FIBRINOLYTIC ACTIVITIES OF A MEDICINAL MUSHROOM: *LIGNOSUS RHINOCEROTIS* (COOKE) RYVARDEN

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ABSTRACT

Ganoderma lucidum, Cordyceps militaris, Lignosus rhinocerotis, Pleurotus giganteus, Pleurotus floridanus and *Auricularia polytricha* were screened for fibrinolytic activity using fibrin plate assay. Fibrinolytic activity was identified in the fruiting bodies of the medicinal mushrooms *L. rhinocerotis* and *A. polytricha*. However, the present study emphasised on the evaluation of the fibrinolytic activity from *L. rhinocerotis* and its partially purified enzyme only. Fibrin plate assay showed 1.2 cm of lytic zone by the crude extracts of *L. rhinocerotis* which doubled in lytic zone size after overnight incubation, which demonstrated the stability of the fibrinolytic enzyme. The extract was initially dialysed using 12 kDa cellulose membrane and the solution was freeze-dried and stored under -20^{o}C prior to use. Aqueous two phase system (ATPS), a single-step purification method was applied to partition and concentrate the protein. In addition, pre-chilled acetone was used to remove contaminants and precipitate the protein through centrifugation at 13,000 – 15,000 g. A PEG 8000/ phosphate system comprising of 4.0 g of 50% of polyethylene-glycol (PEG), 2.9 g of 40% of phosphate solution and pH 7.0 resulted in the concentration of fibrinolytic enzyme in the top phase, with specific activity 151.61 U / mg (2.67 fold). The molecular mass of the partially purified enzyme was identified through SDS-PAGE and estimated to be between 55 kDa and 60 kDa. Native PAGE was carried out for gel excision in which band of interested (partially purified enzyme) was excised and purified from polyacrylamide gel using elution buffer. Then, the eluted sample was tested on fibrin plate and resulting in the confirmation of the fibrinolytic effect.

ABSTRAK

Ganoderma lucidum, Cordyceps militaris, Lignosus rhinocerotis, Pleurotus giganteus, Pleurotus floridanus dan *Auricularia polytricha* ditapis melalui asai fibrinolitik. Aktiviti fibrinolitik telah dikenalpasti dalam janabuah cendawan perubatan *L. rhinocerotis* dan *A. polytricha*. Walau bagaimanapun, kajian ini akan menekankan penilaian aktiviti fibrinolitik daripada *L. rhinocerotis* dan enzim yang separa tulen sahaja. Asai fibrinolitik daripada ekstrak mentah *L. rhinocerotis* menunjukkan zon penguraian sebesar 1.2 cm dan membesar dua kali ganda selepas eraman semalaman, di mana ia menunjukkan kestabilan enzim fibrinolitik tersebut. Ekstrak tersebut telah didialisis dengan menggunakan 12 kDa membran selulosa sebelum larutan tersebut dikeringkan secara sejuk-beku. Selepas proses pengeringan, ekstrak tersebut disimpan di dalam -20^{o}C sebelum mengguna. Sistem dua fasa cair yang merupakan cara satu langkah penulenan telah digunakan untuk memisah dan menumpukan protein. Secara tambahan, aseton sejuk digunakan untuk mengeluarkan bendasing dan memendakkan protein melalui pengemparan pada 13,000–15,000 *g*. Sistem yang menggunakan PEG 8000/ phosphate mengandungi 4 g daripada 50% polietilena glikol (PEG), 2.9 g daripada 40% fosfat larutan dan pH 7.0, telah memberi keputusan di mana enzim fibrinolitik telah ditumpukan ke fasa atas dengan aktiviti spesifik 151.61 U / mg (2.67 ganda). Jisim molekul enzim yang ditulenkan telah dianggarkan oleh SDS-PAGE dan anggaran jisim molekul adalah 55 kDa dan 60 kDa. Native PAGE telah digunakan untuk pemotongan gel di mana enzim yang separa tulen dipotong keluar dan gel dibersihkan dengan menggunakan penampan. Aktiviti fibrinolitik telah dikenalpasti daripada enzim-enzim yang dibersihkan.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

µg	Microgram
µg/mL	Microgram per microlitre
µl	Microlitre
µM	Micromolar
µmole	Micromole
ANOVA	Analysis of Variance
APS	Ammonium persulfate
ATPS	Aqueous two phase system
BSA	Bovine serum albumin
cm	Centimeter
g	Gravity
g	Gram
HCl	Hydrochloric acid
kDa	Kilo Dalton
LSD	Least significant difference
M	Molar
mg	Milligram
mg/ml	Milligram per millilitre
ml	Millilitre
mM	Millimolar
N	Normality
NaCl	Sodium chloride
nm	nanometer
OD	Optical density
PEG	Polyethylene glycol
rpm	Rotation per minute
SDS	Sodium dodecyl sulfate-polyacrylamide
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Tris-HCl	Tris hydrochloride
U	Unit
U/mg	Unit per milligram
v/v	Volume per volume
w/v	Weight per volume

CHAPTER I

INTRODUCTION

Mushrooms are commonly used as food, food flavouring substances and traditional medicines in eastern countries. They are reported to be high in protein, with a significant content of essential amino acid, but low in fat (Guillamon *et al.*, 2010). Besides, mushrooms supply a large amount of carbohydrates, fiber, vitamins (B1, B2, B12, C and D) and mineral ions (Ca, K, Mg, Na, P, Cu, Fe, Mn and Se).

The amino acid composition of mushroom proteins is comparable to animal protein, and it can counterbalance the high consumption of animal proteins especially in developed countries. Besides, edible mushroom is a unique food which contains many different bioactive compounds such as eritadenine (Mattila *et al*., 1999) and phenolic compounds (Barros *et al*., 2007 & 2008) that may be beneficial in cardiovascular disease treatment. Cardiovascular disease is major cause of death and therefore, there is worldwide interest in discovering the potential therapeutic drugs, supplements, and food for better health and lifestyle.

In recent years, variety of mushrooms have been studied for their pharmacological effect and reported to have great benefits for human health. These mushrooms or their extracts which can be used as therapeutic agents are generally known as medicinal mushrooms. Typically, medicinal mushrooms are edible. Nevertheless, edible mushrooms may not necessarily possess therapeutic properties and are usually used only as food.

According to the world health statistics 2012 by World Health Organization (WHO), the annual number of deaths due to cardiovascular disease will increase from 17 million in 2008 to 25 million in 2030 (World Health Organization, 2012). In Malaysia, the mortality rate from cardiovascular disease in 2008 is 263 deaths per 100,000 population, and the majority caused by ischaemic heart disease (OECD/WHO, 2012). Cardiovascular disease has long been the leading cause of death in developed countries. It becomes increasingly prevalent in Asian countries, comprising about one third of all deaths in Asians. Cardiovascular disease includes a range of diseases that are related to circulatory system. The most prevalent diseases are ischaemic heart disease (or heart attack) and cerebrovascular disease (or stroke). These two diseases have contributed to three quarters of all cardiovascular deaths in Asian countries (OECD/WHO, 2012).

Atherosclerosis is a condition in which fatty material collects along the walls of arteries. The fatty material thickens and hardens with calcium deposition, and eventually blocks the arteries. Once the blood vessels are injured, platelets tend to aggregate and react with fibrin to form thrombus. Thrombus is abnormal blood clot formed within the blood vessel that obstructs the flow of blood and nutrients to vital tissue. When there is phenomenon of reduced blood supply to heart muscle, the syndrome is known as Ischaemic heart disease.

In biological system, thrombosis can be prevented through fibrinolysis. The insoluble fibrin fiber is hydrolyzed into fibrin degradation products by plasmin, which is generated from plasminogen by plasminogen activators such as the tissue

plasminogen activator, vascular plasminogen activator, blood plasminogen activator, urokinase, Hageman factor and streptokinase-plasminogen complex (Shen *et al.*, 2007). Thrombolytic agents have been extensively used in therapeutic treatment of thrombosis nowadays. The commonly used thrombolytic agents include streptokinase, urokinase, alteplase, reteplase and tenecteplase. Thrombolytic drugs breakdown dangerous blood clots in blood vessels, improve blood flow, and prevent damage to tissues and organs. However, these thrombolytic agents are of relatively high cost and exhibit low specificity to fibrin. Besides, all of these thrombolytic agents could cause undesirable side effect such as bleeding complications, which is the most common consequences of anticoagulant therapy. Streptokinase is capable of eliciting antigenic response in human since the protein is obtained from streptococci cultures. The usage of streptokinase is previously prohibited or contraindicated because of the risk of anaphylaxis. Streptokinase is also relatively non- specific thrombolytic agent which can lead to systemic fibrinolysis and lysis of normal hemostatic plugs. Hence, the search for safe thrombolytic agents from other sources is necessary. Fortunately, fibrinolytic enzymes were discovered in insects (Hahn *et al*., 1999), earthworm (Cho *et al*., 2004), snake venom (Sun *et al*., 2006) and food-grade microorganism (Wang *et al*., 2006).

For mushrooms, some studies reported that mushrooms do have therapeutic benefit for cardiovascular disease. The hypocholesterolemic action of edible mushrooms has been reported in the early work of Kaneda and Tokuda (1966). *Lentinus edodes* (Berk.) Singer*, Auricularia polytricha* (Mont.) Sacc.*, Flammulina velutipes* (Curtis) Singer and *Agaricus bisporus* (J.E.Lange) Imbach have been reported to have cholesterol lowering properties. On the other hand, some mushrooms have hypotensive effect when blood pressure is already high (Kabir *et al*., 1987 & 1988;

Miyazawa *et al*., 2008). Besides, the properties of mushrooms such as antioxidant (Cheung *et al*., 2003; Wong & Chye, 2009) and anti-inflammatory (Jose & Janardhanan, 2004; Khohno *et al*., 2008) might be helpful in the management of heart and blood circulation.

Today, several mushrooms had been studied for their fibrinolytic activities and the corresponding fibrinolytic enzymes were also identified: *Schizophyllum commune* (Fr) (Lu *et al*., 2010), *Armillariella mellea* (Vahl) P. Karst (Kim and Kim, 1999), *Grifola frondosa* (Dicks.) Gray (Nonaka *et al.,* 1997), *Pleurotus ostreatus* (Jacq.) P. Kumm. (Shen *et al.*, 2007) and *Fomitella fraxinea* (Bull.) Imazeki (Lee *et al*., 2006). Differing from plasminogen activators such as streptokinase and urokinase, these fibrinolytic enzymes are plasmin-like proteins which directly perform fibrinolytic action. An ideal thrombolytic drug should be effective in breaking down fresh and older thrombi, be rapid in its action with complete dissolution of the thrombus, be able to be given as a bolus and be safe without hypotensive, allergic or immunogenic reactions (Thomson, 1999).

According to current dietary recommendations for health, mushrooms are considered as appropriate choice and its consumption can affect some known cardiovascular risk biomarkers. Besides the known hypocholestrolemic effect, the presence of antioxidant and anti-inflammatory compound in mushrooms may synergistically contribute to reduce the atherosclerosis risk. Compared to other sources, mushrooms are considered as a good choice to treat or prevent cardiovascular disease as it can be included in our daily diet and suitable for vegetarians. Although some

mushrooms had been consumed for therapeutic purpose in past, they have yet to scientifically verified and reported. On the other hand, therapeutic effects of mushrooms obtained from different geographical region might act differently. Therefore, in this study, medicinal mushrooms investigated include *Ganoderma lucidum* (Curtis) P. Karst, *Cordyceps militaris* (L.) Fr., and *Lignosus rhinocerotis* (Cooke) Ryvarden. For edible medicinal mushrooms, *Pleurotus giganteus* (Berk.) Karun. & K.D. Hyde, *Pleurotus floridanus* (Singer) and *A. polytricha* were studied.

OBJECTIVES:

The objectives were to:

a. screen the selected edible and medicinal mushrooms for their fibrinolytic activities.
b. isolate and purify the fibrinolytic enzymes from the mushroom.
c. characterize the fibrinolytic enzyme by estimation of the molecular mass.

CHAPTER II

LITERATURE REVIEW

Mushrooms are universally consumed as food, food favouring substances and traditional medicines in eastern countries due to its tastiness, high nutritional values, and pharmacological properties (Chang & Miles, 1989; Lindequist *et al.*, 2005). It is well established that mushroom extracts contain a wide variety of compounds such as protein, polysaccharides, fibre, lectins, and polyphenols, and each of the compounds consists its own pharmacological effects (Smiderle *et al.*, 2010).

Lignosus rhinocerotis (Figure 2.1), also known as "tiger milk mushroom", belongs to the Polyporaceae family and is one of the most important medicinal mushrooms used by the indigenous people of Southeast Asia and China. In Malaysia, the mushroom is locally named as "*cendawan susu rimau*" which means tiger's milk mushroom. It is traditionally used by the natives in peninsular Malaysia as traditional medicine to cure cough, fever, asthma, food poisoning and as a general tonic (Lee *et al.*, 2011). In China, *L. rhinocerus* sclerotium is an expensive folk medicine used by traditional Chinese physicians to treat liver cancer, chronic hepatitis, and gastric ulcer (Wong & Cheung, 2008). It has more than 15 medicinal uses, however, there are limited usage of this mushroom because of its low availability. The mushroom is difficult to cultivate and previously, was only available from forest. Successful cultivation of the mushroom was carried out in agar, solid, and spawn medium with good yield, therefore contributing to large quantity production of the mushroom for investigation and therapeutic purpose (Lee *et al.*, 2011). In recent years, variety of mushrooms have been tested for their pharmacological effect and reported to have great

benefits for human health. The sclerotium of the mushroom is the part of the medicinal value and the extracts from it was reported to have *in vitro* antiproliferative effect on leukemic cells and immunodulatory activity (Lai *et al.*, 2008; Wong *et al.*, 2011).

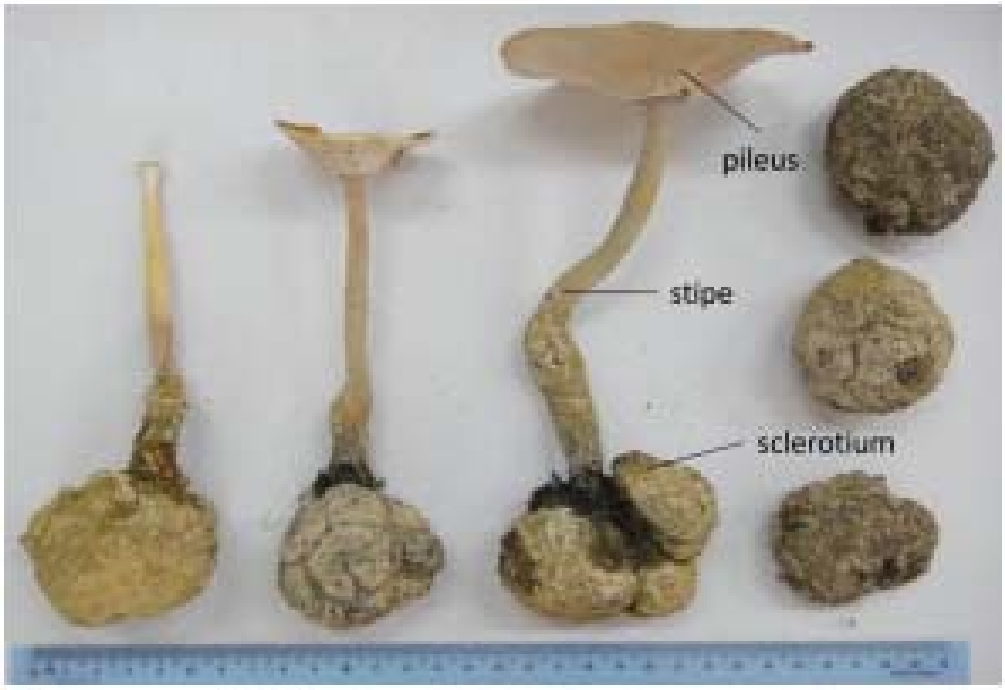


Figure 2.1: *Lignosus rhinocerotis*. Adapted from Noorlidah *et al*., 2013.

Mushrooms are reported to be an important source of thrombolytic agents as they exert hematological, antiviral, antitumorigenic, hypotensive and hepatoprotective effects (Shen *et al.*, 2007). Many fibrinolytic enzymes were identified in the fruiting body of different medical mushrooms such as metalloprotease (AmMEP) from *Armillaria mellea* (Kim & Kim, 1999), aminopeptidase from *Grifola frondosa* (Nonaka *et al.,* 1997), metalloprotease (PoMEP) from *Pleurotus ostreatus* (Shen *et al*., 2007) and two novel fibrinolytic proteases from *Fomitella fraxinea* (Lee *et al.*, 2006). In year 2010, a novel fibrinolytic protease was discovered from *Schizophyllum commune*, a widely distributed medicinal mushroom, from its mycelium (Lu *et al.*, 2010). The

mushroom was cultured with fermentation technology, and protein purification was carried out by cross-flow filtration and fast performance liquid chromatography (FPLC) system.

Fibrinolytic activity was determined by the fibrin plate assay in which fibrin clot was made in petri dish at room temperature by 1.5% agarose, 0.2% human fibrinogen and 10 U of human thrombin, the assay was modified from the method described by Astrup and Mullertz, (1952). For the determination of protease activity, azocasein assay was used, where azocasein hydrolysis was used as an measure of fibrinolytic activity. Activity was determined by measuring the release of acid-soluble material after protease digestion of azocasein. The protease isolated was fractionated through Superdex 75 10/300 GL column and SDS-PAGE. This enzyme showed 21.32 kDa in molecular mass and monomeric form in protein structure. The effect of temperature and pH for optimal protease activity was being estimated as well, the optimal protease activity displayed at the condition of pH 5.0 and 45^{o}C. The activity was enhanced by magnesium and inhibited by EDTA. Despite of low production ratio and recovery rate, the fibrinolytic protease revealed 9.29 fold in specific activity after purification, and showed greater activity than human plasmin.

2.1 Cardiovascular disease

Cardiovascular diseases are a group of heart and blood vessels disorders, including rheumatic heart disease, hypertensive heart disease, ischemic heart disease, cerebrovascular disease, inflammatory heart disease, congenital heart disease and heart

failure. Diseases like acute myocardial, valvular heart disease and stroke is the leading cause of death in developed countries (Lu *et al.*, 2010).

2.1.1 Fibrinolysis mechanism

Fibrinolysis is the aseptic dissolution of fibrin brought about by the direct action of a mechanism existing in normal blood (Macfarlane & Biggs, 1948). Generally, dissolutions take days to week to complete, but the action may be accelerated as a result of natural changes occurring in the living subject or of experimental procedures as to occur within a few hours or minutes (Macfarlane & Biggs, 1948). This acceleration phenomenon had raised the interest of scientists, though a particular set of factors have been studied as being apparently those mainly responsible, it is recognized that others may be involved.

Under physiological conditions, fibrinolysis is highly regulated mechanism that integrates with the coagulation system through several direct molecular links. Both coagulation and fibrinolysis are precisely regulated by measured participation of substrates, activators, inhibitors, cofactors and receptors. Besides preventing blood loss, these co-ordinated molecular events insure blood fluidity (Cesarman-Maus & Hajjar, 2005). Plasminogen (PLG) is a circulating plasma zymogen which can be converted to plasmin by both tissue plasminogen activator (tPA) and by urokinase (uPA). Through positive feedback mechanism, plasmin cleaves both tPA and uPA from single chain to more active two-chain polypeptides. Fibrin, which is the substrate to plasmin, bind with both plasminogen and tPA on its surface to regulate its own degradation and therefore, localizing and enhancing plasmin generation. Plasminogen activator (tPA) is a weak

activator in the absence of fibrin, its catalytic efficiency for PLG activation is enhanced by at least two orders of magnitude in the presence of fibrin. In other words, the affinity between tPA and plasminogen is low in the absence of fibrin, and vice versa. The mechanism of fibrinolytic system is illustrated in Figure 2.2 below.

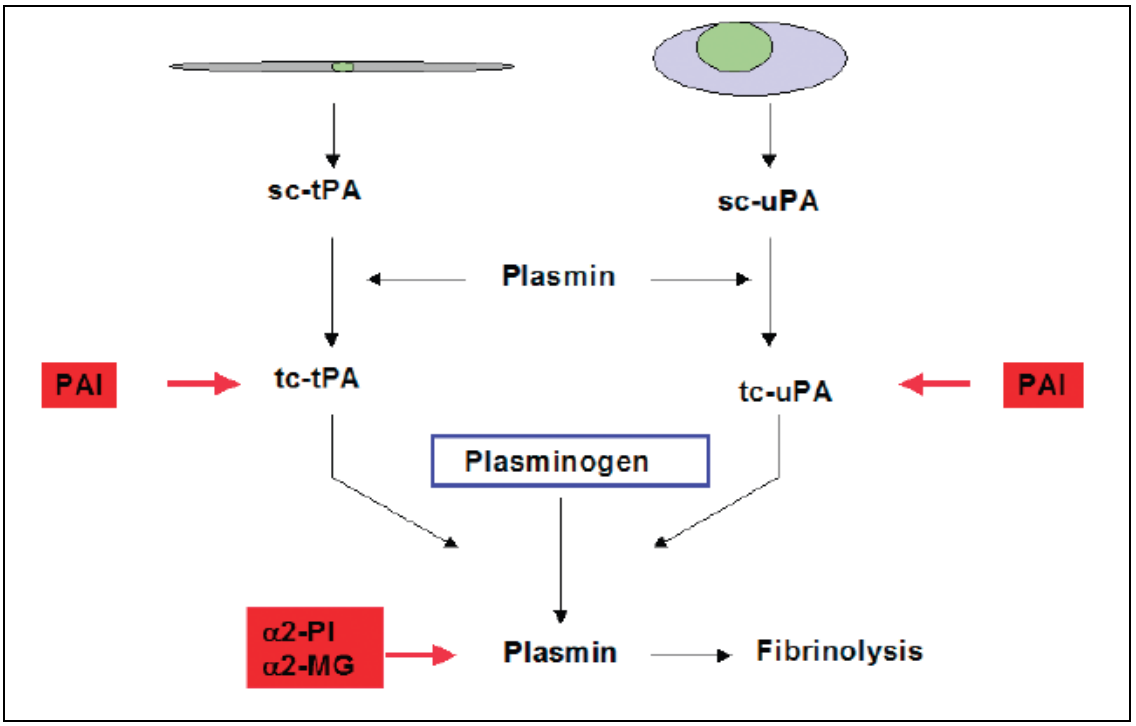


Figure 2.2: Overview of the mechanism of fibrinolytic system. The zymogen plasminogen is converted to the active serine protease, plasmin, through the action primarily of two-chain tissue plasminogen activator (tc-tPA) or two-chain urokinase (tc-uPA). These activators are secreted as single-chain (sc-tPA and scuPA) forms from endothelial cells, and from renal epithelium, monocyte/macrophages, or endothelial cells respectively. Both tPA and uPA can be inhibited by plasminogen activator inhibitor-1 (PAI), while plasmin is inhibited by its major inhibitor, a2-plasmin inhibitor (a2-PI), and to a lesser extent by a2-macroglobulin (a2-MG). Once plasmin is generated, it converts single chain tPA and uPA to double chain forms. It is then rapidly inhibited unless it remains bound to fibrin or to its cell surface receptors. Adapted from Cesarman-Maus and Hajjar (2005).

Plasmin is capable of lysing fibrin, and of digesting fibrinogen, gelatin and casein (Macfarlane and Biggs, 1948). It consists of two fibrinolytic action modes as it degrades both fibrin and fibrinogen with different mechanisms. When plasma antiplasmin activity is overhelmed, as when plasminogen activators are used for the treatment of thrombosis, circulating fibrinogen may be degraded by plasmin as shown in Figure 2.3 (Cesarman-Maus & Hajjar, 2005).

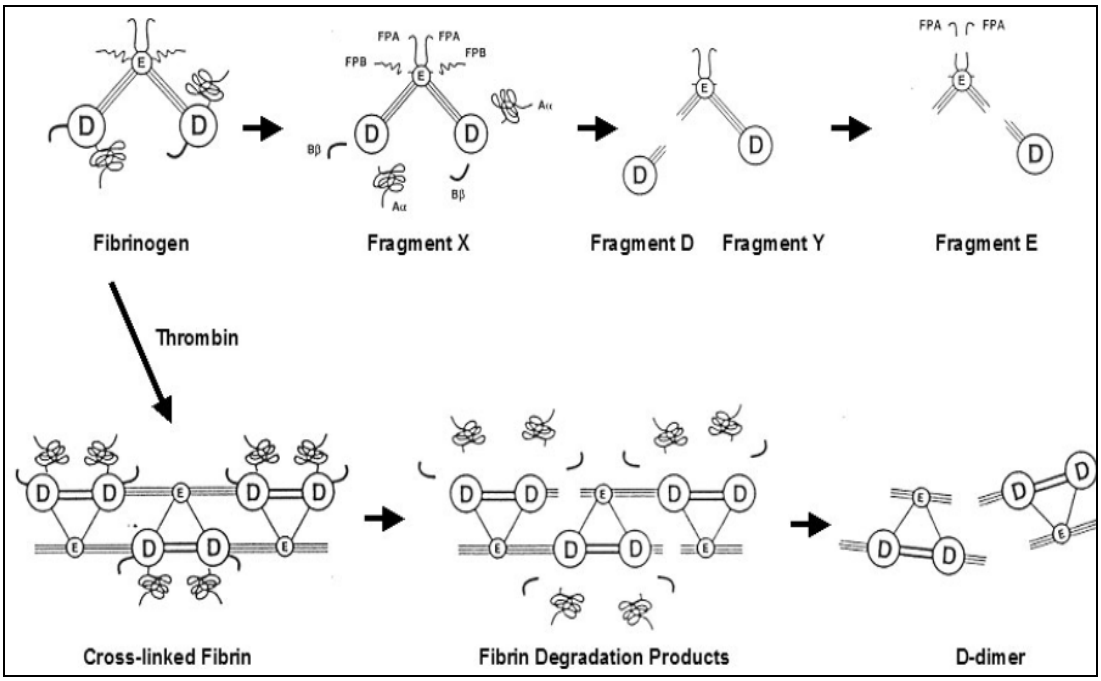


Figure 2.3: Degradation of fibrinogen and cross-linked fibrin by plasmin. Top panel: Plasmin initially cleaves the C-terminal regions of the a- and b-chains within the D-domain of fibrinogen, releasing the Aα- and Bβ-fragments. In addition, a fragment-containing fibrinopeptide B (FPB) from the N-terminal region of the b-chain is also released, giving rise to the intermediate fragment known as 'fragment X'. Subsequently, plasmin cleaves the three polypeptide chains that connect the D- and E-domains, giving rise to fragments D, Y and E. Bottom panel: Fibrinogen can also be polymerized by thrombin to form fibrin. When degrading cross-linked fibrin, plasmin initially cleaves the C-terminal region of the a- and b-chains within the D-domain. Subsequently, some of the connecting regions between the D- and E-domains are severed. Fibrin is ultimately solubilized upon hydrolysis of additional peptide bonds within the central portions of the coiled-coil connectors, giving rise to fibrin degradation products such as D-dimer. Adapted from Cesarman-Maus and Hajjar, 2005.

Plasmin act on the proteolytic cleavage sites on fibrinogen, and subsequently give rise to fragments [Aα, Bβ and fragment fibrinopeptide B (FPB)] for the C- and N-termini of fibrinogen's three polypeptide chains. The resulting molecule is called fragment X which represents a clottable form of fibrinogen. Additional cleavage results in releasing other peptides, and in a series of subsequent reactions, plasmin may further cleave the three polypeptide chains that convert the D- and E- domains. Some of these fragments inhibit the spontaneous polymerization of fibrinogen (Cesarman-Maus & Hajjar, 2005). On the other hand, when fibrin cross-linked by factor XIII, is degraded by plasmin and subsequently fragments known as D-dimers are released (Cesarman-Maus & Hajjar, 2005). In clinical therapy, assays for cross-linked D-dimer fragments are employed to identify disseminated intravascular coagulation-like states associated with excessive plasmin-mediated fibrinolysis (Cesarman-Maus & Hajjar, 2005).

2.1.2 Atherosclerosis

Atherosclerosis is a progressive disease characterized by the accumulation of lipids and fibrous elements in the large arteries, constitutes the single most important contributor to the growing burden of cardiovascular diseases (Libby, 2002). Several mechanisms involved in the antiatherosclerotic effect have been reported (Figure 2.4) (Guillamon *et al.*, 2010). Research in the last two decades has discovered that inflammatory and oxidative processes are common features in several cardiovascular conditions, such as atherosclerosis (Guillamon *et al.*, 2010). Abnormal blood clot called thrombus within the vascular system is formed by the aggregation of fibrin, and the formation of fibrin is triggered from a precursor fibrinogen through the proteolytic action of thrombin. Consequently, it obstructs the flow of blood and nutrients to vital tissue.

In biological system, thromboses can be prohibited through fibrinolysis. The insoluble fibrin fiber is hydrolyzed into fibrin degradation products by plasmin, which is generated from plasminogen by plasminogen activators such as the tissue plasminogen activator, vascular plasminogen activator, blood plasminogen activator, urokinase, Hageman factor and streptokinase-plasminogen complex (Shen *et al.*, 2007).

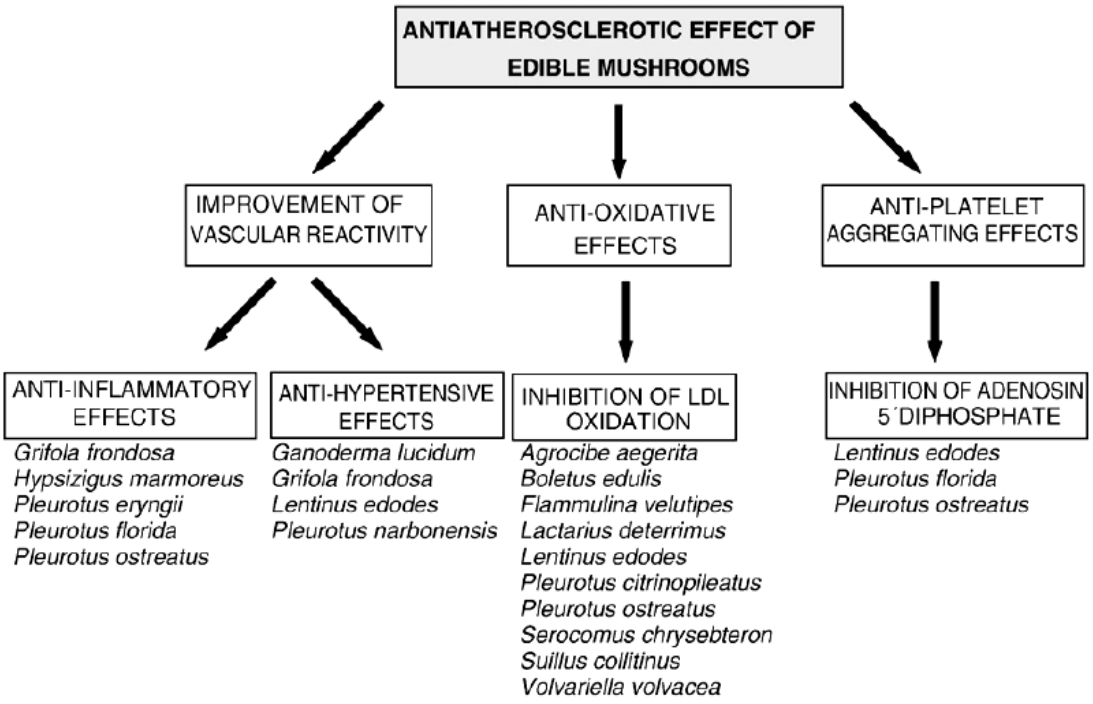


Figure 2.4: Antiatherosclerotic effects and potential involved mechanisms of different edible mushrooms. Adapted from Guillamon *et al.*, 2010.

Today, thrombolytic agents have been extensively used in therapeutic treatment of thrombosis. Thrombolytic agents are classified into: plasminogen activator and plasmin-like protein according to their fibrinolysis mechanisms. Plasminogen activators such as tissue-type plasminogen activator (tPA), urokinase-type plasminogen activator and bacterial plasminogen activator streptokinase, activate the zymogen plasminogen to generate plasmin for fibrinolysis system. In clinical therapy, plasminogen activators are the most widely used among the thrombolytic agents

available. Despite the extensive use, all of these plasminogen activators have undesirable side effects such as resistance to reperfusion, the occurrence of acute coronary reocclusion, and bleeding complications (Kim & Kim, 1999). Besides the relatively high cost, the thrombolytic agents also exhibit low specificity for fibrin (Shen *et al.*, 2007). Plasmin-like proteins which perform fibrinolytic actions directly were discovered from snake venom, earthworm, microorganisms and fermented foods like Japanese *natto*, Korean *chungkook-jang* and Chinese *douchi* (Shen *et al.*, 2007).

2.2 Aqueous two phase system (ATPS)

Aqueous two phase system (ATPS) is an ideal technology combining or integrating clarification, concentration, and partial purification into one single step, and therefore it is prevalent as it is able to shorten and reduce the purification process of protein. Comparing to the conventional liquid-liquid extraction, ATPS has the advantage of preserving the targeted biomolecule with high water content of both phases (70-85% w/w), high biocompatibility and low interfacial tension, low degradation of biomolecules, good resolution, high separation yield, relatively high capacity, ease of scale-up, low material costs and the possibility of polymer and salt recycling (Ramyadevi *et al.*, 2012). Hence, ATPS have been studied extensively on a laboratory scale for the partitioning of whey milk protein, lysozyme (Su & Chiang, 2006), amino acids and peptides (Ramyadevi *et al.*, 2012).

Recently, the potential application of aqueous two phase systems (ATPS) has been demonstrated for the recovery of recombinant protein expressed in plants such as tobacco and seeds (Gu & Glatz, 2007; Platis & Labrou, 2006). Ibarra-Herrera *et al.*

(2011) reported that potential application of selected ATPS as primary step in the recovery process of recombinant human proteins expressed in plants green-tissue, alfalfa. The partition using PEG 8000/phosphate systems comprising of 16.1% (w/w) of polyethylene glycol (PEG), 10% phosphate, tie-line length of 35.7% (w/w), volume ratio equal to one and pH of 7.0 resulted in the potential recovery of 88% of the rhG-CSF (granulocyte-colony stimulating factor) in the top phase. Meanwhile, there was concentration of 93% of alfalfa contaminant proteins at the interface and bottom phase.

Biopharmaceutical products have been discovered in plant species including tobacco, potato, rice, soybean alfalfa, tomato and lettuce (Ma *et al.*, 2003; Stoger *et al.*, 2002). Bioproducts were found to have therapeutic value or diagnostics proteins, industrial proteins, nutritional supplements such as minerals, vitamins, carbohydrates and biopolymers (Ibarra-Herrera *et al.*, 2011). Several reports have revealed that therapeutic proteins produced by the bioreactors obtained from plants consist of several advantages. These advantages include the lack of animal pathogenic contaminants, low cost, larger production level, the presence of natural storage organs such as seeds and tubers, and existing technology for harvesting and processing of plant material (Ibarra-Herrera *et al.*, 2011).

CHAPTER III

MATERIALS AND METHODS

3.1 Preparation of crude extract

Lyophilised powder of *L. rhinocerotis* (Tiger milk mushroom), *C. militaris, G. lucidum, A. polytricha, P. floridanus and P. giganteus* were available in the lab and used in this study. For each sample, 10g of the freeze- dried powder was initially suspended in 200 ml of 20mM Tris-hydrochloride buffer (pH 8.0) [Tris-HCl buffer (pH 8.0)] and then physically mixed in an ice bath for 30 minutes. The suspension was then centrifuged at 10,000 *g* for 30 minutes at 4 ± 2 ^{o}C to remove cell debris. Then, the supernatant was dialysed two to three times using a cellulose membrane with molecular weight 12,000 kDa cut off range, against distilled water. The dialysed crude extract was stored at -20 ± 2 ^{o}C prior to use.

3.2 Preliminary fibrinolytic assay (fibrin plate method)

The fibrin plate method used was a modified method from Kim and Kim (1999). Raw fibrin (bovine blood) was freeze-dried and 0.6 % (w/v) freeze-dried fibrin powder was dissolved in distilled water followed by homogenation at 11,000 *g* for 30 seconds. The homogenate was then centrifuged at 2,800 *g* for 6 minutes to spin down the un-dissolved fibrin. The supernatant was collected as fibrin solution and warmed up to 45 ± 2 ^{o}C. Agarose was prepared at 2 % (w/v) with distilled water and maintained at 55 ± 2 ^{o}C. Fibrin solution was mixed with 2 % agarose in 1:1 ratio and then poured into petri dishes. The clot was allowed to stand for one hour at room temperature. After the medium solidified, a well was made in each of the plates with borer (5mm). Different amount of freeze-dried crude extract was dissolved in 20 mM Tris-HCl buffer to obtain

different concentration of the crude extract. Twenty microliters (20 µl) of crude extract/s was dropped into the well and then incubated at 37±2 ^{o}C for one hour. The lytic zone (the diameter of clear transparent zone) was measured. The steps were repeated by using 3mg/ml plasmin as positive control and Tris-HCl as negative control.

3.3 Aqueous two phase systems (ATPS)

The optimised system used was referred from the study of Ibarra-Herrera *et al*. (2011). Four grams of polyethylene glycol 50 % (PEG 50 %) was added with 2.9 g of phosphate 40 % and 1 g of sample. The system was topped up to 10 g by adding 2.1 g of distilled water, and then mixed the solution by inverting the tube. Then, the tube was centrifuged at 1,500 *g* for 10 minutes. The top and bottom phase was separated into two different tubes. A blank was prepared by replacing the 1 g of sample with 1 g of Tris-HCl.

3.4 Acetone precipitation of proteins

Partially purified enzyme from ATPS was precipitated by using acetone precipitation of protein method (Wessel & Flugge, 1984). A required volume of acetone was cooled to -20 ^{o}C. Protein sample was placed in acetone-compatible tube. Four times of the sample volume of cold (-20^{o}C) acetone was added. The tube was mixed using vortex and incubated for 60 minutes at -20 ^{o}C. Then, the tube was centrifuged 10 minutes at 13,000 - 15,000 *g*. The supernatant was decanted and disposed properly, being careful to not dislodge the protein pellet. The acetone was allowed to be evaporated from the uncapped tube at room temperature for 30 minutes. Pellet was prevented from being over-dried as it may not dissolve properly. Appropriate amount of distilled water was

added for the downstream process and mixed thoroughly through vortex to dissolve protein pellet.

3.5 Fibrinolytic activity assay (Folin-spectrophotometry method)

The crude extract and partially purified enzyme were further evaluated by Folin-spectrophotometer method (Yun *et al*., 2003) for their fibrinolytic capacity. Fibrin was dissolved in 0.1 M Mcllvain buffer (pH 7.0) at concentration of 0.6 % (w/v). The solution was then homogenised at 11,000 *g* for 30 seconds followed by centrifugation at 2,800 *g* for 10 minutes at room temperature. Supernatant was collected as fibrin solution.

After that, 0.3 ml crude extract or partially purified enzyme was added to 0.6 ml fibrin solution and incubated at 40^{o}C for 10 minutes. The reaction was terminated by adding 0.6 ml of 0.55 M trichloro-acetic acid solution for 10 minutes. Mixture was then centrifuged at 2,800 *g* for 10 minutes to get the supernatant. One milliliter of supernatant was mixed with 1 ml of 0.4 M sodium carbonate and subsequently 0.2 ml (1 N) of Folin-Ciocalteau reagent. The mixture was maintained at room temperature for 30 minutes and absorbance reading was then taken at 660 nm. A blank was prepared in the same way except that 0.3 ml of enzyme sample was replaced with an equivalent amount of Mcllvain buffer (pH 7.0) – for crude extract fibrinolytic assay. Another blank was prepared in the same way except that 0.3 ml of enzyme sample was replaced with an equivalent amount of ATPS blank – for partially purified enzyme fibrinolytic assay. The fibrinolytic activity was calculated by comparison with a standard curve generated using tyrosine. Crude extract or partially purified enzyme (0.2 ml) was

mixed thoroughly with 5 ml of Coomassie Brilliant Blue reagent. Blank was only 0.2 ml of distilled water with 5 ml of Coomassie Brilliant Blue reagent. Absorbance was taken at 595 nm and the amount of the soluble protein in the sample was calculated by using the protein calibration plot.

The protease activity was determined in terms of Units, which was the amount in micromoles of tyrosine released from casein per minute. The calculation for protease activity (U/ml) was done by using the formula below:

$$\textbf{Protease activity (U/ml)} = \frac{L-\text{tyrosine released}(\mu\text{g})}{0.3\,\text{ml extract}} \div 10\,\text{minutes of incubation}$$

While, ***L*-tyrosine released (µg)** = $\frac{y}{0.025}$

The specific activity was determined by dividing the protease activity with the soluble protein obtained. For the calculation of soluble protein (mg/ml), the formula used was,

$$\textbf{Soluble protein (mg/ml)} = \frac{\text{y}}{0.007} \div \frac{1}{0.2\text{ml}} \div \frac{1\text{mg}}{1000\mu\text{g}}$$

Based on the standard plots, equation for *L*-tyrosine standard plot was y = 0.025x (Figure A.1) whereas equation for bovine serum albumin standard plot was y = 0.007 (Figure A.2).

3.5.1 Preparation of *L*-tyrosine calibration plot

The calibration plot (Folin & Marenzi, 1929) was prepared in order to estimate the amount of *L*-tyrosine released at 660 nm on spectrophotometer. *L*-tyrosine at concentrations from 25 to 200 µM was prepared to generate a calibration plot. One milliliter of different concentrations of *L*-tyrosine solution was mixed with 1 ml of 0.4 M sodium carbonate and subsequently 0.2 ml of 1 N Folin-Ciocalteu reagent. After 30 minutes, absorbance readings for the mixtures were taken at 660 nm. A blank was prepared in the same way except that *L*-tyrosine was replaced with 1 ml of 0.1 M Mcllvain buffer (pH 7.0). The absorbance was determined at 660 nm with spectrophotometer.

Preparation of *L*-tyrosine solution for calibration plot was summarised in the Table 3.1. Conversion of µM of *L*-tyrosine to µg tyrosine was needed as the unit of fibrinolytic activity was defined in µg tyrosine released. Therefore, the *L*-tyrosine calibration plot was plotted with changes of absorbance against concentrations of *L*-tyrosine (µg). The calculation method for the conversion was described as below:

- Molecular weight of L-tyrosine = 181.19 g / mol
- Mole = $\frac{x}{\text{molecular weight}}$
- 1 mole = 181.19g
- Mole = $\frac{\text{MV}}{1000\ \text{ml}}$
- M = mol x 1000 ml / V
- 1 M = 181.19 g

- Hence 25, 50, 75, 100, 150 and 200 µM of *L*-tyrosine were converted to become 4.53, 9.06, 13.59, 18.12, 27.18 and 36.24 µg respectively.

The changes in absorbance of the crude extracts and partially purified enzyme were translated into *L*-tyrosine released using the *L*-tyrosine calibration plot with the following formula:

$$L\text{- tyrosine released (µg)} = \frac{y}{0.025}$$

Table 3.1: Summary of experimental procedures for preparation of calibration plot with *L*-tyrosine solution (µM) and the changes of absorbance reading at 660 nm

Concentration of *L*-tyrosine (µM)	***L*-tyrosine stock solution (ml)**	**Weight of *L*-tyrosine (µg)**	**0.1M Mcllvain buffer (ml)**	**Absorbance reading at 660nm**
0	0.000	0.000	1.000	0.000
25	0.050	4.530	0.950	0.116
50	0.100	9.060	0.900	0.226
75	0.150	13.590	0.850	0.342
100	0.200	18.120	0.800	0.460
150	0.300	27.180	0.700	0.688
200	0.400	36.240	0.600	0.893

3.5.2 Preparation of protein calibration plot

According to the standard method by Bradford (1976), bovine serum albumin solution (BSA) with 10 to 100 µg/ml of protein was prepared and then the final volume was made up to 1 ml with distilled water in each test tube. Blank contained only 1 ml distilled water without BSA. Five milliliters of Coomassie Brilliant Blue reagent was

added to each test tube and mixed thoroughly. After 30 minutes, absorbance readings for the mixtures were taken at 595 nm and the weight of protein was plotted against the change of absorbance to generate a standard curve. The absorbance readings for different concentrations of bovine serum albumin are summarised in the Table 3.2.

Table 3.2: Standard calibration graph of determination of soluble protein

Concentration of bovine serum albumin (µg/ml)	Absorbance reading at 595 nm
0	0.075
10	0.120
20	0.218
30	0.305
40	0.356
50	0.472
60	0.537
70	0.606
80	0.688
90	0.795
100	0.075

3.6 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

Protein samples were analyzed with SDS polyacrylamide gel electrophoresis. Protein samples were concentrated and precipitated using pre-chilled acetone solution before the SDS-PAGE analysis. Glass plate and spacer were cleaned and assembled "sandwich" in a casting frame. The lower edges of glass plates and spacers were well aligned to make sure there is no leaking of gel. Then, the whole frame was assembled in a casting stand. Fresh ammoniumpersulfate (APS) solution was prepared in 10% and before use only. Acrylamide gel made up of 12% was used in this study. Both resolving and stacking gel mixture were prepared as shown in Table 3.3.

Resolving gel was poured until 2 cm free from the top of the short plate, followed by a quick but steady pipette of distilled water onto the top to create a smooth top surface. After 30 minutes, stacking gel solution was prepared when the resolving gel had polymerized. Then, distilled water was poured off from the top of the resolving gel completely. APS and TEMED were then added into the stacking gel solution and mixed well by pipetting up and down gently to avoid bubbles. Then, stacking gel solution was poured to the top of the resolving gel and comb was inserted. The stacking gel was left for 30 minutes for it to polymerize. The gel sandwich was attached to the electrode assembly. When one gel was used, the gasket was turned from the other side around and acryl block was put on that side. Then, running buffer was poured inside the gel assembly until the buffer covered and be in contact with the gel. After make sure there is no leakage, buffer was poured into the tank to cover the bottom of the gel. Samples were prepared by mixing 15µl of sample and 5µl of sample buffer. Then, electrophoresis was run with 110V for 1 hour 30 minutes. After electrophoresis, polyacrylamide gel was stained with coomassie brilliant blue R-250 for 16-18 hours.

Table 3.3: Preparation of 12% SDS-PAGE gel

Solutions	Resolving gel	Stacking gel
Distilled water	4.4. ml	5.9 ml
Buffer Tris-HCl (1.5M, pH 8.8)	2.5 ml	-
Buffer Tris-HCl (0.5M, pH 6.8)	-	2.5 ml
10% SDS	0.1 ml	0.1 ml
40% bis-acrylamide	3.0 ml	1.5 ml
10% APS	50.0 µl	50.0 µl
TEMED	10.0 µl	15.0 µl

3.7 Protein elution from polyacrylamide gel

3.7.1 Identification and excision of band of interest

Instead of SDS-PAGE, the band of interest was obtained from native PAGE. Native PAGE was carried out same method as SDS-PAGE without the presence of SDS. After gel electrophoresis, clean scalpel was used to cut off a strip on the right or left of the gel. Then, the strip was placed in a tray for staining while the rest of the gel in a container which had filled with distilled water. The cut strip of gel was stained using coomassie brilliant blue R-250 and this strip was used as the 'reference' gel strip. After 30 minutes, the stained strip of gel was aligned with the unstained gel portion and the band of gel that aligns with the stained protein of interest in the reference strip was cut out. The entire remaining gel after excision of bands was stained to determine the accuracy of excision.

3.7.2 Protein elution from the gel matrix

Excised gel pieces were placed in clean screw-up culture or microcentrifuge tubes. Then, 1ml of elution buffer was added so that the gel pieces were completely immersed. Gel pieces were crushed using a clean pestle and incubated in a rotary shaker at 30^{o}C overnight. After that, the tube was centrifuged at 5,000-10,000 *g* for 10 minutes and the supernatant was carefully pipetted into a new microcentrifuge tube. The eluted sample used for fibrin plate assay was prepared from the excised band of interest from native page. A total of 25µl of eluted sample was loaded on each fibrin plate and incubated at 37^{o}C for one hour.

3.8 Statistical Analysis

Mean values of triplicate data for all the parameters tested were obtained and subjected to one-way analysis of variance (ANOVA). The significant was tested by using the multiple range tests at 95% least significant difference (LSD). The least significant difference was conducted to detect any significant difference in fibrinolytic activity of *L.rhinocerotis* enzymes (Appendix B, Table B1 to Table B4).

CHAPTER IV

RESULTS

4.1 Preliminary fibrinolytic assay (fibrin plate method)

Among the six mushroom samples, both *L. rhinocerotis* and *A. polytricha* showed fibrinolytic activity (lytic zone observed) in fibrin plates. As the fibrinolytic activity of *A. polytricha* is well documented, this study focused on the potential of *L. rhinocerotis* as a fibrinolytic agent. Several concentrations of the sample were tested in the preliminary screening, and the observations are summarized in Table 4.1.

According to Table 4.1, there was no lytic zone observed in the fibrin plate that contained 0.2 mg/ml of crude extract. For the 0.3 mg/ml crude extract, one centimeter of lytic zone was observed which indicated the lowest concentration for fibrinolytic activity in this study. The observation showed that crude extracts from 0.4 mg/ml increasing to 0.7 did not increase the lytic zone, which was 1.2 cm in diameter. The diameter of lytic zone for positive control was one centimetre, while no lytic zone was found in negative control (Tris-HCl buffer).

Table 4.1: Diameter of lytic zone (cm) observed in 18 plates after one hour of incubation at 37±2^{o}C

Concentration of crude extract (mg/ml)	Average of diameter of lytic zone (cm)
0.2	-
0.3	1.0 ± 0.05
0.4	1.2 ± 0.05
0.5	1.2 ± 0.02
0.6	1.2 ± 0.03
0.7	1.2 ± 0.02

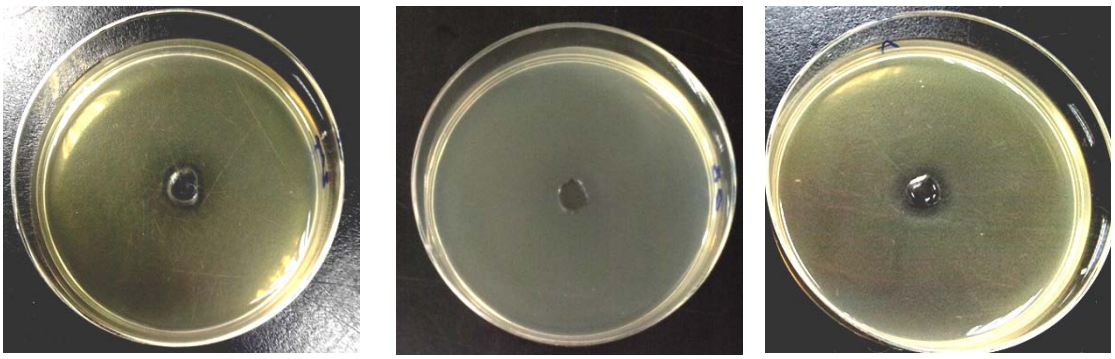
4.2 Fibrin plate assay for crude extract and partially purified enzyme

The sample was partially purified through aqueous two phase system (ATPS) by using the optimized system (Ibarra-Herrera *et al.*, 2010). In order to determine which phase would concentrate the fibrinolytic enzyme, fibrin plate assay was carried out for both top and bottom phase of aqueous two phase system. From the result summarised in Table 4.2 and Figure 4.1, the lytic zone observed in crude extract and top phase of ATPS was 1.2 cm and 1.0 cm respectively.

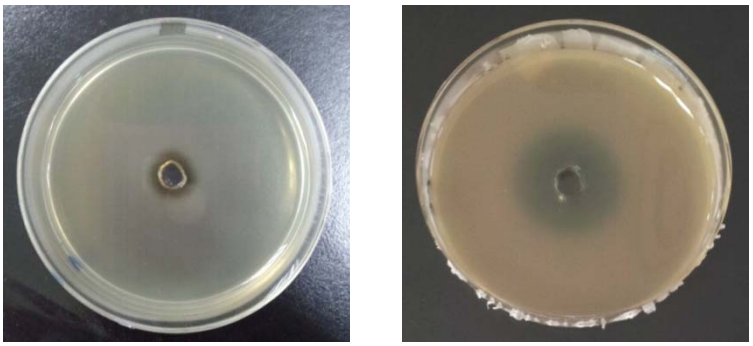
For positive control, one centimetre of lytic zone was observed. While, there was no lytic zone for sample bottom phase from ATPS, top and bottom phase of ATPS blank, and negative control (Tris-HCl buffer). The present research found that the incubation's duration of the assay influenced the diameter of lytic zone, the overnight plate of crude extract showed a double- increased of diameter (2.6cm) [Figure 4.1(e)].

Table 4.2: Fibrin plate assay for crude extract and partially purified enzyme

Sample	Diameter of lytic zone (cm)
Crude extract	1.2 ± 0.01
Human plasmin (positive control)	1.0 ± 0.01
Top phase of ATPS	1.0 ± 0.01
Bottom phase of ATPS	-
Top phase of blank	-
Bottom phase of blank	-
Tri-HCl buffer (Negative control)	-



(a) (b) (c)



(d) (e)

Figure 4.1: Lytic zone in fibrin plates after the application of samples. (a) 3 mg/ml of human plasmin (positive control); (b) Tris-HCl buffer (negative control); (c) ATPS top phase; (d) 3 mg/ml of crude extract; (e) 3 mg/ml of crude extract incubated for 16 hours. Fibrin plate was prepared from raw fibrin solution and 2% (w/v) agarose solution that mixed in 1:1 ratio. Samples (20 µl) were loaded in the well made by borer and incubated at 37 ± 2^{o}C for one hour.

4.3 Fibrinolytic assay (Folin-spectrophotometric method)

Crude extract and partially purified enzyme were evaluated for their fibrinolytic activity by Folin-spectrophotometric method. Prior to the evaluation of fibrinolytic activity, standard curve for *L*-tyrosine (Appendix A, Figure A.1) and bovine serum albumin was plotted (Appendix A, Figure A.2). Based on the standard plots, equation for *L*-tyrosine standard plot was y = 0.025x (Appendix A, Figure A.1) whereas equation for bovine serum albumin standard plot was y=0.007 (Appendix A, Figure A.2). Specific activity

and purification fold of crude extract and partially purified enzyme are summarized in Table 4.3.

According to Table 4.3, the protease activity of crude extract was 2.817 U/ml which was higher than ATPS top phase and bottom phase, 1.520 U/ml and 0.007 U/ml respectively. However, the protease activity of crude extract was lower than the protease activity of acetone precipitated ATPS top phase, which was 4.907 U/ml.

Table 4.3: Specific activity and purification fold of crude extract and partially purified enzyme from *L. rhinocerotis*

Sample	Protease activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg)	Purification fold
Crude extract	2.817 (± 0.002)	0.050 (± 0.003)	56.850	1.000
ATPS top phase	1.520 (± 0.240)	0.015 (± 0.004)	93.850	1.650
ATPS bottom phase	0.007 (± 0.010)	0.005 (± 0.007)	1.390	0.020
Acetone precipitated protein (ATPS top phase)	4.907 (± 0.350)	0.032 (± 0.004)	151.610	2.670

The concentration of protein was measured using Bradford protein assay. Crude extract contained the highest amount of protein, 0.05 mg/ml, whereas ATPS bottom phase contained the lowest amount of protein, 0.005 mg/ml. ATPS top phase contained 0.015 mg/ml of protein which was higher than ATPS bottom phase but lower than crude extract and acetone precipitated ATPS top phase (0.0324 mg/ml).

For specific activity, acetone precipitated ATPS top phase showed the highest activity which was 151.61 U/mg, followed by 93.85 U/mg from ATPS top phase, 56.85 U/mg from crude extract and the lowest specific activity was 1.39 U/mg from ATPS bottom phase. The partially purification method resulted in a 2.67 fold purification of the enzyme.

Based on Table B.1 in Appendix B, the sample was said to be normally distributed since all the p-values were greater than the significant level of 0.05 in the normality test. Levene Test was not significant as the p-value was greater than 0.05, we could assume that the data showed homogeneity of variances (Appendix B, Table B.2). For ANOVA test, the p-value was less than 0.05, which showed that there were significant differences between the groups (Appendix B, Table B.3). Using Duncan post-hoc test, the degree of specific activity was determined where acetone precipitated protein showed the highest activity while ATPS bottom as the lowest (Appendix B, Table B.4).

4.4 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

The molecular mass of the partially purified enzyme was estimated by SDS-PAGE. SDS-PAGE analysis of crude extract and partially purified enzyme and were shown in Figure 4.2(a) and Figure 4.2(b) respectively. The SDS-PAGE image shown in Figure 4.2(a) illustrates the proteins present in crude extract of *L. rhinocerotis*. Based on the SDS-PAGE image shown in Figure 4.2(b), the partially purified enzyme showed double bands on SDS-PAGE, with the molecular weight of 55 kDa and 60 kDa approximately.

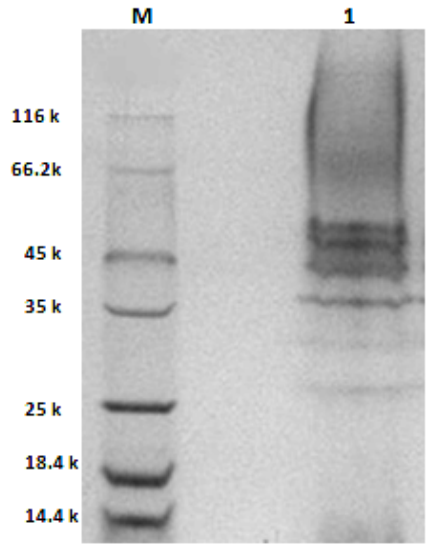


Figure 4.2(a): Molecular mass determination of crude extract on SDS-PAGE. Lane: M = Protein molecular weight standards; 1=Crude extract.

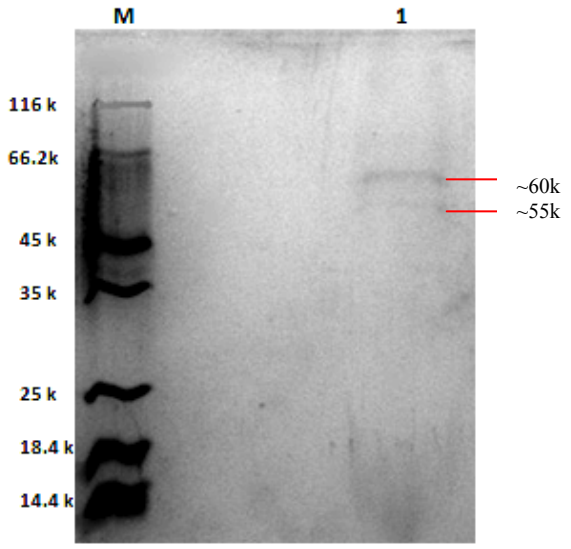
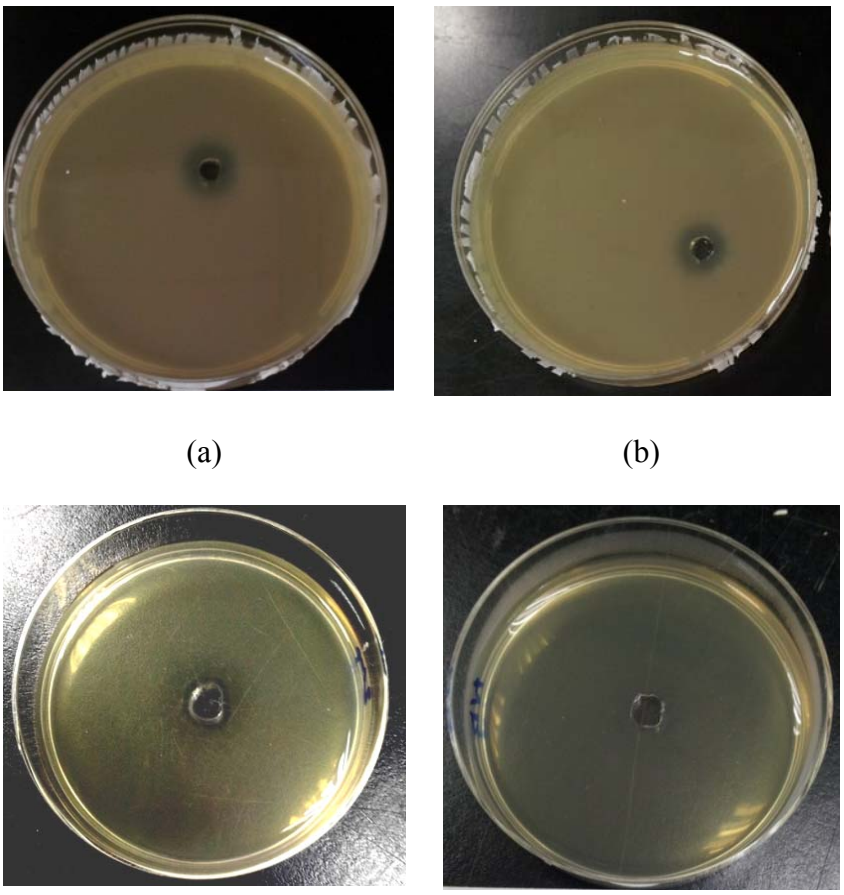


Figure 4.2(b): Molecular mass determination of partially purified enzyme on SDS-PAGE. Lane: M = Protein molecular weight standards; 1= Partially purified enzyme.

4.5 Elution of protein from band of interest

The partially purified enzyme was the fibrinolytic enzyme that present in *L. rhinocerotis*. In order to reconfirm the presence of the fibrinolytic effect, biological test was carried out. In this study, fibrin plate assay was used as the biological test. The band strips of interest obtained from native page gel were crushed gently and eluted by using buffer (50 mM Tris-HCl, 150 mM NaCl; pH7.5). The supernatant of the eluted protein were tested on fibrin plates, and as a result, the eluted protein showed visible digestive zone, with measurement of diameter 1.3 cm and 1.2 cm respectively [Figure 4.3 (a) and (b)].



(a) (b)

(c) (d)

Figure 4.3: Fibrin plate assay of eluted protein. (a) eluted protein of ~60kDa band; (b) eluted protein of ~55kDa band; (c) elution buffer (negative control).

CHAPTER V

DISCUSSION AND CONCLUSIONS

5.1 Preparation of crude extracts

The supernatant of crude extract was dialyzed in order to remove protein of smaller molecular weight. Cellulose membrane with 12 kDa cut off range was used in the dialysis process since the molecular mass of fibrinolytic enzyme obtained from a related source, honey mushroom (*A. mellea*) fruiting bodies was 18 kDa (Kim and Kim, 1999). Besides, fibrinolytic protease purified from *S. commune*, one of the medical mushrooms was reported to have 21.32 kDa (Lu *et al*., 2010). In addition, the molecular mass of fibrinolytic metalloprotease discovered from *P. ostreatus* was estimated to be 32 kDa (Shen *et al*., 2007).

According to Dixon *et al*. (1979a), drying in high vacuum at low temperature from the frozen state was useful in producing an active and soluble powder which can be stored at room temperature. Hence, freeze-dried sample was preferred in this study since fibrinolytic enzyme can be preserved better during the freeze-drying process. Besides, the crude extract of freeze-dried fruit bodies showed the highest fibrinolytic activity using the fibrin plate assay compared to the fresh and oven-dried fruit bodies obtained in a previous study by Kho (2008). Hence, the dialyzed crude extract used in this study was freeze-dried.

One of the factors that affect the protein stability during the preparation of crude extracts was temperature, especially high temperature. Due to thermal stability, many

proteins could be slowly denatured above 25^{o}C. Hence, the preparation of crude extracts in this study was performed on an ice bath (4^{o}C).

Besides temperature, constant pH of buffer was essential to maintain protein stability (Hames and Hooper, 2000). In this study, Tris-HCl buffer with constant pH of 8.0 was used. Tris buffer was preferred since it is comparatively inexpensive, very freely soluble in water, inert in the enzymatic system and had a high buffer capacity. In addition, the sample powder was physically mixed instead of blending and homogenizing so as to minimize frothing and at the same time, maintaining the stability of the protein.

Many proteins could be denatured by contact with the air-water interface, and significant fraction of protein may be lost through adsorption to surfaces. Therefore, the sample powder and solvent buffer were mixed in 1:20 ratio to make sure that enzyme was relatively concentrated and fully dissolved (Mohammad Ali et al., 2014).

5.2 Preliminary fibrinolytic assay (fibrin plate method)

In this study, increased concentration of the *L. rhinocerotis* sample resulted in the increment of the size of the lytic zone, which indicating increase of the fibrinolytic activity. At the concentration of 0.4 mg/ml to 0.7, the lytic zones observed were constant which was 1.2 cm in diameter. In fact, the slight increase of the fibrinolytic activity could not be accurately determined through the fibrin plate assay. Hence, quantitative assay was carried out in this study. Fibrinolytic effect of traditional

medicinal mushrooms, *G. lucidum* and *C. militaris*, has been previously reported (Kumaran *et al*., 2011 and Cui *et al*., 2008). However, there was no fibrinolytic activity observed with this mushroom and this might be due to the geographical factors that could affect the properties of the mushrooms.

The fibrin plate assay used in this study was modified from the method of Kim and Kim (1999) in which raw bovine blood collected from local abattoir was utilized as raw blood fibrin. In place of the purified protein that available in market, raw bovine blood was preferred to be used due to several reasons. Besides low material cost, raw bovine blood could form blood clot naturally which was considered as a more suitable model for fibrin clot formation in this study. The utilization of whole bovine blood may reduce the loss of any co-factor and co-enzyme which involved in the fibrin clot formation (Kho, 2008).

In the fibrin plate assay, clear transparent regions indicated hydrolysis of fibrin, and its lytic zone diameter was directly proportional to the potency of the fibrinolytic activity. A borer was used to make well in fibrin plate so that sample was loaded in the center of fibrin plate without spreading, as an even circular shape of lytic zone was necessary for diameter estimation. The incubation temperature was set at 37^{o}C to give optimal enzymatic activity.

During the preparation of fibrin plates, the fibrin agarose mixture must be poured evenly in every plate in terms of the thickness of the fibrin agar. The ideal thickness of the fibrin agar was the thickness in which the level was parallel to the

height of 20µl of sample loaded in the well. This is to ensure the accuracy and reproducibility of the estimation of fibrinolytic activity.

5.3 Purification of fibrinolytic enzyme

Crude extracts are complex mixture which contains substances that will interfere with the downstream applications. Hence, purification is necessary to extract certain specific enzyme from the complex mixture. The conventional methods of purification of biomolecules involve several steps of operations and therefore are usually expensive. Moreover, some quantity of target molecule is lost in each step of purification and resulting in a big overall loss. The aqueous two phase system (ATPS) is an alternative method for the separation of biomolecules which reduces number of steps and also the cost of operation. The technique is used in recovery and purification of many biological products including proteins, genetic material, bionanoparticles, cells and organelles (Andrews and Asenjo, 2010; Rito-Palomares, 2004).

Complete phase separation was achieved when two phases were formed. The top phase was polymer phase and the bottom phase was the salt phase. The fibrinolytic enzyme should be concentrated in one of the phases and the contaminants in the other. The phase preferences were attributed to the hydrophilic nature of the proteins present in the extracts and the effect of system parameters on the partition behaviour of the proteins (Ibarra-Herrera *et al*., 2011). Parameters were manipulated to partition the enzyme in polymer (PEG with molecular weight of 8000 g mol^{-1}) because the presence of salt may interfere the downstream applications. Based on Ibarra-Herrera *et al*. (2011), ATPS using PEG with molecular weight of 8000 g mol^{-1} can be easily

implemented for the recovery of hydrophobic proteins that exhibit top phase preference. The fibrinolytic protease from *Streptomyces* sp. DPUA1576 was partitioned preferentially to the PEG rich phase under these conditions (Medeiros e Silva *et al*., 2013). In addition, protease extraction through ATPS was reported from *A. bisporus*, in which the protease was also partitioned to the PEG rich phase (Deloisa *et al*., 2009).

Due to cost considerations, the aqueous two phase polymer/salt system was preferred over the other systems. Polymer/salt systems have advantages including larger differences in density, greater selectivity, lower viscosity, lower cost and larger relative size of the drops (Chunha and Aznar, 2009). Polyethylene glycol (PEG) was used as the polymer in ATPS because it is available at low cost and able to form a two-phase system with salts. In addition, PEG can significantly enhance the refolding of proteins to recover the activity (Cleland *et al*., 1992).

There are several factors that influence the recovery of biomolecules from a mixture by ATPS such as phase components and their concentration, tie-line length (TLL), pH, temperature, and sample concentration. In order to optimize the ATPS, many number of experiments have to be carried out. This is laborious, time consuming and increases the overall cost. Different systems selected for ATPS could allow differential partitioning between target protein and contaminants. Therefore, the system selected for the evaluation of the partition behaviour of the proteins from *L. rhinocerotis* was based on the previous experiences by Aguilar and Rito-Palomares (2008). The strategy behind the selection of the experimental systems was well explained in another paper of Rito-Palomares (2004). According to Aguilar *et al*.

(2006), the system tie-line length (TLL) that represents the length of the line which connects the compositions of the top and bottom phases in a phase diagram for a defined system, was calculated and described. In addition, the application of the selected system on other mushroom samples was investigated in the laboratory recently and showed ideal results.

Using ATPS, it is necessary to precipitate and concentrate the proteins after the partitioning step. In this study, acetone precipitation of proteins was employed to remove undesirable substances which may interfere with downstream applications and analyses. After centrifugation to pellet the precipitated proteins, the supernatant which contained the interfering substances was discarded. The acetone precipitation of protein step was performed once only instead of repeating the purification step to prevent sample loss in each cycle of precipitation.

5.4 Fibrin plate assay for crude extract and partially purified enzyme

Fibrinolytic activity was observed in both the crude extract and partially purified enzyme of *L. rhinocerotis* when assayed by using fibrin plate after one hour of incubation. In comparison, fibrinolytic activity of the culture filtrate from *C. militaris* was observed after 5 hours when it was incubated in fibrin plate (Cui *et al*., 2008). Therefore, the result explains that the fibrinolytic enzyme found in *L. rhinocerotis* is higher than in *C. militaris* a property might be helpful in the thrombolytic therapy in the future. On the other hand, the present study found that the fibrinolytic activity of *L. rhinocerotis* was stable overnight. A 2x increase of the lytic zone was observed after 16 hours which demonstrate the stability of the enzyme derived from *L. rhinocerotis*. The

partially purified enzyme obtained in the top phase of ATPS had a slightly smaller diameter lytic zone on the fibrin plate than the crude extract of freeze dried *L. rhinocerotis*. The result may due to the amount of the fibrinolytic enzyme recovered was reduced throughout each of the purification process.

5.5 Fibrinolytic assay (Folin-spectrophotometric method) for crude extracts and partially purified enzyme preparations

The fibrinolytic protease from *L. rhinocerotis* in top phase of ATPS showed the highest specific activity after partitioning and precipitation by cold acetone (151.61 U/mg). It is because protein was concentrated through the precipitation action on the partially purified enzyme produced by ATPS, while a number of contaminants in the sample had been eliminated after partitioning by ATPS. This assay showed that the purification fold in the top phase of ATPS (1.65 fold) was higher than in the bottom phase (0.02 fold). It was demonstrated that fibrinolytic enzyme was well partitioned into top phase. The top phase of ATPS is usually the polymer (PEG) which was hydrophobicity, and hence, the hydrophobic protein preferably partitioned in the top phase of ATPS (polymer phase). There was a small detection of activity in the bottom phase of ATPS and some of them had no activity at all. It may be caused by technical error during pipetting out the bottom phase solution. The bottom phase solution might have mixed with some amount of the ATPS top phase solution causing a small detection of fibrinolytic activity. As compared to the recent study by Mohamed Ali et al. (2014), the fibrinolytic enzyme from a mushroom was also partitioned to the top phase of the ATPS and with good recovery outcome.

From the result shown in Table 4.3, the specific activity of ATPS top phase protein after acetone precipitation was 2.67 fold. In the report by Kim and Kim (1999), the specific activity of the fibrinolytic enzyme from the sample *A. mellea* was 2.41 fold after ammonium sulphate precipitation. As a comparison, the purification fold found in this study is slightly higher than the ammonium sulphate purification method. Hence, ATPS may be considered as a better option since the recovery of the fibrinolytic enzyme in mushroom using ammonium sulphate extraction method was low (Mohamed Ali et al., 2014). The utilization of ATPS for primary recovery of phytase produced by *S. commune* was reported in a recent study by Salmon et al., (2014) with considerably high purification fold (5.43) of protein, which indicated the efficiency of the method.

The Enzyme Commission recommended that specific activity should be given as unit per mg protein. Therefore, Folin-Ciocalteau reagent was used in the present study to estimate the quantity of fibrinolytic enzyme more accurately. This reagent reacted on chromogenic nature of the amino acid (tyrosine) side chain was sensitive, enabling it to detect 0.01 to 0.1 mg of protein in the assay (Dixon and Webb, 1979b).

Folin-spectrophotometric method was used in the assay of fibrinolytic activity because of its simplicity, sensitivity and ease. According to Dixon and Webb (1979b), enzyme unit is defined in term of the change of absorbance in a certain time of incubation with other reaction conditions being constructed. Ten minutes of incubation time at 40^{o}C was used in this study. These conditions were sufficient to assay as a fibrinolytic activity because of short reaction time.

In this assay, casein was the substrate and when protease broke peptide bonds of casein, the amino acid tyrosine was released along with other amino acids and peptide fragments. Folin's reagent reacted primarily with free tyrosine to produce a blue colored chromophore, which was quantifiable and measurable as an absorbance value at 660 nm in a UV spectrophotometer. The absorbance values generated by the activity of protease were compared to a standard curve. The amount of tyrosine released was proportional to the protease activity. In other words, the more the chromophore were generated, the greater the protease activity.

5.6 Molecular mass estimation for partially purified enzyme

The molecular weights of the partially purified enzyme were confirmed as 55kDa and 60kDa after the fibrin plate assay and gel elution. The highest molecular weight of fibrinolytic enzyme was reported in *G. lucidum* (100kDa), whereas the lowest in *Pleurotus eryngii* (14kDa) (Choi and Sa, 2000; Lu and Chen, 2012). The molecular weights found in this study were closest to the molecular weight reported in *A. polytricha*, which is 66kDa (Mohamed Ali et al., 2014). The molecular weight of the fibrinolytic enzyme found in this study did not match to the reported ones. In fact, the molecular weight of the fibrinolytic protease might differ from diverse species (Choi et al., 2011).

The purpose of the SDS (sodium dodecyl sulfate) detergent was to denature the proteins and dissociate them from each other. Besides, it solubilizes all proteins and then covered the proteins with negative charges. During SDS-PAGE, all proteins migrate downwards to the positively charged electrode (anode). Good quality of SDS

was used to ensure that sharp protein bands were observed. Poor quality or old SDS would cause protein with stained background along the individual gel tract with indistinct protein band. The concentration of SDS should not be too high as high concentration of SDS might interfere with Coomassie Blue staining.

5.7 Native PAGE and excision of band of interest

Both of the bands on native PAGE were eluted in order to detect the enzyme activity. The partially purified enzyme from *L. rhinocerotis* exhibited fibrinolytic activity. In order to reconfirm the presence of the fibrinolytic effect, its biological activity was assayed. In this study, fibrin plate assay was used as the biological assay. The bands of interest (55kDa and 60kDa) were excised from native page gel, crushed gently and the protein eluted by using buffer (50 mM Tris-HCl, 150 mM NaCl; pH7.5). Native PAGE was employed instead of SDS-PAGE for gel excision because active and non-denaturing conditions were necessary for biological assay. In the present study, the eluted protein showed fibrin digestion and confirmed the fibrinolytic activity of *L. rhinocerotis*.

5.8 Recommendations for future studies

Future study may focus on the large scale of the protein purification through aqueous two phase system (ATPS) in the considerations of commercial use of the product. In addition, blood parameters such as C-reactive protein, fibrinogen, leukocyte count, monocyte count, total cholesterol, high density lipoprotein-cholesterol, LDL-cholesterol and triglycerides in animal models should be evaluated in order to characterize the *in*

vivo potential of this enzyme to reduce the risk of the cardiovascular disease (Boudjeltia *et al*., 2006).

5.9 Conclusions

Lignosus rhinocerotis and *A. polytricha* were found to exhibit fibrinolytic activities, while *P. giganteus* and *P. floridanus* did not show any fibrinolytic activity. However, *G. lucidum* and *C. militaris* which were recognized traditional medicinal mushrooms were found to have no fibrinolytic activity.

This was the first report of fibrinolytic activity of *L. rhinocerotis*. The fibrin plate assay result had shown that *L. rhinocerotis* had the potential to lyse the fibrin clot. Besides, the fibrinolytic effect remained after overnight (16 hours), which demonstrated the stability of the fibrinolytic activity. The specific activity of the crude extract evaluated through Folin-spectrophotometric method was 56.85 U/mg.

The fibrinolytic enzyme in crude extract from *L. rhinocerotis* was partially purified through aqueous two phase system (ATPS) and subsequently concentrated by using cold acetone. Fibrin plate assay was employed to examine fibrinolytic activity of the ATPS partitions (top and bottom phases). The assay demonstrated that the fibrinolytic enzyme showed more affinity to top phase compared to the bottom phase. After evaluation by using the Folin-spectrophotometric method, the specific activity of the partially purified enzyme was 151.61 U / mg, which represented a 2.67 of purification fold.

The molecular mass of the partially purified enzyme estimated through SDS-PAGE and resulted was 55 kDa and 60 kDa approximately. Native PAGE and gel purification step was carried out and confirmed the fibrinolytic activity of the partially purified enzyme.

CHAPTER VI

REFERENCES

Aguilar O. and Rito-Palomares M. (2008). Processing of soybean (Glycine max) extracts in aqueous two-phase systems as a first step for the potential recovery of recombinant proteins. *Journal of Chemical Technology and Biotechnology*. 83: 386–293.

Aguilar O., Albiter V., Serrano-Carreon L. and Rito-Palomares M. (2006). Direct comparison between ion-exchange chromatography and aqueous two-phase processes for the partial purification of penicillin acylase produced by *E. coli*. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*. 835: 77-83.

Andrews B.A. and Asenjo J.A. (2010). Theoretical and experimental evaluation of hydrophobicity of proteins to predict their partitioning behaviour in aqueous two-phase systems. *Separation Science and Technology*. 45: 2165-2170.

Astrup T. and Mullertz S. (1952). The fibrin plate method for estimating fibrinolytic activity. *Archieves of Biochemistry and Biophysics.* 40:346-351.

Barros L., Cruz T., Baptista P., Estevinho L.M., Ferreira I.C.F.R. (2008). Wild and commercial mushrooms as source of nutrients and neutraceuticals. *Food and Chemical Toxicology*. 46: 2742-2747.

Barros L., Ferreira M.J., Queirós B., Ferreira I.C.F.R. and Baptista P . (2007). Total phenols, ascorbic acid, β-carotene and lycopene in Portuguese wild edible mushrooms and their antioxidant activities. *Food Chemistry*. 103: 413-419.

Boudjeltia K.Z., Guillaume M., Henuzet C. Delree Cauchie P., Remacle C., Ducobu J., Vanhaeverbeek M. and Brohee D. (2006). Fibrinolysis and cardiovascular risk factors: Association with fibrinogen, lipids and monocyte count. *European Journal of Internal Medicine*. 17: 102-108.

Bradford M.M. (1976). A rapid and sensitive for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*. 72: 248-254.

Cui L., Dong M.S., Chen X.H. Jiang M., Lv X. and Yan G.J. (2008). A novel fibrinolytic enzyme from *Cordyceps militaris*, a chinese traditional medicinal mushroom. *World Journal of Microbiology and Biotechnology*. 24: 483-489.

Cesarman-Maus G. and Hajjar K.A. (2005). Molecular mechanisms of fibrinolysis. *British Journal of Haematology*. 129: 307–321.

Chang S. T. and Miles P. G. (1989). *Edible mushrooms and their cultivation*. Boca Raton, USA: CRC Press.

Cheung L.M., Cheung P.C.K. and Ooi V.E.C. (2003). Antioxidant activity and total phenolics of edible mushroom extracts. *Food Chemistry*. 81: 249-255.

Cho H.S., Choi E.S., Lim H.G. and Lee H.H. (2004). Purification and characterization of six fibrinolytic serine-proteases from earthworm *Lumbricus rubellus*. *Journal of Biochemistry and Molecular Biology*. 37: 199-205.

Choi D., Cha W., Park N., Kim H., Lee J.H., Park J.S. and Park S. (2011). Purification and characterization of a novel fibrinolytic enzyme from sporocarps of Korean *Cordyceps militaris*. *Bioresource Technology*. 3: 3279-3285.

Choi H.S. and Sa Y.S. Fibrinolytic and antithrombotic protease from *Ganoderma lucidum*. *Mycologia*. 92:545-552.

Chunha E. V. C. and Aznar M. (2009). Liquid-liquid equilibrium in aqueous two-phase (water + PEG 8000 + salt): Experimental determination and thermodynamic modelilng. *Journal of Chemical and Engineering Data*. 54: 3242-3246.

Cleland J.L., Hedgepeth C. and Wang D.I.C. (1992). Polyethylene glycol enhanced refolding of bovine carbonic anhydrase B. *Journal of Biological Chemistry*. 267: 13327-13334.

Deloisa K. M., Hernandez M.R.T. and Palomares M. R. (2009).Recovery of laccase from the residual compost of *Agaricus bisporus* in aqueous two-phase systems. *Process Biochemistry*. 44: 435-439.

Dixon M. and Webb E.C. (1979a). *Enzyme techniques*. London: Longman Group Limited.

Dixon M. and Webb E.C. (1979b). *Enzyme isolation*. London: Longman Group Limited.

Folin O. and Marenzi A.D. (1929). Tyrosine and tryptophane determinations in one-tenth gram of protein. *Journal of Biological Chemistry*. 83: 89-102.

Gu Z.R. and Glatz C.E. (2007). Aqueous two-phase extraction for protein recovery from corn extracts. *Journal of Chromatography B*. 845: 38-50.

Guillamon E., Garcia-Lafuente A., Lozano M., D'Arrigo M., Rostagno M.A., Villares A. and Martinez J.A. (2010). Edible mushrooms: Role in the prevention of cardiovascular diseases. *Fitoterapia*. 81: 715-723.

Hahn B.S., Cho S.Y., Wu S.J., Chang I.M., Baek K.H., Kim Y.C. and Kim Y.S. (1999). Purification and characterization of a serine protease with fibrinolytic activity from *Tenodera sinensis* (praying mantis). *Biochimica et Biophysica Acta*, 1430: 376-386.

Hames B.D. and Hooper N.M. (2000). *Section B – Amino acids and proteins*. Guildford, United Kingdom: BIOS Scientific Publishers Limited.

Ibarra-Herrera C.C., Aguilar O. and Rito-Palomares M. (2011). Application of an aqueous two phase systems strategy for the potential recovery of a recombinant protein from alfalfa (*Medicago sativa*). *Separation and Purification Technology*. 77:94-98.

Imazeki R. and Hongo T. (1989). *Colored illustrations of mushrooms of Japan*. Osaka: Hoikusha Publishing.

Jose N., Ajith T.A. and Janardhanan K.K. (2004). Methanol extract of the oyster mushroom, *Pleurotus floridanus*, inhibits inflammation and platelet aggregation. *Phytotherapy Research*. 18: 43-46.

Kabir Y., Kimura S. and Tamura T. (1988). Dietary effect of *Ganoderma lucidum* mushroom on blood pressure and lipid levels in spontaneously hypertensive rats (SHR). *Journal of Nutritional Science Vitaminology*. 34: 433-438.

Kabir Y., Yamaguchi M. and Kimura S. (1987). Effect of shiitake (*Lentinus edodes*) and maitake (*Grifola frondosa*) mushrooms on blood pressure and plasma lipids of spontaneously hypertensive rats. *Journal of Nutritional Science Vitaminology*. 33: 341-346.

Kaneda T. and Tokuda S. (1966). Effect of various mushroom preparations on cholesterol levels in rats. *Journal of Nutrition*. 90: 371-376.

Kho Y.S. (2008). Antimicrobial, antioxidant and fibrinolytic activities of *Auricularia auricular-judae* (FR.) Quél. Dissertation of master degree of science, Faculty of Science, University of Malaya.

Khohno K., Miyake M., Sano O., Tanaka-Kataoka M., Yamamoto S., Koya-Miyata S., Arai N., Fujii M., Watanabe H. and Ushio S. (2008). Anti-inflammatory and immunomodulatory properties of 2-amino-3H-phenoxazin-3-one. *Biological and Pharmaceutical Bulletin*. 31: 1938-1945.

Kim J.H and Kim Y.S. (1999). A fibrinolytic metalloprotease from the fruiting bodies of an edible mushroom, *Armillariella mellea*. *Bioscience, Biotechnology, and Biochemistry*. 63:2130-2136.

Kumaran S., Palani P., Nishanthi R., Srimathi S. and kaviyarasan V. (2011). Purification of an intracellular fibrinolytic protease from *Ganoderman lucidum* Vk12 and its susceptibility to different enzyme inhibitors. *Tropical Journal of Pharmaceutical Research*. 10: 413-420.

Lai C.K.M., Wong K.H. and Cheung P.C.K. (2008). Antiproliferative effects of sclerotial polysaccharides from *Polyporus rhinocerus* cooke (Aphyllophoromycetideae) on different kinds of leukemic cells. *International Journal of Medicinal Mushrooms*. 10: 255-264.

Lai W.H.,Siti Murni M.J., Fauzi D., Abas Mazni O. and Saleh N.M. (2011). Optimal culture conditions for mycelia growth of *Lignosus rhinocerotis*. *Mycobiology*. 39: 92-95.

Lee J.S., Baik H.S. and Park S.S. (2006). Purification and characterization of two novel fibrinolytic proteases from mushroom, *Fomitella fraxinea*. *Journal of Microbiology and Biotechnology*. 16: 264-271.

Lee L.M., Tan N.H., Fung S.Y., Tan C.S. and Ng S.T. (2011). The antiproliferative activity of sclerotia of *Lignosus rhinocerotis* (Tiger Milk mushroom). *Evidence-Based Complementary and Alternative Medicine.* 10.1155/2012/697603.

Lee S.Y., Kim J.S., Kim J.E., Sapkota K., Shen M.H. , Kim S., Chun H.S., Yoo J.C., Choi H.S., Kim M.K. and Kim S.J. (2005). Purification and characterization of fibrinolytic enzyme from cultured mycelia of *Armillaria mellea*. *Protein Expression and Purification*. 43: 10-17.

Libby P. (2002). Inflammation in atherosclerosis. *Nature*. 420: 868-74.

Lindequist U., Niedermeyer T. H. J. and Julich W. D. (2005). The pharmacological potential of mushrooms. *Evidence-Based Complementary and Alternatice Medicine.* 2: 285-299.

Lu C. and Chen S. (2012). Fibrinolytic enzymes from medicinal mushrooms. *Protein Structure*. 15: 338-362.

Lu C.L., Chen S.W. and Chen S.N. (2010). Purification and characterization of a novel fibrinolytic protease from *Schizophyllum commune*. *Journal of Food and Drug Analysis*. 18: 69-76.

Ma J.K.C., Drake P.M.W. and Christou P. (2003). The production of recombinant pharmaceutical proteins in plants. *Nature Reviews Genetics*. 4: 794-805.

Macfarlane R. G. and Biggs R. (1948). Fibrinolysis. Its mechanism and significance. *Blood.* 3: 1167-1187.

Mattila P., Konko K., Eurola M., Philava J.M., Astola J., Vahteristo L., Hietaniemi V., Kumpulainen J., Valtonen M. and Piironen V. (2001). Contents of vitamins, mineral elements, and some phenolic compounds in cultivated mushrooms. *Journal of Agriculture and Food Chemistry*. 49: 2343 – 2348.

Medeiros e Silva G.M., Araujo Viana Marques D., Porto T.S., Filho J.L.L., Teixeira J.A.C, Junior A.P. and Porto A.L.F. (2013). Extraction of fibrinolytic protease from *Streptomyces* sp. DPUA1576 using PEG-phosphate aqueous two-phase systems. *Fluid Phase Equilibria*. 339: 52-57.

Miyazawa N., Okazaki M. and Ohga S. (2008). Antihypertensive effect of *Pleurotus nebrodensis* in spontaneously hypertensive rats. *Journal of Oleo Science*. 57: 675-681.

Mohamed Ali S., Ling T.C., Sekaran M., Tan Y.S., Jegadeesh R. and Vikineswary S. (2014). Recovery and partial purification of fibrinolytic enzymes of *Auricularia polytricha* (Mont.) Sacc by an aqueous two-phase system. *Separation and Purification Technology*. 122: 359-366.

Nonaka T., Naoshi D., Yohichi H., and Koji T. (1997). Amino acid sequences of metalloendopeptidases specific for acyl-lysine bonds from *Grifola frondosa* and *Pleurotus ostreatus* fruiting bodies. *Journal of Biological Chemistry*. 272: 30032-30039.

Noorlidah A., Muhammad Z.D.H., Lau B.F. and Suffian M.A. (2013). Domestication of a wild medicinal sclerotial mushroom, *Lignosus rhinocerotis* (Cooke) Ryvarden. *Industrial Crops and Products*. 47: 256-261.

OECD/WHO. (2012). Mortality from cardiovascular disease. Health at a Glance: Asia/Pacific 2012. *OECD Publishing*. Retrieved from: http://dx.doi.org./10.1787/9789264183902-8-en.

Platis D. and Labrou N.E. (2006). Development of an aqueous two phase partitioning system for fractionating therapeutic proteins from tobacco extract. *Journal of Chromatography A*. 1128: 114-124.

Ramyaderi D., Subathira A. & Saravanan S. (2012). Central composite design application for optimization of aqueous two phase extraction of protein from shrimp waste. *Journal of Chemical and Pharmaceutical Research*. 4: 2087-2095.

Rito-Palomares M. (2004). Practical application of aqueous two-phase partition to process development for the recovery of biological products. *Journal of Chromatography B*: *Analytical Technologies in the Biomedical and Life Sciences*. 807: 3 -11.

Salmon D.N.X., Walter A., Porto T.S., Moreira K.A., Souza Vandenberghe L.P., Soccol C.R., Porto A.L.F. and Spier M.R. (2014). Aqueous two-phase extraction for partial purification of *Schizophyllum commune* phytase produced under solid-state fermentation. *Biocatalyst and Biotransformation*. 32: 45-52.

Shen M.H., Kim J.S., Kumar S., Park S.E., Choi B.S., Kim S., Lee H.H., Kim C.S., Chun H.S., Ryoo C.I. and Kim S.J. (2007). Purification, characterization and

cloning of fibrinolytic metalloprotease from *Pleurotus ostreatus* mycelia. *Journal of Microbiology and Biotechnology*. 17: 1271-1283.

Smiderle F. R., Sassaki G. L., Arkel J. van, Iacomini M., Wichers H. J. and Griensven L. J. van. (2010). High molecular weight glucan of the culinary medicinal mushroom *Agaricus bisporus* is an *α*-Glucan that forms complexes with low molecular weight galactan. *Molecules*. 15: 5818–5830.

Stoger E., Sack M., Fischer R. and Christou P. (2002). Plantibodies: applications, advantages, and bottlenecks. *Current Opinion in Biotechnology*. 13: 161-166.

Su C.K. and Chiang B.H. (2006). Partitioning and purification of lysozyme from chicken egg white using aqueous two-phase system. *Process Biochemistry*. 41: 257–263.

Sun M.Z., Liu S.Q. and Greenaway F.T. (2006). Characterization of a fibrinolytic enzyme (ussurenase) from *Agkistrodon blomhoffii ussurensis* snake venom: Insights into the effects of Ca^{2+} on function and structure. *Biochimica et Biophysica Acta*. 1764: 1340-1348.

Tan C.S. (2009). *Lignosus rhinocerotis* (Cooke) Ryvarden 1972. *Encyclopedia of Life*. Retrieved from: http://www.eol.org/pages/192772.

Thomson A. (1999). New thrombolytic drugs. *Australian and New Zealand Journal of Medicine*. 29: 433-435.

Wang C.T., Ji B.P., Li B., Nout R., Li P.L., Ji H. and Chen L.F. (2006). Purification and characterization of fibrinolytic enzyme of *Bacillus subtilis* DC33, isolated from Chinese traditional Douchi. *Journal of Industrial Microbiology and Biotechnology*. 33: 750-758.

Wessel D. and Flugge U.I. (1984). A method for the quantitative recovery of protein in dilute solution in the presence of detergent and lipids. *Analytical Biochemistry*. 138: 141-143.

WHO. (2012). World health statistics. *World Health Organization*. Retrieved from: www.who.int.

Wong J.Y. and Chye F.Y. (2009). Antioxidant properties of selected tropical wild edible mushrooms. *Journal of Food Composition and Analysis*. 22: 269-277.

Wong K. H. and Cheung P. C. K.. (2008). *Sclerotia: emerging functional food derived from Mushrooms*. Hoboken, NJ, USA: John Wiley & Sons.

Wong K.H., Lai C.L.M. and Cheung P.C.K. (2011). Immunomodulatory activities of mushroom sclerotial polysaccharides. *Food Hydrocolloids*. 25: 150–158.

Yun G.H., Lee E.T. and Kim S.D. (2003). Purification and characterization of a fibrinolytic enzyme produced from *Bacillus amyloliquefaciens* K42 isolated from Korean soy sauce. *Korean Journal of Microbiology and Biotechnology*. 31: 284-291.

Appendix A: MEDIA AND METHODS

A.1 Reagents

20mM Tris-HCl buffer (pH 8.0), 0.1 M citric acid, 0.2 M disodium hydrogen phosphate, 0.1 M Mcllvain buffer (pH 7.0), 0.55 M trichloro-acetic acid solution, 0.4 M sodium carbonate, 1 N Folin-Ciocalteus reagent, and *L*-tyrosine, 95% ethanol, Coomassie Brilliant Blue solution and bovine serum albumin stock solution. 40% phosphate stock solution and 50% PEG stock solution

A.2 Preparation of reagents

A.2.1 20 mM Tris-HCl buffer (pH 8.0)

- Molecular weight of trizma = 121.14 g/mol
- To prepare 1000 ml,

  $$\text{Mole} = \frac{20\times10^{-3}\,\text{M}\,(1000\text{ml})}{1000\,\text{ml}}$$

  $$= 0.02 \text{ mol}$$
- 0.02 mole = $\frac{x}{121.14\,\text{g / mol}}$
- x = 2.4228 g
- To prepare 20 mM Tris-HCl buffer (pH 8.0), 2.4228 g of trizma was dissolved in 900 ml of distilled water and pH was adjusted to pH 8.0 with HCl before the final volume was brought to 1000 ml with distilled water

A.2.2 0.1 M citric acid solution

- Molecular weight of citric acid = 210.14 g / mol
- To prepare 100 ml,

  $$\text{Mole} = \frac{0.1\,\text{M}\times100\,\text{ml}}{1000\,\text{ml}}$$

  $$= 0.01 \text{ mol}$$
- 0.01 mole = $\frac{x}{210.14\,\text{g / mol}}$
- x = 2.1014 g
- To prepare 0.1 M citric acid solution, 2.1014 g of citric acid was dissolved in 100 ml of distilled water.

A.2.3 0.2 M disodium hydrogen phosphate solution

- Molecular weight of disodium hydrogen phosphate = 141.96 g / mol
- To prepare 1000 ml,

  $$\text{Mole} = \frac{0.2\,\text{M}\times1000\,\text{ml}}{1000\,\text{ml}}$$

= 0.2 mol

- 0.2 mole = $\frac{x}{141.96\,\text{g / mol}}$
- X = 28.392 g
- To prepare 0.2 M disodium hydrogen phosphate solution, 28.392 g of disodium hydrogen phosphate was dissolved in 1000 ml of distilled water.

A.2.4 0.1 M Mcllvain buffer (pH7.0)

- To prepare 100 ml of 0.1 M of Mcllvain buffer (pH7.0), 17.65 ml of 0.1 M citric acid was mixed thoroughly with 82.35 ml of 0.2 M disodium hydrogen phosphate solution.

A.2.5 0.55 M trichloro-acetic acid solution

- Molecular weight of trichloro-acetic acid = 163.39 g / mol
- To prepare 100 ml,
  Mole = $\frac{0.55\,\text{M} \times 100\,\text{ml}}{1000\,\text{ml}}$
  = 0.055 mol
- 0.055 mole = $\frac{x}{163.39\,\text{g /mol}}$
- X = 8.9865 g
- To prepare 0.55 M trichloro-acetic acid solution, 8.9865 g of trichloro-acetic acid was dissolved in 100 ml of distilled water.

A.2.6 0.4 M sodium carbonate

- Molecular weight of sodium carbonate = 105.99 g / mol
- To prepare 100 ml,
  Mole = $\frac{0.4\,\text{M} \times 100\,\text{ml}}{1000\,\text{ml}}$
  =0.04 mol
- 0.04 mole = $\frac{x}{105.99\,\text{g / mol}}$
- x = 4.2396 g
- To prepare 0.4 M sodium carbonate, 4.2396 g of sodium carbonate was dissolved in 100ml of distilled water.

A.2.7 1 N Folin-Ciocalteu reagent

- To prepare 1 N Folin-Ciocalteu reagent, Folin-Ciocalteu reagent stock (2 N) was mixed evenly with distilled water in 1:1 ratio and kept in dark prior to use.

A.2.8 *L*-tyrosine stock solution

- Molecular weight of *L*-tyrosine = 181.2 g / mol
- To prepare 1000 ml,

$$\text{Mole} = \frac{500\times10^{-6}\ \text{M}\,(1000\,\text{ml})}{1000\,\text{ml}}$$
$$= 0.0005\ \text{mol}$$

- $0.0005\ \text{mole} = \frac{x}{181.2\,\text{g / mol}}$
- X = 0.0906 g
- To prepare 500 µM *L*-tyrosine, 0.0906 g of *L*-tyrosine was dissolved in 1000 ml of 0.1 M Mcllvain buffer (pH 7.0)

A.2.9 95% ethanol

- 95 ml of pure ethanol (100%) was mixed thoroughly with five milliliters of distilled water.

A.2.10 Coomassie Brilliant Blue solution

- To prepare 1 L of Coomassie Brilliant Blue solution, approximately 100 mg of Coomassie Brilliant Blue G-250 was dissolved in 50 ml of 95% ethanol. After that, 100ml of 85 % (w / v) phosphoric acid was added into this mixture and brought up to final volume of 1 L.

A.2.11 Bovine serum albumin stock solution

- 0.01 % (w / v) of bovine serum albumin stock solution was prepared by dissolving 0.01 g of bovine serum albumin in 100 ml of distilled water.

A.2.12 40% phosphate stock solution

- To prepare 100g,
  21.7g of K_2HPO_4 mixed with 18.3 g of KH_2PO_4 and 60 g of distilled water.

A.2.13 50% PEG stock solution

- To prepare 80 g,
  40 g of PEG 8000 was mixed with 40 g of distilled water.

A.3 *L*-tyrosine calibration plot (Folin & Marenzi, 1929)

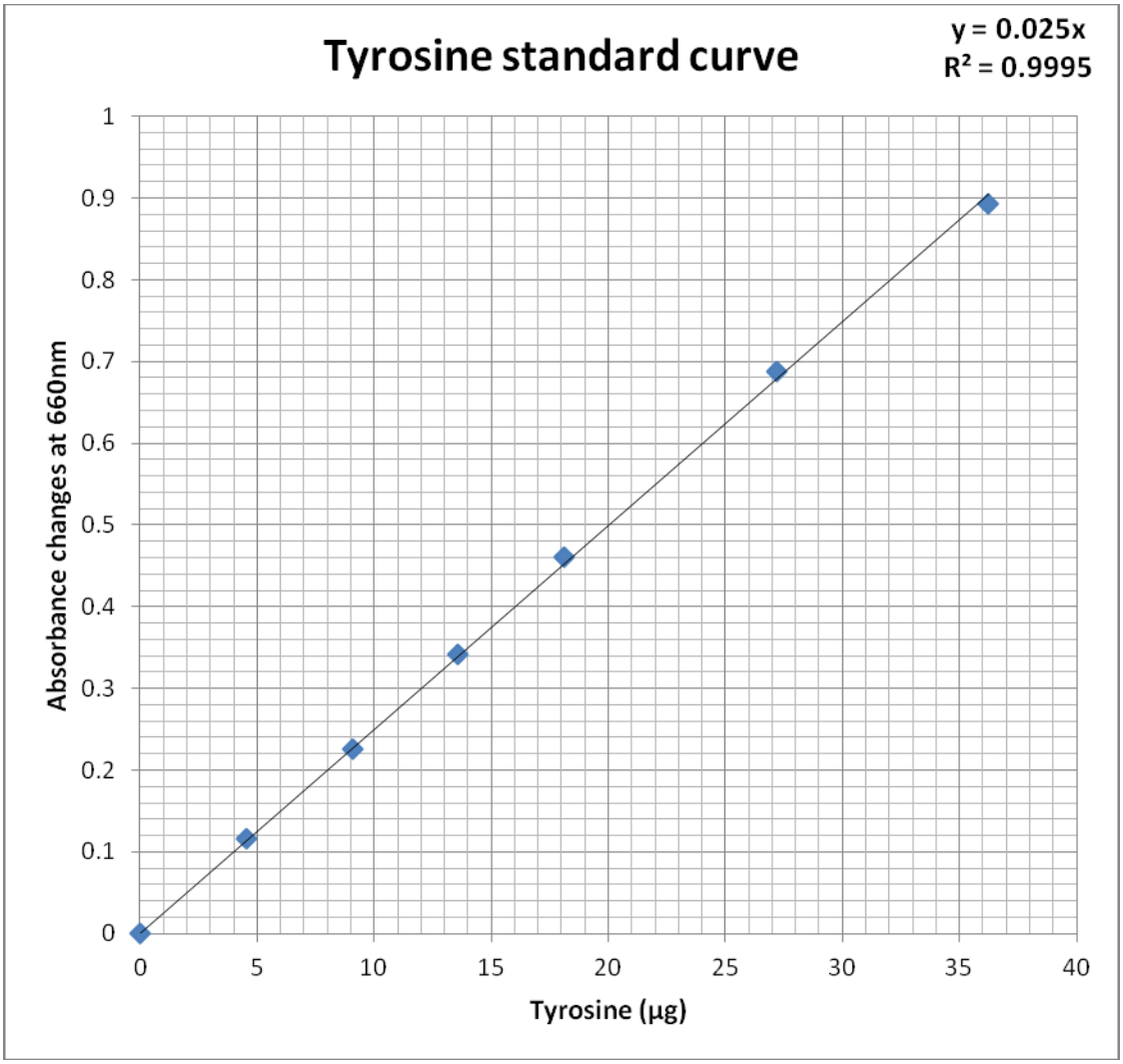


Figure A.1: *L*-tyrosine calibration plot for fibrinolytic activity

A.4 Protein calibration plot (Bradford, 1976)

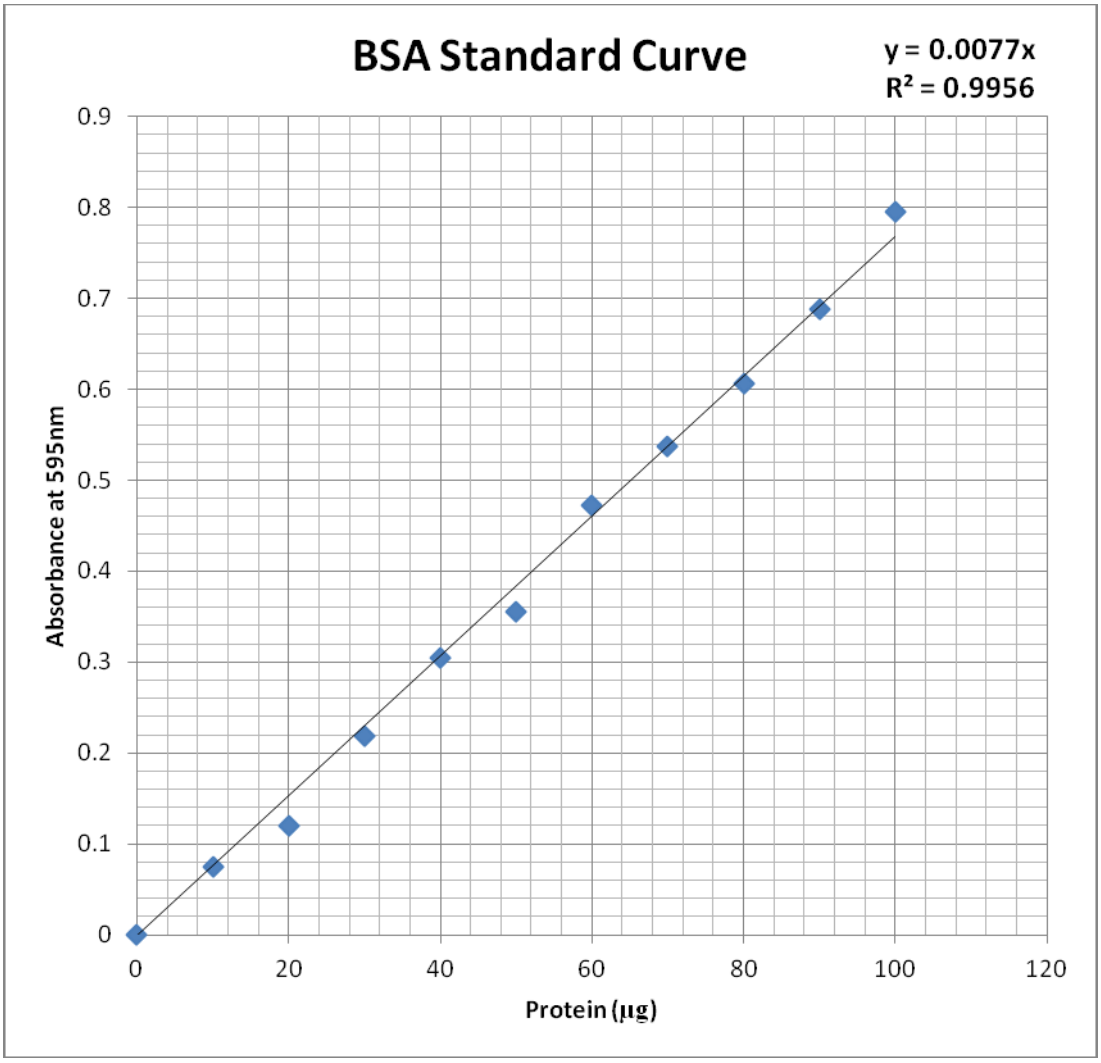


Figure A.2: Protein calibration plot for determination of soluble protein

Appendix B: STATISTICAL ANALYSIS

B.1 Normality test and Homogeneity of Variances

Table B.1: Normality test - Fibrinolytic activities of crude extract and partially purified enzymes from *L. rhinocerotis*

	Samples	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Specific_activity	Crude extract	.369	3	.	.789	3	.088
	ATPS Top	.312	3	.	.896	3	.374
	ATPS Bottom	.185	3	.	.998	3	.923
	Acetone precipitated protein	.233	3	.	.979	3	.722

a. Lilliefors Significance Correction

B.1.1 Test of homogeneity of variances

Table B.2: Test of homogeneity of variances - Fibrinolytic activities of crude extract and partially purified enzymes from *L. rhinocerotis*

Levene Statistic	d.f. 1	d.f. 2	Sig.
3.312	3	8	.078

B.2 One-way ANOVA

Table B.3: ANOVA - Fibrinolytic activities of crude extract and partially purified enzymes from *L. rhinocerotis*

	Sum of Squares	d.f.	Mean of square	F-ratio	Sig. level #
Between Groups	35906.922	3	11968.974	1348.695	.000
Within Groups	70.996	8	8.874		
Total	35977.917	11			

p = 0.0000 (denotes a statistically significant difference)

B.3 Post-Hoc for ANOVA

Table B.4: Duncan post-hoc test - Fibrinolytic activities of crude extract and partially purified enzymes from *L. rhinocerotis*

	Samples	N	Subset for alpha = 0.05			
			1	2	3	4
Duncan[a]	ATPS Bottom	3	1.3900			
	Crude extract	3		56.8467		
	ATPS Top	3			93.8500	
	Acetone precipitated protein	3				151.6100
	Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets were displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

Appendix C: RAW DATA

Table C.1: Diameter of lytic zone (cm) observed in 18 plates after one hour of incubation at 37±2^{o}C

Concentration of crude extract (mg/ml)	Replicate	Diameter of lytic zone (cm)
0.2	1	-
	2	-
	3	-
0.3	1	1.00
	2	1.00
	3	1.09
0.4	1	1.20
	2	1.10
	3	1.20
0.5	1	1.23
	2	1.21
	3	1.19
0.6	1	1.23
	2	1.21
	3	1.18
0.7	1	1.19
	2	1.21
	3	1.23

Table C.2: Fibrin plate assay for crude extract and partially purified enzyme

Sample	Replicate	Diameter of lytic zone (cm)
Crude extract	1	1.21
	2	1.23
	3	1.22
Human plasmin (positive control)	1	1.40
	2	1.20
	3	1.30
Top phase of ATPS	1	1.04
	2	1.02
	3	1.03
Bottom phase of ATPS	1	-
	2	
	3	
Top phase of blank	1	-
	2	
	3	
Bottom phase of blank	1	-
	2	
	3	
Tri-HCl buffer (Negative control)	1	-
	2	
	3	

Table C.3: Fibrinolytic activities of crude extract and partially purified enzymes from *L. rhinocerotis*

Sample	Replicate	Protease activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg)	purification fold
Crude extract	1	2.790	0.047	59.360	1.000
	2	2.840	0.051	55.686	
	3	2.830	0.051	55.490	
ATPS top phase	1	1.510	0.016	92.073	1.650
	2	1.060	0.011	92.982	
	3	1.930	0.020	96.500	
ATPS bottom phase	1	0.007	0.005	1.400	0.020
	2	0.007	0.006	1.167	
	3	0.008	0.005	1.600	
Acetone precipitated protein	1	5.000	0.034	147.059	2.670
	2	4.820	0.031	157.003	
	3	4.900	0.033	150.769	