FIBRINOLYTIC ACTIVITIES OF MYCOSYNTHESIZED SELENIUM NANOPARTICLES WITH SELECTED CULINARY MUSHROOMS

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2017

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DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

INSTITUTE OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2017

UNIVERSITY OF MALAYA

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ABSTRACT

Fibrinolytic drugs are commonly used in medical applications to treat various diseases such as cardiovascular diseases (CVD). Currently, the fibrinolytic drugs used in clinical application results in undesirable side effects. Hence, seeking fibrinolytic drugs from natural sources is becoming more favourable. Recently, many edible mushrooms have become attractive sources of biological active compounds including fibrinolytic enzymes. In this study, the fibrinolytic activities of freeze-dried basidiocarps of ten edible mushrooms were quantified using the Folin-spectrophotometric technique and the fibrin plate assay. The fibrinolytic activity in crude protein extracts was recorded in the range from 6 to 48 U/mL in all ten mushrooms. The fibrinolytic enzymes from the crude protein extracts have been recovered using an aqueous two-phase system (ATPS). The ATPS fraction of *L. edodes* recorded highest fibrinolytic activity of 54.28 U/mg. The fibrinolytic enzymes from *L. edodes* were further analysed through gel electrophoresis study. A 50 kDa of a fibrinolytic enzyme from *L. edodes* was revealed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The crude protein extracts and ATPS fraction of *L. edodes* were selected for selenium nanoparticles (SeNPs) mycosynthesis. The mycosynthesised SeNPs were then characterised by UV-Vis spectrophotometer, Field Emission Scanning Electron Microscopy (FESEM), Energy Dispersive X-ray (EDX), Transmission Electron Microscopy (TEM) and Fourier Transform Infrared Spectroscopic (FT-IR). The absorption peaks at 220 nm and 280 nm were recorded in UV-Vis spectrophotometer confirmed the formation of selenium nanoparticles. The mycosynthesised SeNPs using crude protein extract and ATPS fraction of *L. edodes* revealed that the selenium particles appear to be encapsulated by fibrinolytic enzymes in FESEM and HR-TEM morphology test. The EDX and FT-IR analysis confirmed the presence of Se and fibrinolytic enzyme in mycosynthesised SeNPs using crude protein extract and ATPS fraction of *L. edodes* respectively.

ABSTRAK

Ubat berkaitan fibrinolitik biasa digunakan dalam aplikasi perubatan untuk merawat pelbagai jenis penyakit terutamanya sakit jantung. Namun demikian, penggunaan ubat-ubatan fibrinolitik secara klinikal mengundang kepada kesan sampingan yang tidak diingini. Oleh itu, pencarian kepada ubat alternatif yang mengantikan ubat fibrinolitik telah menjadi tumpuan utama kepada para penyelidik-penyelidik dunia. Baru-baru ini, banyak cendawan yang boleh dimakan telah menjadi sumber tarikan para penyelidik untuk digunakan sebagai ubat alternatif. Dalam cendawan yang boleh dimakan mengandungi pelbagai sebatian aktif biologi termasuk enzim fibrinolitik. Dalam kajian ini, sepuluh cendawan yang boleh dimakan digunakan untuk menganalisa aktiviti-aktiviti fibrinolitik. Sepuluh Basidiokarpa yang telah dikeringkan diukur menggunakan teknik Folin-spektrofotometri dan plat fibrin untuk mengetahui aktiviti fibrinolitik tersebut. Aktiviti-aktiviti fibrinolitik dalam ekstrak protein mentah dicatatkan dalam julat 6-48 U / mL di dalam semua sepuluh cendawan yang dikaji. Enzim fibrinolytic daripada ekstrak protein mentah telah disucikan menggunakan sistem dua fasa akueus (ATPS). Menurut proses pensucian tersebut, cendawan *L. edodes* mencatatkan aktiviti fibrinolytic tertinggi iaitu 54.28 U / mg. Enzim fibrinolytic daripada cendawan *L. edodes*. telah dianalisa melalui kajian gel elektroforesis dan didapati enzim fibrinolitik yang mempunyai berat molekul sebanyak 50 kDa telah didedahkan oleh gel *sodium dodecyl sulfate polyacrylamide gel electrophoresis* (SDS-PAGE). Ekstrak protein mentah dan ATPS daripada cendawan *L. edodes* telah dipilih untuk disintesis nanopartikel selenium (SeNPs). Nanopartikel yang disintesis kemudiannya dianalisakan menggunakan *UV-Vis spectrophotometer, Field Emission Scanning Electron Microscopy* (FESEM), *Energy Dispersive X-ray* (EDX), *Transmission Electron Microscopy* (TEM) dan *Fourier Transform Infrared Spectroscopic* (FT-IR). Puncak penyerapan pada 220 nm dan 280 nm yang telah direkodkan oleh *UV-Vis spectrophotometer* mengesahkan pembentukan

nanopartikel *selenium*. Gambar-gambar daripada FESEM dan HR-TEM mendedahkan bahawa zarah *selenium* kelihatan terkandung oleh enzim fibrinolitik. Analisa-analisa EDX dan FT-IR turut mengesahkan kehadiran Se dan enzim fibrinolitik dalam SeNPs yang dihasilkan menggunakan ekstrak protein mentah dan ATPS.

ACKNOWLEDMENT

The project has been extensive, but in the first place exciting, instructive and pleasurable. Without help, support and encouragement from several persons, it wouldn't have been possible for me to complete this project.

First of all, I would like to thank my supervisor, Professor Dr. Vikineswary Sabaratnam for guiding and teaching me the importance of using curiosity as the driving force behind research. I am grateful to her for invaluable lessons about the importance of guidance and for the freedom she granted to me during this project.

I specially thank my co-supervisor, Professor Dr. Ling Tau Chuan, for his invaluable assistance and suggestions, which were indispensable for the successful accomplishment of my study.

I am also indebted to Madam Chang May Heng and my laboratory mates from Plant Pathology and Mycology Laboratory, Institute of Postgraduates Studies, University of Malaya, for their technical assistance and tolerance to my insisted needs.

Finally, I thank my parents, family and my friends for their continuous encouragement, patience and support.

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LIST OF SYMBOLS AND ABBREVIATIONS

α	alpha
ATPS	aqueous two-phase system
β	beta
cm	centimeter
°C	degree Celsius
Fe11	ferrous
Fe	ferric
g	gravity
g	gram
g/L	gram per liter
>	greater than
HCl	hydrochloric acid
HMWK	high molecular-weight kininogen
kDa	kilo Dalton
L	liter
LDL	low-density lipoprotein
In	natural log
<	less than
K_2HPO_4	dipotassium phosphate
KH_2PO_4	potassium orthophosphate
µg	microgram
µg/mL	microgram per microlitre
µL	microliter
µM	micromolar

µ mole	micromole
M	molar
mg	milligram
mg / mL	milligram per milliliter
mg/L	milligram per liter
mL	milliliter
mm	millimeter
mmole	millimole
mM	millimolar
nm	nanometer
OD	optical density
%	percentage
±	plus minus
pH	hydrogen ion concentration
RNA	ribonucleic acid
rpm	rotation per minute
R	R-squared
SeNPs	Selenium nanoparticles
TLL	tie-line lenght
U	unit
v/V	volume per volume
w/V	weight per volume

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CHAPTER 1: INTRODUCTION

Cardiovascular diseases (CVD) are one of the most prevalent causes of morbidity and mortality in the world. According to World Health Organization (WHO), 17 million people die every year from CVD and it is expected to increase to 23 million deaths in 2013. There are several factors that lead to CVD and the name is in a cluster of disorders afflicting the heart and blood vessels, including hypertension (high blood pressure), coronary heart disease (heart attack), cerebrovascular disease (stroke), heart failure and peripheral vascular disease. However, thromboembolic disorders are one of the foremost vascular disease causing CVDs (World Health Statistics, 2013).

The name thromboembolic disorders originated from thrombosis and its main complication; embolism. Deep vein thrombosis and myocardial infarction are the common types of thromboembolic disorders. Initially, deep vein thrombosis develops by the formation of a blood clot (thrombosis) in a deep vein, particularly in leg veins (femoral or the popliteal vein). If a thrombus (blood clot) breaks apart (embolus), it is transported through the circulation to microcirculation. However, this thrombosis can take place in both arteries and veins (Engelmann & Massberg, 2013). If the deposition of dislodged thrombus occurs in the pulmonary artery in the lungs, the blood circulation is blocked and causes pulmonary embolism. Besides, the thrombus also can travel to coronary arteries and lead to myocardial infarction (Goldhaber & Bounameaux, 2012).

Myocardial infarction, commonly known as a heart attack is another type of thromboembolic disorders. The myocardial infarction occurs when heart tissues are damaged prior to blockages in blood vessels by a thrombus. Left ventricular thrombus is a common complication of acute myocardial infarction (Delewi et al., 2012). The formation of thrombus on the walls of the ventricle may lead to blockage in a blood

vessel (Reeder et al., 1981). The primary risk increases if the thrombus detaches from the ventricular wall and travels through the circulation and blocking other blood vessels (Delewi et al., 2012). In the United States, more than 2,000 patients die every day due to clot formation in coronary, triggering heart attack (Roger et al., 2011). However, in Malaysia the coronary heart disease deaths reached 29,363 or 23.10% of total deaths in 2014 (WHO, 2014). Thus, the thromboembolic disorders are a major healthcare concern and the occurrence of its complications continues to rise at an alarming pace, although, effective fibrinolytic approaches have been developed to combat this cardiovascular epidemic.

Fibrinolytic drugs such as tissue type plasminogen activator, urokinase type plasminogen activator, and the bacterial plasminogen activator are well-known fibrinolytic agents used for clinical and medicinal applications. However, these drugs are expensive as well as results in undesirable side effects such as internal haemorrhage and allergic reactions. Moreover, it also has limitations in fibrinolytic activity towards fibrin (Blann et al., 2002). Hence, search for fibrinolytic agents from other natural sources such as plants, herbs and mushroom, which are enormous in diversity coupled with ease industrial production are more favourable (Singh et al., 2014).

Recently, edible mushrooms have become attractive as functional food and sources of biologically active metabolites (Hawksworth, 1991 & Wasser, 1999). Many such compounds are beneficial as they are reported to show antitumor, antiviral, antibacterial, antihypotensive, immunostimulation, antioxidative and anticoagulation activities (Thakur et al., 2013). Also, the fibrinolytic proteases have previously been purified and characterised from edible or medicinal mushrooms such as *Fomitella fraxinea* (Bull.) Imazeki (Lee et al., 2006), *Pleurotus ostreatus* (Jacq.) P. Kumm. (Choi & Shin, 1998), *Armillaria mellea* (Vahl) P. Kumm. (Lee et al., 2005), *Ganoderma lucidium* (Curtis) P.

Karst. (Choi & Sa, 2000), *Flammulina velutipes* (Curtis) Singer (Park et al., 2007), *Tricholoma saponaceum* (Fr.) P. Kumm. (Kim & Kim, 2001), *Auricularia polytricha* (Mont.) Sacc (Mohamed Ali, 2014) and *Pleurotus eryngii* (DC.) Quél. (Cha et al., 2010). Most of these enzymes are either metalloproteases or serine proteases.

Several reports had shown that the mushrooms contained therapeutic proteins such as fibrinolytic proteases, which can degrade the blood clot (Kim et al, 2009; Kim & Kim, 1999). However, the deliveries of therapeutic proteins in human body is a big challenge for pharmaceutical technologists (Muheem et al., 2014). The therapeutic potential and clinical application of these therapeutic proteins are frequently hampered by various obstacles such as poor solubility, insufficient *in vitro* stability (shelf life), too low bioavailability, too short *in vivo* stability (half-life) and lack of large scale production (Muller & Keck, 2004).

The nanotechnology focuses on formulating biocompatible nanocomposites such as nanoparticles using therapeutic agents. Nanotechnology is being developed progressively as a mode of delivery of therapeutic drugs, including small molecular weight drugs and macromolecules such as proteins, peptides by either localized or targeted to the tissue of interest (Moghimi et al., 2001). Since these systems are submicron in size and polymeric, they have multidimensional benefits in drug delivery such as delivering the drug to target cell or tissue and can also improve bioavailability, sustain drug/gene effect in the target tissue, solubilize drugs for intravascular delivery, and improve the stability of therapeutic agents against enzymatic degradation (nucleases and proteases), especially protein, peptide, and nucleic acid drugs (Moghimi et al., 2001; Patravale et al., 2012).

Numerous nanoparticles, for instance titanium oxide, silver, gold, cadmium selenide and carbon nanoparticles, have been used in catalysis, stain-resistant clothing, sunscreens, cosmetics, and electronics (Nel et al., 2006). Recently, developments in medical applications using metal nanoparticles is gaining interest (Sahoo et al., 2007). Apart from drug delivery, the metal nanoparticles are also used in medical diagnosis and imaging. (Lewinski et al., 2008). For instance, the silver nanoparticles are distinguished for antimicrobial activity in wound healing (Wright et al., 1999). Also clinical treatment of rheumatoid arthritis was reported by using gold nanoparticles (Aaseth et al., 1998).

Recently, selenium nanoparticles (SeNPs) have become more attractive as drug delivery agents due to its excellent biological activities (Gao et al., 2002) and low toxicity (Huang et al., 2003). Further, selenium has the ability to prevent free radicals from damaging cells and tissues *in vivo* as well as improve the activity of the seleno-enzyme, especially glutathione peroxidase (Baker et al, 1993). Besides, the selenium is well known as an antioxidant and catalyst for the production of active thyroid hormone. Selenium is required for the proper operation of the immune system, and appears to be a key nutrient in counteracting the development of virulence and inhibiting HIV progression to AIDS (Rayman, 2000). Since selenium is an essential element required by humans, it has been used as a nutrient as well as a supplement. However, very few studies have been reported on the synthesis of SeNPs with protein from mushrooms extract (Dobias et al., 2011).

OBJECTIVES

The objectives were to:

a. estimate the fibrinolytic activity in crude protein extract and partially purified fraction (ATPS fraction) of selected edible mushrooms.
b. mycosynthesise selenium nanoparticles using crude protein extract and ATPS fraction of selected mushrooms.
c. characterise the mycosynthesised selenium nanoparticles of the selected mushroom.
d. assess fibrinolytic activity of mycosynthesised SeNPs.

CHAPTER 2: LITERATURE REVIEW

2.1 Mushrooms

Mushroom is a macrofungi that can be seen by the naked eye and picked by hand (Chang and Miles, 1992). Mushroom have a distinctive fruiting body which can be classified as Basidiomycetes or Ascomycetes. However, this classification is only applicable as a workable term to estimate the number of mushrooms on the earth (Hawksworth, 2001).

Mushroom belongs to the kingdom of Fungi (Stephenson, 2010). Mushrooms can be grouped into four categories according to Cheung and Peter (2008), which are: (1) edible mushrooms that are fleshy and edible (e.g., *Agaricus bisporus*); (2) medicinal mushrooms used in medicinal applications (e.g., *Ganoderma lucidum*); (3) poisonous mushrooms proven to be or suspected of being poisonous (e.g., *Amanita phalloides*); and (4) "other mushrooms" or miscellaneous category, for which the properties remain undefined. Certainly, this approach of classifying mushrooms is not absolute and not mutually exclusive. Many kinds of mushrooms are not only edible but also possess tonic and medicinal qualities.

Edible mushrooms are widely consumed and used as cooking materials since ancient period in China, Japan and Korea (Kim & Song, 2014). Due to its attractive texture and taste, it is popularly consumed as a human daily food in numerous countries (De Silva et al., 2012). Initially, only the basidiocarp portions were consumed, but now the mycelia are also being processed and widely commercialized (Smith et al., 2002; Lindequist et al., 2005; Hyde et al., 2010).

The cultivated or wild mushrooms have enormous prospects as natural sources of medicine with high nutritional and economic values. Moreover, various studies have reported that the mushrooms are a momentous source of nutritious food as well as income in many countries (Wong et al., 2001).

2.1.1 Nutritional and Medicinal Value of Mushrooms

Mushrooms are rich in digestible proteins with protein content somewhat less than most meats and milk and above most vegetables (Sullivan et al, 2002).

The protein content in mushrooms varies from 10-40% on a dry weight basis. All the essential amino acids are included in this content, but the sulphur-containing amino acids, cysteine and methionine are restrictive (Choi et al., 2004; Breene, 1990). Fresh mushrooms comprise 3-21% carbohydrates and 3-35% fibre on a dry weight basis. Thus, a substantial proportion of the carbohydrate in mushrooms contains dietary fibre which cannot easily be digested by humans and which function essentially as dietary fibre; in this way the calorific value of most mushrooms is low (Sullivan et al, 2002).

Several thousand years ago, many edible and certain non-edible mushrooms have been recognised to have medicinal properties (Bensky and Gamble, 1993; Hobbs, 1995). The historical evolution of the usage of these mushrooms is scarce and wild medicinal mushrooms were certainly not consumed as whole mushroom, but as hot water extracts, concentrates, liquors or powders and used in medicines, tinctures, teas, soups and herbal formulae (Sullivan et al., 2002).

The medicinal mushrooms are usually consumed as powdered concentrates or extracts in hot water, and the extract is concentrated and used as a drink or freeze-dried or spray-dried granular powders for easier handling, consumption and transportation (Mizuno et

al., 1995). As such, these dried or liquid concentrates, powdered mushrooms, processed as capsules can be consumed as dietary supplements or nutritional supplements with potential health benefits. Regular consumption of these concentrates is believed to boost the immune response in the human body, thereby increasing resistance to disease and in some cases causing regression of the disease state (Chang and Buswell, 1996).

In the past few decades, the scientific research on medicinal mushrooms, especially in Japan, China and Korea, further extended the traditional use of mushrooms to a wide and promising medicinal applications (Table 2.1) (Wasser and Weis, 1999a). While much focus has been drawn to anti-cancer properties and various immunological potential of these mushrooms apart from other potential therapeutic properties such as antioxidants, anti-hypertensive, Cholesterol-lowering, liver protection, anti-fibrotic, anti-inflammatory, anti-diabetic, anti-viral and anti-microbial (Sullivan et al., 2002).

Besides, mushrooms play vital roles in inhibiting the platelet aggregation as well as prevent heart disease (Ghaly et al., 2011). Therefore, large production of mushrooms in a bigger scale is required in order to achieve the high demands of the global market (Guillamon et al., 2010).

Table 2.1: Cross index of medically active higher Basidiomycetes mushrooms and their medicinal properties (Wasser and Weis, 1999a)

1	Antifungal 2	Antiinflammatory 3	Antitumour 4	Antiviral (e.g. anti-HIV) 5	Antibacterial & Antiparasitic 6	Blood pressure regulation 7	Cardiovascular disorders 8	Hypercholesterolemia, hyperlipidemia 9	Antidiabetic 10	Immunomodulating 11	Kidney tonic 12	Hepatoprotective 13	Nerve tonic 14	Sexual potentiator 15	Chronic bronchitis 16
Auriculariales			+			+	X	X							X
Auricularia auricula-judas (Bull.) Wettst.															
Tremellales		+	+					+	+	+		+			X
Tremella fuciformis Berk.						+									+
Tremella mesenterica Rits.:Fr.															
Polyporales															
Schizophyllum commune Fr.:Fr.		X	X		X					X	X	X			
Dendropolyporus umbellatus (Pers.:Fr.)			X							X		X			X
Jül.	+		X	X	X	X			X	X		+			+
Grifola frondosa (Dicks.:Fr.) S.F. Gray															
Fomes formentarius (L.:Fr.) Fr.			+		+										
Fomitopsis pimicola (Schw.:Fr.) P. Karst.		+	+		+							+			
Trametes versicolor (L.:Fr.) Lloyd			X	X	X						X	X			
Piptoporus betulinus (bull.:Fr.) P. Karst.	+		+		+										
Hericium erinaceus (bull.:Fr.) Pers.			+							X			X		X
Inonotus obliquus (Pers.:Fr.) Bond.et Sing.		X	X							X		X			
Lenzites betulina (L.:Fr.) Fr.			+				+								
Laetiporus sulphurous (Bull.:Fr.) Murr.	+		+												
Ganodermatales															
Ganoderma lucidum (Curt.:Fr.) P.Karst		X	X	X	X	X	X			X	X	X	X	X	X
Ganoderma applanatum (Pers.) Pat.			+	+	+					+					
Agaricomycetideae															
Agaricales s.l.															
Pleurotaceae															
Lentinus edodes (Berk.) Sing.		X	X	X	X	X		X	X	X	X	X		X	
Pleurotus ostreatus (Jacq.:Fr.) Kumm.			+	+	+			+					+		
Pleurotus pulmonarius (Fr.:Fr.) Quél	+		+					+							
Tricholomataceae															
Flammulina velutipes (Curt.:Fr.) P.Karst.	+	X	X	+						X					
Oudemansiella mucida (Schrad.:Fr.) v. Höhn.	X														
Armillariella mellea (Vahl.:Fr.) P.Karst.	+					X	X						X		
Hypsizygus marmoreus (Peck) Bigel.			X												
Marasmius androsaceus (L.:Fr.) Fr.		X											X		
Agaricaceae															
Agaricus bla\ei Murr.			X												
Agaricus bisporus (J.Lge) Imbach			+							X	X				
Pluteaceae															
Volvariella volvacea (Bull.:Fr.) Sing.			+	+	+			+							
Bolbitiaceae															
Agrocybe aegerita (Brit.) Sing.	+		+					+					+		

X = commercially developed mushroom product (drug or dietary supplement)

\+ = non commercially developed mushroom product.

2.1.2 Bioactive Compounds in Medicinal Mushroom

Recent studies have reported interesting bioactive macromolecules, such as polysaccharides, proteins, polysaccharide-protein complexes, isolated and characterized from medicinal mushrooms. (Ferreira et al., 2010; Quang et al., 2006 and Wasser, 2010). These isolated bioactive compounds have potential activity such as antioxidative, antitumor, antiviral, antimicrobial, and immunomodulatory agents. Besides, the isolated polysaccharides from mushrooms have been tested in clinical trials, including by Phase I, II, and III and successfully used to treat various diseases, especially cancer (Xu et al., 2011).

Another important constitute from functional components in mushrooms is bioactive proteins. Generally, mushrooms produce a large number of proteins and peptides with potential biological activities, such as lectins, fungal immunomodulatory proteins (FIP), ribosome inactivating proteins (RIP), antimicrobial proteins, ribonucleases, and laccases. These proteins have contributed towards the development of new pharmaceutical products which can cure human diseases (Xu et al., 2011). However, the major drawback in isolating these proteins from mushrooms is requiring a high yield for isolation. Moreover, the isolating process also consumes long time and high cost. Thus, it is important to develop new techniques such as genetic engineering for mass production of these bioactive proteins.

On the other hand, the mechanistic action of isolated bioactive compounds (e.g. immunomodulation, antiproliferation, antivirus, antimicrobes, etc.) is still poorly understood, although numerous reports have been published for the isolation, purification and functions of these compounds. The new ‘omic’ technologies like proteomics should be promising in this aspect (Li et al., 2010). Certainly, more discoveries of the relationship

structure-activity relationship are highly needed, which may lead to designs of new therapeutically drugs to human diseases (Xu et al., 2011).

2.2 Selected Edible Mushrooms

2.2.1 *Lentinula Edodes* (Berk.) Pegler

Lentinula edodes (Berkely) Pegler (shiitake) is a well-known edible mushroom in Japan. It is classified in the family of Marasmiaceae and is naturally distributed in Southeast Asia. The cultivation of this mushroom is stress-free and usually grown in deciduous logs. It is also widely being cultivated in bags using wood substrates (Moonmoon et al., 2011) or wheat straw (Mata and Savoie, 1998). Fresh and dried shiitake has been used in cooking apanese miso soup or Chinese vegetarian dishes due to its unique aroma, taste and high nutritional values. The mushroom *L. edodes* also have potential therapeutic properties that can cure depressed immune function, cancer, environmental allergies, fungal infection, diabetes and respiratory illness such as flu and cough (Bisen et al., 2010). Moreover, promising pharmacological effects like antitumor (Finimundy et al., 2013), antioxidant (Turlo et al., 2010) and immunomodulatory activities (Xu et al., 2014) also have been reported in *L. edodes.*

Figure 2.1: *Lentinula edodes*

Source: www.freshcropmushrooms.com

2.2.2 *Flammulina velutipes* (Curtis) Singer

Flammulina velutipes is a member of the family of Tricholomataceae. It is commonly known as enokitake (in Japanese "The Snow Peak Mushroom") and 'cendawan jarum emas' or 'golden needle mushroom' (in Malaysia). The regions of temperate climate are favorable for its growth, and low-temperature for fruiting (Lou et al., 1983).

Flammulina velutipes is a delicious mushroom usually served in soups, stir-fries, salads and other dishes due to its flavourful taste and crispy nature. On fresh weight basis, the *F. velutipes* is made up about 89.2% of moisture, 17.6% of crude protein, 1.9% of crude fat, 73.1% of carbohydrate, 3.7% of crude fibre, and 7.4% of ash (Crisan and Sands, 1978).

The bioactive compounds such as peroxidase, superoxide dismutase isolated from *Flammulina velutipes* have been reported to prevent from diseases like cancer and coronary heart disease. Further, the consumption of this mushroom on a regular basis helps in preventing liver damage and gastroenteric ulcers (Ying, 1987; Yoshioka et al., 1973). In addition, consuming both mycelium and basidiocarp of *F. velutipes* could be recommended for formulating antioxidative dietary supplements (Bao et al., 2009; 2008).

Figure 2.2: *Flammulina velutipes*

Source: Mushroom Research Centre (MRC), University of Malaya (UM)

2.2.3 *Pleurotus pulmonarius* (Fr.) Quél.

More than 1000 species of *Pleurotus* sp. have been described all over the world, in more than 25 related and/or confused genera. However, only approximately 50 were recognized fully as a valid species of *Pleurotus*. These including *P. ostreatus*, *P. cornucopiae*, *P. florida*, *P. eryngii*, *P. cystidiosis*, *P. flabellatus*, *P. cornucopie* and *P. ostreatoroseus* (Andrade et al., 2010). The *P. pulmonarius* are often called as *P. sajor caju* by many of the mushroom growers, manufacturers and cultivators. Researchers also believe that *P. pulmonarius* is a synonym of the species *P. sajor caju*. Besides, it is being also being recognised by several other names such as *P. sapidus* (Shnyreva et al., 2012).

Pleurotus pulmonarius is known as the oyster mushroom due to the shell-like, spatulate pileus with eccentric or lateral stipe (Ohga, 2000). Currently, *Pleurotus pulmonarius* is rapidly cultivated worldwide due to high demand on its consumption (Gern et al., 2008). Alam et al. (2008) reported that there are different nutritional values in t different parts of cultivated mushrooms. However, according to Icons of Medicinal Fungi from China, the basidiocaps of this mushroom is the part that contains most medicinal properties and also low in calories, carbohydrate, and calcium (Ying et al., 1987; Miles and Chang, 2004).

Pleurotus pulmonarius and its extracts are believed to have promising medicinal applications for a wide range of conditions. Apart from having potential anti-diabetic property, *P. pulmonarius* was able to decrease the triglyceride level and total plasma cholesterol. Thus, the chance of suffering from atherosclerosis or other cardiovascular and artery related disorders can be reduced. *P. pulmonarius* also has active ingredients, which affect the renin-angiotensin system and results in hypertensive effects. (Chang & Buswell, 1996).

Figure 2.3: *Pleurotus pulmonarius*

Source: MRC, UM

2.2.4 *Pleurotus floridanus* (Singer)

Pleurotus floridanus is one of the edible oyster mushrooms, which is cultivated in large scale around the world. *P. floridanus* contains high therapeutic value and contain a large portion of protein, fat and ash on dry weight basis (Rout et al., 2006). According to Jose et al. (2004), *P. floridanus* show platelet aggregation inhibiting activities in human.

Figure 2.4: *Pleurotus floridanus*

Source: MRC, UM

2.2.5 *Pleurotus cystidiosus* O.K. Mill.

Pleurotus cystidiosus also known as the 'summer oyster mushroom' (Ohiratake in Japanese) is a member of the mushroom genus *Pleurotus* (Jacq.Fr.) P. Kumm (Basidiomycotina. Pleurotaceae). This edible species tastes like an oyster, but is of vegetable nature and has high commercial importance like other *Pleurotus* species such as *P. pulmonarius*. The *Pleurotus cystidiosus* contain darkly pigmented arthroconidia like two other *Pleurotus* species such as *P. ausiralis* (Cooke, 1892) and *P. purpureoolivaceus* (Segedin et al., 1995). Thus, it mycelium or basidiomata appeared as black colour (Petersen et al., 1997; Zervakis, 1998).

Pleurotus cystidiosus have high nutritional content and bioactive compounds. The methanol extract of *P. cystidiosus* reported to have antioxidant property (Yang et al., 2002). According to Wasser and Reshetnikov (2002), *P. cystidiosus* possess cholesterol lowering ability in hypercholesterolemic rats. *P. cystidiosus* was also shown to have antinociceptive (Kudahewa et al., 2008) and anti-fungal activities (Menikpurage et al., 2009).

Figure 2.5: *Pleurotus cystidiosus*

Source: MRC, UM

2.2.6 *Pleurotus salmoneostramineus* Lj.N. Vassiljeva

Pleurotus salmoneostramineus, also known as pink oyster mushroom, grows on dead deciduous plants (Murakami and Takemaru, 1990). It is well-known because of attractive colour, sustainable yield, delicious taste and unique texture. According to Shibata et al. (1997), the presence of chromoprotein in *P. salmoneostramineus* gives the pink colour appearance, which is involved in the photosynthetic function. *P. salmoneostramineus* is also rich in carbohydrates, protein, fibre, lipids, vitamins, and contains abundant amounts of essential amino acids (Shibata et al., 1997; Bao et al., 2004). Kim et al. (2009) reported that the *P. salmoneostramineus* showed antioxidant and antitumor activity. Besides, it also has cholesterol reducing agents in blood (Wang et al., 2000).

Figure 2.6: *Pleurotus salmoneostramineus*

Source: MRC, UM

2.2.7 *Hericium erinaceus* (Bull.: Fr.) Pers.

Hericium erinaceus (Bull.: Fr.) Pers. is a member of the basidiomycetous fungus and well known as a traditional medicine or food in China and Japan. This mushroom is known as Houtou (monkey head mushroom) in China due to the close resemblance of fruiting body to the head of a baby monkey. The hot water extract from dried fruiting

bodies are used as a tonic drink (Yang and Jong, 1989). It is also pickled in brewing wine to produce a health drink (Mizuno, 1999).

The medicinal properties of *H. erinaceus* have been widely studied. Both the fruiting bodies and mycelia of *H. erinaceus* have been reported to contain bioactive polysaccharides, which exhibit various pharmacological activities including immunomodulatory effect, antitumor, hypoglycaemic and anti-aging properties (Zhang et al., 2007). Besides the polysaccharides, an ergosterol derivative isolated from *H. erinaceus* has been reported to have anti-inflammatory (Keyzers and Davies-Coleman, 2005) and antimicrobial properties (Lu et al., 2000).

Figure 2.7: *Hericium erinaceus*

Source: MRC, UM

2.2.8 *Agaricus bisporus* (J.E. Lange) Imbach

Agaricus bisporus is an edible basidiomycete mushroom typically known as common mushroom, button mushroom, white mushroom, table mushroom, Portobello mushroom, cremini, crimini mushroom, Swiss brown mushrooms, Roman brown mushrooms, Italian brown, Italian mushroom, or cultivated mushroom (Have et al., 2003). The mushroom grows in grasslands in India, Europe and North America. White button mushroom (*Agaricus bisporus*) (WBM) contributes to 90% of the total mushroom consumed in the United States, it is one of the most highly cultivated mushroom in the world (Grove,

1981). The original wild form is characterised by a brownish cap and dark brown gills, but more familiar is the current variant with white cap, stalk and flesh and brown gills (Parslew et al., 1999).

Agaricus bisporus is an essential sources of trace elements like sodium, potassium, and phosphorus, conjugated linoleic acid and antioxidants (Winer et al., 2002). It can reduce the oestrogen levels in the human body by inhibiting aromatase and reducing the susceptibility to breast cancer. It also possesses the possible immune system enhancing properties (Loganathan et al., 2009).

Figure 2.8: *Agaricus bisporus* (white)

Figure 2.9: *Agaricus bisporus* (brown)

Source: MRC, UM

2.2.9 *Grifola frondosa* (Dickson: Fries) Gray

Grifola frondosa (Dickson: Fries) Gray (Maitake) is also called as the king of mushrooms and the hen of the woods is a Basidiomycete fungus belonging to the order Aphyllopherales, and family Polyporaceae (Stamets, 1993). *Grifola frondosa* is traditionally consumed as a medicine called 'keisho" by the Chinese. It has been commonly used for improving the ailment of spleen and stomach, calming the nerve and the mind, and treating hemorrhoids. *Grifola frondosa* is often dried and used in the production of healthy foods, including Maitake tea, whole Maitake powder, powders of

hot water extracts of Maitake, Maitake granules, and Maitake drinks (Mizuno et al., 1995).

The liquid-cultured mycelium and badiocarps of *G. frondosa* have been reported to contain potential anti-tumor polysaccharides. These polysaccharides have been identified as glucans (e.g. β-1,6- and β-1,3-) (Lee et al., 2003). These bioactive β-glucans has a promising cytotoxic effect on prostatic cancer cells in vitro, leading to apoptosis (Fullerton et al., 2000).

Figure 2.10: *Grifola frondosa*

Source: http://www.hokto-kinoko.co.jp/en/products/

2.3 Fibrinolytic Activity

2.3.1 Haemostasis

Haemostasis is an equal balance process of blood clotting and the dissolution of the blood clots with the subsequent repair of the injured tissues and is a well-balanced interaction of blood cells, the vascular system, plasma proteins and low molecular weight components (Schaller et al., 2008). It plays vital roles in restrictive blood losses after trauma by inducing vasoconstriction, formation of platelet plugs, and activation of coagulation cascade as well as activate the complement system and fibrinolysis to

maintain the delicate balance of the body system. Hence, the haemostasis process permits the repair of vessels as well as controls the excess blood loss from the body (Gale, 2011).

2.3.2 Blood Coagulation and Fibrin Formation

Blood coagulation can be initiated either by the extrinsic (tissue factor pathway) or intrinsic (contact pathway) pathways (Figure 2.1). The intrinsic pathway is activated when tissue factors are released from the injury site. While, intrinsic system is triggered when contact with a negatively charged surface. When the tissue cuts, the blood vessel will be raised up and committed to intact endothelial barrier. It effects blood to be exposed to these sub-endothelial structures, the membrane-bound protein tissue factor will be in contact with plasma containing factor VII or VIIa. Then, on the cell surface, the tissue factor-VIIa complex was formed, subsequently; factor X will be converted to Xa while factor IX will be converted to IXa (Norris, 2003; Esmon, 2004).

Generally, little or no negative charge is expressed by the phospholipid on the cell's outer leaflet of the plasma membrane. However, this scenario changes when complement activation, apoptosis or necrosis were brought by inflammatory reactions. The inflammatory reactions start and intensify the coagulation system. This commencement of intrinsic or contact pathway will stimulate the factor IX and factor XII. Once factor IX and factor XII is bound to the negatively charged surface, factor XI will be converted to factor XIa while factor XII will be converted to Xlla (Norris, 2003 and Esmon, 2004).

In plasma, factor VIII is usually present as a non-covalent complex with von Wiilebrand factor. Nevertheless, von Willebrand factor binding to platelets and attached to the surface of injured endothelium assists activation of factor VIII to VIIIa. Besides, factor IX is converted to IXa by either the extrinsic or intrinsic pathway to form factor VIIIa complex, calcium and phospholipids. This complex then activates the factor Xa

from X. This complex known as "tenase" and the key to haemostasis. On the other hand, prothrombin will be converted to thrombin when factor Xa is bound to factor Va. Thrombin then breaks soluble fibrinogen to fibrin monomers followed by polymerization into stable, cross-linked fibrin clot. These haemostatic plugs (fibrin clots) are ropes to seal the site of injury and protect broken tissue during wound healing (Norris, 2003 and Esmon, 2004).

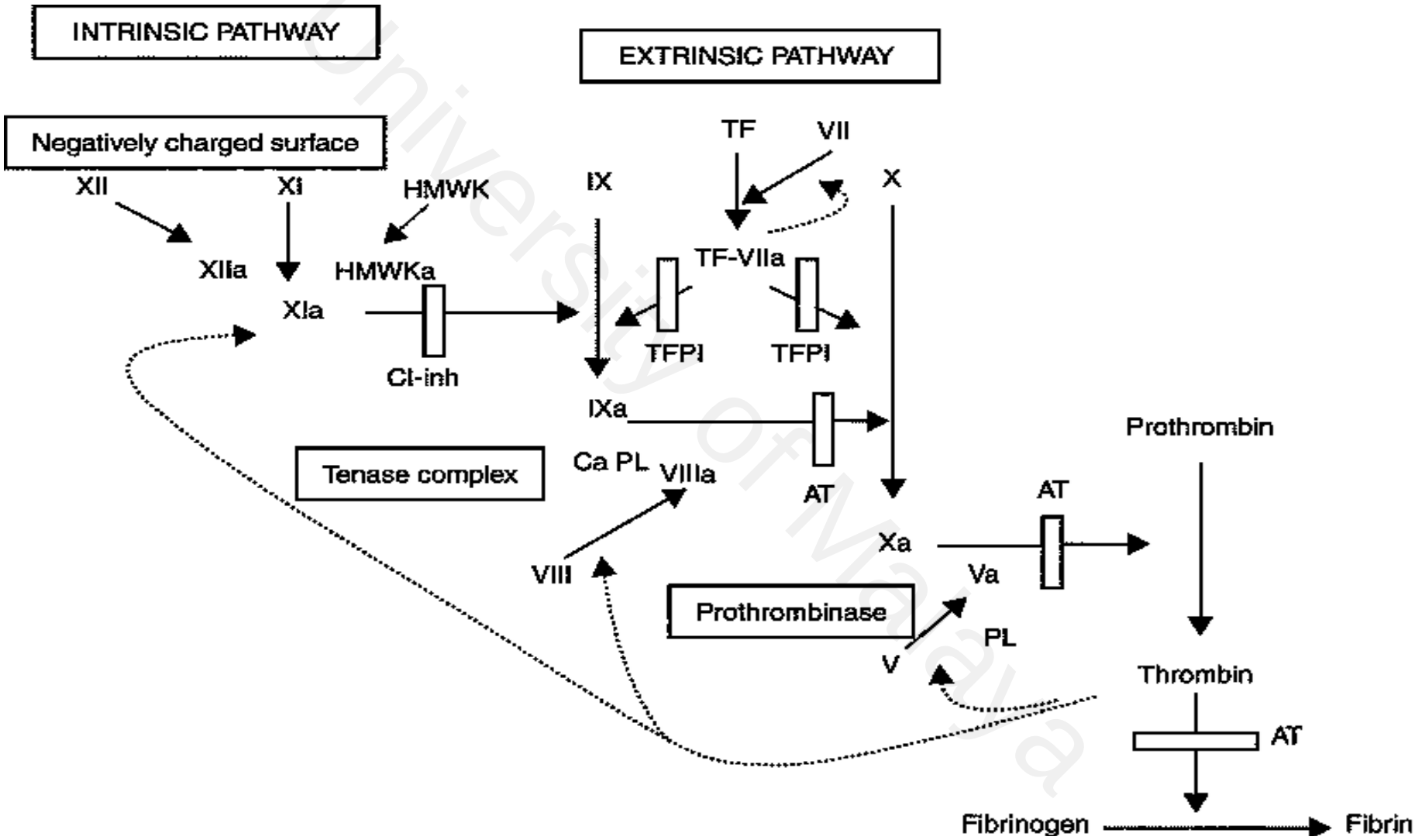

Figure 2.11: The coagulation cascade showing both intrinsic and extrinsic activation, inhibitors and feedback activation (dashed lines). (Norris, 2003) *HMWK is high molecular-weight kininogen. *C1-inh is C1-inhibitor. *TF is tissue factor. *TFPI is tissue factor pathway inhibitor. *PL is phospholipids. *Ca is calcium. *AT is antithrombin.

2.3.3 Fibrinolysis

Fibrinolysis is a process that controls the formation of a fibrin clot. It is a cascade mechanism of enzymes that break the fibrin clot into fibrin fragments, also known as fibrin degradation products, when activated. This is a crucial mechanism that maintains the haemostatic balance in the human body.

The lysis of fibrin to fibrin degradation products is powered by an enzyme called plasmin. This enzyme is present as plasminogen before the lysis process is activated. The active protease plasmin formation occurs by cleavage of the plasminogen molecule at the Arg561–Val bond. This cleavage would happen when the plasminogen bind to fibrin via lysine binding sites on the heavy-chain portion. However, this mechanism is highly influenced by the presence of plasminogen activators and their inhibitors (Norris, 2003).

2.3.4 Thrombosis

Thrombosis is a multifactorial disease that happens due to dysregulation of haemostatic pathways and uncontrollable thrombus formation (Norris, 2003). The unconditional intensity of the stimulus is a major reason for thrombosis. Then, the natural anticoagulant mechanism ability gets distorted. The susceptibility of people to get arterial thrombosis depends on several environmental and genetic factors. Besides, reports state that the diet and smoking also increase the incidence of this disease (Colman, 1995).

Thrombosis are considered as an acute illness in the arterial diseases which links to myocardial infarction, stroke, venous thromboembolic disorders, and also account for considerable morbidity and mortality. Moreover, venous thrombosis is the second prominent cause of death among patients with cancer (Furie et al., 2008).

In the bloodstream, the wall with its inner lining of endothelium is difficult to maintain a vasculature. The endothelium holds three thromboregulators named as nitric oxide, prostacyclin, and the ectonucleotidase CD394, which together deliver a defense against thrombus formation. Collagen in the tissue factor and sub-endothelial matrix provide the maintenance of a closed circulatory system. When the endothelium is disrupted or the bloodstream wall is ruptured, tissue factor and collagen become available to the flowing blood and activates the formation of a thrombus. The visible collagen triggers the activation and accumulation of platelets, whereas visible tissue factor initiates the generation of thrombin, which not only transforms fibrinogen to fibrin, but also activates platelets (Furie et al., 2008).

When the endothelium was exposed, this area is rapidly enclosed by thrombus consisting of platelets and fibrin. The thrombus contains platelets and thrombin will greatly enhance the lipid uptake of macrophages and lead to the formation of foam cell and subsequently atherosclerosis. Hence, it can fasten the development of atherosclerotic plaques. Furthermore, fibroblasts and smooth muscle cells synthesis connective tissues and incorporate lipids into the cells. All this scenario leads to the formation of a fibrous capsule or fibro-muscular lesion of a lipid-rich plaque. When plaque breaks apart, tissue factor and collagen on the bottom of the plaque will be exposed. Subsequently, platelet will be stimulated followed by both intrinsic and extrinsic coagulation pathway activation. Prothrombinase activity will be increased by activating platelets, which then will further promote thrombin production. More platelet aggregation will be promoted by thrombin, which is a strong agonist for platelet. This may encourage in the reduction of lumen size due to the formation of a larger thrombus and causes serious clinical symptoms such as ischemia and/or necrosis (Furie, 2009).

2.3.5 Thrombolysis and Its Clinical Aspect

Thrombosis is an important mechanism in treatment for acute myocardial infarction and stroke. Recanalization of the occluded coronary artery is an early potential of thrombolytic agent. However, in recent descent, it delivers the basis for the development of thrombolytic therapy in acute myocardial infarction (Collen and Lijnen, 1991). In the past, anticoagulant and antiplatelet drugs are widely used to treat cardiovascular diseases.

Anticoagulation is the mechanism that hinders one or more steps in the fibrin formation cascade that inhibits the synthesis of certain clotting factors. In ancient times, heparin and coumarin has been reported as famed anticoagulant compounds.

Heparin is necessary to be given parentally, rather by continuous intravenous infusion and has short-life (Fitzmaurice et al., 2002). Heparin results in serious side effects such as haemorrhage, osteoporosis, thrombocytopenia, and hypersensitivity. Pregnant women are not allowed to take heparin for the reason that stillbirth and pre-mature labor may occur. On the other hand, coumarins cause bleeding in nervous and gastrointestinal system and provoke the biosynthesis of vitamin K-dependent coagulation factors, which may inhibit the action of certain tissue factors.

Streptokinase and alteplase are also used as thrombolytic drugs in serious illness such as stroke. Likewise, the urokinase, pro-urokinase and ancrod have been developed as anticoagulant drugs. The filtration of β-haemolytic streptococci cultures produce Streptokinase, and is weakly antigenic. Streptokinase activates the fibrinolysis system by inducing the dissolution of intravascular thrombi and emboli. Fibrin degradation will be induced by decreasing the level of plasminogen and fibrinogen simultaneously. The biological and elimination half-life of streptokinase is around 80 min. However, the fibrin and fibrin degradation fragments following fibrinolysis have an additional anticoagulant effect. So, the thrombin time may be prolonged for 4 h or more.

Urokinase is an active protease extracted from culturing human foetal renal cell. It has a half-life of 2.5 hours approximately, which can generate plasmin from plasminogen. It has greater specificity compared to streptokinase and less systemic effects, and is not antigenic.

Alteplase is a tissue plasminogen activator and produced synthetically by recombinant DNA technology. It acts as a serine protease to boost conversion of plasminogen to plasmin and supported by fibrin. When fibrin is absent, the plasminogen conversion will decrease and induce local fibrinolysis with limited systemic proteolysis. Thus, the fibrinogen levels are reduced. Alteplase disappears from the plasma with an initial half-life of less than 5 min.

Ancrod is a purified extract from the Malayan pit viper's venom. It stimulates the defibrinogenation by splitting fibrinopeptide A from fibrinogen. Further, the viscosity of blood reduces due to reduction in fibrinogen, and depleting the substrates required for thrombus formation by anticoagulation action. Platelet activation by fibrinogen and stimulation of endogenous plasminogen activators can cause some local thrombolysis then it is reduced. However, ancrod is not a true thrombolytic drug in clinical aspect (Kennedy, 2000).

Acetylsalicylic acid (ASA) commonly known as aspirin is the most widely prescribed agents in medicine. It is an effective antiplatelet drug which significantly reduces the risk of myocardial infarction and stroke. Aspirin can inhibit the platelet within an hour due to its systemic bioavailability. Aspirin is generally metabolized by the liver and has a half-life of 15 to 20 minutes.

2.4 Aqueous Two-Phase Systems (ATPS)

Aqueous two-phase system (ATPS) is a striking alternative to facilitate the adoption of bioprocess based on plants as production systems (Aguilar and Rito-Palomares, 2008). Based on previous reports, the ATPS has high potential for the recovery and purification of biological compounds, including proteins, genetic material, bio-nanoparticles, cells and organelles (Silva et al., 2009; Benavides, 2006; Rito-Palomares, 2004). It is very economical since costly unit operations can be avoided, such as chromatography, throughout the separation process. The efficiency of the initial capture and purification steps are the main aspect to determine the overall production cost. This efficiency is based on the production of large feed volumes by removing biomass solids and oils and concentrating the protein as well (Menkhaus et al., 2004).

The effective separation of a particular protein can be achieved by exploiting the following properties individually or in conjunction (Andrews and Asenjo, 1989);

i. Hydrophobicity, where the hydrophobicity of proteins is considered in phase system and arranged hydrophobic properties of a phase system accordingly.
ii. Electrochemical, where the molecules or particles are separated according to their charge by using the electrical potential between the phases.
iii. Size-dependent partitioning, where the surface area of the molecules (proteins) or particles or molecular size of the proteins is the dominating factor.
iv. Bio-specific affinity, where the affinity between sites on the protein and ligand attachment are used to exploit the separation.
v. Conformation-dependent, where the determining factor is the conformation of the proteins.

2.4.1 Protein Separation in Aqueous Two-Phase Systems (ATPS)

The ATPS has the capacity to purify proteins from other proteins or to separate proteins from cell debris. In bottom phase, the partitioning matter must be more soluble and particulate, while at top phase, the partitioning matter is usually proteins since less polar and more hydrophobic phase (Andrews and Asenjo, 1989). Separation of proteins in ATPS can be attained by changing the type of ions in the system, the average molecular weight of the polymers, the ionic strength of the salt phase or by adding an additional salt such as sodium chloride (NaCl). Polyethylene glycol (PEG) and dextran are the most commonly used polymers to construct ATPS. The ATPS comprises a polymer and a high concentration of salt also have a comparable exclusion phenomenon (e.g. PEG and phosphate, sulphate, citrate), since the salt will capture a large amount of the water in the system (Asenjo and Andrews, 2011).

However, several factors must be considered in designing the ATPS with effective protein separation. The following are the factors which determine the effective partitioning of a protein in aqueous two-phase systems:

i. molecular weights/size of polymers;
ii. concentration of polymer;
iii. ionic strength of the salt;
iv. pH;
v. additional salt used such as NaCl that increases the hydrophobicity
vi. resolution of the system.

Essentially, the aqueous two-phase system is the best way to separate and purify the desired proteins, but certain mechanism of partitioning is still undefined. However, in clear view, the physico-chemical properties and the composition of the system are the

main aspects which affect the partitioning of proteins in ATPS (Asenjo and Andrews, 2011).

2.5 Nanotechnology

Nanotechnology is a multidisciplinary field that is used to obtain some unique properties of the materials with diameter < 100 nm. These particles not only nano in size, also have high bioavailability with high surface area to mass ratio, and show considerable surface activity along with Plasmon resonance bands (Vithiya & Sen, 2011). In recent years, nanotechnology has become important in numerous fields such as energy, health care, environment, agriculture, etc.

In medical diagnostic applications, nanoparticles can be successfully attached to a single strand of a non-destructive DNA (Nakao et al., 2003). Besides that, in neuroscience, the new interface was designed, which allows to interact with the cells and their tissues at the molecular level (Silva, 2008). Furthermore, the great achievements in the area of nanotechnology application in the development of technologies and approaches for delivering drugs and other small molecules such as drugs that cross the blood brain barrier to carry out specific function as needed (Silva, 2008). Metal nanoparticles as a drug delivery system became very important in the nanotechnological research compared to using non-functional vehicle. The study of the unique characteristics of the nanoparticle is needed before optimising it. These unique characteristics generally depend on the structure, synthesis method, capping agents and stability (Singh et al., 2011).

2.5.1 Selenium Nanoparticles

Selenium is an important trace element in human dietary. Selenium plays a vital role in the production of active thyroid hormone (Vanderpas et al., 1990), also well known as an antioxidant (Diplock, 1992). Selenium is an essential element for the proper functioning of the immune system and acts as a key nutrient in counteracting the development of virulence and inhibiting HIV progression to AIDS (Rayman, 2000). Besides, a proper selenium intake is associated with reduced cancer risk. Many clinical trials are now being executed to confirm or refuse this hypothesis (Rayman, 2000).

Recently, selenium nanoparticles are becoming more attractive due to their excellent biological activities and low toxicity. It is being used as nutritional supplements such as selenomethionine and selenium-enriched yeast, due to their excellent bioavailability and lower toxicity in all forms (Griffiths et al., 1976) . Selenomethionine can be non-specifically integrated into proteins in place of methionine, but concerns have been raised that selenomethionine could potentially cause accumulation of selenium in tissues to toxic levels (Waschulewski & Sunde, 1988). According to Wang et al. (2007), the selenium nanoparticles were less toxic compared to the selenomethionine. Selenium nanoparticles can be used as an antioxidant with reduced risk of selenium toxicity. Besides that, selenium nanomaterial crystals have been employed as enhancing and settled materials for H_2O_2 biosensor due to high surface-to-volume ratio, good adhesive ability and biocompatibility (Wang et al., 2010). Selenium nanoparticles also act as anti-proliferative activity and have excellent cellular uptake characteristics (Wu et al., 2012).

2.5.2 Preparation of Nanoparticles

Nanoparticles synthesis has been the core subject of a lot of studies due to its commercial importance and applications. Their unique characteristics and extensive applications have led to numerous methods being developed for the nanoparticle synthesis in various shapes and sizes (Kannan and Subbalaxmi, 2011). Nanoparticles can be prepared by three different methodsviz. chemical, physical and biological method. Usually, nanoparticles prepared by ion sputtering, solvothermal synthesis, reduction and sol-gel technique are the commonly used physical and chemical methods (Singh et al., 2011).

A chemical method is the most popular method in nanoparticle preparation due to its fast process with a fairly good control on the size distribution. Reduction of metal particles by using a chemical reducing agent such as sodium borohydride (Jana et al., 2000) or sodium citrate (Cao, Hu, & Jiang, 2009) is the most commonly used chemical method. However, chemical method is energy intensive, employs toxic chemicals (Parashar et al., 2009), cost ineffective and produced hazardous waste (Gade et al., 2010).

On the other hand, physical methods were time consuming and still under development (Gade et al., 2010). Nanoparticle synthesis through thermal decomposition is an example of the physical method. This method produces monodispersed nanoparticles. Synthesis of nanoparticles is reliant on several factors including size of nanoparticles, inherent properties of the drug, surface characteristics such as charge and permeability, degree of biodegradability, biocompatibility and toxicity, drug release profile desired and antigenicity of the final product (Mohanraj & Chen, 2007).

Basically, there are two types of approaches for nanoparticle synthesis namely top-down approach and bottom-up approach (Figure 2.2). Top down approach involves the breaking down of the bulk material into smaller nano-sized structures or particles.

Bottom-up approach denotes the build-up of a material from the lowest: atom-by-atom, molecule-by-molecule, or cluster-by-cluster. Bottom-up approach produced less waste, which is also known as alternative tactic.

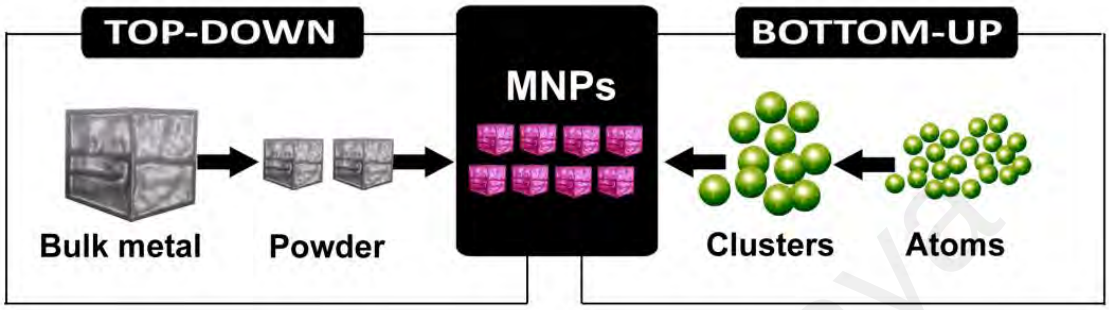

Figure 2.12: Scheme of Top-Down and Bottom-Up method for synthesis of the metal nanoparticles (MNPs). (Domènech et al., 2012)

2.5.3 Biosynthesis of Nanoparticles (Biological Method)

Biosynthesis of nanoparticles are an environmentally friendly and sustainable way of producing nanocomposites. Since the physical and chemical methods are expensive, alternative cheaper pathways for the synthesis of nanoparticles is required and that can be obtained from biological method. Many biological sources such as fungi, bacteria, actinomycetes, algae, plant materials, even viruses can be used in nanoparticle synthesis as shown in Table 2.2.

Biosynthesis of nanoparticles are a redox reaction using the bottom-up approach. The synthesis of nanoparticles is considered as green chemistry since the solvent and medium used, the choice of an environmentally benign reducing agent and the choice of stabilising agent, is a non-toxic material (Singh et al., 2011). The biotechnological method for synthesis of nanoparticles by using organisms is fully a green chemistry principle which has two different methods enzymatic reduction or non-enzymatic reduction. Intracellular and extracellular nanoparticles are the two different types of synthesised nanoparticles

with respect to its environmental conditions. Biosynthesis of nanoparticles carry several advantages against other methods like:

i. reaction takes place in ambient condition similiar to the environment, tolerable range of material sources present and good nature of reduction to form nanoparticle (Singh et al., 2011).
ii. the shape and size of nanoparticles can be manipulated by controlling the pH or the temperature of the reaction mixture (Gericke and Pinches, 2006).
iii. scale up abilities by using fungi (Deepak et al., 2011).

2.5.4 Mycosynthesis of nanoparticles

Mycosynthesis of nanoparticles have unique benefits compared to bacteria. Fungi have high tolerance and metal bioaccumulation ability as well as easy to culture on a large scale by solid substrate fermentation, thus it produces large amounts of biomass. Besides, fungi can easily hydrolyse metal particles to nanoparticles due to its ability to produce large amount of extracellular enzymes (Table 2.3). For instance, the reduction of the silver metal by *Verticillum* sp. was able to produce 2-20 nm sized particles. Similarly, 7-12 nm sized gold nanoparticles were produced by the extracellular synthesis by *Thermomonospora sp.* (Sastry et al., 2003). Moreover, Wu et al. (2012) reported that the mushroom extracts contain polysaccharide-protein complex that can act as reducing agent to form capping and helps to reduce the size of the selenium nanoparticles (SeNPs). The same report stated that the SeNPs without the polysaccharide-protein complexes as capping agent will aggregate and become bigger in size which is not suitable for cellular uptake.

Table 2.2: Metallic nanoparticles from different organisms (Thakkar et al., 2010)

Microorganism	Type of nanoparticle	Location	Size range (nm)
(A) Bacteria			
Pseudomonas stuteri	Ag	Intracellular	-200
Miorganella Sp.	Ag	Extracellular	20-30
Lactobacillus strains	Ag and Au	Intracellular	—
Plectanema boryanum (Cyanobacteria)	Ag	Intracellular	1-10 1-100
Escherichia coli	CdS	Intracellular	2-5
Clostridium thermoaceticum	CdS	Intracellular and extracellular	—
Actinobacter Spp.	Magnetite	Extracellular	10-40
Shewanella algae	Au	Intracellular, pH » 7 Extracellular, pH - 1	10-20 50-500
Rhodopseudomonas capsulata	Au	Extracellular, pH - 7 Extracellular, pH - 4	10-20 50-400
Escherichia coli DHSa	Au	Intracellular	25-33
Clostridium thermoaceticum	CdS	Intracellular and extracellular	—
Aclinobacter spp.	Magnetite	Extracellular	0-40
Shewanella algae	Au	Intracellular, pH - 7 Extracellular, pH - 1	10-20 50-500
Rhodopseudomonas capsulata	Au	Extracellular, pH - 7 Extracellular, pH - 4	10-20 50-400
Escherichia coli DHSa	Au	Intracellular	25-33
Thermomonospora sp.	Au	Extracellular	8
Rhodococcus Sp.	Au	Intracellular	5-15
Klebsiella pneumoniae	Ag	Extracellular	5-32
Pseudomonas aeruginosa	Au	Extracellular	15-30
Shewanella oneidensis	Uranium (IV)	Extracellular	—
(B) Yeast			
MKY3	Ag	Extracellular	2-5
Candida glabrata and *Schizosaccharomyces pombe*	CdS	Intracellular	200
(C) Plant and plant extracts			
Azadirachla indica(Neem)	Ag, Au, and Ag/Au bimetallic	Extracellular	50-100
Geranium leaves plant extract	Ag	—	16-40
Lemongrass plant extract	Au	—	200-500
Avena saliva (Oat)	Au	Extracellular	5-85
Alfalfa sprouts	Ag	Intracellular	2-20
Aloe vera	Au	Extracellular	50-350
Cinnamomum camphora	Au and Ag	Extracellular	55-80
(D) Algae			
Sargassum wightii	Au	Extracellular	8-12
Chlorella wlgaris	Au	—	9-20

Table 2.3: List of metal nanoparticles synthesised from different fungi (Gade et al., 2010)

Name of organism	Nanoparticle synthesized	Intracellular/ Extracelluar	Size (nm)
Schizosaccharomyces pombe	CdS	Intracellular	2-2.5
Torulopsis sp.	PbS	Intracellular	2-5
Verticillium sp.	Silver & Gold	Intracellular	2-20
	Silver	Intracellular	2-25
Thtrmomonospora sp.	Gold	Extracellular	7-12
Phoma sp.3.2883	Silver	Extracellular	70-75
F. oxysporum	Silver	Extracellular	5-15
F. oxysporum	Silica & Titania	Extracellular	5-15
	Silica	Extracellular	2-10
	Zuronia	Extracellular	3-11
F. oxysporum	Silver	Extracellular	20-50
F. oxysporum f. sp. *fycopersici*	Platinum	Intracellular & Extracellular	10-100
F. oxysporum	Magnetite	Extracellular	20-50
F. oxysporum PTCC 5115	Silver	Extracellular	-
Colleiofrichum sp.	Gold	Extracellular	20-40
Yeast strain MKY_3	Silver		2-5
Trichothtcium sp.	Gold	Intracellular & Extracellular	-
A. fundgat us	Silver	Extracellular	5-25
F. oxysporum viz. strain 5115, 23, 24, 25 and 30	Silver	Extracellular	5-60
A. flavus	Silver	Extracellular	8.92 ± 1.61
F. semitectum	Silver	Extracellular	10-60
F. acuminatum	Silver	Extracellular	4-50
A. niger	Silver	Extracellular	15-20
Cladosporium cladosporioides	Silver	Extracellular	10-100
F. solani	Silver	Extracellular	5-35
P. glomerata	Silver	Extracellular	60-80
Trichoderma asperellum	Silver	Extracellular	13-18
Penicillium sp.	Silver	Extracellular	16-40
S. cerevisiae	Titania oxide	Extracellular	-
Altemaria altemata	Silver	Extracellular	20-60

CHAPTER 3: MATERIALS AND METHODS

3.1 Fungal Strains

The basidiocarps of *Lentinula edodes* (shiitake)*, Flammulina velutipes* (enoki), and *Agaricus bisporus* (white and brown button mushroom) were purchased from local markets in Malaysia. While, *Pleurotus floridanus, P. pulmonarius, P. salmoneostramineus, P. cystidiosus,* and *Hericium erinaceus* were obtained from mushroom farm (Ganofarm Sdn Bhd, Tanjung Sepat, Malaysia). The maitake mushroom (*Grifola frondosa*) was obtained from University of Aizu, Japan. The basidiocarps were freeze-dried (Christ Alpha 1-4 LD plus) for 48 h. Samples were powdered and stored at -20 ± 2 °C prior to use. All basidiocarps samples were identified and confirmed by Mushroom Research Center (MRC), University of Malaya.

3.2 Preparation of Crude Extracts from Basidiocarps

The powdered samples were suspended in 20 mM Tris-hydrochloride buffer (pH 8.0) in a 1:20 ratio and then physically ground with mortar and pestle for 30 min in an ice bath and centrifuged at 10,000 g for 30 min. The supernatant was designated as the crude protein extract (Kim & Kim, 1999).

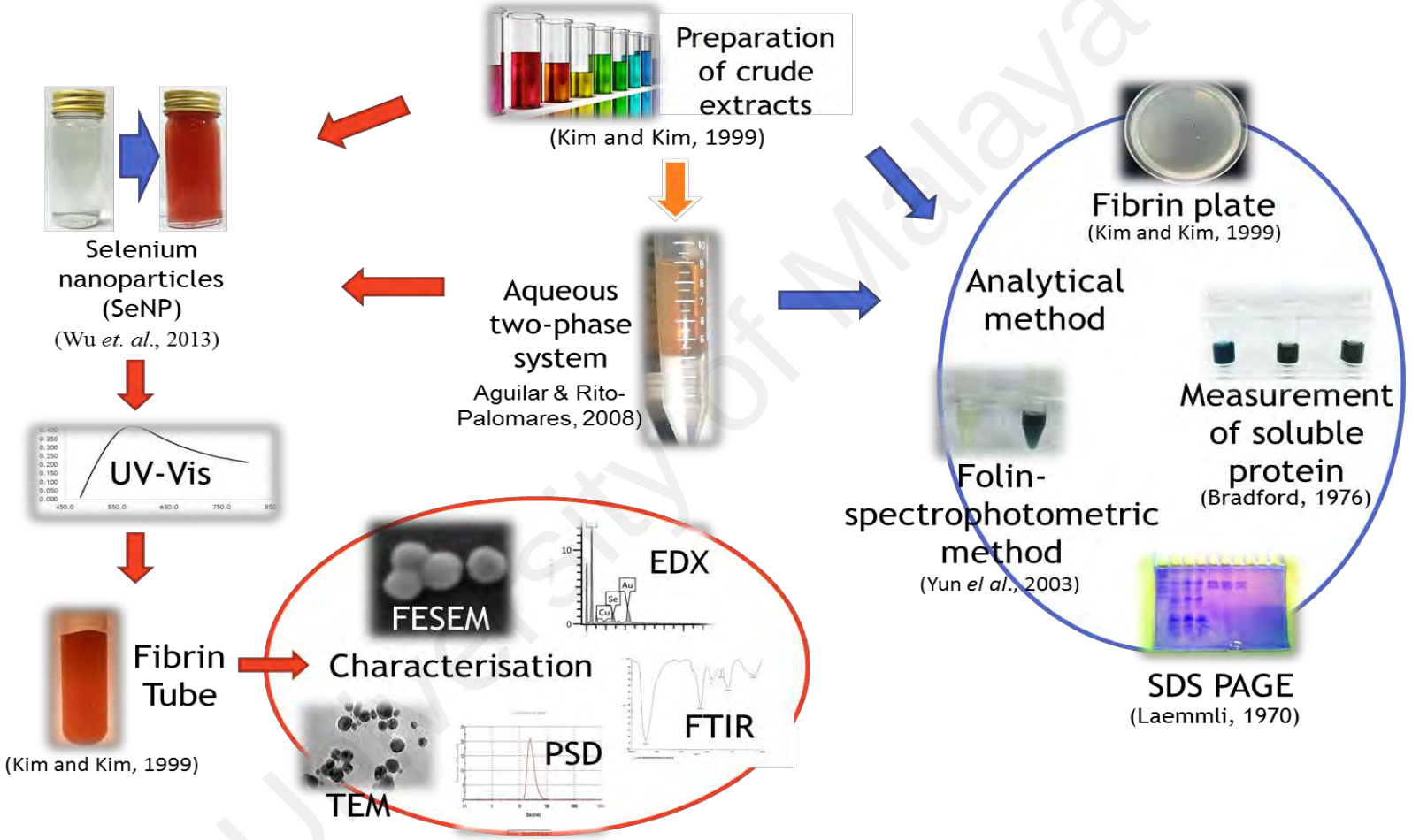

Figure 3.1 Flowchart of experimental design.

3.3 Recovery of Fibrinolytic Enzyme from Basidiocarps

3.3.1 Aqueous Two-Phase System (ATPS) Extraction

The fibrinolytic enzymes from the crude extracts of all ten edible mushrooms were recovered by the aqueous two-phase separation method. The polyethylene glycol (PEG) and the phosphate based aqueous two-phase system (ATPS) were used based on a previous study (Mohamed Ali et al., 2014). The crude extracts were added to 20.0% (w/w) of PEG 8000 and 11.6% (w/w) of potassium phosphate (pH 7.0). The ATPS was gently mixed and left until two phases were formed. The top phase of samples was carefully extracted using micropipette and assayed for fibrinolytic activity by using the Folin-spectrophotometric method and fibrin plate test.

3.3.2 Protein Precipitation

The precipitation of protein from the top phase of ATPS fractionion was carried out according to Wessel and Flügge (1984). Cold acetone (-20 °C) was added into tubes containing the top phase of the ATPS fractionion with 4:1 ratio. The solutions were mixed and incubated at −20 °C for an hour. Then, centrifuged at 10,000 g for 10 min. The pellets obtained were air-dried for 30 minutes after removing the supernatant. The precipitated protein pellets were dissolved in a glass-distilled water (250 μL) prior to use.

3.4 Analytical Methods

3.4.1 Fibrin Plate Method

The fibrinolytic activities in crude protein extracts and ATPS fractions of all ten edible mushrooms were assessed in fibrin plate assay according to the modified method of Kim and Kim (1999). The freeze-dried fibrin powder was prepared and dissolved with 0.6% w/v of distilled water. The mixture was homogenised at 11,000 g for 30 seconds, followed by centrifugation at 10,000 g for six min. The supernatant denoted as fibrin solution was collected and warmed up to 45 ± 2 °C. Subsequently, the agarose was prepared in 2% (w/v) of distilled water and maintained at 55 ± 2 °C.

The prepared fibrin solution was mixed with 2% (w/v) agarose in 1:1 ratio and poured into a petri-dish. The clot was left for an hour at room temperature. Then, 20 µL of samples (crude protein extracts and ATPS fractions of fibrinolytic enzyme) were carefully dropped in the fibrin film in the petri-dish and incubated at 37 ± 2°C. After one hour, the clear transparent region denoted as lytic zone was measured. Replacing samples with 2.28 mg/mL of plasmin and 50 mg/mL of aspirin served as positive controls. While, distilled water (dH_2O) was used as negative control. In this study, a lytic zone was observed on fibrin plate when fibrin was hydrolysed and the diameter and intensity of lytic zones measured was directly proportional to the strength of the fibrinolytic activity.

3.4.2 Folin-Spectrophotometric Method (Quantitative Assay)

The fibrinolytic activities of crude protein extracts and ATPS fractions of all the ten edible mushrooms were determined by the Folin-spectrophotometric method (Yun et al., 2003). The fibrin was dissolved at a concentration of 0.6% (w/v) in 0.1 M McIlvaine buffer (pH 7.0). Further, the solution was homogenized at 11,000 g for 30 seconds,

followed by centrifugation at 10,000 g for 10 min at room temperature. The supernatant was collected as fibrin solution and 0.6 mL of fibrin solution was added to 0.3 mL of either crude extracts or partial purified fibrinolytic enzymes (50 mg/3 mL).

Once incubated at 40 ± 2°C for 10 min, the reaction was terminated by adding 0.6 mL of 0.55 M trichloroacetic acid (TCA) solution. The mixture was left for 10 min before centrifugation and the supernatant was mixed with one mL of 0.4 M sodium carbonate (Na_2CO_3) and 0.2 mL of 1N Folin–Ciocalteau reagent. A blank was prepared equivalently and the sample was replaced with 0.3 mL of McIlvaine buffer (pH 7.0). Aspirin (50 mg/mL) was used as positive control. The mixture was incubated for 30 min at room temperature and the absorbance was read at 660 nm.

The total activity was measured by comparing to the standard curve generated using tyrosine. One unit (U) per mL of enzyme activity was defined as one µg tyrosine released/mL extract/minute of incubation. Specific activity was measured by total activity/total protein and expressed as U/mg of protein.

The fibrinolytic activity was calculated based on a standard curve generated by using tyrosine (Appendix A). The fibrinolytic activity was calculated according to equation as follow:

$$Fibrinolytic\ activity\ \left(\frac{U}{ml}\right) = \frac{Abs_{660nm}}{0.0329} \times \frac{1}{0.3\ mL} \times \frac{1}{10\ min}$$

While, the specific fibrinolytic activity was calculated as:

$$Specific\ fibrinolytic\ activity\ \left(\frac{U}{mg}\right) = \frac{Fibrinolytic\ activity\ \left(\frac{U}{mL}\right)}{Soluble\ protein\ \left(\frac{mg}{mL}\right)}$$

3.4.3 Measurement of Soluble Protein

The soluble proteins in the crude protein extracts and ATPS fractions of all the edible mushrooms were estimated. Bovine serum albumin was the standard (Bradford, 1976). The crude protein extracts and ATPS fractions (0.2 mL) were mixed thoroughly with 5 mL of Coomassie Brilliant Blue reagent and maintained at room temperature for 15 min. The sample was replaced by 0.2 mL of distilled water and mixed with 5 mL of Coomassie Brilliant Blue reagent for the blank. The amount of protein in the sample was calculated by using the protein calibration plot after reading the absorbance at 595 nm.

Soluble protein concentrations in ten edible mushrooms were measured according to Bradford (1976) (Appendix A). The bovine serum albumin (BSA) was used as the standard curve to calculate the soluble protein concentration by using equation as below:

$$Soluble\ protein\ \left(\frac{mg}{ml}\right) = \frac{Abs_{590nm}}{0.006} \times \frac{1}{0.2\ mL} \times \frac{1\ mg}{1000\ \mu g}$$

3.5 Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis (SDS–PAGE)

The molecular weight of crude protein extracts and ATPS fractions were determined by SDS–PAGE according to a modified method of Laemmli, 1970. The sample (crude extracts and ATPS fractions) was mixed with a 10× sample buffer containing 1 M Tris–HCl, 30% (v/v) glycerol, 0.06% (w/v) SDS, 16% (v/v) 2-mercaptoethanol and 0.06% (w/v) bromophenol blue in 9:1 ratio. Then, the samples of 20 µL/well were loaded in an acrylamide gel consisting 12% (w/v) resolving gel and 4.5% (w/v) stacking gel. The gel was electrophoresed in Tris–glycine running buffer (30 mM Tris, 144 mM glycine, 0.1%

(w/v) SDS) at 80 V for approximately 120 min. Prestained protein molecular weight markers from Thermo Scientific ranged from 10 to 260 kDa were used. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 and destained with a destaining buffer of 10% (v/v) methanol and 10% (v/v) acetic acid.

3.6 Preparation of Selenium Nanoparticles (SeNPs)

Selenium nanoparticle of fibrinolytic enzymes was prepared according to Wu et al. (2013). The *L. edodes* was selected based on its prominent fibrinolytic activity. Thus, the crude protein extracts and ATPS fractions of *L. edodes* (0.8 % w/v) mixed with one millilitre of 25 mM of sodium selenite solution. One millilitre of 100 mM of freshly prepared ascorbic acid was added dropwise into the resulting mixture. Then, the mixture was reconstituted to a final volume of 25 mL with Milli-Q water, and reacted for 24 h at 4 ºC. However, the control (SeNPs-AA) was prepared equivalently without mixture of either crude protein extracts or ATPS fractions of *L. edodes*. The reacted mixture was dialyzed by using cellulose tubes against Milli-Q water water for 12 hours.

3.7 Characterisation of Mycosynthesised Selenium Nanoparticles (SeNPs).

3.7.1 UV-Visible Spectrum Analysis of SeNPs

The mycosynthesised selenium nanoparticles with crude protein extracts (MySeNPs-CE) and ATPS fractions (MySeNPs-ATPS) of *L. edodes* were primarily characterized by UV-visible spectroscopy (UV-160A, Shimadzu). Distilled water was designated as blank for both SeNPs-CE and SeNPs-ATPS. The absorbance readings were recorded and analysed in a spectrum range between 200 to 800 nm at room temperature (Ramamurthy et al., 2013).

3.7.2 Particle Size Distribution of SeNPs

The particle size distributions of selenium nanoparticles were measured by laser diffractometry using a nano size particle analyser (Malvern Instrument, USA). The laser diffraction method was used to determine the particles sizes in MySeNPs-CE, MySeNPs-ATPS and SeNPs-AA solutions which were dispersed in deionised water (Wu et al., 2013).

3.7.3 Morphological Analysis of SeNPs by High Resolution Transmission Electron Microscopy (HR-TEM)

The transmission electron microscopy (TEM) samples were prepared on 300 mesh copper grids coated with Formvar film. A drop of MySeNPs-CE, MySeNPs-ATPS and SeNPs-AA solutions were carefully placed on the separate copper grid surface and the samples were dried by critical point drying prior to examination in the electron microscope. Then, the nanostructures in SeNP solutions were observed and characterised using a high-resolution transmission electron microscope (HRTEM, JEOL JEM-2100F, operated at 200 kV) (Prasath et al., 2013).

3.7.4 Morphological Analysis of SeNPs by Field Emission Scanning Electron Microscopy (FESEM)

Freshly prepared selenium nanoparticles (MySeNPs-CE, MySeNPs-ATPS and SeNPs-AA) were dropped on 300 mesh copper grid coated with Formvar film. Then, the copper grids were incubated in critical point dryer for three days. The dried samples on copper grids were examined for surface morphology by field-emission scanning electron microscope (FE-SEM, FEI, Quanta FEG 450) operated at 5-10 kV (Raman et al., 2015).

3.7.5 Elemental Identification of Mycosynthesised Nanoparticles

Elemental analysis of the synthesised selenium nanoparticles (MySeNPs-CE, MySeNPs-ATPS and SeNPs-AA) were performed using an energy-dispersive X-ray spectroscope (EDX, X-MAX, Oxford instrument), which was attached to a field emission scanning electron microscope (FE-SEM, FEI, Quanta FEG 450) (Wu et al., 2013).

3.7.6 Functional Group Identification

The functional groups present in crude protein extract of *L. edodes,* ATPS fractions of *L. edodes* as well as synthesised selenium nanoparticles (MySeNPs-CE, MySeNPs-ATPS and SeNPs-AA) were identified by Fourier Transform-Infrared spectrophotometer (FT-IR, PerkinElmer Spectrum 400) in the range between 4,000 and 400 cm^{-1} (Prasath et al., 2013). All samples were freeze dried and mixed separately with potassium bromide (KBr) at a ratio of 1:10, followed by data scanning by FT-IR spectrophotometer.

3.8 Statistical Analysis

Data are expressed as the mean ± SEM of triplicate individual values. The data for all the parameters obtained were subjected to one-way analysis of variance (ANOVA). Statistical significance was set at $p < 0.05$ using Duncan's multiple range test (DMRT) (Duncan, 1955).

CHAPTER 4: RESULTS

4.1 Extraction, Recovery and Characterisation of Fibrinolytic Enzymes from Edible Mushrooms

4.1.1 Fibrinolytic Activity of Crude Extracts from Edible Mushrooms

Crude extracts were obtained from basidiocarps of *L. edodes, F. velutipes, A. bisporus* (white and brown button mushroom), *P. floridanus, P. pulmonarius, P. salmoneo stramineus, P. cystidiosus, H. erinaceus* and *G. frondosa* according to the method of Kim & Kim (1999).

The specific fibrinolytic activity of the crude extracts in ten edible mushrooms based on Folin-spectrophotometric method is given in Figure 4.1. Among the ten mushrooms, the extract of *L. edodes* (shiitake) had a significantly ($p < 0.05$) higher specific fibrinolytic activity of 48.06 U/mg compared other mushroom extracts. The lowest fibrinolytic activity of 6.14 U/mg was recorded in the extract of *P. cystidiosus*. Among the four *Pleurotus spp*., the extract of *P. salmoneostramineus* showed a significantly ($p < 0.05$) higher specific fibrinolytic activity of 15.08 U/mg compared to the extracts of *P. floridanus* (9.64 U/mg), *P. pulmonarius* (9.01 U/mg) and *P. cystidiosus* (6.14 U/mg). Further, the specific fibrinolytic activity recorded in *P. salmoneostramineus* (15.09 U/mg) and *H. erinaceus* (14.77 U/mg) were not significantly different.

The specific fibrinolytic activity of *A. bisporus* (white) was recorded 41% higher (26.76 U/mg) compared to *A. bisporus* (brown) (10.89 U/mg). Besides, the specific fibrinolytic activities of other mushrooms were in the range of 9-25 U/mg. It is evident from the Figure 4.1 that the crude sample of *L. edodes* exhibited a 50% higher specific fibrinolytic activity compared to other mushroom samples tested.

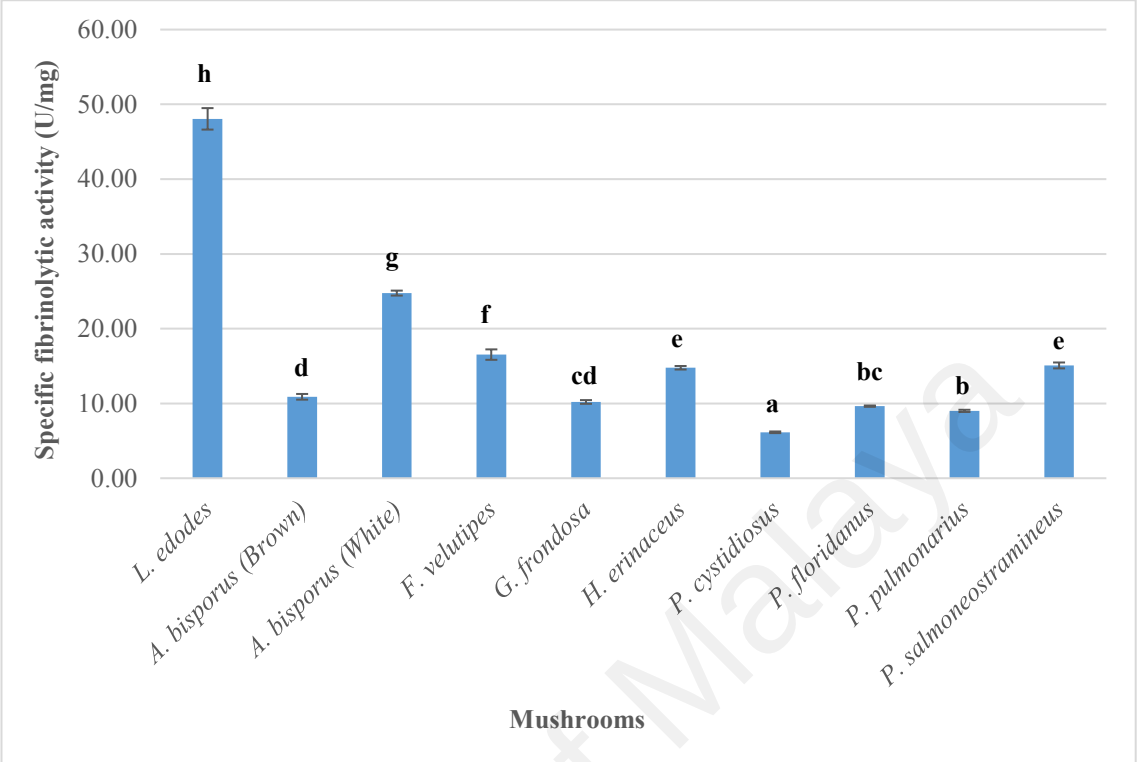

Figure 4.1: The fibrinolytic activity of crude protein extracts of selected edible mushrooms.

The data are presented as means of triplicate data ± standard error; Values with the same alphabet are considered not significant difference ($p > 0.05$).

4.1.2 Recovery of Fibrinolytic Enzyme from Edible Mushrooms by ATPS

The specific fibrinolytic activity of the recovered fibrinolytic enzymes by ATPS method were determined again by the Folin-spectrophotometric method (Figure 4.2). The highest fibrinolytic activity was recorded in *L. edodes* (54.28 U/mg), followed by *P. floridanus* (42.93 U/mg). Whereas, *G. frondosa* showed the lowest fibrinolytic activity with 4.63 U/mg. The ATPS fractions of *A. bisporus* (white and brown) showed lesser fibrinolytic activity compared to other basidiocarps.

The fibrinolytic activities in ATPS fractions of *P. pulmonarius, P. floridanus* and *P. cystidiosus* had increased to 77%, 69% and 65% respectively, as when compared to its

crude extracts. These ATPS fractions had recovered the higher fibrinolytic enzymes compared to others. The *A. bisporus* (white), however, exhibited a 72% decrease in the fibrinolytic activity (72%) after the ATPS recovery. The ATPS fractions of *G. frondosa* and *A. bisporus* (brown) also recorded a decrease in fibrinolytic activities with 55% and 16% respectively compared to its crude fibrinolytic activity. However, the fibrinolytic activities of other ATPS fractions (*L. edodes, F. velutipes* and *H. erinaceus*) showed an increase (~10%) after ATPS recovery.

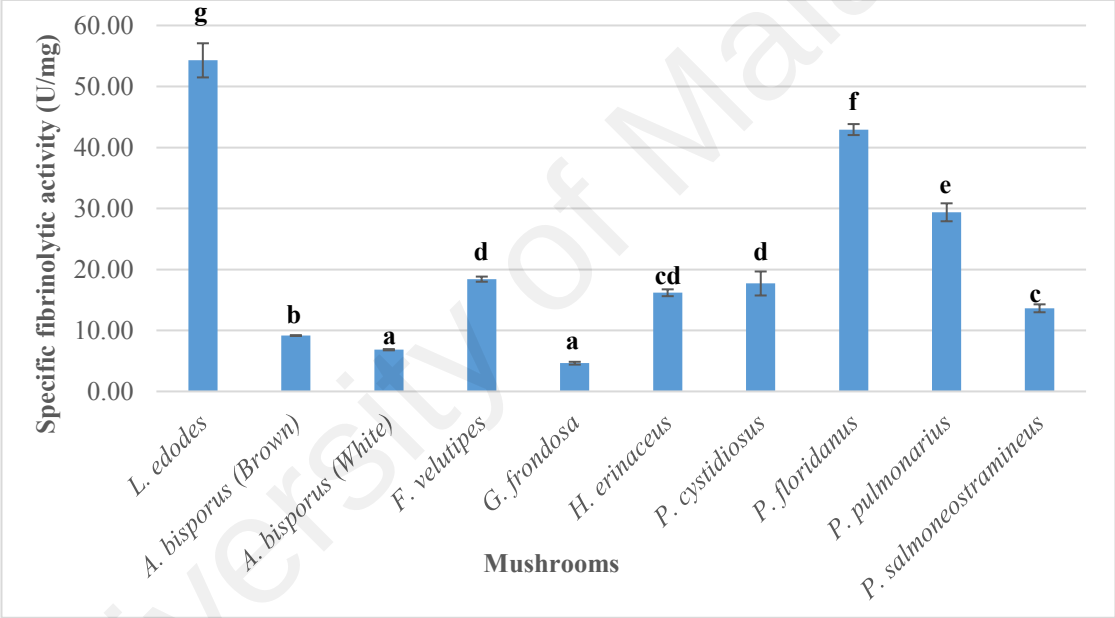

Figure 4.2: Recovery of fibrinolytic activity of various edible mushrooms on ATPS.

The data are presented as means of triplicate data ± standard error; Values with the same alphabet are considered not significant ($p > 0.05$).

4.1.3 Preliminary Assay of Fibrinolytic Enzyme – Fibrin Plate Assay

The fibrinolytic activity of all the ten mushrooms crude extracts and ATPS fractions were subjected to fibrin plate assay (Table 4.1). The crude extracts of *L. edodes, P. pulmonaris, F. velutipes, A. bisporus* (white & brown), and *P. salmoneo stramineus*

showed strong lytic zones with diameters ranging from 2-3 cm. These were significantly higher compared to the positive control plasmin (1.3 ± 0.1 cm). The crude extracts of *A. bisporus* (white button) and *P. salmoneostramineus* showed strong lytic zone with highest diameters of 3.0 ± 0.1 cm and 2.8 ± 0.1 cm, respectively on fibrin plates. On the contrary, the crude extract of *P. cystidiosus* did not show any lytic activity. However, the ATPS fraction of *P. cystidiosus* showed mild lytic zones with diameter of 3.2 ± 0.2 cm. The specific fibronolytic activity of *P. cystidiosus* was recorded lowest in crude extract, then, after ATPS fraction it was increased up to 65 % in Figure 4.1 and Figure 4.2. This influenced the lytic zone on fibrin plates test too. In overall, the diameters of lytic zones recorded in all of ATPS fractions were 1-2 cm higher compared to the crude extracts except for *A. bisporus* (white).

Table 4.1: The fibrinolytic activity of edible mushrooms on fibrin plate.

Samples	Diameter of lytic zone (cm)	
	Crude extract	**ATPS fraction**
dH_2O (negative control)	NA	NA
Plasmin (positive control)	1.3 ± 0.1 *	NA
A. bisporus (brown button)	2.3 ± 0.1 *	2.5 ± 0.5 *
A. bisporus (white button)	3.0 ± 0.1 *	2.9 ± 0.1 *
F. velutipes	2.2 ± 0.2 *	2.8 ± 0.6 *
G. frondosa	2.3 ± 0.1 *	2.5 ± 0.1 *
H. erinaceus	1.4 ± 0.2 **	2.4 ± 0.3 **
L. edodes	1.9 ± 0.1 *	2.6 ± 0.5 *
P. cystidiosus	NA	3.2 ± 0.2 **
P. floridanus	1.8 ± 0.1 *	2.7 ± 0.3 *
P. pulmonarius	2.6 ± 0.1 *	3.0 ± 0.2 *
P. salmoneo stramineus	2.8 ± 0.1 *	3.0 ± 0.1 *

The data are presented as means of triplicate data ± standard error.
'NA' – no activity, '*' – clear lytic zone, '**' – fuzzy lytic zone.

4.1.4 Molecular Weight of Fibrinolytic Enzyme

Based on the activity of the extracts in fibrinolytic assay and fibrin plate assay after ATPS recovery, two out of the ten mushrooms (*L. edodes* and *P. floridanus*) were selected for determination of molecular weight.

The molecular weights of the fibrinolytic enzymes from *L. edodes* and *P. floridanus* were determined by SDS-PAGE electrophoresis (Figure 4.3). The crude extract of *L. edodes* showed seven bands in the range ~9 kDa to ~120 kDa. Among them, two bands observed on top region of gel had molecular weight of ~45 kDa and ~50 kDa. Another two bands that appeared on the bottom region, had a molecular weight of ~9 kDa and ~10 kDa. However, the ATPS fraction of *L. edodes* showed only a single band at ~50 kDa.

The crude extracts of *P. floridanus* revealed five bands with the molecular weight of approximately 30 kDa, 35 kDa, 40 kDa, 130 kDa and 140 kDa. After partial purified by using ATPS, two bands with the molecular weight of ~45 kDa and ~ 140 kDa were observed on SDS-PAGE gel. The band observed at ~140 kDa was denser compared to the band at ~45 kDa (Lane E, Figure 4.3).

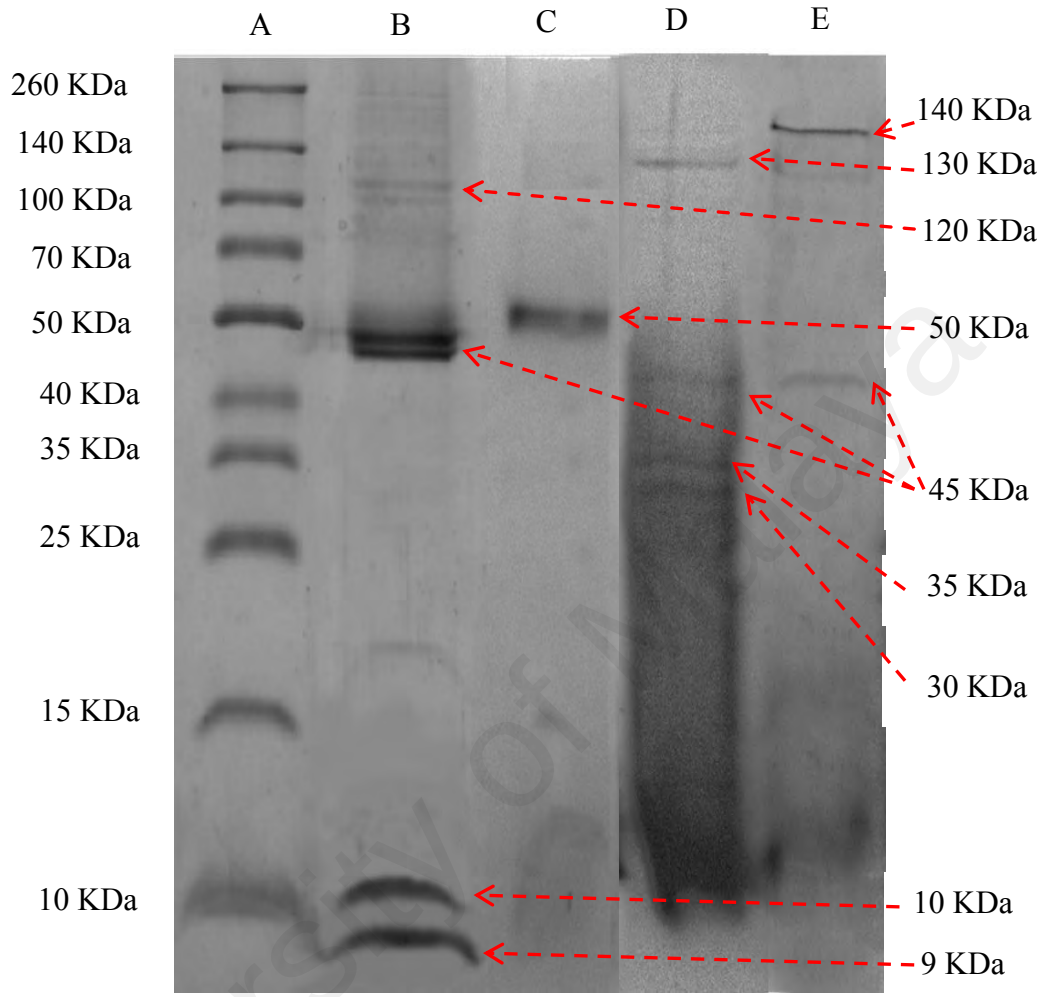

Figure 4.3: Molecular weight analysis of fibrinolytic enzyme from crude extracts and ATPS extracts of *L. edodes* and *P. floridanus.* Lane A, protein marker with molecular weight ranged from 10 kDa – 260 kDa. Lane B, crude extract of *L. edodes.* Lane C, ATPS extract of *L. edodes.* Lane D, crude extract of *P. floridanus.* Lane E, ATPS extract of *P. floridanus*.

4.2 Mycosynthesis and Characterisation of Mycosynthesised Selenium Nanoparticles

4.2.1 Reduction of Mycosynthesised Selenium Nanoparticles

Mycosynthesis of selenium nanoparticles was achieved by using the crude extracts and ATPS fraction of *L. edodes*. Figure 4.4 (A), shows the stacked UV-Visible spectra of selenium nanoparticle formed from the reaction of aqueous sodium selenite (Na_2SeO_3) and ascorbic acid with fibrinolytic enzymes from crude extracts (MySeNPs-CE) and ATPS fraction (MySeNPs-ATPS) of *L. edodes*.

Addition of fibrinolytic enzyme to the aqueous sodium selenite (Na_2SeO_3) and ascorbic acid resulted in colour change from dark brick red to orange, which indicated the formation of selenium nanoparticles (Se^0) in the reaction mixture (Figure 4.4 (B)). The absorption peaks at 220 nm and 280 nm were recorded in all selenium nanoparticle samples. Another peak was observed at wavelength of 570 nm in ascorbic acid synthesised nanoparticles (control) and this peak intensity was reduced in MySeNPs-CE. However, the MySeNPs-ATPS showed no peak at 570 nm.

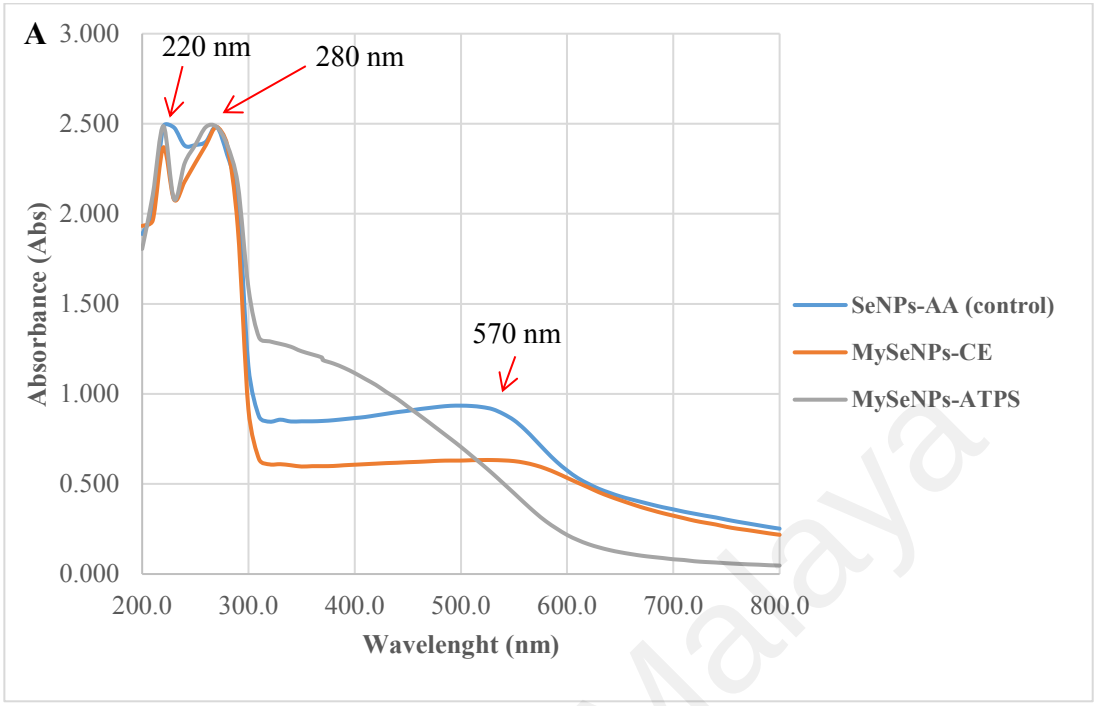

Figure 4.4: Mycosynthesis of selenium nanoparticles from crude extract and ATPS fraction of *L. edodes*. (A) UV-Visible Spectroscopy of mycosynthesised selenium nanoparticles. (B) Formation of selenium nanoparticles from crude extract and ATPS fraction of *L. edodes*. SeNPs-AA – Selenium nanoparticles with ascorbic acid (control); MySeNPs-CE – Selenium nanoparticles with fibrinolytic enzyme from crude extracts of *L. edodes*; MySeNPs-ATPS – Selenium nanoparticles with fibrinolytic enzyme from ATPS extracts of *L. edodes*.

4.2.2 Morphological Analysis and Elemental Identification

Morphology and elemental composition of mycosynthesised nanoparticles from *L. edodes* (crude extract and ATPS fraction) have been examined by the Field Emission Scanning Electron Microscopy (FESEM) and Energy-Dispersive X-Ray Spectroscope (EDX) analysis. From the FESEM ultra-high magnification images, it was observed that the Selenium nanoparticles with fibrinolytic enzyme from crude extracts of *L. edodes* (MySeNPs-CE) were spherical in shape and highly agglomerated (Figure 4.5). However, in control, the Selenium nanoparticles with ascorbic acid (SeNPs-AA) were appeared with image of slightly agglomerated also some as single spherical particles (Figure 4.6). In contrast, the FESEM image of MySeNPs-ATPS were dispersed individually without any clumping (Figure 4.7). In Figure 4.8 (A), the representative image from MySeNPs-ATPS showed a thin layer on the surface of selenium nanoparticles (Se^0) and its appeared slightly transparent. However, a smaller nano ball (Se^0) without encapsulation was observed on SeNPs-AA in Figure 4.8 (B). Hence, the high magnification image (600 000 X) of MySeNPs-ATPS shows the selenium nanoparticles (Se^0) were encapsulated by the fibrinolytic enzymes of *L. edodes.*

The elemental compositions of SeNPs-AA, MySeNPs-CE and MySeNPs-ATPS were analysed using EDX. The EDX spectrum of all samples confirmed the presence of selenium with peak at 0.5 to 3.5 keV (33-57% of weightage). The EDX spectrum of SeNPs-AA, MySeNPs-CE and MySeNPs-ATPS showed a strong Carbon signal (2-10.5 keV) along with weak signals from Copper and Aurum (0.1-2.8 keV). A weak signal of atom Oxygen was also observed on EDX spectrum of MySeNPs-ATPS with energy peak of 0.3 keV (Figure 4.7).

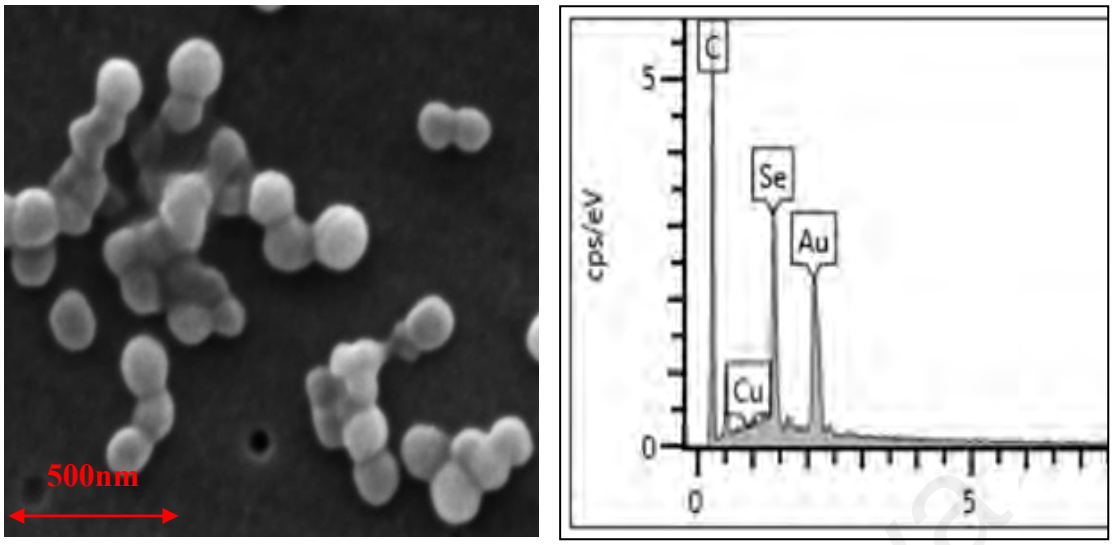

Figure 4.5: Representative FESEM image and EDX analysis of MySeNPs-CE

MySeNPs-CE – Selenium nanoparticles with fibrinolytic enzyme from crude extracts of *L. edodes*

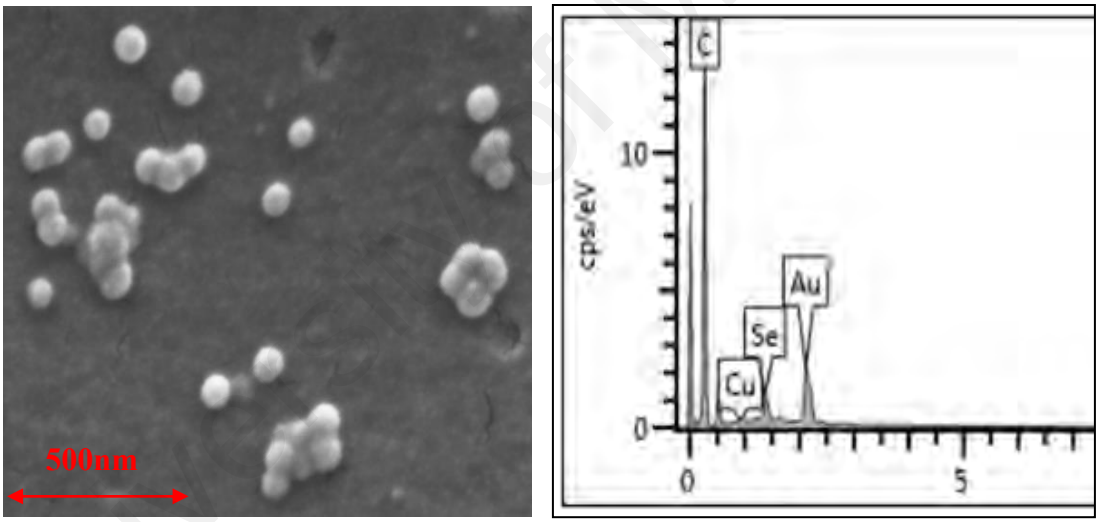

Figure 4.6: Representative FESEM image and EDX analysis of SeNPs-AA

SeNPs-AA – Selenium nanoparticles with ascorbic acid (control).

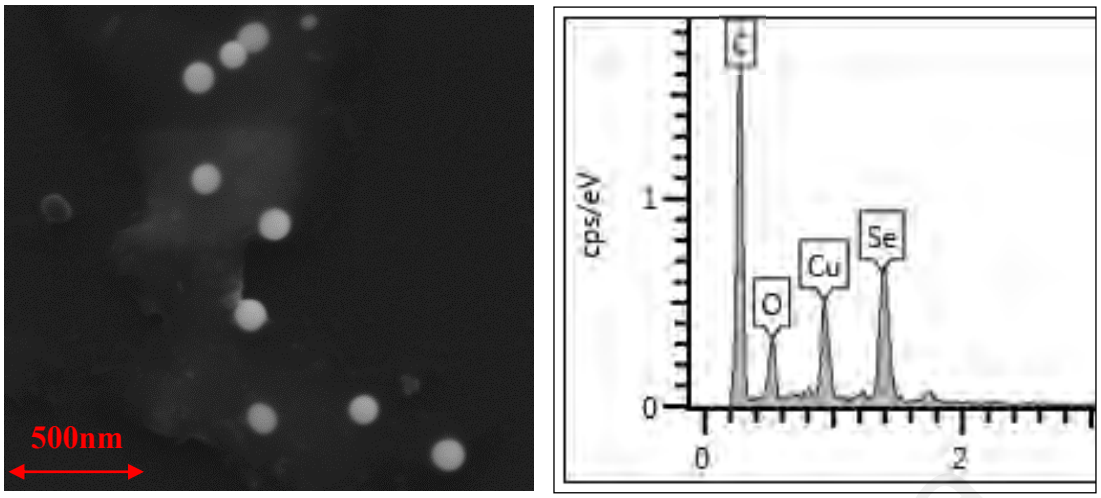

Figure 4.7: Representative FESEM image and EDX analysis of MySeNPs-ATPS

MySeNPs-ATPS – Selenium nanoparticles with fibrinolytic enzyme from ATPS extracts of *L. edodes.*

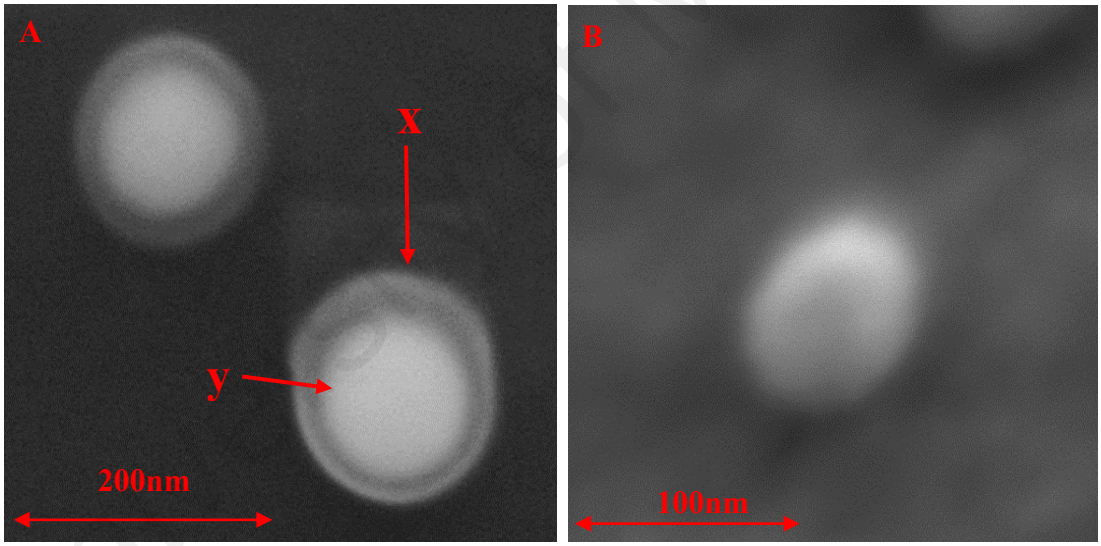

Figure 4.8: Representative FESEM image of (A) MySeNPs-ATPS and (B) SeNPs-AA (control) under high magnification. (x) Capping of fibrinolyic enzymes. (y) Elemental Selenium.

MySeNPs-ATPS – Selenium nanoparticles with fibrinolytic enzyme from ATPS extracts of *L. edodes.*

4.2.3 Particles Size Distribution and High Resolution Morphological Examination

The particles size distribution of mycosynthesised selenium nanoparticles was determined by a laser diffractometry particle size analyser (Malvern Instrument, USA) and the results are presented in Figure 4.9. The particles size distribution of control (SeNPs-AA) recorded with 28.37 nm Figure 4.9 (A). However, the PSD result of MySeNP-CE in Figure 4.9 (B) recorded the highest particle size (281.30 nm). The particles size of MySeNPs-ATPS in Figure 4.9 (C) was recorded with size of 64.53 nm, which is 56% higher than SeNPs-AA. The particle size was further supported by HRTEM images taken by JEOL JEM-2100F which is depicted in Figure 4.10.

The morphology of mycosynthesised nanoparticles observed in High Resolution Transmission Electron Microscopy (HR-TEM) images were similar to the Field Emission Scanning Electron Microscopy (FESEM). Figure 4.10 (A) showed monodispersed spherical particles of SeNPs-AA with size of ~80 nm. Whereas, the Figure 4.10 (B) showed aggregated spherical particles of MySeNPs-CE with an approximate size of 280 nm. In Figure 4.10 (C), the morphology of MySeNPs-ATPS showed that the particles were spherical and slightly agglomerated. When magnification was set to 50,000x, the particle of MySeNP-ATPS was observed as a smooth spherical core (selenium) capped by rough surface structure. The rough surface structure were observed in Figure 4.10 (D) indicating the fibrinolytic enzymes.

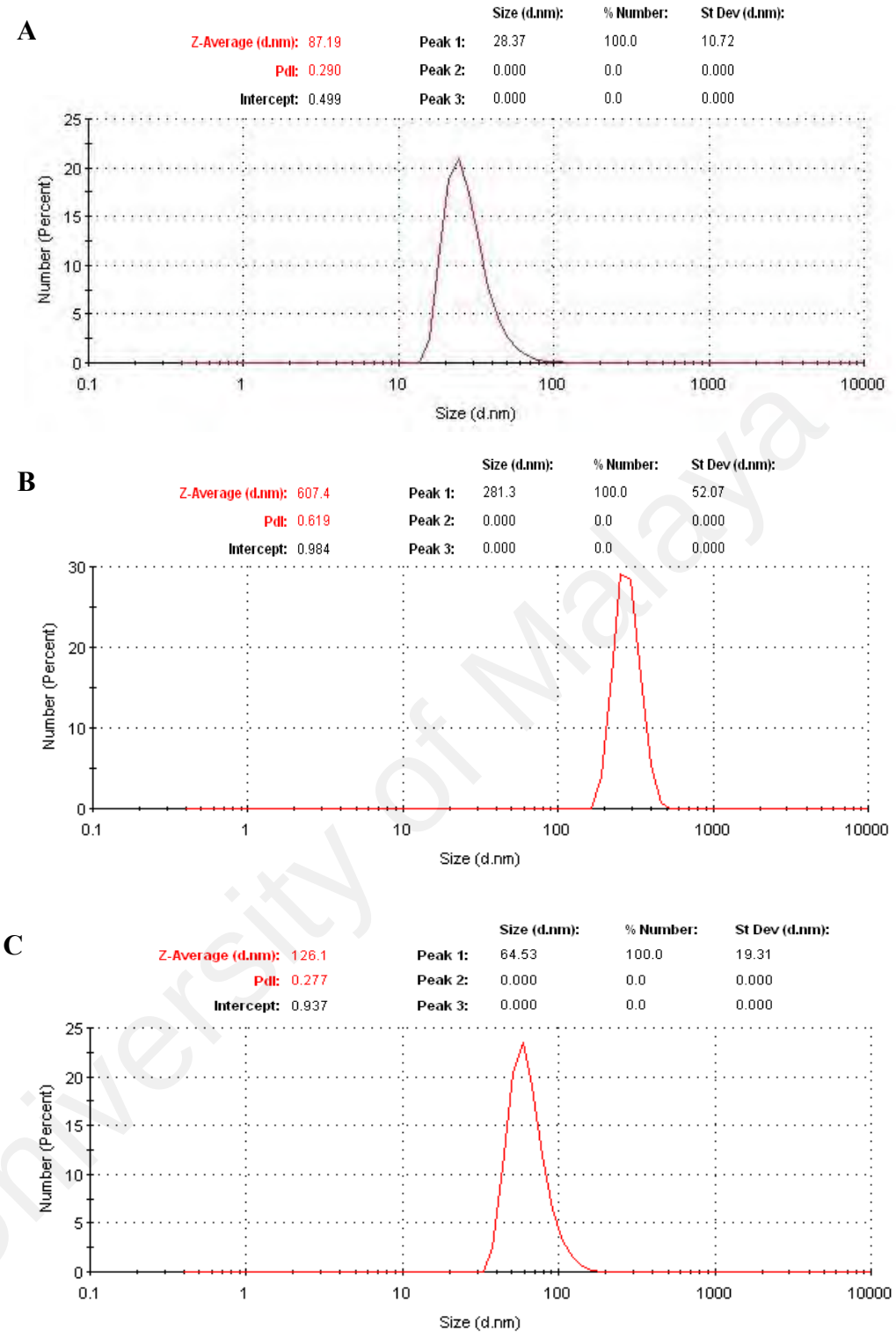

Figure 4.9: Particles size distribution of mycosynthesised nanoparticles. (A) SeNPs-AA. (B) MySeNPs-CE. (C) MySeNPs-ATPS.

SeNPs-AA – Selenium nanoparticles with ascorbic acid (control); MySeNPs-CE – Selenium nanoparticles with fibrinolytic enzyme from crude extracts of *L. edodes*; MySeNPs-ATPS – Selenium nanoparticles with fibrinolytic enzyme from ATPS extracts of *L. edodes*.

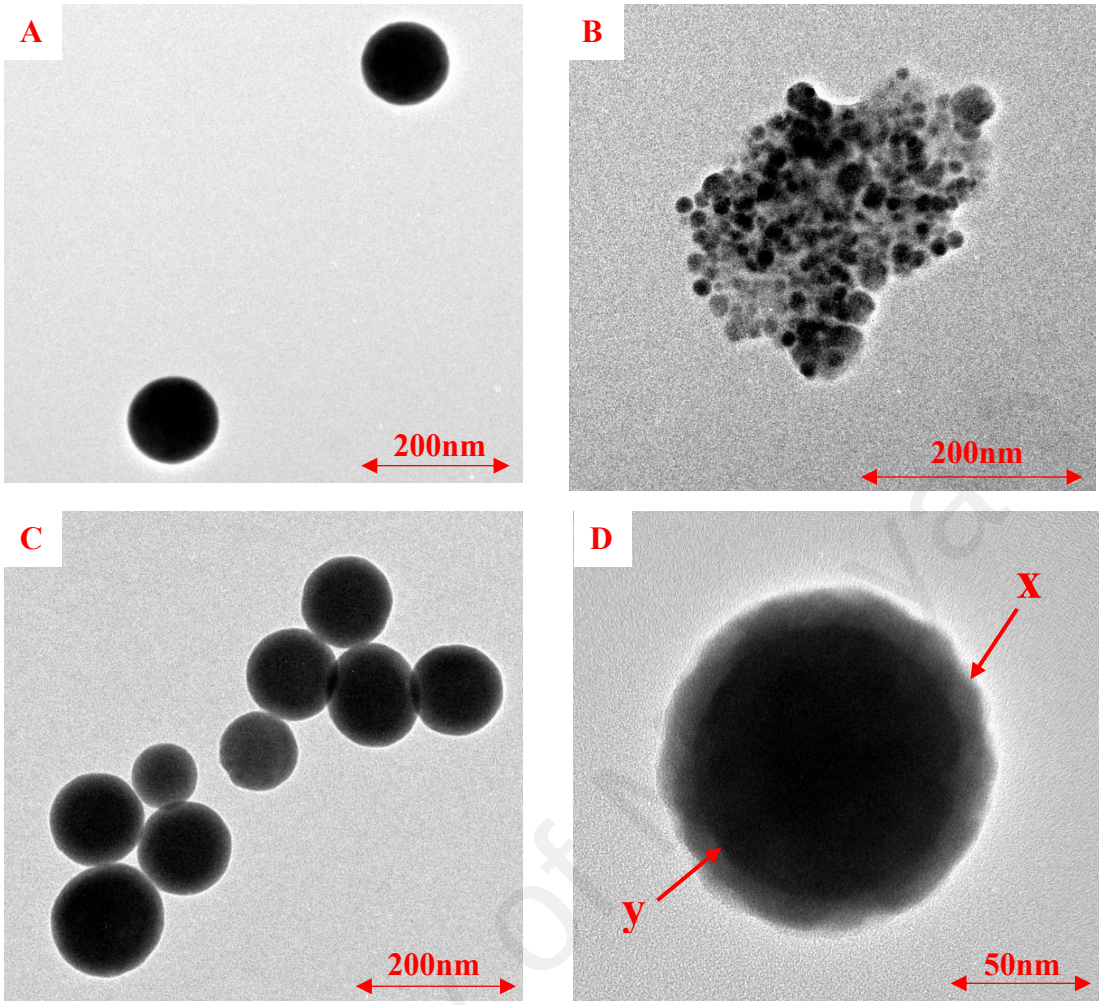

Figure 4.10: Representative HRTEM images of mycosynthesised selenium nanoparticles form *L. edodes.* (A) SeNPs-AA. (B) MySeNPs-CE. (C & D) MySeNPs-ATPS. (x) Capping of fibrinolyic enzymes. (y) Elemental Selenium.

SeNPs-AA – Selenium nanoparticles with ascorbic acid (control); MySeNPs-CE – Selenium nanoparticles with fibrinolytic enzyme from crude extracts of *L. edodes*; MySeNPs-ATPS – Selenium nanoparticles with fibrinolytic enzyme from ATPS extracts of *L. edodes*.

4.2.4 Fourier Transform Infrared Spectroscopy (FTIR) Analysis

The functional groups of mycosynthesised selenium nanoparticles were identified by using FTIR spectrum (Figure 4.11). The peaks observed in SeNPs-AA in Figure 4.11 (A) was different from MySeNPs-CE and MySeNPs-ATPS in Figure 4.9 (B) and Figure 4.9 (C) respectively. Whereas, the FTIR spectrum of MySeNPs-CE and MySeNPs-ATPS showed virtually similar peaks.

The strong peaks at 3408 cm^{-1} and 1627 cm^{-1} show OH and C=O vibration of SeNPs-AA in Figure 4.11 (A) and represents the presence of ascorbic acid. The FTIR spectra of MySeNPs-ATPS showed a broad peak at 3224 cm^{-1}, assigned for NH_3^+ stretching vibration (Figure 4.9 (C)). A strong peak at 1586 cm^{-1} and 1023 cm^{-1} was also observed, which are characteristic peaks for NH bend and CO, respectively. The MySeNPs-CE shared similar peaks with SeNPs-ATPS (Figure 4.9 (B)). A peak at 3300 cm^{-1} was recorded in MySeNPs-CE is assigned for NH_3^+ stretching vibration. Whereas, the peaks at 1606 cm^{-1} and 1026 cm^{-1} are assigned for NH bend and CO, respectively. Therefore, the formation of mycosynthesised selenium nanoparticles (MySeNPs-CE and MySeNPs-ATPS) showed the presence of functional groups from fibrinolytic enzymes.

Table 4.2: The Characteristic Absorption of mycosynthesised selenium nanopartilces.

Sample	Characteristic Absorption (cm^{-1})	Functional Group
SeNPs-AA (Control)	3408	O-H, Stretch
	1627	C=O, Vibration
MySeNPs-CE	3300	NH_3^+, Stretching Vibration
	1606	N-H, Bend
	1026	C-O, Stretch
MySeNPs-ATPS	3224	NH_3^+, Stretching Vibration
	1586	N-H, Bend
	1023	C-O, Stretch

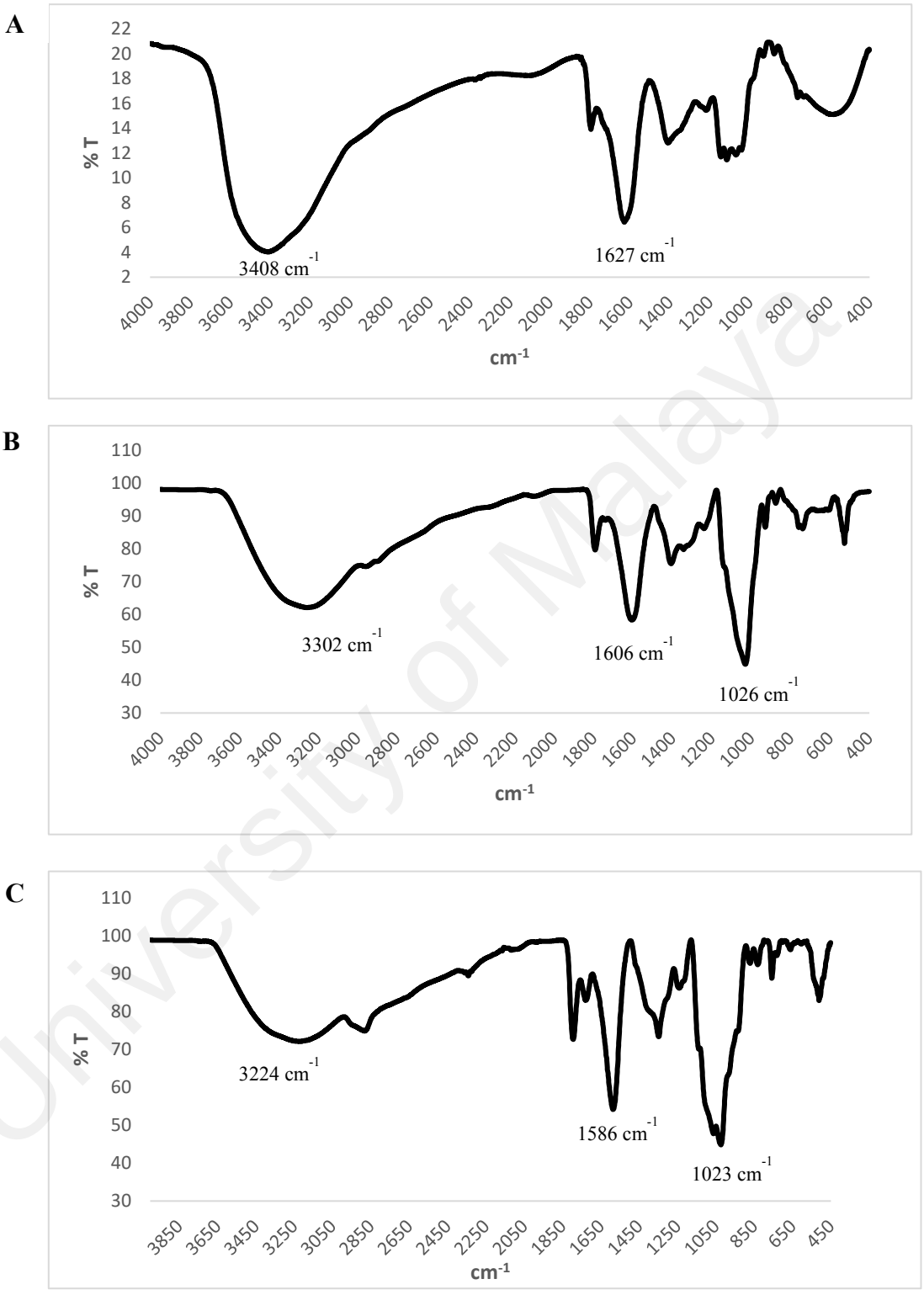

Figure 4.11: Fourier transform infrared spectroscopy (FTIR) analysis for mycosynthesised selenium nanopartilces. (A) SeNPs-AA. (B) MySeNPs-CE. (C) MySeNPs-ATPS.

SeNPs-AA – Selenium nanoparticles with ascorbic acid (control); MySeNPs-CE – Selenium nanoparticles with fibrinolytic enzyme from crude extracts of *L. edodes*; MySeNPs-ATPS – Selenium nanoparticles with fibrinolytic enzyme from ATPS extracts of *L. edodes.*

4.3 Fibrinolytic Activity of Mycosynthesised Selenium Nanoparticles

The modified fibrin assay was conducted to confirm the fibrinolytic activity of mycosynthesised selenium nanoparticles from crude extract and ATPS fraction of *L. edodes*. In fibrin test tubes, the SeNPs-CE and SeNPs-ATPS showed spots of lysis throughout the fibrin agar after 48 hours of incubation (Figure 4.12 (A) & (B)). On the other hand, the crude and ATPS extract of *L. edodes* showed clear zone approximately 3-5 mm on top of fibrin agar (Figure 4.12 (C) & (D)). However, there was no lytic zone observed in tube added with SeNPs-AA (Figure 4.12 (E)).

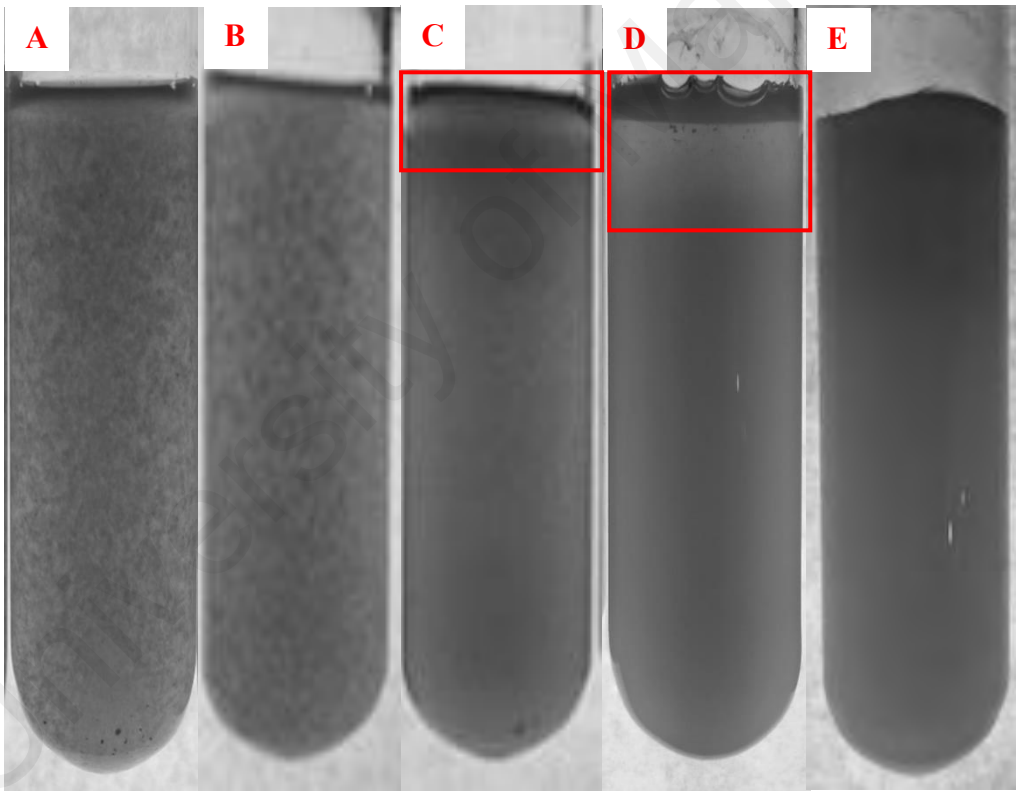

Figure 4.12: Fibrinolytic activity of mycosynthesised selenium nanoparticles on fibrin plates. (A) MyMySeNPs-CE (B) MyMySeNPs-ATPS (C) Crude extracts of *L. edodes*. (D) ATPS fraction of *L. edodes*. (E) SeNPs-AA (Control).

SeNPs-AA – Selenium nanoparticles with ascorbic acid (control); MyMySeNPs-CE – Selenium nanoparticles with fibrinolytic enzyme from crude extracts of *L. edodes*; MyMySeNPs-ATPS – Selenium nanoparticles with fibrinolytic enzyme from ATPS extracts of *L. edodes*.

CHAPTER 5: DISCUSSION & CONCLUSION

5.1 Preparation of Protein Extracts

5.1.1 Fibrinolytic Activities in Crude Protein Extracts of Edible Mushrooms

In the present study, ten edible mushrooms were randomly chosen to identify the fibrinolytic components. *Lentinula edodes* showed the highest fibrinolytic activity (48.06 U/mg) among the tested mushrooms. Based on previous study, the crude extract of *P. ostreatus* had recorded 40 U/mg of fibrinolytic activity (Choi & Shin, 1998). In the present study, fibrinolytic activity of *L. edodes* was ten times (10x) higher compared to *P. ostreatus*. Besides, the fibrinolytic activity in crude proteins extracts of all edible mushrooms were significantly high ($p > 0.05$) compared to crude protein extracts of *Armillariella mellea* (Kim & Kim, 1999) and *Cordyceps militaris* (Cui et al., 2008), which showed only 0.17 U/mg and 0.02 U/mg, respectively.

It has been reported that *L. edodes* is rich in selenium (Ogra, Ishiwata, Encinar, Łobiński, & Suzuki, 2004) and contains sulphur compounds like lenthionine that has the capability to break blood clot (Shimada, Komamura, Kumagai, & Sakurai, 2004) and enhance the fibrinolytic activity. Similarly, *A. bisporus* (white) has also been described as a selenium-enriched mushroom (Van Elteren, Woroniecka, & Kroon, 1998). On the other hand, it was reported by Masukawa, Goto & Iwata (1983) that selenium able to inhibit platelet aggregation and control the enzymatic activity of glutathione peroxidase. Hence, the selenium components present in both *L. edodes* and *A. bisporus* (white) might be the active constituent for the high fibrinolytic activity of its crude extracts.

5.2 Partially Purification of Extract

Downstream processing of fibrinolytic enzyme from crude protein extracts is an important step of production. Usually this process results low yield and is expensive (Naganagouda & Mulimani, 2008). Aqueous two phase system (ATPS) is a most economical downstream processing technique that was recently developed for biomolecules recovery. This technique is straight forward and requires fairly simple equipment, which is also easy to operate (van Berlo et al., 1998). Moreover, the conditions for separation on a large scale are unchanged from small scale, thus easy in scale-up and highly reliable.

In this study, polymer and salt based ATPS were used to recover the fibrinolytic enzymes from the crude protein extracts of all the ten edible mushrooms. According to Chunha and Aznar (2009), the polymer/salt systems have better recovery towards protein by forming larger differences in density and also have greater selectivity on desired proteins. Besides, the polymer/salt based ATPS creates lower viscosity as well as lower cost compared to other techniques. In the present study, polyethylene glycol (PEG) was used as the polymer and phosphate as the salt. When PEG was mixed with phosphate, two aqueous phases were formed due to high viscosity. The top phase was the polymer and the bottom phase was the salt. Thus, complete phase separation occurred by concentrating the fibrinolytic enzyme in one of the phases and the contaminants in the other.

Parameters in ATPS are usually manipulated to partition the fibrinolytic enzymes in polymer phase (PEG with molecular weight of 8000 g mol^{-1}). This helps to reduce the interference of salt in downstream process (Ibarra-Herrera et al., 2011). Moreover, 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 (Ibarra-Herrera et al.,

2011). Fibrinolytic enzymes extracted from *Streptomyces sp* (Medeiros e Silva et al., 2013) and *Auricularia polytricha* (Mohamed Ali et al, 2014) by using ATPS also partitioned into the top phase as well.

5.2.1 Fibrinolytic Activities in Partially Purified Extracts

The recovery of fibrinolytic enzymes from the ten edible mushrooms were carried out by ATPS which was optimised in our previous study (Mohamed Ali et al., 2014). The highest fibrinolytic activity of 54.28 U/mg, after ATPS recovery, was observed in *L. edodes*, which is significantly high compared to its crude extract. The recovery of fibrinolytic enzymes from *L. edodes* was 1.13-fold.

Surprisingly, *P. floridanus* showed the highest recovery by a purification fold of 4.45-fold. Similarly, the recovery of fibrinolytic activity from *P. pulmonaris* (29.37 U/mg) and *P. cystidiosus* (17.70 U/mg) were 3.26-fold and 2.88-folds, respectively. A similar ATPS (PEG/phosphate) fibrinoloytic enzyme recovery of 1.51-fold from *Streptomyces* sp. was reported (Silva et al., 2013), which is nearly three times lower than *P. floridanus, P. cystidiosus* and *P. pulmonarius* compared to this study**.**

Drastic reduction on fibrinolytic activities were recorded on ATPS fraction of *A. bisporus* (white and brown) and *G. frondosa* as compared to its crude extracts. It is evident from the current results that the fibrinolytic enzymes of *A. bisporus* (white and brown) and *G. frondosa* may have slightly different fibrinolytic enzymes characteristics compared to others. Post-translational modification of amino acids in protein causes changes in the chemical nature of an amino acid, which also gives characteristics such as polar, non-polar, hydrophobic and hydrophilic (Brennan & Barford, 2009). The top phase in ATPS creates more hydrophobic nature due to viscosity of PEG (8000 g mol^{-1}). These

hydrophobic environments cause the fibrinolytic enzymes have low affinity towards top phase in ATPS. Hence, the fibrinolytic activities reduced after ATPS recovery in *A. bisporus* (white and brown) and *G. frondosa.*

5.3 Preliminary Assay of Fibrinolytic Enzyme – Fibrin Plate Assay

Fibrinolytic activity was confirmed by using fibrin plate in both the crude protein extracts and partially purified extracts of all the ten edible mushrooms. The ATPS recovered samples of all mushrooms showed higher lytic zones than plasmin (positive control) and crude extracts. The diameter of lytic zones from ATPS fractions of all mushrooms were between of 2.50 and 3.00 cm (Table 4.1). The observed lytic zone of ATPS samples in the present study is higher compared to the purified enzyme lytic zones of *Cordyceps militaris* (Cui et al., 2008) and *Auricularia polytricha* (Mohamed Ali et al., 2014), which was measured as 1.95 cm and 1.03 cm, respectively.

5.4 Molecular Weight of Fibrinolytic Enzyme

Based on molecular weight of fibrinolytic enzymes analysis, the crude protein extracts of *L. edodes* showed five bands on SDS-PAGE gel with molecular weight ~120 kDa, ~50 kDa, ~45 kDa, ~10 kDa and ~9 kDa. After ATPS recovery, only a band with molecular weight of ~50 kDa was observed. This band was very close to purified extracts of *H. erinaceum* (51 kDa) and *Cordyceps militaris* (52 kDa) based on previous reports (Choi et. al., 2013; Kim et al., 2007).

The molecular weight of fibrinolytic activity was determined based on the activity on fibrinolytic assay and fibrin plate assay after ATPS recovery. Two (*L. edodes* and *P. floridanus*) out of the ten mushrooms were selected for SDS-PAGE analysis. The

molecular weight of crude protein extracts and ATPS fractions varied in these two mushrooms studied. According to Lu & Chen (2012), the molecular weight of fibrinolytic enzymes from mushrooms ranged from 14 to 100 kDa. In basidiocarp, the molecular weight of fibrinolytic enzymes ranges from 24-52 kDa (Choi & Shin, 1998; Kim et al., 2006). In the present study, the ATPS protein extract of *L. edodes* showed a ~50 kDa band and it was very close to purified extracts of *H. erinaceum* (51 kDa) and *Cordyceps militaris* (52 kDa) based on previous reports (Choi et. al., 2013; Kim et al., 2007).

Besides, the ATPS extract of *P. floridanus* showed band size of 140 kDa, which is larger compared to the range of 24-52 kDa as reported before (Choi & Shin, 1998; Kim et al., 2006). The fibrinolytic enzyme with largest molecular weight (100 kDa) was reported from *Ganoderma lucidum* (Choi & Sa, 2000). Another band of 45 kDa was observed from ATPS extract of *P. floridanus*. This band is slightly similar to fibrinolytic enzyme extracted from *Perenniporia fraxinea* (42 kDa) (Kim et al., 2008).

5.5 Mycosynthesis of Selenium Nanoparticles

The fibrinolytic enzymes extracted from *L. edodes* was used to synthesise selenium nanoparticles (SeNPs) with Sodium Selenite (Na_2SeO_3) and ascorbic acid ($C_6H_8O_6$) as a reducing agent. Ascorbic acid is biocompatible and has good reducing properties forming a spherical nanoparticle having a small size range compared to others (Malhotra et al., 2014). When sodium selenite is allowed to react with ascorbic acid in ratio of 1:1 without fibrinolytic enzyme, the selenium was reduced to elemental selenium (Se^0). As a result, the colourless solution turned into brick red, which indicated the occurrence of reduction reaction to form elemental selenium nanoparticles (SeNPs-AA) (Zhang et al., 2004). The mechanism involved in the formation of nanoparticles is (Se^0) given below:

$$\text{Aq. } Na_2SeO_3 \rightarrow 2Na^+ + SeO_3^{2-} \quad \text{(a)}$$

$$\text{Aq. } C_6H_8O6 \rightarrow C_6H_7O_6^- + H^+ \quad \text{(b)}$$

$$2Na^+ + SeO_3^{2-} + C_6H_7O_6^- + H^+ + H_2O \rightarrow Se^0 + 2Na^+ + C_6H_7O_6^- + H_2O \quad \text{(c)}$$

The biosynthesis of selenium nanoparticles by using fibrinolytic enzymes of crude proteins (MySeNPs-CE) and ATPS fractions (MySeNPs-ATPS) of *L. edodes* were indicated by colour change from colourless to orange. This observation pointed out the formation of amorphous spherical selenium nanoparticles as well as absorption features of the particles in the visible region of the spectra after mixing with fibrinolytic enzyme (Ingole et al., 2010; Lin & Wang, 2005).

From Figure 4.4, the SeNPs reacted fibrinolytic enzyme from crude protein extract and ATPS fractions exhibited absorption maxima at 220 nm and 280 nm respectively. These two peaks attributed to the formation of Se^0 particles in SeNPs-CE, SeNPs-ATPS and SeNPs-AA solutions (Praharaj et al., 2006). The colour change occurs in SeNPs were due to the surface plasma resonance of Se^0 particles (Ramamurthy et al., 2013). Besides, the absorption intensity of the spectra at 570 nm was recorded in SeNPs-AA, and not in MySeNPs-CE and MySeNPs-ATPS. This indicating the effective reduction reaction occur to form elemental selenium (Se^0) as well as creats high capping capacity of fibrinolytic enzymes from crude proteins extracts and ATPS fractions of *L. edodes* on elemental selenium (Se^0) produced.

5.6 Characterisation of Mycosynthesised Selenium Nanoparticles

5.6.1 Morphological Analysis and Elemental Identification

The morphologies and structures of the biosynthesised SeNPs with fibrinolytic enzymes from crude and ATPS fractions were examined by FESEM. The spherical nano-ball structure was observed in all type of SeNPs (Figure 4.5, Figure 4.6 & Figure 4.7) However, the FESEM image of MySeNPs-CE showed spherical nano-ball with highly agglomerated. Whereas, the MySeNPs-ATPS shared almost similar monodispersed spherical morphology image with SeNPs-AA. The SeNPs-AA appeared to be more monodispersed.

Generally, surface chemistry of nanoparticles plays an important role in aggregation. The aggregation kinetics on surface of nanoparticle creates specific interactions such as hydrogen bonding between nanoparticles. Then, the nanoparticles tend to attach one another to achieve stability (Pranami, 2009). The crude protein extract of *L. edodes* used in mycosynthesising MySeNPs-CE contains numerous proteins compared to ATPS extract (partially purified extract) in MySeNPs-ATPS. Therefore, the specific attraction on surface of MySeNPs-CE was higher and leads to form severe aggregation than MySeNPs-ATPS.

The composition of nano-ball in all selenium nanoparticles samples was confirmed by EDX analysis. It is evident from EDX results that the major percentage (33-57%) belongs to selenium. The selenium nanocrystallites displayed optical absorption bands, peaking at 0.5 to 3.5 keV, which is typical of the absorption of metallic selenium nanocrystallites (Oremland et al., 2004). Apart from that, the existence of carbon and oxygen elements were recorded by EDX, which belongs to reducing agents or capping agents (ascorbic acid or fibrinolytic enzyme). Similar signals of carbon and oxygen were recorded in EDX spectra of mushroom protein complexes in Wu et al., 2012). Besides, two atom signals

were measured by EDX which is assigned for gold and copper used as coating agent and copper grids, respectively.

5.6.2 Particles Size Distribution and High Resolution Transmission Electron Microscope (HRTEM) Analysis

Biosynthesised selenium nanoparticles were observed by high resolution TEM and the particles size was measured by particles size distribution. As observed in Figure 4.10 and Figure 4.11, the particle size of MySeNPs-CE and MySeNPs-ATPS was increased compared to SeNPs-AA. The surface encapsulation by fibrinolytic enzymes in MySeNPs-CE and MySeNPs-ATPS are major reasons for the increased particles size compared to SeNPs-AA. In addition, the aggregation of particles observed in MySeNPs-CE contributed to highest particle size distribution recorded (Figure 4.9). The enlargements of selenium nanoparticles after biosynthesising have been reported in surface decoration of selenium nanoparticles by mushroom polysaccharides-protein complexes by Wu et al. (2012).

The HR-TEM image explained in detail about the encapsulation of fibrinolytic enzyme on Se^0. The presence of rough structure on surface of Se^0 describe the encapsulation of fibrinolytic enzyme in MySeNPs-ATPS (Figure 4.11 (C & D)) which was absent in SeNP-control (Figure 4.11 (A)). Besides, the particle size of MySeNPs-ATPS was increased due to the encapsulation compared to SeNPs-AA in Figure 4.11 (A & C). In general, fibrinolytic enzyme is a protein and is built by globular structures with a large number of hydroxyl and imino groups (Manning, 1995). These groups have larger affinity toward the surface of Se^0 in order to form good dispersible and stable nanoparticles (Wu et al., 2012).

5.6.3 Fourier Transform Infrared Spectroscopy (FTIR) Analysis

The FTIR spectrum studies were carried out to investigate the possible bioreducing functional groups present in MySeNPs-CE and MySeNPs-ATPS compared to SeNPs-AA. The proficient synthesis of SeNPs was achieved when one or more bioreducing functional groups are present in crude and ATPS fractions (Ramamurthy et al., 2013). The peaks at 3302 cm^{-1}, 1606 cm^{-1} and 1026 cm^{-1} recorded in MySeNPs-ATPS were assigned for stretching and vibrating of NH_3^+, NH and CO, respectively. Similar bioreducing functional groups were recorded in MySeNPs-CE with peaks at 3224 cm^{-1}, 1586 cm^{-1} and 1023 cm^{-1}. These bioreducing functional groups were thus confirmed to be the fibrinolytic enzymes presents in crude and ATPS fractions of *L. edodes*.

Fibrinolytic enzymes are proteases which are made up of amino acids (Manning, 1995). The amino acids play important roles in stabilising protein structures and in catalysing enzymatic reactions. The functional group presents in amino acids reflects its capabilities in building protein structure. The major functional groups found in amino acids are NH_3^+, NH, and CO (Barth, 2000). These functional groups also recorded in both MySeNPs-CE and MySeNPs-ATPS FTIR analysis. Thus, the presence of reducing groups in fibrinolytic enzymes is responsible for the reduction of SeNPs and results in effective SeNPs formation.

5.7 Fibrinolytic Activity of Mycosynthesised Selenium Nanoparticles.

The selenium nanoparticles have excellent bioavailability in living cells and even higher than inorganic selenium (Bell & Cowey, 1989). Thus, the efficiency of cellular uptake towards biosynthesised selenium nanoparticles is higher (Wang & Lovell, 1997). The nanometre dimension of selenium nanoparticles is the main parameter for its

promising bioavailability (Wang et al., 2007). Similarly, the biosynthesised MySeNPs-CE and MySeNPs-ATPS showed better penetration on fibrin tubes (Figure 4.12)**.** The spots of lysis throughout the fibrin agar was observed (Figure 4.12) indicating the penetration of selenium nanoparticles encapsulated by fibrinolytic enzymes from crude and ATPS fractions of *L. edodes* through 2 % (w/v) of agarose, which can break fibrin. The structure of fibrin (blood clot) becomes weak by this way and also removes the blood clot from blood vessels. Hence, the fibrinolytic enzymes become more effective by SeNPs biosynthesis.

5.8 Recommendations for Further Studies

Future study may focus on the further purification and characterisation of fibrinolytic by using advanced techniques such as Fast Protein Liquid Chromatography (FPLC), Liquid Chromatography (LC/MS Q-TOF) or Matrix Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF).

The fibrinolytic protein structure may further characterised by using 3D-JIGSAW (for comparative modeling) and 3D-PSSM (for fold recognition). Besides, the biosynthesised selenium nanoparticles with partially purified fibrinolytic enzymes from *L. edodes* can be further characterised the *in vivo* potential of this enzyme to reduce the risk of the cardiovascular disease can be evaluated from 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 (Boudjeltia et al., 2006).

5.9 Conclusion

To conclude, the crude extracts from *Lentinula edodes, Pleurotus floridanus* Singer*, Pleurotus pulmonarius, Flammulina velutipes, Agaricus bisporus* (white and brown variety)*, Pleurotus salmoneostramineus, Hericium erinaceus, Pleurotus cystidiosus* and *Grifola frondosa* were successfully acquired. Then, the fibrinolytic enzymes in crude extracts from all ten edible mushrooms were successfully concentrated by ATPS method.

The crude extracts and ATPS extracts of all the ten edible mushrooms exhibited fibrinolytic activities in both Folin-spectrophotometric technique (quantitative assay) and the fibrin plate assay (qualitative assay). Among the ten edible mushrooms, the crude extract and ATPS extract of *L. edodes* showed significantly high ($p < 0.05$) specific fibrinolytic activity i.e. 48.06 U/mg and 54.28 U/mg, respectively. The molecular weight of the fibrinolytic enzyme of *L. edodes* was estimated to be 50 kDa by SDS-PAGE.

Biosynthesis of selenium nanoparticles (SeNPs) was achieved by using fibrinolytic enzymes from crude (MySeNPs-CE) and ATPS extract (MySeNPs-ATPS) of *L. edodes* under a simple redox system. The UV-Vis spectra recorded the reduction of selenium in MySeNPs-CE and MySeNPs-ATPS. Additionally, the MySeNPs-CE and MySeNPs-ATPS showed higher absorption on fibrin tube method.

Morphology of biosynthesised SeNPs was observed by FESEM and HR-TEM. The FESEM and HR-TEM revealed the morphology of the spherical SeNPs with surface encapsulation by fibrinolytic enzymes. The PSD analysis recorded the particles size of SeNPs was in the range of 20-280 nm. The presence of fibrinolytic enzyme in MySeNPs-CE and MySeNPs-ATPS were determined by FTIR spectra.

In this study, the penetration of selenium nanoparticles encapsulated by fibrinolytic enzymes from crude protein and ATPS fraction of *L. edodes* through 2 % (w/v) of agarose was observed. This process helped to break the fibrin.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Publication

Ali, S. M., Ling, T. C., Muniandy, S., Tan, Y. S., Raman, J., & Sabaratnam, V. (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.

Paper Presented

Ali, S. M., Ling, T. C., Muniandy, S., Tan, Y. S., Raman, J., Lakshmanan, H., Sabaratnam, V. (2015, December 8). Nanoparticles from Mushroom Extract as Fibrinolytic Agents. International Congress of the Malaysian Society for Microbiology 2015, Bayview Beach Resort, Penang, Malaysia. Poster Presentation.

Ali, S. M., Ling, T. C., Muniandy, S., Tan, Y. S., Raman, J., Sabaratnam, V. (2014, June 18). Recovery of Fibrinolytic Enzymes from Edible Mushrooms. Monash Science Symposium 2014, Monash University. Poster Presentation.

Seminar

Ali, S. M., Ling, T. C., Muniandy, S., Tan, Y. S., Raman, J., Lakshmanan, H., Sabaratnam, V. (2015, June 24). Nanoparticles of Selected Mushrooms as Fibrinolytic Agents. ISB Seminar 2015, University of Malaya. Oral presentation.