: This solution must be freshly prepared prior use, and cannot be re-used)
Trypsin working solution 12.5 ng/ µl	1 µl stock solution + 79 µL 10 mM NH_4HCO_3 (Note: This solution must be freshly prepared prior use, and cannot be re-used)

c) Procedure

A. Gel piece destaining and washing:

(1) Destainingvof silver stained gel

1. Gel band or spot was cut into smaller pieces.

2. Gel pieces was covered with ~150-200 µl de-staining solution, incubated at room temperature 15 min or until brownish colour was removed. Further to short spun, and the solution were removed. The gel was washed briefly with 300 µl MilliQ and the solution was removed.

3. The gel pieces were washed with 300 µl MilliQ for 10 min, to remove de-staining solution residual. The procedure was repeated.

4. The gel pieces were washed with 200 µl 50 % ACN for 15 min and the solution was removed. The procedure was repeated.

5. The gel was shrank by adding 150 µl 100 % ACN and briefly vortexed. The solution was removed.

6. Gel was re-hydrated in 50 µl 10 mM NH_4HCO_3 for 5-10 min. The solution was removed.

7. The gel was shrank by adding ~150-200 µl of 100 % ACN 5-10 min. Further to short spin and the solution was removed.

8. The shrank gel was dried in Speed Vac. for 5 min at < 30ºC. (Gel can be stored at 20ºC if required).

(2) Reduction and Alkylation for 1-D gel

1. 50 µl or sufficient amount of freshly prepared 10 mM DTT/ 10 mM NH_4HCO_3 was added to cover gel pieces and incubated at 56ºC for 40-60 min.

2. The tubes were chilled to RT. Further to short spin and DTT solution was removed.

3. The same volume of freshly prepared 55 mM IAA / 10 mM NH_4HCO_3 were added. Incubated at room temp (in DARK) for 30-45 min.

4. Further to short spin and IAA solution was removed at once.

5. 300 µl MilliQ was used to wash gel for 10 min. The solution was removed and the procedure was repeated.

6. 200 µl 50 % ACN was added to wash gel for 15 min. The solution was removed and the procedure was repeated.

7. 150 µl 100 % ACN was added to shrink the gel, and the tube was briefly vortexed. The solution was removed.

8. The gel was re-hydrated in 50 µl 10 mM NH_4HCO_3 for 5-10 min.

9. 50 µl 100 % ACN was added to further equilibrate gel for 15 min more. Short spin and the solution were removed.

10. The gel was shrank by adding ~150-200 µl ACN. Short spin and the solution was removed.

11. The shrunk gel was dried in Speed Vac. for 10-15 min at < 30ºC.

B. In Gel-digestion:

1. 15-20 µl (depends on gel size, volume that adequate to cover the gel piece) of 12.5 ng/µl trypsin working solution was added to samples, or add until gel pieces are covered by the trypsin working solution.

2. The samples were pre-incubated for 30-40 min at 4ºC to ensure complete re-swelling of gel pieces. If the gel pieces appear white or opaque after pre-incubation, additional 10 µl was added and incubated for another 30 min.

3. The remaining trypsin supernatant was removed and 15-20 µl (depends on gel size, volume that adequate to cover the gel piece) of ice-cold 10 mM NH_4HCO_3 was added to keep the gel wet during the enzymatic cleavage.

4. The gel was incubated for 14-16 hours at 37ºC.

C. Peptides Extraction:

1. The sample tubes/ plate has been a short spun.

2. The solution was extracted in a new tube (extracts).

3. The gel was extracted again by adding 20 µl 5 % formic acid: 50 % ACN (= 1:1 volume of 10 % formic acid + 100 % ACN), vortex or mixing in the shaker for 15 min. the removed solution were all recovered in the extracts tube. The procedure was repeated once.

4. The gel was extracted again with 20 µl 100 % ACN.

5. The solution was recovered in the extracts tube.

6. The solution was concentrated to ~10-20 µl, in Speed Vac for ~ 1 to 1.5 hour at room temp (*do not > 30ºC). The solution was prevented from totally dried out. If the solution dried out, recovered peptides by adding ~ 15 µl 0.1 % FA with sonication for 1 min.

7. The recovered peptides are ready for further processing or store < -20°C.

D. Zip Tip Protocol

1. The tip was wet by with 10 µl 50 % ACN /0.1 % FA for 5 times.

2. The tip was washed again with 10 µl of 0.1 % FA for 5 times.

3. The sample was filled into tip by pipetting fully into and out of tip >30 times. The liquid was expelled.

4. The tip was washed with 10 µl 0.1 % FA to remove contaminants for 3 times.

5. The peptides was eluted in 4 µl 50 % ACN /0.1 % FA by pipetting fully into and out of tip >30 times in new tube.

6. The eluted peptides can be stored < -20°C for further use.

Note: During the Zip-Tip procedure, please be careful and do not introduce any air bubbles as this may block the c18 mini-column at the tip-end.

APPENDIX B: EXPERIMENTAL DATA

1.0 *In-vitro* Screening of Solvent Extracts and Protein Fractions from *P. pulmonarius* Basidiocarps for Anti-diabetic Enzymes Inhibitory Assays

Table 1.1: *In-vitro* Screening for *Saccharomyces cerevisiae* Alpha Glucosidase Inhibitory Activity Assay

a) Crude extracts at 25 mg/ml concentration

Samples	Replicates			Average	Percentage of inhibition (%)
	1	2	3		
Voglibose	1.329	1.44	1.433	1.4	5.23
Crude aqueous extract	0.457	0.524	0.427	0.46	73.50
Polysaccharide extract	0.256	0.27	0.274	0.277	85.82
Methanol extract	1.752	1.693	1.668	1.70	2.61
Dichloromethane extract	0.158	0.142	0.2	0.17	91.13

b) Protein fractions at 25 µg/ml concentration

Samples	Replicates			Average	Percentage of inhibition (%)
	1	2	3		
Voglibose	1.329	1.64	1.433	1.47	5
F10	1.85	1.896	1.81	1.85	1
F20	2.223	2.087	2.16	2.16	0
F30	1.45	1.505	1.346	1.43	24
F40	1.801	1.712	1.758	1.76	7
F50	1.604	1.797	1.876	1.76	6
F60	1.578	1.446	1.5	1.51	20
F70	1.856	1.675	1.723	1.75	7
F80	1.576	1.55	1.547	1.56	17
F90	1.58	1.588	1.577	1.58	16
F100	1.936	1.95	1.959	1.95	0

Table 1.2: *In-vitro* Screening for Porcine Pancreatic Alpha Amylase Inhibitory Activity Assay

a) Crude extracts at 25 mg/ml concentration

Samples	Replicates			Average	Percentage of inhibition (%)
	1	2	3		
Acarbose	0.321	0.351	0.336	0.336	20.90
Crude aqueous extract	0.186	0.244	0.265	0.23	87.74
Polysaccharide extract	0.967	1.014	0.962	0.98	48.10
Methanol extract	1.066	1.089	1.092	1.08	42.73
Dichloromethane exract	1.336	1.311	1.332	1.33	29.82

b) Protein fractions at 25 µg/ml concentration

Samples	Replicates			Average	Percentage of inhibition (%)
	1	2	3		
Acarbose	0.321	0.351	0.336	0.336	20.9
F10	0.377	0.377	0.392	0.382	10.1
F20	0.35	0.379	0.37	0.366	13.8
F30	0.39	0.376	0.396	0.387	8.9
F40	0.397	0.381	0.367	0.381	10.4
F50	0.376	0.364	0.321	0.357	16.0
F60	0.351	0.341	0.344	0.345	18.7
F70	0.292	0.271	0.317	0.293	31.0
F80	0.393	0.415	0.303	0.370	12.9
F90	0.339	0.355	0.327	0.302	29.0
F100	0.221	0.256	0.265	0.247	41.8

2.0 Bioassay-guided Purification of Selected Active $(NH_4)_2SO_4$ Protein Fractions by Reverse Phase High Performance Liquid Chromatography (RP-HPLC)

Table 2.1: Alpha glucosidase inhibitory assay of fractionated protein fractions

Samples (Protein fraction)	Replicates			Average	Percentage of inhibition (%)
	1	2	3		
Voglibose	1.567	1.72	1.799	1.70	13
Peak 1	1.548	1.758	1.575	1.63	17
Peak 2	1.715	1.386	1.673	1.59	18
Peak 3	1.5	1.4	1.46	1.46	25
Peak 4	1.557	1.537	1.447	1.51	22
Peak 5	1.441	1.61	1.806	1.62	17
Peak 6	1.577	1.53	1.623	1.58	19
Peak 7	1.632	1.656	1.731	1.67	14
Peak 8	1.602	1.552	1.382	1.51	22
Peak 9	1.51	1.651	1.61	1.59	18
Peak 10	1.625	1.555	1.67	1.62	17

Table 2.2: Alpha amylase inhibitory assay of fractionated protein fractions

Samples (Protein fraction)	Replicates			Average	Percentage of inhibition (%)
	1	2	3		
Acarbose	1.941	1.931	1.905	1.926	2.051542896
Peak 1	1.905	2.016	1.984	1.968	0
Peak 2	2.081	2.005	1.969	2.018	0
Peak 3	1.864	1.95	1.916	1.910	2.848423194
Peak 4	1.94	1.945	1.95	1.945	1.068158698
Peak 5	1.909	1.95	1.954	1.938	1.441166497
Peak 6	1.959	1.964	1.969	1.964	0.1017294

APPENDIX C: ONE-WAY ANOVA analysis

1.0 *In-vitro* Screening of Solvent Extracts and Protein Fractions from *P. pulmonarius* Basidiocarps for Anti-diabetic Enzymes Inhibitory Assays

1.1. *In-vitro* Screening for *Saccharomyces cerevisiae* Alpha Glucosidase Inhibitory Activity Assay

1.1.1. The statistical data of one-way ANOVA on percentage inhibition of *Saccharomyces cerevisiae* alpha glucosidase assay activity by aqueous, polysaccharide, methanol and dichloromethane extracts at 25 mg/ml.

```
One-way ANOVA: Percentage of inhibition versus crude extracts (mg/ml)

Source  DF       SS       MS       F      P
C1       4  5.92051  1.48013  806.37  0.000
Error   10  0.01836  0.00184
Total   14  5.93887
S = 0.04284   R-Sq = 99.69 %   R-Sq(adj) = 99.57 %

Individual 95 % CIs For Mean Based on Pooled StDev
Level  N    Mean   StDev  --------+---------+---------+---------+-

1      3  1.4007  0.0622                            (*)

2      3  0.4693  0.0497        (*)
3      3  0.2667  0.0095    (*)
4      3  1.7043  0.0431                                  (*)

5      3  0.1667  0.0300  (*)
--------+---------+---------+---------+-
0.50      1.00      1.50      2.00

Pooled StDev = 0.0428
```

Level indicates
1: voglibose
2: aqueous extracts
3: polysaccharide extracts
4: methanol extracts
5: dicholoromethane extracts

1.1.2. The statistical data of one-way ANOVA on percentage inhibition of *Saccharomyces cerevisiae* alpha glucosidase activity assay by $(NH_4)_2SO_4$ protein fractions (F10-100) samples and voglibose at 25 µg/ml protein concentration

```
One-way ANOVA: Percentage of inhibition versus protein fractions (mg/ml)

Source  DF      SS     MS      F      P
C1      10  2034.0  203.4  12.55  0.000
Error   22   356.6   16.2
Total   32  2390.6
S = 4.026   R-Sq = 85.08 %   R-Sq(adj) = 78.31 %

Individual 95 % CIs For Mean Based on Pooled StDev
Level  N    Mean  StDev  ------+---------+---------+---------+---

 1     3  19.333  8.622                          (-----*-----)

 2     3   0.167  0.289  (-----*-----)
 3     3   0.000  0.000  (-----*-----)
 4     3  21.000  4.583                            (-----*-----)

 5     3   3.467  2.501      (-----*-----)
 6     3   4.400  6.528       (------*-----)
 7     3  17.333  4.041                       (-----*-----)

 8     3   4.333  4.041       (-----*-----)
 9     3  14.333  1.155                     (-----*-----)

10     3  13.000  0.000                   (-----*-----)

11     3   0.000  0.000  (-----*-----)
                         ------+---------+---------+---------+---

                             0.0       8.0      16.0      24.0

Pooled StDev = 4.026
```

Level indicates

1: voglibose
2: F10
3: F20
4: F30
5: F40
6: F50
7: F60
8: F70
9: F80
10: F90
11: F100

1.2. *In-vitro* Screening for Porcine Pancreatic Alpha Amylase Inhibitory Activity Assay

1.2.1. The statistical data of one-way ANOVA on percentage inhibition of porcine pancreatic alpha amylase activity by crude aqueous, polysaccharide, methanol and dichloromethane extracts at 25 mg/ml

```
One-way ANOVA: Percentage of inhibition versus crude extracts (mg/ml)

Source  DF       SS       MS       F      P
C1       4  7365.04  1841.26  541.04  0.000
Error   10    34.03     3.40
Total   14  7399.07
S = 1.845   R-Sq = 99.54 %   R-Sq(adj) = 99.36 %

Individual 95 % CIs For Mean Based on Pooled StDev
Level  N    Mean  StDev  ---------+---------+---------+---------+

1      3  25.000  3.000  (*)
2      3  87.767  2.178                                    (*)
3      3  48.083  1.509             (*)
4      3  42.733  0.757           (*)
5      3  29.967  0.651     (*)
                         ---------+---------+---------+---------+

                                  40        60        80       100

Pooled StDev = 1.845
```

Level indicates
1: acarbose
2: aqueous extracts
3: polysaccharide extracts
4: methanol extracts
5: dicholoromethane extracts

1.2.2. The statistical data of one-way ANOVA on inhibition of porcine pancreatic alpha amylase activity by 10 %-100 % protein fractions and acarbose at 25 µg/ml

```
One-way ANOVA: Percentage of inhibition versus protein fractions (mg/ml)

Source  DF      SS     MS     F      P
C1      10  2789.4  278.9  9.46  0.000
Error   22   648.7   29.5
Total   32  3438.1
S = 5.430   R-Sq = 81.13 %   R-Sq(adj) = 72.56 %
Individual 95 % CIs For Mean Based on Pooled StDev
Level  N    Mean   StDev  ----+---------+---------+---------+-----

 1     3  25.000   3.000           (-----*----)
 2     3  14.667   2.309   (----*-----)
 3     3  18.000   3.606      (----*----)
 4     3  13.333   2.517  (----*-----)
 5     3  14.667   3.512   (----*-----)
 6     3  21.000   6.245        (-----*----)
 7     3  22.667   1.528         (-----*----)
 8     3  35.000   5.000                    (----*-----)
 9     3  17.000  13.229     (----*-----)
10     3  24.000   3.000           (----*----)
11     3  45.000   5.292                          (-----*----)

                         ----+---------+---------+---------+-----

                            12        24        36        48

Pooled StDev = 5.430
```

Level indicates

1: acarbose
2: F10
3: F20
4: F30
5: F40
6: F50
7: F60
8: F70
9: F80
10: F90
11: F100

1.3. Bioassay-guided Purification of Selected Active $(NH_4)_2SO_4$ Protein Fractions by Reverse Phase High Performance Liquid Chromatography (RP-HPLC).

1.3.1. The statistical data of one-way ANOVA on Alpha Glucosidase Inhibitory Assay of fractionated protein fraction

```
One-way ANOVA: Percentage of inhibition versus protein fractions (mg/ml)

Source  DF      SS     MS      F      P
C1      10  849.32  84.93  26.52  0.000
Error   22   70.46   3.20
Total   32  919.78

S = 1.790   R-Sq = 92.34 %   R-Sq(adj) = 88.86 %
Individual 95 % CIs For Mean Based on Pooled StDev
Level  N    Mean  StDev  -------+---------+---------+---------+--
 1     3  10.000  2.000  (---*--)
 2     3  20.200  0.721                   (---*--)
 3     3  13.690  1.078        (---*--)
 4     3  25.860  2.583                            (--*---)
 5     3  22.777  2.984                        (---*---)
 6     3  27.597  0.981                             (---*---)
 7     3  19.547  2.350                    (---*--)
 8     3  16.313  0.696             (--*---)
 9     3  15.913  2.108            (---*--)
10     3  22.267  1.184                        (--*---)
11     3  15.897  1.076            (--*---)
                          -------+---------+---------+---------+--
                              12.0      18.0      24.0      30.0

Pooled StDev = 1.790
```

Level indicates
1: voglibose
2: F10
3: F20
4: F30
5: F40
6: F50
7: F60
8: F70
9: F80
10: F90
11: F100

1.3.2. The statistical data of one-way ANOVA on Alpha Amylase Inhibitory Assay of fractionated protein fraction

```
One-way ANOVA: Percentage of inhibition versus protein fractions (mg/ml)

Source  DF       SS      MS       F      P
C1       6  5328.05  888.01  757.03  0.000
Error   14    16.42    1.17
Total   20  5344.48

S = 1.083   R-Sq = 99.69 %   R-Sq(adj) = 99.56 %

Individual 95 % CIs For Mean Based on Pooled StDev

Level  N    Mean  StDev  -+---------+---------+---------+--------
1      3   1.233  0.410   (*)
2      3   2.180  1.888    *)
3      3  46.567  2.043                                  (*)
4      3   0.673  0.356   *)
5      3   1.269  0.254   (*)
6      3   0.790  0.225   (*
7      3   0.301  0.250  (*)
                         -+---------+---------+---------+--------
                          0        15        30        45

Pooled StDev = 1.083
```

Level indicates
1: acarbose
2: F10
3: F20
4: F30
5: F40
6: F50
7: F60
8: F70
9: F80
10: F90
11: F100

APPENDIX D: MALDI-TOF/TOF MS ANALYSIS DATA

1.0 MALDI-TOF/TOF MS Analysis of Five Protein Bands Obtained From Active Peak 3-RPF Silver-Stained Electrophoregram by In-Gel Digestion Protocol.

Analysis Information

Report Type	Protein-Peptide Summary by Spot	Analysis Type	Combined (MS+MS/MS)
Sample Set Name	2012-03-29 [GRC-001-2012-02-17-1]	Database	NCBInr
Analysis Name	MedicineUofM-TanEngChong[Lentus NCBI]	Creation Date	03/30/2012 14:58:45
Reported By	03/30/2012 15:30:10 - admin	Last Modified	03/30/2012 15:24:22
MS Acq. : Proc. Methods	(Unspecified) : (Unspecified)		
Interpretation Method	(Unspecified)		

Gel Idx/Pos	109/E8	Instr./Gel Origin	Ak115/2012-02-17-1	Process Status	Analysis Succeeded
Plate [#] Name	[1] GRC-001	Instrument Sample Name		Spectra	6

Rank	Protein Name	Accession No.	Protein Score	Protein Score C. I. %	Total Ion Score	Total Ion C. I. %	Protein MW	Protein PI	Spot Name	Pep. Count	User name	Department
1	RecName: Full=Trehalose phosphorylase; AltName: Full=Trehalose synthase; Short=TSase; Flags: Precur	gi|74626081	18	60.973	10	99.549	83999.8	6.38	A1	4	Tan Eng Chong	Medicine, U of Malaya

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. %	Modification	Rank	Result Type
929.505	929.5025	-0.0025	-3	737	744	EGETKLPR	10	99.549			Mascot
929.505	929.5025	-0.0025	-3	737	744	EGETKLPR					Mascot
1172.5919	1172.631	0.0391	33	486	495	GSHLNRGEFR					Mascot
1232.6093	1232.5973	-0.012	-10	586	595	EYAHDIVVMR					Mascot
1320.639	1320.6016	-0.0374	-28	204	215	TLDEQSDSAARK					Mascot

Rank	Protein Name	Accession No.	Protein Score	Protein Score C. I. %	Total Ion Score	Total Ion C. I. %	Protein MW	Protein PI	Spot Name	Pep. Count	User name	Department
2	RecName: Full=Glyceraldehyde-3-phosphate dehydrogenase; Short=GAPDH	gi|30580405	11	0			36156.7	6	A1	3	Tan Eng Chong	Medicine, U of Malaya

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. %	Modification	Rank	Result Type
904.4734	904.4702	-0.0032	-4	56	64	GSIEAKDGK					Mascot
997.5425	997.5554	0.0129	13	106	115	ASAHLKGGEK					Mascot
1179.5939	1179.6183	0.0244	21	1	11	MVNVGINGFGR			Oxidation (M)[1]		Mascot

Rank	Protein Name	Accession No.	Protein Score	Protein Score C. I. %	Total Ion Score	Total Ion C. I. %	Protein MW	Protein PI	Spot Name	Pep. Count	User name	Department
3	HSP100 [Lentinus sajor-caju]	gi|62998570	9	0			99394	5.71	A1	6	Tan Eng Chong	Medicine, U of Malaya

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. %	Modification	Rank	Result Type

Analysis Information

Report Type	Protein-Peptide Summary by Spot	Analysis Type	Combined (MS+MS/MS)
Sample Set Name	2012-03-29 [GRC-001-2012-02-17-1]	Database	NCBInr
Analysis Name	MedicineUofM-TanEngChong[Lentus NCBI]	Creation Date	03/30/2012 14:58:45
Reported By	03/30/2012 15:30:11 - admin	Last Modified	03/30/2012 15:24:22
MS Acq. : Proc. Methods	(Unspecified) : (Unspecified)		
Interpretation Method	(Unspecified)		

	929.5713	929.5025	-0.0688	-74	209	215	VIRILCR				Carbamidomethyl (C)[6]			Mascot	
	929.5713	929.5025	-0.0688	-74	209	215	VIRILCR				Carbamidomethyl (C)[6]			Mascot	
	958.5567	958.5109	-0.0458	-48	536	543	IEQLEAKK							Mascot	
	972.5261	972.4604	-0.0657	-68	569	576	WTNIPVSR							Mascot	
	1045.5636	1045.5784	0.0148	14	232	241	TSIAEGLAQR							Mascot	
	1179.6157	1179.6183	0.0026	2	526	535	FYAVPEVQAR							Mascot	
	1664.849	1664.7787	-0.0703	-42	168	182	IESKTAEQGFDALQK							Mascot	
4	WUN [Lentinus sajor-caju]				gi|6018116		8	0		5174.7	10.43	A1	1	Tan Eng Chong	Medicine, U of Malaya
	Peptide Information														
	Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence			Ion Score	C. I. %	Modification		Rank	Result Type
	1016.5306	1016.5024	-0.0282	-28	23	30	LDICSPRR					Carbamidomethyl (C)[4]			Mascot
5	RNA polymerase II second largest subunit [Lentinus sajor-caju]				gi|194306409		7	0		33383	8.34	A1	3	Tan Eng Chong	Medicine, U of Malaya
	Peptide Information														
	Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence			Ion Score	C. I. %	Modification		Rank	Result Type
	997.5425	997.5554	0.0129	13	35	43	HNTITNGLK								Mascot
	998.5604	998.5797	0.0193	19	1	7	LFRMLFR					Oxidation (M)[4]			Mascot
	998.5604	998.5797	0.0193	19	1	7	LFRMLFR					Oxidation (M)[4]			Mascot
	1131.5575	1131.4962	-0.0613	-54	83	92	CNTPLGREGK					Carbamidomethyl (C)[1]			Mascot
6	RecName: Full=Antimicrobial ribonuclease				gi|71153693		6	0		1015.5	6.13	A1	1	Tan Eng Chong	Medicine, U of Malaya
	Peptide Information														
	Calc. Mass	Obsrv. Mass	± da	± ppm	Start	End	Sequence			Ion	C. I. %	Modification		Rank	Result Type

Analysis Information

Report Type	Protein-Peptide Summary by Spot	Analysis Type	Combined (MS+MS/MS)
Sample Set Name	2012-03-29 [GRC-001-2012-02-17-1]	Database	NCBInr
Analysis Name	MedicineUofM-TanEngChong[Lentus NCBI]	Creation Date	03/30/2012 14:58:45
Reported By	03/30/2012 15:30:11 - admin	Last Modified	03/30/2012 15:24:22
MS Acq. : Proc. Methods	(Unspecified) : (Unspecified)		
Interpretation Method	(Unspecified)		

						Seq.	Seq.			Score						
	1016.4868	1016.5024	0.0156	15	1	10	DNGEAGRAAR								Mascot	
7	heat-induced catalase [Lentinus sajor-caju]				gi|13183346			5	0		60039	6.77	A1	2	Tan Eng Chong	Medicine, U of Malaya
	Peptide Information															
	Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence			Ion Score	C. I. %	Modification			Rank	Result Type
	904.4417	904.4702	0.0285	32	413	419	NQERMAR								Mascot	
	1111.5314	1111.5563	0.0249	22	518	528	CNIGGPPGPSR					Carbamidomethyl (C)[1]			Mascot	
8	laccase 3 [Lentinus sajor-caju]				gi|11036960			5	0		18143.7	5.23	A1	1	Tan Eng Chong	Medicine, U of Malaya
	Peptide Information															
	Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence			Ion Score	C. I. %	Modification			Rank	Result Type
	1529.7383	1529.6859	-0.0524	-34	86	98	GVFVVYDPEDPHR								Mascot	
9	translation elongation factor 1 alpha [Lentinus sajor-caju]				gi|170517001			4	0		20207.6	7.07	A1	1	Tan Eng Chong	Medicine, U of Malaya
	Protein Group															
	translation elongation factor 1 alpha [Lentinus sajor-caju]				gi|170516999						20175.6	7.9800 000190 7349				
	Peptide Information															
	Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence			Ion Score	C. I. %	Modification			Rank	Result Type
	966.5255	966.4963	-0.0292	-30	115	122	ETSNFIKK								Mascot	

Analysis Information

Report Type	Protein-Peptide Summary by Spot	Analysis Type	Combined (MS+MS/MS)
Sample Set Name	2012-03-29 [GRC-001-2012-02-17-1]	Database	NCBInr
Analysis Name	MedicineUofM-TanEngChong[Lentus NCBI]	Creation Date	03/30/2012 14:58:45
Reported By	03/30/2012 15:30:11 - admin	Last Modified	03/30/2012 15:24:22

MS Acq. : Proc. Methods (Unspecified) : (Unspecified)
Interpretation Method (Unspecified)

Gel Idx/Pos	110/E9	Instr./Gel Origin	Ak115/2012-02-17-1	Process Status	Analysis Succeeded
Plate [#] Name	[1] GRC-001	Instrument Sample Name		Spectra	3

Rank	Protein Name	Accession No.	Protein Score	Protein Score C. I. %	Total Ion Score	Total Ion C. I. %	Protein MW	Protein PI	Spot Name	Pep. Count	User name	Department
1	HSP100 [Lentinus sajor-caju]	gi|62998570	14	0			99394	5.71	A2	4	Tan Eng Chong	Medicine, U of Malaya

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. %	Modification	Rank	Result Type
929.5713	929.5075	-0.0638	-69	209	215	VIRILCR			Carbamidomethyl (C)[6]		Mascot
951.4815	951.4728	-0.0087	-9	577	584	LMSSEKEK					Mascot
1045.5636	1045.5819	0.0183	18	232	241	TSIAEGLAQR					Mascot
1179.6157	1179.6185	0.0028	2	526	535	FYAVPEVQAR					Mascot

2	RecName: Full=Glyceraldehyde-3-phosphate dehydrogenase; Short=GAPDH	gi|30580405	13	0			36156.7	6	A2	2	Tan Eng Chong	Medicine, U of Malaya

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. %	Modification	Rank	Result Type
904.4734	904.4762	0.0028	3	56	64	GSIEAKDGK					Mascot
1179.5939	1179.6185	0.0246	21	1	11	MVNVGINGFGR			Oxidation (M)[1]		Mascot

3	RNA polymerase II second largest subunit [Lentinus sajor-caju]	gi|194306409	6	0			33383	8.34	A2	1	Tan Eng Chong	Medicine, U of Malaya

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. %	Modification	Rank	Result Type
1065.6163	1065.5663	-0.05	-47	235	243	HIENLVRGK					Mascot

Analysis Information

Report Type	Protein-Peptide Summary by Spot	Analysis Type	Combined (MS+MS/MS)
Sample Set Name	2012-03-29 [GRC-001-2012-02-17-1]	Database	NCBInr
Analysis Name	MedicineUofM-TanEngChong[Lentus NCBI]	Creation Date	03/30/2012 14:58:45
Reported By	03/30/2012 15:30:12 - admin	Last Modified	03/30/2012 15:24:22
MS Acq. : Proc. Methods	(Unspecified) : (Unspecified)		
Interpretation Method	(Unspecified)		

4	catalase [Lentinus sajor-caju]	gi|28558774	5	0	43768.6	6.19	A2	1	Tan Eng Chong	Medicine, U of Malaya

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. %	Modification	Rank	Result Type
904.5284	904.4762	-0.0522	-58	87	94	LLCSVGKK			Carbamidomethyl (C)[3]		Mascot

5	heat-induced catalase [Lentinus sajor-caju]	gi|13183346	5	0	60039	6.77	A2	1	Tan Eng Chong	Medicine, U of Malaya

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. %	Modification	Rank	Result Type
904.4417	904.4762	0.0345	38	413	419	NQERMAR					Mascot

6	RecName: Full=Trehalose phosphorylase; AltName: Full=Trehalose synthase; Short=TSase; Flags: Precur	gi|74626081	4	0	83999.8	6.38	A2	1	Tan Eng Chong	Medicine, U of Malaya

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. %	Modification	Rank	Result Type
929.505	929.5075	0.0025	3	737	744	EGETKLPR					Mascot

Analysis Information

Report Type	Protein-Peptide Summary by Spot	Analysis Type	Combined (MS+MS/MS)
Sample Set Name	2012-03-29 [GRC-001-2012-02-17-1]	Database	NCBInr
Analysis Name	MedicineUofM-TanEngChong[Lentus NCBI]	Creation Date	03/30/2012 14:58:45
Reported By	03/30/2012 15:30:12 - admin	Last Modified	03/30/2012 15:24:22
MS Acq. : Proc. Methods	(Unspecified) : (Unspecified)		
Interpretation Method	(Unspecified)		

Gel Idx/Pos	111/E10	Instr./Gel Origin	Ak115/2012-02-17-1	Process Status	Analysis Succeeded
Plate [#] Name	[1] GRC-001	Instrument Sample Name		Spectra	2

Rank	Protein Name	Accession No.	Protein Score	Protein Score C. I. %	Total Ion Score	Total Ion C. I. %	Protein MW	Protein PI	Spot Name	Pep. Count	User name	Department
1	RNA polymerase II second largest subunit [Lentinus sajor-caju]	gi|194306409	8	0			33383	8.34	A3	1	Tan Eng Chong	Medicine, U of Malaya

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. %	Modification	Rank	Result Type
1036.6011	1036.608	0.0069	7	234	241	RHIENLVR					Mascot

Rank	Protein Name	Accession No.	Protein Score	Protein Score C. I. %	Total Ion Score	Total Ion C. I. %	Protein MW	Protein PI	Spot Name	Pep. Count	User name	Department
2	RecName: Full=Glyceraldehyde-3-phosphate dehydrogenase; Short=GAPDH	gi|30580405	8	0			36156.7	6	A3	1	Tan Eng Chong	Medicine, U of Malaya

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. %	Modification	Rank	Result Type
904.4734	904.4921	0.0187	21	56	64	GSIEAKDGK					Mascot

Rank	Protein Name	Accession No.	Protein Score	Protein Score C. I. %	Total Ion Score	Total Ion C. I. %	Protein MW	Protein PI	Spot Name	Pep. Count	User name	Department
3	RecName: Full=Trehalose phosphorylase; AltName: Full=Trehalose synthase; Short=TSase; Flags: Precur	gi|74626081	8	0	2	89.7	83999.8	6.38	A3	1	Tan Eng Chong	Medicine, U of Malaya

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. %	Modification	Rank	Result Type
929.505	929.5153	0.0103	11	737	744	EGETKLPR	2	89.7			Mascot
929.505	929.5153	0.0103	11	737	744	EGETKLPR					Mascot

Rank	Protein Name	Accession No.	Protein Score	Protein Score C. I. %	Total Ion Score	Total Ion C. I. %	Protein MW	Protein PI	Spot Name	Pep. Count	User name	Department
4	catalase [Lentinus sajor-caju]	gi|28558774	7	0			43768.6	6.19	A3	1	Tan Eng Chong	Medicine, U of Malaya

Analysis Information

Report Type	Protein-Peptide Summary by Spot	Analysis Type	Combined (MS+MS/MS)
Sample Set Name	2012-03-29 [GRC-001-2012-02-17-1]	Database	NCBInr
Analysis Name	MedicineUofM-TanEngChong[Lentus NCBI]	Creation Date	03/30/2012 14:58:45
Reported By	03/30/2012 15:30:12 - admin	Last Modified	03/30/2012 15:24:22
MS Acq. : Proc. Methods	(Unspecified) : (Unspecified)		
Interpretation Method	(Unspecified)		

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. %	Modification	Rank	Result Type
904.5284	904.4921	-0.0363	-40	87	94	LLCSVGKK			Carbamidomethyl (C)[3]		Mascot

5	HSP100 [Lentinus sajor-caju]	gi|62998570	7	0	99394	5.71	A3	2	Tan Eng Chong	Medicine, U of Malaya

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. %	Modification	Rank	Result Type
929.5713	929.5153	-0.056	-60	209	215	VIRILCR			Carbamidomethyl (C)[6]		Mascot
929.5713	929.5153	-0.056	-60	209	215	VIRILCR			Carbamidomethyl (C)[6]		Mascot
1045.5636	1045.5952	0.0316	30	232	241	TSIAEGLAQR					Mascot

6	heat-induced catalase [Lentinus sajor-caju]	gi|13183346	6	0	60039	6.77	A3	1	Tan Eng Chong	Medicine, U of Malaya

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. %	Modification	Rank	Result Type
904.5284	904.4921	-0.0363	-40	87	94	LLCSVGKK			Carbamidomethyl (C)[3]		Mascot

Analysis Information

Report Type	Protein-Peptide Summary by Spot	Analysis Type	Combined (MS+MS/MS)
Sample Set Name	2012-03-29 [GRC-001-2012-02-17-1]	Database	NCBInr
Analysis Name	MedicineUofM-TanEngChong[Lentus NCBI]	Creation Date	03/30/2012 14:58:45
Reported By	03/30/2012 15:30:12 - admin	Last Modified	03/30/2012 15:24:22

MS Acq. : Proc. Methods (Unspecified) : (Unspecified)
Interpretation Method (Unspecified)

Gel Idx/Pos	112/E11	**Instr./Gel Origin**	Ak115/2012-02-17-1	**Process Status**	Analysis Succeeded
Plate [#] Name	[1] GRC-001	**Instrument Sample Name**		**Spectra**	6

Rank	Protein Name	Accession No.	Protein Score	Protein Score C. I. %	Total Ion Score	Total Ion C. I. %	Protein MW	Protein PI	Spot Name	Pep. Count	User name	Department
1	RecName: Full=Trehalose phosphorylase; AltName: Full=Trehalose synthase; Short=TSase; Flags: Precur	gi|74626081	32	98.132	32	99.993	83999.8	6.38	A4	1	Tan Eng Chong	Medicine, U of Malaya

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. %	Modification	Rank	Result Type
929.505	929.5182	0.0132	14	737	744	EGETKLPR	32	99.993			Mascot
929.505	929.5182	0.0132	14	737	744	EGETKLPR					Mascot

Rank	Protein Name	Accession No.	Protein Score	Protein Score C. I. %	Total Ion Score	Total Ion C. I. %	Protein MW	Protein PI	Spot Name	Pep. Count	User name	Department
2	RecName: Full=Glyceraldehyde-3-phosphate dehydrogenase; Short=GAPDH	gi|30580405	11	0			36156.7	6	A4	2	Tan Eng Chong	Medicine, U of Malaya

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. %	Modification	Rank	Result Type
904.4734	904.4826	0.0092	10	56	64	GSIEAKDGK					Mascot
1179.5939	1179.6449	0.051	43	1	11	MVNVGINGFGR			Oxidation (M)[1]		Mascot

Rank	Protein Name	Accession No.	Protein Score	Protein Score C. I. %	Total Ion Score	Total Ion C. I. %	Protein MW	Protein PI	Spot Name	Pep. Count	User name	Department
3	HSP100 [Lentinus sajor-caju]	gi|62998570	10	0			99394	5.71	A4	3	Tan Eng Chong	Medicine, U of Malaya

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. %	Modification	Rank	Result Type
929.5713	929.5182	-0.0531	-57	209	215	VIRILCR			Carbamidomethyl (C)[6]		Mascot
929.5713	929.5182	-0.0531	-57	209	215	VIRILCR			Carbamidomethyl (C)[6]		Mascot
1045.5636	1045.5999	0.0363	35	232	241	TSIAEGLAQR					Mascot

Analysis Information

Report Type	Protein-Peptide Summary by Spot	Analysis Type	Combined (MS+MS/MS)
Sample Set Name	2012-03-29 [GRC-001-2012-02-17-1]	Database	NCBInr
Analysis Name	MedicineUofM-TanEngChong[Lentus NCBI]	Creation Date	03/30/2012 14:58:45
Reported By	03/30/2012 15:30:13 - admin	Last Modified	03/30/2012 15:24:22
MS Acq. : Proc. Methods	(Unspecified) : (Unspecified)		
Interpretation Method	(Unspecified)		

	1179.6157	1179.6449	0.0292	25	526	535	FYAVPEVQAR							Mascot	
4	RNA polymerase II second largest subunit [Lentinus sajor-caju]					gi|194306409		8	0		33383	8.34	A4	2 Tan Eng Chong	Medicine, U of Malaya
	Peptide Information														
	Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence			Ion Score	C. I. %	Modification		Rank	Result Type
	982.5654	982.4955	-0.0699	-71	1	7	LFRMLFR							Mascot	
	1036.6011	1036.5985	-0.0026	-3	234	241	RHIENLVR							Mascot	
5	laccase 4 [Lentinus sajor-caju]					gi|11036962		5	0		49579.8	4.92	A4	1 Tan Eng Chong	Medicine, U of Malaya
	Peptide Information														
	Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence			Ion Score	C. I. %	Modification		Rank	Result Type
	1455.7478	1455.6874	-0.0604	-41	277	289	YSFVLTANQAVDK							Mascot	
	1455.7478	1455.6874	-0.0604	-41	277	289	YSFVLTANQAVDK							Mascot	
6	catalase [Lentinus sajor-caju]					gi|28558774		5	0		43768.6	6.19	A4	1 Tan Eng Chong	Medicine, U of Malaya
	Peptide Information														
	Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence			Ion Score	C. I. %	Modification		Rank	Result Type
	904.5284	904.4826	-0.0458	-51	87	94	LLCSVGKK					Carbamidomethyl (C)[3]		Mascot	
7	heat-induced catalase [Lentinus sajor-caju]					gi|13183346		4	0		60039	6.77	A4	1 Tan Eng Chong	Medicine, U of Malaya
	Peptide Information														
	Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence			Ion Score	C. I. %	Modification		Rank	Result Type

Analysis Information

Report Type	Protein-Peptide Summary by Spot	Analysis Type	Combined (MS+MS/MS)
Sample Set Name	2012-03-29 [GRC-001-2012-02-17-1]	Database	NCBInr
Analysis Name	MedicineUofM-TanEngChong[Lentus NCBI]	Creation Date	03/30/2012 14:58:45
Reported By	03/30/2012 15:30:13 - admin	Last Modified	03/30/2012 15:24:22

MS Acq. : Proc. Methods (Unspecified) : (Unspecified)
Interpretation Method (Unspecified)

904.4417	904.4826	0.0409	45	413	419	NQERMAR	Mascot

Analysis Information

Report Type	Protein-Peptide Summary by Spot	Analysis Type	Combined (MS+MS/MS)
Sample Set Name	2012-03-29 [GRC-001-2012-02-17-1]	Database	NCBInr
Analysis Name	MedicineUofM-TanEngChong[Lentus NCBI]	Creation Date	03/30/2012 14:58:45
Reported By	03/30/2012 15:30:13 - admin	Last Modified	03/30/2012 15:24:22
MS Acq. : Proc. Methods	(Unspecified) : (Unspecified)		
Interpretation Method	(Unspecified)		

Gel Idx/Pos **Plate [#] Name**	113/E12 [1] GRC-001	**Instr./Gel Origin** **Instrument Sample Name**	Ak115/2012-02-17-1					**Process Status** **Spectra**	Analysis Succeeded 6			
Rank	**Protein Name**	**Accession No.**	**Protein Score**	**Protein Score C. I. %**	**Total Ion Score**	**Total Ion C. I. %**	**Protein MW**	**Protein PI**	**Spot Name**	**Pep. Count**	**User name**	**Department**
1	RecName: Full=Trehalose phosphorylase; AltName: Full=Trehalose synthase; Short=TSase; Flags: Precur	gi|74626081	18	55.191	7	99.41	83999.8	6.38	A5	5	Tan Eng Chong	Medicine, U of Malaya

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. %	Modification	Rank	Result Type
929.505	929.5217	0.0167	18	737	744	EGETKLPR	7	99.41			Mascot
929.505	929.5217	0.0167	18	737	744	EGETKLPR					Mascot
957.4999	957.5398	0.0399	42	234	243	NPVTVDAGGK					Mascot
963.5258	963.5347	0.0089	9	512	519	DYIVQVAR					Mascot
973.5312	973.5645	0.0333	34	268	275	LADELREK					Mascot
1232.6093	1232.6355	0.0262	21	586	595	EYAHDIVVMR					Mascot

2	RNA polymerase II second largest subunit [Lentinus sajor-caju]	gi|194306409	12	0			33383	8.34	A5	4	Tan Eng Chong	Medicine, U of Malaya

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. %	Modification	Rank	Result Type
973.4996	973.5645	0.0649	67	82	89	RCNTPLGR			Carbamidomethyl (C)[2]		Mascot
1036.6011	1036.579	-0.0221	-21	234	241	RHIENLVR					Mascot
1065.6163	1065.5487	-0.0676	-63	235	243	HIENLVRGK					Mascot
1131.5575	1131.5708	0.0133	12	83	92	CNTPLGREGK			Carbamidomethyl (C)[1]		Mascot

3	heat-induced catalase [Lentinus sajor-caju]	gi|13183346	10	0			60039	6.77	A5	4	Tan Eng Chong	Medicine, U of Malaya

Analysis Information

Report Type	Protein-Peptide Summary by Spot	Analysis Type	Combined (MS+MS/MS)
Sample Set Name	2012-03-29 [GRC-001-2012-02-17-1]	Database	NCBInr
Analysis Name	MedicineUofM-TanEngChong[Lentus NCBI]	Creation Date	03/30/2012 14:58:45
Reported By	03/30/2012 15:30:14 - admin	Last Modified	03/30/2012 15:24:22

MS Acq. : Proc. Methods (Unspecified) : (Unspecified)
Interpretation Method (Unspecified)

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. %	Modification	Rank	Result Type
904.5284	904.4902	-0.0382	-42	87	94	LLCSVGKK			Carbamidomethyl (C)[3]		Mascot
963.4781	963.5347	0.0566	59	2	9	PTQEVFDK					Mascot
994.5428	994.5457	0.0029	3	158	165	RDPQTHLK					Mascot
999.4854	999.5418	0.0564	56	232	240	TLDGETAHR					Mascot

4	RecName: Full=Glyceraldehyde-3-phosphate dehydrogenase; Short=GAPDH	gi|30580405	10	0	36156.7	6	A5	3	Tan Eng Chong	Medicine, U of Malaya

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. %	Modification	Rank	Result Type
904.4734	904.4902	0.0168	19	56	64	GSIEAKDGK					Mascot
963.5145	963.5347	0.0202	21	246	253	LEKPADYK					Mascot
1179.5939	1179.6415	0.0476	40	1	11	MVNVGINGFGR			Oxidation (M)[1]		Mascot

5	HSP100 [Lentinus sajor-caju]	gi|62998570	10	0	99394	5.71	A5	7	Tan Eng Chong	Medicine, U of Malaya

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. %	Modification	Rank	Result Type
929.5713	929.5217	-0.0496	-53	209	215	VIRILCR			Carbamidomethyl (C)[6]		Mascot
929.5713	929.5217	-0.0496	-53	209	215	VIRILCR			Carbamidomethyl (C)[6]		Mascot
957.5363	957.5398	0.0035	4	246	254	DVPASLISR					Mascot
963.4993	963.5347	0.0354	37	517	525	DITTASDLK					Mascot
969.5475	969.5513	0.0038	4	73	82	AGGDPAVVKR					Mascot
972.5261	972.5507	0.0246	25	569	576	WTNIPVSR					Mascot
1045.5636	1045.6014	0.0378	36	232	241	TSIAEGLAQR					Mascot

Analysis Information

Report Type	Protein-Peptide Summary by Spot	Analysis Type	Combined (MS+MS/MS)
Sample Set Name	2012-03-29 [GRC-001-2012-02-17-1]	Database	NCBInr
Analysis Name	MedicineUofM-TanEngChong[Lentus NCBI]	Creation Date	03/30/2012 14:58:45
Reported By	03/30/2012 15:30:14 - admin	Last Modified	03/30/2012 15:24:22
MS Acq. : Proc. Methods	(Unspecified) : (Unspecified)		
Interpretation Method	(Unspecified)		

	1179.6157	1179.6415	0.0258	22	526	535	FYAVPEVQAR							Mascot	
6	catalase [Lentinus sajor-caju]				gi|28558774			9	0		43768.6	6.19	A5	3 Tan Eng Chong	Medicine, U of Malaya

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. %	Modification	Rank	Result Type
904.5284	904.4902	-0.0382	-42	87	94	LLCSVGKK			Carbamidomethyl (C)[3]		Mascot
963.4781	963.5347	0.0566	59	2	9	PTQEVFDK					Mascot
994.5428	994.5457	0.0029	3	158	165	RDPQTHLK					Mascot
7	WUN [Lentinus sajor-caju]				gi|6018116			8	0		5174.7	10.43	A5	1 Tan Eng Chong	Medicine, U of Malaya

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. %	Modification	Rank	Result Type
1104.5028	1104.5662	0.0634	57	30	39	RSSSADDPNR					Mascot
8	translation elongation factor 1 alpha [Lentinus sajor-caju]				gi|170517001			4	0		20207.6	7.07	A5	1 Tan Eng Chong	Medicine, U of Malaya

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. %	Modification	Rank	Result Type
972.5876	972.5507	-0.0369	-38	1	8	AWVLDKLK					Mascot

Analysis Information

Report Type	Protein-Peptide Summary by Spot	Analysis Type	Combined (MS+MS/MS)
Sample Set Name	2012-03-29 [GRC-001-2012-02-17-1]	Database	NCBInr
Analysis Name	MedicineUofM-TanEngChong[Lentus NCBI]	Creation Date	03/30/2012 14:58:45
Reported By	03/30/2012 15:30:14 - admin	Last Modified	03/30/2012 15:24:22
MS Acq. : Proc. Methods	(Unspecified) : (Unspecified)		
Interpretation Method	(Unspecified)		

Gel Idx/Pos Plate [#] Name	114/E13 [1] GRC-001	Instr./Gel Origin Instrument Sample Name	Ak115/2012-02-17-1	Process Status Spectra	Analysis Succeeded 6

Rank	Protein Name	Accession No.	Protein Score	Protein Score C. I. %	Total Ion Score	Total Ion C. I. %	Protein MW	Protein PI	Spot Name	Pep. Count	User name	Department
1	heat-induced catalase [Lentinus sajor-caju]	gi|13183346	12	0			60039	6.77	24	5	Tan Eng Chong	Medicine, U of Malaya

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. %	Modification	Rank	Result Type
903.5046	903.5226	0.018	20	116	123	DPRGFALK					Mascot
904.5284	904.5135	-0.0149	-16	87	94	LLCSVGKK			Carbamidomethyl (C)[3]		Mascot
920.4366	920.5009	0.0643	70	413	419	NQERMAR			Oxidation (M)[5]		Mascot
985.5213	985.5549	0.0336	34	461	470	NVAGHFGGVK					Mascot
1050.667	1050.6312	-0.0358	-34	496	506	AVGAKPVAPLK					Mascot

2	HSP100 [Lentinus sajor-caju]	gi|62998570	10	0			99394	5.71	24	5	Tan Eng Chong	Medicine, U of Malaya

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. %	Modification	Rank	Result Type
901.4988	901.5337	0.0349	39	279	286	AVLNEVEK					Mascot
969.5475	969.5643	0.0168	17	73	82	AGGDPAVVKR					Mascot
1004.5775	1004.5721	-0.0054	-5	783	790	IDEIVIFR					Mascot
1070.6429	1070.6066	-0.0363	-34	605	614	AVANAIRLSR					Mascot
1179.6157	1179.662	0.0463	39	526	535	FYAVPEVQAR					Mascot

3	RecName: Full=Glyceraldehyde-3-phosphate dehydrogenase; Short=GAPDH	gi|30580405	7	0			36156.7	6	24	2	Tan Eng Chong	Medicine, U of Malaya

Analysis Information

Report Type	Protein-Peptide Summary by Spot	Analysis Type	Combined (MS+MS/MS)
Sample Set Name	2012-03-29 [GRC-001-2012-02-17-1]	Database	NCBInr
Analysis Name	MedicineUofM-TanEngChong[Lentus NCBI]	Creation Date	03/30/2012 14:58:45
Reported By	03/30/2012 15:30:15 - admin	Last Modified	03/30/2012 15:24:22

MS Acq. : Proc. Methods (Unspecified) : (Unspecified)
Interpretation Method (Unspecified)

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. %	Modification	Rank	Result Type
904.4734	904.5135	0.0401	44	56	64	GSIEAKDGK					Mascot
1179.5939	1179.662	0.0681	58	1	11	MVNVGINGFGR			Oxidation (M)[1]		Mascot

4	catalase [Lentinus sajor-caju]	gi|28558774	6	0	43768.6	6.19	24	2	Tan Eng Chong	Medicine, U of Malaya

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. %	Modification	Rank	Result Type
903.5046	903.5226	0.018	20	116	123	DPRGFALK					Mascot
904.5284	904.5135	-0.0149	-16	87	94	LLCSVGKK			Carbamidomethyl (C)[3]		Mascot

5	laccase 5 [Lentinus sajor-caju]	gi|32399649	5	0	57360.2	6.09	24	2	Tan Eng Chong	Medicine, U of Malaya

Peptide Information

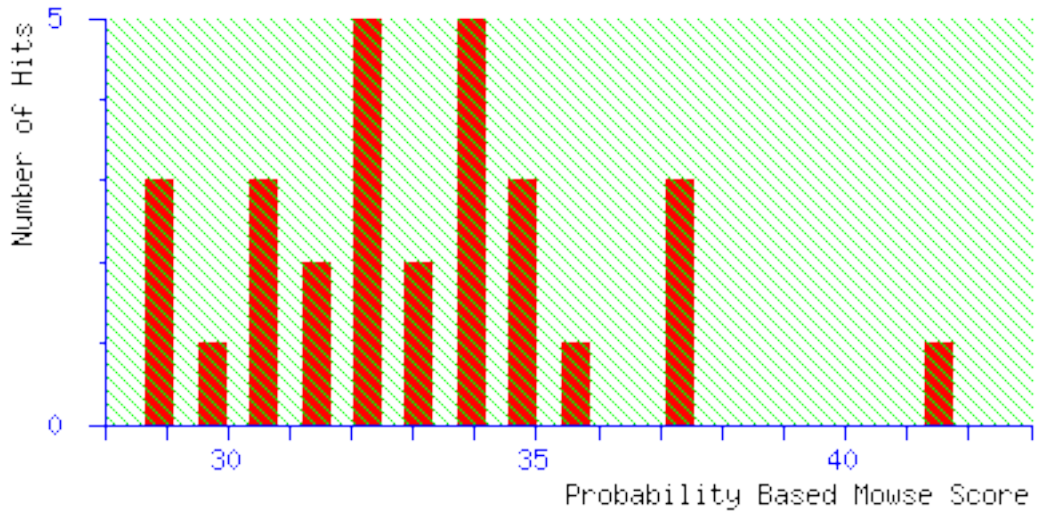
Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. %	Modification	Rank	Result Type
935.5097	935.5205	0.0108	12	279	285	IANYWIR					Mascot
1020.5988	1020.6204	0.0216	21	215	222	VLWGVKYR					Mascot

2.0 MALDI-TOF/TOF MS Analysis of Peak 3-RPF Protein by Using In-Solution Digestion Protocol.

MATRIX SCIENCE Mascot Search Results

User : Email : Search title : SampleSetID: 1461, AnalysisID: 3387, MaldiWellID: 135821, SpectrumID: 233890, Path=\071211\msms\azwa-re Database : Sprot 080308 (261513 sequences; 95638062 residues) Timestamp : 19 Dec 2011 at 02:51:40 GMT Top Score : 42 for PROF1_PHAVU, Profilin-1 - *Phaseolus vulgaris* (Kidney bean) (French bean)

Probability Based Mowse Score
Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 67 are significant (p<0.05).



Top of Form

Concise Protein Summary Report

		Help
	Significance threshold p< 0.05	Max. number of hits 10

1. PROF1_PHAVU **Mass:** 14172 **Score:** 42 **Expect:** 19 **Queries matched:** 2
   Profilin-1 - *Phaseolus vulgaris* (Kidney bean) (French bean)
   PROF1_RICCO **Mass:** 14199 **Score:** 42 **Expect:** 19 **Queries matched:** 2
   Profilin-1 - *Ricinus communis* (Castor bean)
   PROF1_SOYBN **Mass:** 14091 **Score:** 42 **Expect:** 19 **Queries matched:** 2
   Profilin-1 (GmPRO1) (Allergen Gly m 3.0101) - *Glycine max* (Soybean)
   PROF2_HEVBR **Mass:** 14142 **Score:** 42 **Expect:** 19 **Queries matched:** 2

Profilin-2 (Pollen allergen Hev b 8.0102) - *Hevea brasiliensis* (Para rubber tree)

PROF2_MALDO **Mass:** 14043 **Score:** 42 **Expect:** 19 **Queries matched:** 2

Profilin-2 (GD4-2) (Pollen allergen Mal d 4.0201) - *Malus domestica* (Apple) (Malus sylvestris)

PROF2_SOYBN **Mass:** 14091 **Score:** 42 **Expect:** 19 **Queries matched:** 2

Profilin-2 (GmPRO2) (Allergen Gly m 3.0102) - *Glycine max* (Soybean)

PROF_FRAAN **Mass:** 14058 **Score:** 42 **Expect:** 19 **Queries matched:** 2

Profilin (Allergen Fra a 4) - *Fragaria ananassa* (Strawberry)

PROF_PRUPE **Mass:** 13980 **Score:** 42 **Expect:** 19 **Queries matched:** 2

Profilin (Allergen Pru p 4.02) - *Prunus persica* (Peach)

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2. EX7S_GEOSL **Mass:** 8693 **Score:** 38 **Expect:** 47 **Queries matched:** 2

Exodeoxyribonuclease 7 small subunit (EC 3.1.11.6) (Exodeoxyribonuclease VII small subunit) (Exonuc

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3. Y1491_LACJO **Mass:** 18213 **Score:** 37 **Expect:** 48 **Queries matched:** 2

UPF0090 protein LJ_1491 - *Lactobacillus johnsonii*

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4. GPX3_CEBAP **Mass:** 25517 **Score:** 37 **Expect:** 53 **Queries matched:** 2

Glutathione peroxidase 3 precursor (EC 1.11.1.9) (GSHPx-3) (GPx-3) (Plasma glutathione peroxidase)

GPX3_HUMAN **Mass:** 25489 **Score:** 37 **Expect:** 53 **Queries matched:** 2

Glutathione peroxidase 3 precursor (EC 1.11.1.9) (GSHPx-3) (GPx-3) (Plasma glutathione peroxidase)

GPX3_MACFU **Mass:** 25513 **Score:** 37 **Expect:** 53 **Queries matched:** 2

Glutathione peroxidase 3 precursor (EC 1.11.1.9) (GSHPx-3) (GPx-3) (Plasma glutathione peroxidase)

GPX3_PONPY **Mass:** 25499 **Score:** 37 **Expect:** 53 **Queries matched:** 2

Glutathione peroxidase 3 precursor (EC 1.11.1.9) (GSHPx-3) (GPx-3) (Plasma glutathione peroxidase)

GPX3_HYLLA **Mass:** 25535 **Score:** 37 **Expect:** 55 **Queries matched:** 2

Glutathione peroxidase 3 precursor (EC 1.11.1.9) (GSHPx-3) (GPx-3) (Plasma glutathione peroxidase)

---

5. Y012_METKA **Mass:** 19878 **Score:** 36 **Expect:** 66 **Queries matched:** 2

UPF0254 protein MK0012 - *Methanopyrus kandleri*

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6. Y362_HAEIN **Mass:** 32443 **Score:** 35 **Expect:** 91 **Queries matched:** 2

Putative periplasmic iron-binding protein HI0362 precursor - *Haemophilus influenzae*

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7. VG41_ICHV1 **Mass:** 33871 **Score:** 35 **Expect:** 91 **Queries matched:** 2

Hypothetical gene 41 protein - Ictalurid herpesvirus 1 (IcHV-1) (Channel catfish herpesvirus)

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8. MYP0_HORSE **Mass:** 27467 **Score:** 35 **Expect:** 91 **Queries matched:** 2

Myelin P0 protein precursor (Myelin protein zero) (Myelin peripheral protein) (MPP) - Equus

caballu

MYP0_RAT **Mass:** 27553 **Score:** 35 **Expect:** 91 **Queries matched:** 2

Myelin P0 protein precursor (Myelin protein zero) (Myelin peripheral protein) (MPP) - Rattus norveg

MYP0_HUMAN **Mass:** 27537 **Score:** 34 **Expect:** 1e+002 **Queries matched:** 2

Myelin P0 protein precursor (Myelin protein zero) (Myelin peripheral protein) (MPP) - *Homo sapiens*

MYP0_MOUSE **Mass:** 27604 **Score:** 34 **Expect:** 1e+002 **Queries matched:** 2

Myelin P0 protein precursor (Myelin protein zero) (Myelin peripheral protein) (MPP) - *Mus musculus*

MYP0_BOVIN **Mass:** 24695 **Score:** 34 **Expect:** 1.1e+002 **Queries matched:** 2

Myelin P0 protein (Myelin protein zero) (Myelin peripheral protein) (MPP) - *Bos taurus* (Bovine)

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9. ISCU_HUMAN **Mass:** 17926 **Score:** 34 **Expect:** 97 **Queries matched:** 2

Iron-sulfur cluster assembly enzyme ISCU, mitochondrial precursor (NifU-like N-terminal domain-cont

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10. H2B1_WHEAT **Mass:** 16423 **Score:** 34 **Expect:** 99 **Queries matched:** 2

Histone H2B.1 - *Triticum aestivum* (Wheat)

Search Parameters

Type of search : Sequence Query Enzyme : Trypsin Variable modifications : Carbamidomethyl (C),Oxidation (M) Mass values : MONOISOTOPIC Protein Mass : Unrestricted Peptide Mass Tolerance : ± 100 ppm Fragment Mass Tolerance: ± 0.2 Da Max Missed Cleavages : 1 Instrument type : MALDI-TOF-TOF Query1 (842.57,1+) : MaldiWellID: 135821, SpectrumID: 233898, Query2 (842.57,1+) : <no title>

Mascot: http://www.matrixscience.com/

APPENDIX E: PUBLICATION

1.0 Proceedings for poster presented at the 5th International Peptide Symposium in conjunction with 47th Japanese Peptide Symposium. Date: December 4–9, 2010. Venue: Kyoto International Conference Center. Kyoto, Japan.

Protein with Anti-diabetic activity from *Pleurotus sajor-caju* (Grey Oyster Mushroom)

Nurul Azwa Abd.Wahab, Noorlidah Abdullah & Norhaniza Aminudin

Mushroom Research Centre, Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia
E-mail: azwawahab862gmail.com

The inhibition of α-amylase and α-glucosidase enzymes is a strategy to reduce type 2 diabetes disease. At 25µg/ml, 30% protein fraction strongly suppressed the activity of α-glucosidase while for α-amylase inhibitory assay, 100% fraction showed the highest inhibition.

Keywords: Diabetes Mellitus, α-amylase, α-glucosidase

Introduction

Pleurotus species (oyster mushroom) have been used by human cultures all over the world for their nutritional value, medicinal properties and other beneficial effects.

Oyster mushrooms are a good source of dietary fibre and other valuable nutrients. They also contain a number of biologically active compounds with therapeutic activities. Oyster mushrooms modulate the immune system, inhibit tumour growth and inflammation, have hypoglycaemic and antithrombotic activities, lower blood lipid concentrations, prevent high blood pressure and atherosclerosis, and have antimicrobial and other activities (Gunde-Cimerman, 1999).

As *Pleurotus* species are comparatively easy to grow and the broad adaptability, they are cultivated worldwide and their production has increased rapidly in recent years. It was the third most popular cultivated edible mushrooms in 1997 after Agaricus bisporus and Lentinula edode (Huang, 1997).

Results and Discussion

The percentage of soluble yield (g/100g) of the following different types of extraction. (Figure 1). Aqueous extraction with the highest percentage yield (18.34%) followed by methanol (1%), DCM (0.525%) and polysaccharide (0.27%).

For α-glucosidase inhibitory assay, DCM extracts showed the highest inhibition (91.13%) for extract samples (figure 2). For protein fraction that strongly suppressed the activity of α-glucosidase from *Saccharomyces cerevisiae* is 30% protein fraction with 24.18% inhibition

For α-amylase inhibitory assay (figure 3), crude aqueous extract showed the highest inhibition (87.74%) and for protein fraction that strongly suppressed the activity of porcine pancreatic α-amylase is 100% protein fraction with 41.80% inhibition.

The concentration used for protein is 25µg/ml (1000x lower than 25mg/ml) since lower concentrations did not show any inhibition. Thus, the inhibition activities for 4 extracts were significantly lower than activity of protein fractions.

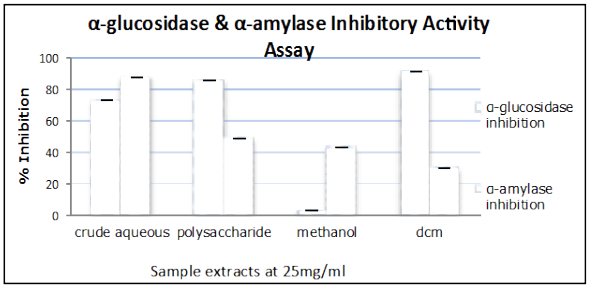

Figure 2: The inhibition of α-glucosidase & α-amylase activity at 25mg/ml by 4 sample extracts

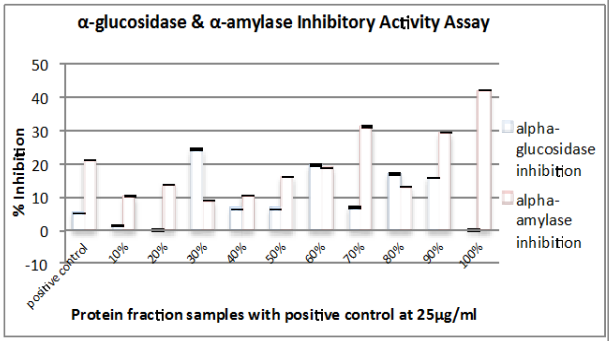

Figure 3: Inhibition of α-glucosidase & α-amylase activity by protein fractions 10%-100% with the positive controls at 25µg/ml

Figure 4: SDS-PAGE of selected active protein fractions

This study was supported by the research grants from

References

1. Bailey CJ, 2003. New Drugs for the Treatment of Diabetes Mellitus.In; Pick up JC.Williams (Ed.s), Textbook of Diabetes, vol.2. 3rd ed. Blackwell Science Ltd.UK, pp. 38-40.