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Fibrinolytic Enzymes from Medicinal Mushrooms

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1. Introduction

Mushrooms, a special group of macrofungi, are not plants and thus do not use photosynthesis for nutrient acquisition. Mushrooms are fleshy, have the spore-bearing fruiting body of fungi, and typically grow above ground on soil, or other food sources. Most mushrooms are Basidiomycota or Agaricomycetes. The fruiting body of mushrooms is an important food and is used in many cuisines worldwide. Additionally, several species have been consumed extensively as a crude drug or folk tonics; in East Asia, these mushrooms are considered as medicinal mushrooms. Some medicinal mushrooms can be cultured and are an abundant source of natural proteins and polysaccharides. These mushrooms have garnered considerable attention for clinical research and for modern scientific and medicinal researches investigating their biological functions (Kino et al., 1989).

1.1 Functional mushrooms

In recent decades, medicinal mushrooms have been used to improve human health and strengthen the immune system, which have become significant issues in medical and pharmacological researchers (Guillamon et al., 2010; Kwok et al., 2005). The activities that have been identified include immunomodulatory and hypocholesterolemic actions, and antitumor, anti-inflammatory, anti-allergic, anticoagulation, and antithrombin activity as well as fibrino(geno)lysis stimulation (Lu et al., 2010b; Wang et al., 1995). Based on the medicinal potentials of edible mushrooms, scientists and businesses have devoted considerable effort to increase the quality of cultivated mushroom. It's estimated that approximately 14,000 species of mushrooms exist (Miles & Chang, 2004); as edible and medicinal mushrooms account for only a small proportion. More than 10 million metric tons of edible and medicinal mushrooms are cultivated annually worldwide. Products from cultivated mushrooms have been used extensively as food additives, health foods, and for medicinal purposes to treat cancers and circulatory disorders (Kim et al., 2003; Mao et al., 2005; Park et al., 2001). Improvements in mushroom cultivation technology have brought countless benefits to commercial production and laboratory applications of mushroom.

1.2 Bioactive compounds in mushrooms

Many bioactive compounds, such as cordycepin, polysaccharides, polysaccharide-peptide complex, ergosterol, mannitol, peptides and protein/protease, in various mushrooms have

chemotherapeutic or medicinal activity (Das et al., 2010). The sources of these bioactive compounds include fruiting body, mycelia, cultivation broth, submerged cultivation mycelia and fermentation derivatives (Cheung, 1996; Fiore & Kakkar, 2003; Kwok et al., 2005; Sugimoto et al., 2007; Wang et al., 1995; Wong et al., 2011; Wu et al., 2010, Yamamoto et al., 2005; Yoon et al., 2003). This chapter focuses on the fibrinolytic enzymes derived from medicinal mushrooms. The function and characterization of these fibrinolytic enzymes are described in detail. Technologies for purification and characterization of fibrinolytic enzyme from *Schizophyllum commune* are discussed.

2. Haemostasis and antithrombotic studies

Cardiovascular diseases are the leading cause of death worldwide. A common cause of cardiovascular diseases is abnormal fibrin accumulation in the blood vessels or a fibrin clot adhering to the unbroken vessel walls of the endoepithelium. An abnormal clot formation is called a "thrombus". Thrombosis can stop blood circulation in vessels (arteries or veins), and may cause a hypoxiation syndrome such as acute myocardial infarction, high blood pressure, ischemic heart, and stroke (Mine et al., 2005). In response to the high mortality rates associated with thrombosis, antithrombotic studies and clinical therapies are progressing rapidly.

2.1 Mechanisms of coagulation

The balance of circulation blood in a liquid or clotted state is called haemostasis, which includes two complementary mechanisms: blood coagulation and fibrinolysis. Coagulation limits blood loss from a damaged vessel via clot formation. After the rehabilitation of the vessel's endoepithelium, fibrinolysis system processes dissolve clots and recover circulating blood (Takada et al., 1994). When a blood vessel is damaged by external force (e.g. a cut or scrape), this damage induces the platelet activation and aggregation of clotting plasma proteins. Platelets adhere to the subendothelium and simultaneously activate a coagulation cascade that induces fibrin production (Heemskerk et al., 2002). This coagulation cascade is the outcome of multiple interdependent interactions among plasma proteins (tissue factors), platelets, prothrombin, thrombin, fibrinogen, and fibrin. Via the intrinsic and extrinsic pathways, soluble fibrinogen is converted into insoluble fibrin. Fibrins construct a mesh structure over the platelet plug, sealing the injury site (Gentry, 2004; Norris, 2003; Wolberg, 2007).

2.2 Fibrinolysis

Under normal conditions, fibrin clots formed in blood vessels should be disassembled rapidly and removed by the fibrinolysis system effectively. The key enzyme in the fibrinolysis system is plasmin, a serine protease, which is activated from proenzyme plasminogen via a tissue-type plasminogen activator (tPA) or a urinary plasminogen activator (u-PA) trigger. The fibrinolysis system is regulated by a number of orchestrated interactions between fibrin, specific inhibitors, and plasminogen; plasminogen activators are indeed necessary that generate clot degradation (Medved & Nieuwenhuizen, 2003). Collen (1999) demonstrated that a fibrin formation can trigger activation of fibrinolytic system and generate of active plasmin; the latter substance may then degrade fibrin into soluble fibrin degradation products (FDPs), followed by clot disintegration (Collen, 1999).

2.3 Clinical thrombolytic agents

Pharmacologic dissolution of an established thrombus is now an accepted therapeutic approach for thrombotic occlusive disease. Intravenous infusion of commercial thrombolytic agents - plasminogen activators (PA), including recombinant tPA (r-tPA), u-PA, rokinase, streptokinase, and anisoylated plasminogen streptokinase-activator complex is effective in restoring blood flow in occluded arteries and veins (Liu et al., 2005). However, these agents are expensive and have a number of drawbacks, such as rapid degradation, uncontrollable acceleration of fibrinolysis and haemorrhage. Widespread systemic activation of fibrinolysis leads to potentially life-threatening side effects. To overcome these risks, a safer thrombolytic agent is need for treating thrombolytic processes.

3. Overview of fibrinolytic enzymes

Fibrinolytic enzymes have been found in natural sources. Their activity resembles that of plasmin, which can degrade fibrin and inhibit fibrin clot formation (Chen et al., 1991; Mihara et al., 1991; Sumi et al., 1987). Moreover, some fibrinolytic enzymes exhibit activity similar to that of a PA. These enzymes may have great potential for antithrombotic therapy. Their specific characteristics, such as fibrinolysis, fibrinogenolysis and the proteolytic effect, alter the balance between coagulation and anticoagulation, resulting in wide-ranging therapeutic applications.

3.1 Fibrinolytic enzymes from animals

Earthworms have been used for their antithrombotic effect in East Asia traditional folk medicine for a thousand years. However, their precise physiological and biochemical mechanism remains unclear. In this century, applications of earthworms have been investigated intensively. In 1991, lumbrokinase (LK), a proteolytic enzyme, was first extracted from the *Lumbricus rubellus* by Mihara et al. (Mihara et al., 1991). Studies of LK demonstrated that these enzymes, which have a molecular weight of 25-32 kDa, found in the earthworm's body cavity and digestive organs, perform PA and plasmin activities. Recent studies have shown that LK enzymes can dissolve blood fibrin clots and inhibit platelets activation and aggregation, such that LK enzymes can be administered to treat stroke patients as well those with cardiovascular diseases (Nakajima et al., 1993; Tang et al., 2002). In 2008, absorption and efficacy of earthworm fibrinolytic enzyme d (EFE-d) was enhanced when delivered in water-in-oil (w/o) microemulsions to rats (Cheng et al., 2008). Intestinal absorption experiments for LK enzymes have also showed that these heterologous proteolytic enzymes have potent properties, facilitating their development as anti-thrombotic drugs (Fan et al., 2001).

Many snake venoms, which consist of a multitude of biologically active proteins and peptides, are lethal to humans by adversely altering haemostasis. These biological molecules have been classified as serine proteases, metalloproteinases, C-type lectins, disintegrins and phospholipases. Each may act selectively on different blood coagulation factors, blood cells, and tissues (Clemetson et al., 2007). Venom proteases may involve activation or inactivation of each factor related to coagulation and fibrinolysis. Notably, a thrombin-like function, which stimulates fibrinogen forward clotting processes, and a fibrino(geno)lytic function which can digest fibrin and fibrinogen, also exist in snake venoms. Further study has showed that venom

contains two groups of fibrino(gen)olytic enzyme, with molecular masses of approximately 25 and 60 kDa, respectively. Fibrino(gen)olytic enzymes have been isolated in snake venom, including those of *Agkistrodon acutus*, *A. contortrix*, *A. rhodostoma*, *A. halys brevicaudus*, *A. piscivorus piscivorus*, *A. piscivorus conami*, and *Crotalus atrox*. The most significant characteristic of the amino acid composition of an enzyme is very high levels of Asx and Glx residues (Hahn et al., 1995). Swenson and Markland (2005) characterized venom fibrino(gen)olytic metalloproteinases and serine proteinases. Two sub-classes of proteinases that have distinct sensibility to enzymatic inhibitors, such as EDTA, a metalloproteinase inhibitor, and phenylmethanesulfonylfluoride (PMSF), a serine proteinase inhibitor. The mechanism of action of venom fibrin(ogen)olytic metalloproteinases and serine proteinases differs, and they target different amino acid sequences in fibrin(ogen). The α -chain and β -chain fibrinogenases can be defined as venom enzymes degrading preferentially (although not exclusively) either the α - or β -chain of fibrinogen, respectively (Swenson & Markland, 2005). Recently, several venom fibrin(ogen)olytic enzymes and genetically recombined venom fibrin(ogen)olytic enzymes have been examined using animal models and a promising result was obtained (Gasmi et al., 1997; Marsh & Fyffe, 1996; Moise & Kashyap, 2008; Toombs, 2001). Based on the high fibrinolytic activity of the fibrinolytic enzyme process in venom, these enzymes are currently examined under examination in preclinical and clinical experiments for their thrombolytic efficacy and haemostatic safety.

3.2 Fibrinolytic enzymes from microbial

Peng et al. (2005) presented an overview of microbial fibrinolytic enzymes. Microbial fibrinolytic enzymes are derivatives from bacteria (e. g. streptomyces, actinomyces, and bacilli), fungi, and algae (Peng et al., 2005). Two well-known plasminogen activators, streptokinase and staphylokinase from *Streptococcus hemolyticus* and *Streptococcus aureus*, were demonstrated to be effective in thrombolytic therapy. In 1997, the streptokinase gene was cloned and its expression in the non-pathogenic *Escherichia coli* was characterized. The recombinant 47.5 kDa protein corrected to native streptokinase has a peptide sequence that was successfully used to treat Thrombus (Avilan et al., 1997). Unlike native streptokinase, the recombinant forms of streptokinase have low antigenicity and high fibrin-selective activity in human circulatory system (Collen & Lijnen, 1994). The recombinant fibrinolytic enzyme produced by recombinant technology is now mass-produced. Additionally, the side effect of the recombinant fibrinolytic enzyme is reduced.

Studies of fermented foods showed that fibrinolytic enzymes may be purified from such sources as Japanese Natto, Korean Chungkook-Jang soy sauce, dochi, fermented shrimp paste, salt-fermented fish, fermented vegetables (e.g., Kimchi), and Indonesia soy products (e.g., Tempeh). Most fermented foods are derived from raw materials such as beans, grains, fish, meat, vegetables, and dairy products. Fermentation is carried out by edible bacteria or fungi (Kim et al., 1996; Sugimoto et al., 2007; Sumi et al., 1987; Sumi et al., 1995; Wong & Mine, 2004). For example, the *Bacillus* genus contains microbial species that usually are used for fermentation during fermented-foods production. Natto a popular soybean food in Japan, is fermented by the microorganism *Bacillus subtilis* natto. The first commercial fibrinolytic enzyme, Nattokinase (NK), was purified and characterized from Natto. Nattokinases, which were found with fibrin and plasmin substrate H-D-Val-Leu-Lys-pNA (S-2251) digestion activity (Sumi et al., 1987), have been investigated extensively worldwide.

In 1993, NK was characterized as a substilin-like serine protease, based on its high sensitivity to protein substrate Suc-Ala-Ala-Pro-Phe-pNA for substilin (Fujita et al., 1993). In 2005, a novel NK protein (NKCP) with both antithrombotic and fibrinolytic effects was discovered. Dose-dependent prolongations of both prothrombin time (PT) and active partial thromboplastin time (APTT) were observed in rats administered NKCP intraduodenally (Omura et al., 2005). Two fibrinolytic enzymes, QK-1 and QK-2, from supernatant of *B. subtilis* QK02 culture broth were purified and characterized in 2004; QK-1 is a plasmin-like serine protease and QK-2 is a subtilisin family serine protease (Ko et al., 2004). A strong fibrinolytic subtilisin doenjang (DJ)-4 was purified from doenjang, a traditional Korean fermented soybean paste (Kim & Choi, 2000). Fibrinolytic recombinant full-subtilisin DJ-4 (rf-subDJ-4) and mature-subtilisin DJ-4 (rm-subDJ-4) were expressed by a pET29 vector system (Choi et al., 2004). The rf-subDJ-4 had higher heat- and acid-resisting (pH 3.0–4.0) properties than native subtilisin DJ-4. Notably, rf-subDJ-4 has the same abilities as the hydrolyzed α -, β -, and γ -chains of fibrinogen; however, rm-subDJ-4 does not. Sumi et al. (1990) further demonstrated that Natto or NK capsules administered orally enhance fibrinolysis in canine plasma. NK has great potential as a drug candidate for treating and preventing thrombus in animal models (Fujita et al., 1995; Sumi et al., 1990). Moreover, as the fibrinolytic enzyme was purified from common edible sources, oral administration is likely safe.

4. Mushroom fibrinolytic enzymes

The fruiting body of mushrooms can produce and disperse a large number of spores within a short period. Spores may create new individuals when in an environment suitable for growth. Previously, the fruiting body was cultured in growing mediums comprising woodchips, straw, sawdust, coffee grounds, logs, and similar organic items. However, after seeding spores or the mycelium of fungi on artificial solid or liquid culture medium under controlled temperature and moisture, now desired mushroom cultures can be successfully created. The products of mushroom cultivation include hyphae, mycelium, fruiting body, culture broth, and derivatives that dissolve in broth. Many fibrinolytic enzymes were discovered from these products recently. Table 1 lists fibrinolytic enzymes derived from mushrooms.

4.1 Fibrin(ogen)olytic enzyme of mushroom

The proteolytic complex from fungus *Flammulina velutipes* was studied by gel chromatography and the activities of the enzyme complex were compared with those of *Aspergillus terricola* and *Streptomyces griseus* proteinases (Morozova et al., 1982). This was the first study of fibrinolytic enzyme from mushrooms and their application as therapeutic agents. Thereafter, fibrin(ogen)olytic proteases were discovered in the fruiting of *Pleurotus ostreatus*, *Armillaria mellea*, *Tricholoma saponaceum*, and *Cordyceps militaris* (Choi & Shin, 1998, Kim & Kim, 1999, 2001; Kim et al., 2006). The mushroom fruiting body can be collected from nature and cultured via a sterilized growth medium made of organic substances for saprophytic utilization. Since air, ground, plants (e.g., trees) and ground water can be pollute, non-polluted substances for growing mediums are becoming rare. Moreover, cultivation of mushroom fruiting body is space occupied and labor-intensive. In the last decade, technology for mushroom submerged path culture has been established in the laboratory and at an industrial level. Mushrooms (fungi) may generate a new generation

Mushroom	Protease	Fibrinogen Degradation	Native Mol. Wt.	Reference
From fruiting body				
<i>Flammulina velutipes</i>	Two protease (no name)	N*	N	(Morozova et al., 1982)
<i>Pleurotus ostreatus</i>	Metalloprotease	+	24 kDa	(Choi & Shin, 1998)
<i>Armillaria mellea</i>	Metalloprotease	+	18.5 kDa	(Kim & Kim, 1999)
<i>Tricholoma saponaceum</i>	TSMEP1	+	18.1 kDa	(Kim & Kim, 2001)
<i>Cordyceps militaris</i>	no name	+	52 kDa	(Kim et al., 2006)
From mycelium				
<i>Ganoderma lucidum</i>	metalloprotease	N	100 kDa	(Choi & Sa, 2000)
<i>Armillaria mellea</i>	AMMP	+	21 kDa	(Lee et al., 2005)
<i>Flammulina velutipes</i>	FVP-1	+	37 kDa	(Park et al., 2007)
<i>Perenniporia fraxinea</i>	metalloprotease	+	42 kDa	(Kim et al., 2008)
From culture broth				
<i>Fomitella fraxinea</i>	FFP1	+	32 kDa	(Lee et al., 2006)
	FFP2	+	42 kDa	
<i>Cordyceps sinensis</i>	CSP	+	31 kDa	(Li et al., 2007)
<i>Fusarium sp. BLB</i>	FP	N	27 kDa	(Ueda et al., 2007)
<i>Schizophyllum commune</i>	no name	+	21.32 kDa	(Lu et al., 2010a)
From recombinant source				
<i>Fusarium sp. BLB</i>	FP	N	28.5 kDa	(Sugimoto et al., 2007)

* N means undetermined

Table 1. Fibrinolytic enzymes derived from mushrooms

when a fresh environment and nutrients are provided. The mycelium may grow stably without pollutants. Choi and Sa (2000) isolated a metalloprotease with fibrinolytic activity from cultured mycelium of *Ganoderma lucidum* (Choi & Sa, 2000). In the following decade, fibrin(ogen)olytic proteases from mushroom mycelium, including metalloprotease from *A. mellea* (AMMP), metalloprotease from *F. velutipes* FVP-1, and metalloprotease from *Perenniporia fraxinea*, were identified (Kim et al., 2008; Lee et al., 2005; Park et al., 2007). Fibrinolytic metalloproteases are sensitive to metalloprotease inhibitors (e.g., EDTA and 1,10-phenanthroline) and metal ions.

Mushrooms grown in medium or submerged broth may differ morphologically. Nutrients and differences between growth mediums and submerged broth contribute to the particular metabolite of mushrooms. Fukushima et al. (1991) reported that when soy sauce oil was the carbon source for *Aspergillus oryzae*, protease secretion was increased significantly during submerged cultivation. Furthermore, specific protease production was stimulated selectively by the oils (Fukushima et al., 1991). Changing culture medium parameters, such as osmotic pressure, salt concentrations, protein content, and the carbon source, markedly alter the extracellular performance of fungi mycelium (Archer et al., 1995; Archer & Peberdy, 1997; Bobowicz-Lassociska & Grajek, 1995; Kadimaliev et al., 2008). Broth for submerged cultivation is rich in mushroom derivatives. Over the last 50 years, mushroom derivatives with biological activities for humans have been investigated extensively. Several extracellular fibrinolytic enzymes have been purified from submerged broth of *Fomitella fraxinea* (i.e., FFP1 and FFP2),

Cordyceps sinensis (i.e., CSP) and *S. commune* (Lee et al., 2006; Li et al., 2007; Lu et al., 2010a; Ueda et al., 2007). Previous studies have demonstrated that the fibrinolytic enzymes may be available in the fruiting bodies of some mushrooms but not in the cultured mycelium (Kim et al., 2008). The purification stratagem for these extracellular fibrinolytic enzymes differs from those of fruiting bodies or mycelium. Fungi derivatives in submerged broth are biological molecules considered important for human health (Papagianni, 2004).

Ueda et al. (2007) first purified a fibrinolytic protease from *Fusarium* sp. BLB (FP), and identified the N-terminal amino acid (Ueda et al., 2007). Sugimoto et al. (2007) cloned an FP gene encoding a novel protease derived from *Fusarium* sp. BLB. The hydrolytic activity of FP toward synthetic peptide substrates is higher than that of proteases from *Bacillus subtilis* natto, *Aspergillus oryzae*, *Streptomyces griseus* and commercial plasmin (Sugimoto et al., 2007). This development of FP from *Fusarium* sp. BLB may demonstrate that manufacturing mushroom fibrinolytic enzymes may be possible in the future. Extracellular secreted protease of fungi is in some cases the fungi metabolite that may reflect cultivation conditions. Nutrition and the physical environment may be the dominant factor for growth of fungi and the rate at which extracellular proteases are produced in a submerged culture system. Base on the critical influence that produce mushroom extracellular secreted protease in submerged culture system, we expect that the high production efficiency of mushroom fibrin(ogen)olytic enzymes can be achieved by well-designed cultivation processes.

4.2 Characteristics of medicinal mushroom fibrin(ogen)olytic enzymes

Fibrin(ogen)olytic enzymes including those from *P. ostreatus*, *A. mellea*, *T. saponaceum*, *C. militaris*, *G. lucidum*, *P. fraxinea*, *F. fraxinea*, *C. sinensis*, *F. velutipes*, *Fusarium* sp. BLB, and *S. commune*, have been identified. Table 2 lists their biochemical properties, including molecular weight, optimal pH, thermal stability, inhibitors, and substrate specificity. The N-terminal sequences of most fibrinolytic enzymes have been determined. These fibrin(ogen)olytic enzymes, except for metalloprotease derived from *A. mella* fruiting body and mycelia, have markedly different N-terminal sequences. The optimal pH for these mushroom fibrin(ogen)olytic enzymes is 5-10; the optimal temperature is 20-60°C. An overview of microbial fibrinolytic enzymes (Table 2) showed that mushroom fibrinolytic enzymes may be classified as serine protease (i.e., inhibited by serine protease inhibitors) and metalloprotease (i.e., inhibited by metalloprotease inhibitors) according to protease inhibitor specificity.

The protease activity of mushroom serine fibrinolytic enzymes can be irreversibly inhibited by PMSF but no other protease inhibitors. Previous studies have showed that most fibrinolytic serine protease from traditional fermented foods belong to subtilisin of *Bacillus* origin such as nattokinase, subtilisin DFE and subtilisin QK-1 (Peng et al., 2005). Via study of N-terminal sequence alignment; a chymotrypsin-like serine protease from mushroom *C. militaris* had high sequence identify with subtilisin PR1J (GeneBank, CAC95048) (Kim et al., 2006); FFP1 from *F. fraxinea* also has 20% identify with Nattokinase and subtilisin E (Lee et al., 2006). Serine fibrinolytic enzymes from mushrooms of *F. fraxinea* (i.e., FFP1), *Fusarium* sp. BLB (i.e., FP), and *C. sinensis* (i.e., CSP) were found to have a broad substrate specificity for synthetic substrates, which including fibrin, fibrinogen, casein, substrates for subtilisin and substrates for plasmin. In laboratory works, fibrinolytic protease from *Fusarium* sp. BLB had higher fibrin degradation and plasminogen activation than Nattokinase (Sugimoto et al., 2007).

Mushroom Protease	Optimal pH and temperature	Ion induced	Ion inhibit	Fibrinogen degrade	Protein inhibitor	N-Terminal sequence	Reference
Serine protease type-							
<i>Cordyceps militaris</i> (52 kDa)	pH 7.4, 37°C	Ca, Mg	Cu, Co	YES	PMSF, PMASF	Yes	(Kim et al., 2006)
<i>Fomitella fraxinea</i> (32 kDa, FFP1)	pH 10, 40°C	N*	N	Yes	PMSF, aprotinin	Yes	(Lee et al., 2006)
<i>Cordyceps sinensis</i> (31 kD, CSP)	pH 7, 40°C	N	Mn, Cu, Hg	Yes	PMSF	Yes	(Li et al., 2007)
<i>Fusarium</i> sp. BLB (27 kDa, FP)	pH 9.5, 50°C	N	N	N	DFP, PMSF	Yes	(Ueda et al., 2007)
<i>Fusarium</i> sp. BLBN (28.5 kDa, FP)	N	N	N	N	N	Yes	(Sugimoto et al., 2007)
Metalloprotease type--							
<i>Flammulina velutipes</i> (metalloprotease)	N	N	N	N	EDTA	N	(Morozova et al., 1982)
<i>Pleurotus ostreatus</i> (24 kDa, metalloprotease)	pH 7.5~8.0, up to 50°C	Zn, Co	N	Yes	1,10-phenanthroline	N	(Choi & Shin, 1998)
<i>Armillaria mellea</i> (18.5 kDa, metalloprotease)	pH 7, 45~55°C	Zn, Co, Mg	Hg	Yes	1,10-phenanthroline, EDTA	Yes	(Kim & Kim, 1999)
<i>Ganoderma lucidum</i> (100 kDa, metalloprotease)	pH 7.0~7.5, up to 60°C	Zn, Co	N	Yes (assume)	1,10-phenanthroline, EDTA	N	(Choi & Sa, 2000)
<i>Tricholoma saponaceum</i> (18.1 kDa, TSMEP1)	pH 7.5~9, 30~40°C	Zn, Co, Mg, Fe	Hg, Cu	Yes	1,10-phenanthroline, EDTA	Yes	(Kim & Kim, 2001)
<i>Armillaria mellea</i> (21 kDa, AMMP)	pH 6, 33°C	Ca, Mg	Cu, Co	Yes	EDTA	Yes	(Lee et al., 2005)
<i>Fomitella fraxinea</i> (42 kDa, FFP2)	pH 5, 40°C	Zn, Co	Cu, Ni	Yes	1,10-phenanthroline, EDTA	Yes	(Lee et al., 2006)
<i>Flammulina velutipes</i> (37 kDa, FVP-1)	pH 6, 20~30°C	Mn, Mg	Cu, Fe ²⁺ , Fe ³⁺	Yes	EDTA, EGTA	Yes	(Park et al., 2007)
<i>Perenniporia fraxinea</i> (42 kDa, metalloprotease)	pH 6, 35~40°C	Mn, Mg	Cu, Fe, Zn	Yes	EDTA	Yes	(Kim et al., 2008)
<i>Schizophyllum commune</i> (21.3 kDa, metalloprotease)	pH 5, 45°C	Mg	Hg, Cu, Co	Yes	EDTA	Yes	(Lu et al., 2010)

*N means undetermined.

Table 2. Review of medicinal mushroom fibrin(ogen)olytic enzymes

Mushroom fibrinolytic metalloproteases were discovered in *F. velutipes*, *P. ostreatus*, *A. mellea*, *G. lucidum*, *T. saponaceum*, *F. fraxinea*, *P. fraxinea* and *S. commune* (Choi & Shin, 1998; Kim & Kim, 1999, 2001; Kim et al., 2008; Lee et al., 2005, Lee et al., 2006; Lu et al., 2010; Morozova et al., 1982; Park et al., 2007). Proteases were purified in the fruiting body, or mycelium, or culture supernatant. The presence of Zn^{2+} was detected in metalloprotease from *P. ostreatus* and *G. lucidum* by mass spectrometry; both of these metalloproteases have Zn^{2+} -dependent protease activity. Additionally, Zn^{2+} -dependent protease activity exists in metalloprotease derived from *F. fraxinea* (FFP2), *A. mellea* and *T. saponaceum* (TSMEP1), with an undefined ion structure. Mg^{2+} -dependent protease activity was found in metalloproteases from *F. velutipes* (FVP-1), *A. mellea* (AMMP), *P. fraxinea* and *S. commune*. The activity of mushroom fibrinolytic metalloproteases can be inhibited by EDTA and 1,10-phenanthroline predominantly. The metal ions dependent activity is not only a character of mushroom fibrin(ogen)olytic enzymes but also can be considered as a critical point for proteases under the clinical application for thrombolytic therapy (Lu & Chen, 2010).

5. Production, purification and characterization of mushroom fibrinolytic enzymes

Streptokinase (SK) from streptococci is a widely used therapeutic agent for acute myocardial infarction. However, the manufacturing capacity of SK from haemolytic streptococci is limited with a high price tag. Pharmacological uses of SK take risks of causing potential myocardium and liver damages due to the residual bacteriohemolysin in the manufacturing process (Zhang et al., 1999). Peng et al. (2005) illustrated that microbial fibrinolytic enzymes, especially those from food-grade microorganisms, have potential to be developed as functional food additives and drugs to prevent or cure thrombotic disease (Peng et al., 2005). Therefore, fibrin(ogen)olytic enzymes from non-toxic mushrooms has gradually become the centre of attention for investigators in thrombolytic therapy. Mushroom of *S. commune* is a ubiquitous white rot and widespread fungus in existence. This medicinal mushroom has been an additive in traditional folk medicine and the subject of genetic analysis. The production, purification and characterization of an fibrin(ogen)olytic protease from *S. commune* were discussed in this section (Lu et al., 2010a; Lu et al., 2010b; Lu & Chen, 2010). The fast growing and easy cultivation raise the universal uses for *S. commune* in science researches and medicinal applications.

5.1 Submerged cultivation of *S. commune*

Schizophyllum commune derivatives have been suspected with antithrombotic effect for human beings by scientists (Okamura-Matsui et al., 2001), yet the detail mechanism is unclear. Base on the high protease and extracellular biological substance production ratio in submerged cultivation (Desrochers et al., 1981), *S. commune* is a good biological resource for scientists whom study the functional substance. In our study, *S. commune* was cultured in YM agar plate (contains peptone, malt extract, dextrose, yeast extract and 0.2% agar) at 25°C for one week. The mycelia grew and covered plate surface, was like white flannelette. Surface of plate agar was pure colour and without contamination. As a seed culture, a piece of plate agar covered with grown mycelia, was transferred to 50ml YM broth for 5 days at 25°C. For mass production, the seed culture then transferred to 15L YM broth for additional

7 days with shaking platform and air exchangeable cover. Colonies of fungi in submerged cultivation grow exponentially, that is different from yeast cells grow at a constant rate. The growth rate increases with time so that the logarithm of the amount of fungus mycelium increases with time.

5.2 Purification of fibrinolytic enzyme from *S. commune*

Mycelia in submerged culture broth were removed by centrifugation at 4°C, 8,200 ×g, for 30min. The supernatant was filtered by 0.45µm membrane and fractionized by cross-flow filtration, to collect the <100-kDa fraction, which was further concentrated by 3-kDa ceramic column filtration. Due to the extracellular polysaccharide with huge molecular weight present in broth, that will obstruct the protein purification. The ceramic cross-flow filtration is done before protein precipitation. Protease purification was then performed by fast performance liquid chromatography. The protein concentration of each eluted fraction was detected with A280 in real time by ÄKTA purifier 10 (GE Healthcare). Measuring activity to azocasein is a general selected detection method to identify target protease fractions. Combination of liquid chromatography and protease activity screening, the purified fraction containing target protease is available.

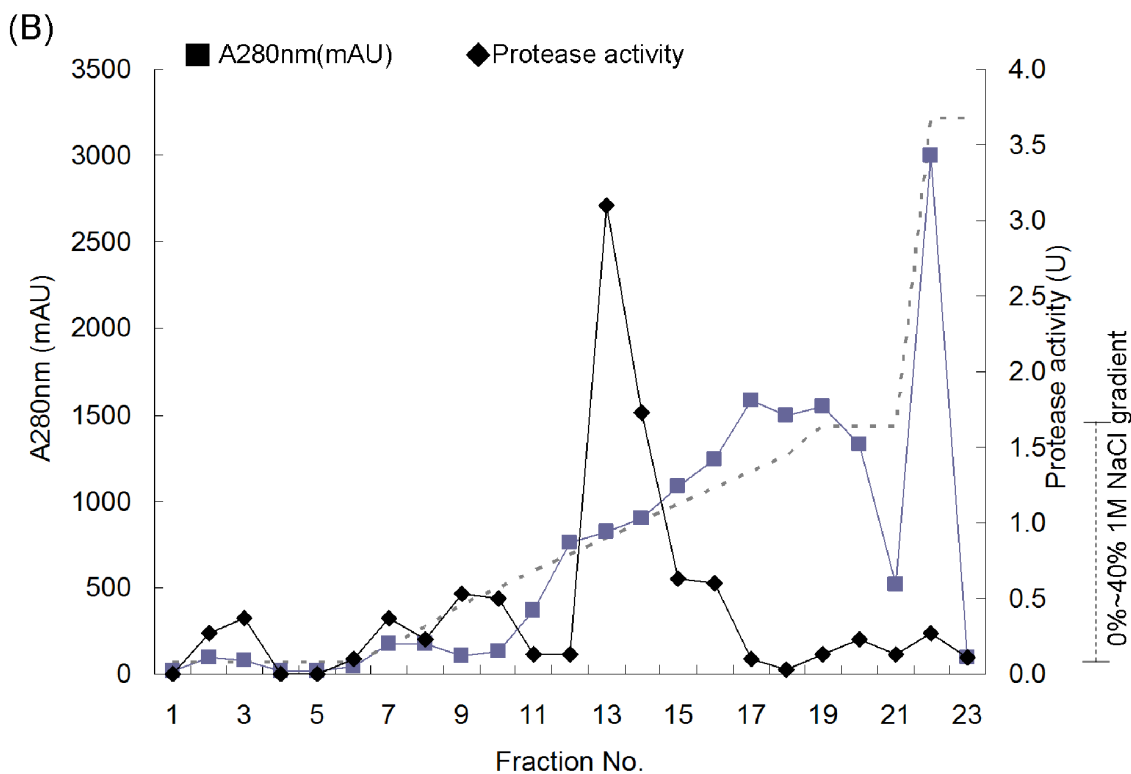
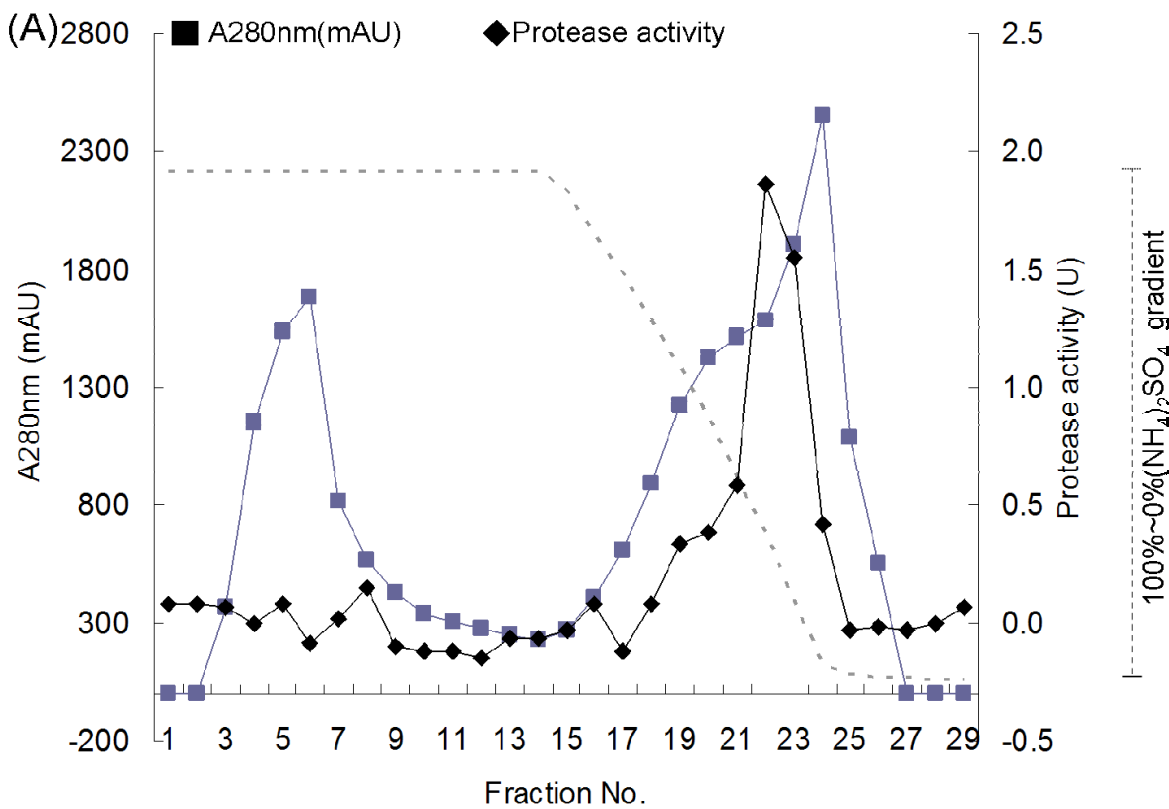
The concentrated fraction (molecular weight 3 -100 kDa) was precipitated by saturated ammonium sulfate at 4°C for 8 hour followed by centrifugation at 10,000×g, 30min at 4°C. The protein pellet was dissolved in ddH₂O and followed by dialysis with FPLC column buffer containing 50mM sodium phosphate (pH 8.0) and 1M (NH₄)₂SO₄ as 1st dialysis substrate. In step 1, a hydrophobic interaction column, Phenyl Sepharose™ High Performance beaded packing column was preequilibrated with column buffer. Equilibrated 1st dialysis substrate was loaded on column at flow rate of 1 ml/min. Figure 1A illustrated the result of elution processes with column buffer without (NH₄)₂SO₄ in a stepwise manner of 60 min interval at flow rate of 1 ml/min. Eluted fractions were analyzed for protease activity to azocasein and recorded (Fig. 1A). The active fractions were pooled; protein precipitated and dialyzed against 20mM Tris (pH 8.0) as 2nd dialysis substrate for next step of chromatography.

In step 2, an ion exchange column, Mono Q™ 5/50 GL column was preequilibrated with 20mM Tris buffer (pH 8.0). The 2nd dialysis substrate was loaded on Mono Q™ 5/50 GL column at flow rate of 1 ml/min. Elution was carried out with the same buffer but containing 1M NaCl, by linear gradient of 20-fold column volume to 40% 1M NaCl. After examination of protease activity to azocasein (see in Fig. 1B), the active fractions were pooled; protein precipitated and dialyzed against 50mM sodium phosphate buffer (pH 8.0) as 3rd dialysis substrate for next step of chromatography.

In step 3, a size exclusion column, Superdex 75 10/300 GL column was preequilibrated with 50mM sodium phosphate buffer. The 3rd dialysis substrate was loaded with same buffer at flow rate of 1.2 ml/min. Eluted fractions were analyzed for protease activity to azocasein (see in Fig. 1C). Figure 2 showed the purification purity by SDS-PAGE (Fig. 2). The purified protease then can be applied for more.

Fibrinolytic enzyme purification from mushroom may divide into two processes. One is the pre-treatment of resource, including the crude protein extraction from fruiting body, or mycelium, or culture supernatant. Another is the chromatography stratagem, which

including the combination of ion exchange, hydrophobic interaction and size exclusion chromatography. Techniques with selectivity are highly independent of protease resources and the enzyme properties.



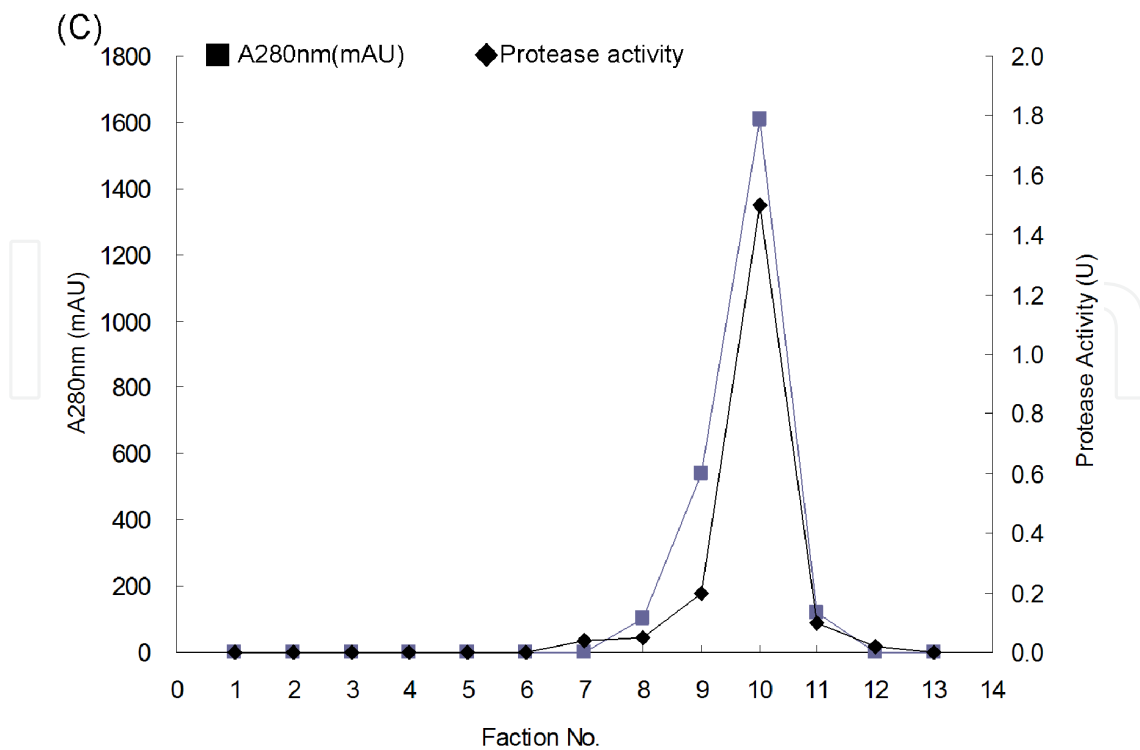


Fig. 1. Chromatography purification of *S. commune* fibrinolytic enzyme (A) Eluted fractions of Phenyl Sepharose™ High Performance column; (B) Eluted fractions of Mono Q column; (C) Eluted fractions of Superdex 75 10/300 GL column.

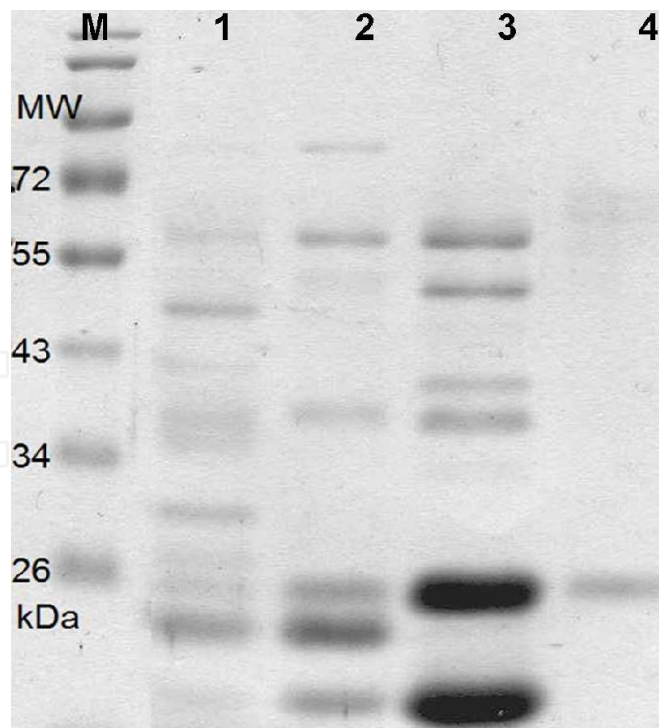


Fig. 2. SDS-PAGE result of chromatographic purification M: protein markers; Lane 1: 1st dialysis substrate; Lane 2: 2nd dialysis substrate; Lane 3: 3rd dialysis substrate; Lane 4: Protein with highest protease activity eluted from Superdex 75 10/300 GL column.

5.3 Characterization of fibrinolytic enzyme from *S. commune*

To study the effects of pH and temperature to fibrinolytic enzyme from *S. commune*, purified enzyme was incubated in various pH and temperature respectively, and the protease activity to azocasein then examined. Optimal protease activity reveal at pH 5.0 and 45°C, the data was illustrated in Fig. 3A and 3B.

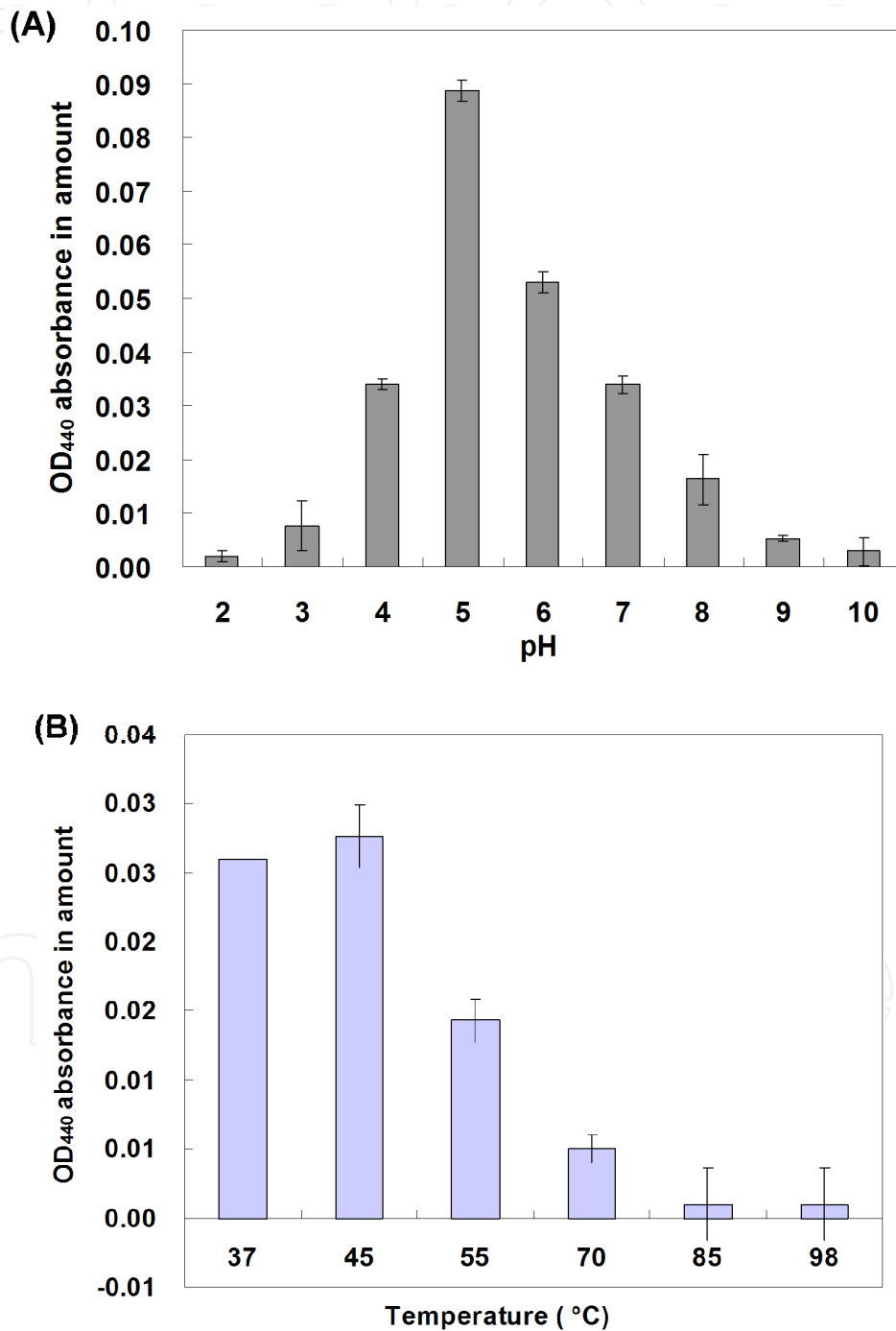


Fig. 3. The effects of pH and temperature on protease activity of fibrinolytic enzyme from *S. commune* (A) pH effects; (B) Temperature effects.

Three replicates of fibrinolytic enzyme were mixed with HgCl_2 , $\text{C}_4\text{H}_6\text{O}_4\text{Zn} \cdot 2\text{H}_2\text{O}$, CuSO_4 , MgCl_2 , CoCl_2 , CaCl_2 , $\text{Pb}(\text{NO}_3)_2$, ddH₂O (control) and protease inhibitors of PMSF (serine protease inhibitor), EDTA (metalloprotease inhibitor), benzamidine hydrochloride hydrate (trypsin, trypsin-like protease inhibitor), pepstatin A (aspartyl peptidases inhibitor), aprotinin (serine protease) and phosphoramidon (endopeptidase inhibitor) respectively. Protease activity of mixed solution was then examined by azocasein assay. Figure 4 demonstrated the relative protease activity to the control treatment (100%=1) (Fig. 4A & 4B). The result indicated that the protease shows Mg^{2+} -dependent activity, and inhibits by EDTA.

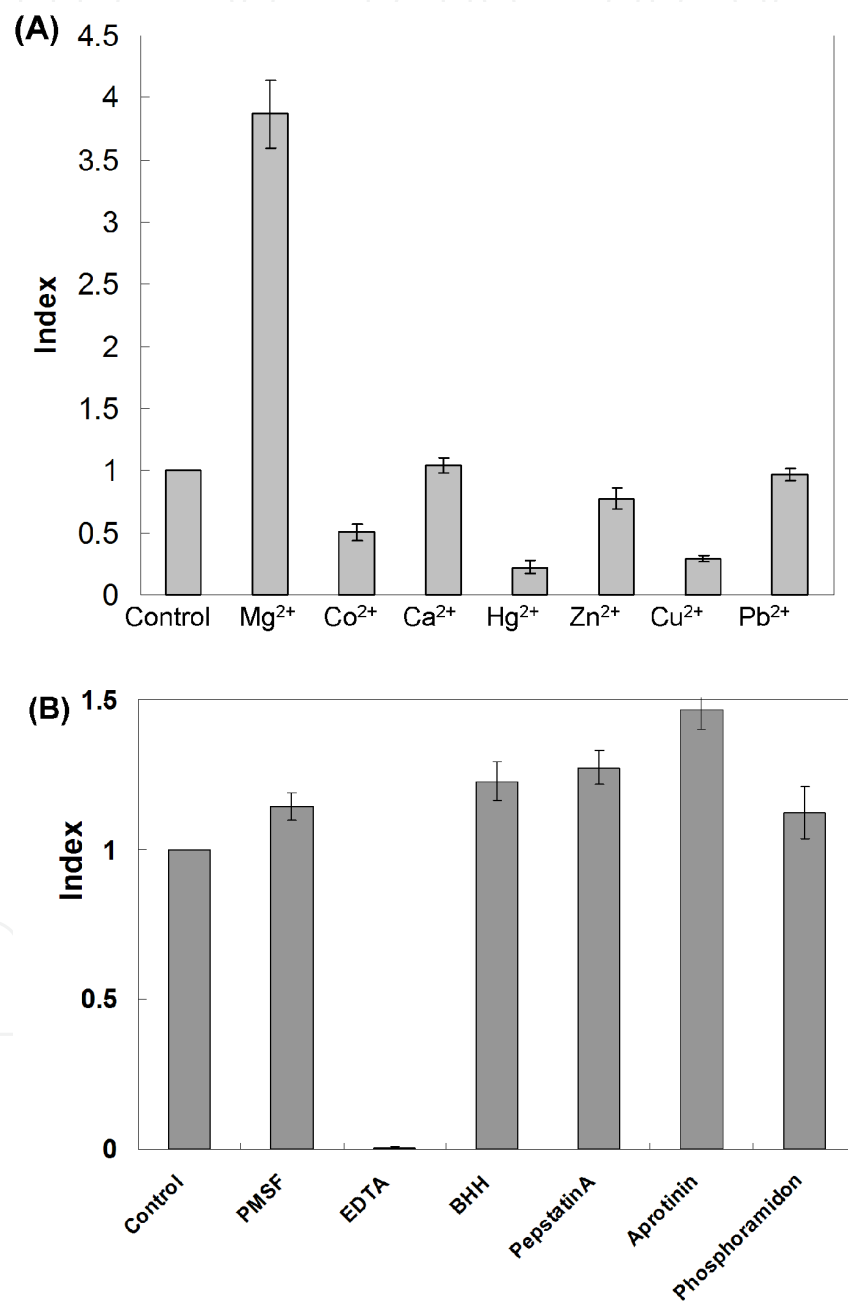


Fig. 4. The effects of divalent cations and protein inhibitors on protease activity of fibrinolytic enzyme from *S. commune* (A) Divalent cations effects; (B) Protein inhibitors effects.

N-terminal sequence of protease is another character for protein identification. However, fibrinolytic enzymes from microorganisms show low homology except the fibrinolytic enzyme from substilisin group, such as substilisin NAT (Nakamura et al., 1992), substilisin E (Wong et al., 1984), substilisin DFE (Peng et al., 2003). Table 3 illustrated the results of N-terminal sequence comparison; it demonstrated the distinctive feature of the fibrinolytic enzyme from *S. commune* to other mushroom.

Enzyme ¹⁾	N-terminal amino acid sequence									
NK & substilisin DFE	A	Q	S	V	P	Y	G	I	S	S
GFMEP		T	Y	N	G	C	S	S	S	S
POMEPE	A	T	F	V	G	C	S	A		
AMMEPE	X ²⁾	X	Y	N	G	X	T	X		
TSMEPE	A	L	Y	V	G	X	S	P		
Fibrinolytic enzyme from <i>S. commune</i>	A	S	Y	N	G	X	S	S		

(1) GFMEPE, POMEPE, AMMEPE and TSMEPE are metalloendopeptidases from *Grifola frondosa*, *Pleurotus ostreatus*, *Armillariella mellea* and *Tricholoma saponaceum*. (2) X means amino acid undetermined.

Table 3. Comparison of N-terminal amino acid sequence of mushroom fibrinolytic enzyme

6. Antithrombotic effect of mushroom fibrinolytic enzymes

Current clinical thrombolytic agents are used to convert plasminogen to active enzyme, plasmin, which degrades fibrin and process antithrombotic effect. It's an effective way to decompose harmful thrombus in circulation system, but the side effect, the excess stimulation of plasmin that may cause haemorrhage within patients. Newly thrombolytic agents now are developed for fibrin-specific property, acting on the surface of thrombus that avoids excessive induction of systemic fibrinolytic system. Fibrinolytic enzymes from medicinal mushroom are novel proteases with fibrin and fibrinogen degradation activity, which directly break the clot and interfere the clotting system. In order to identify the clinical functions and risks of medicinal mushroom fibrinolytic enzymes; mushroom fibrinolytic enzymes were examined by various antithrombotic examinations, such as fibrinolytic assay, fibrinogenolytic assay, plasminogen activation assay, and the dynamic tracking of blood clot formation.

6.1 Fibrinolytic activity assay and clot degradation test

Fibrinolytic activity was determined by artificial fibrin plate assay, which synthesized by the method described by Astrup and Mullertz (Astrup & Mullertz, 1952). Fibrin plate was made at room temperature in a petri dish containing 1.5% agarose, 0.2% human fibrinogen, and 10U thrombin. Purified protease were loaded on a fibrin plate, and incubated for 24 hours. Plasmin from human plasma (3U/mg protein) was applied as a positive control. The result showed that the purified *S. commune* fibrinolytic enzyme display stronger fibrinolytic activity than commercial plasmin (Fig. 5).

Rat blood was withdrawn without anticoagulant and stayed for clot formation in a tube. A piece of clot was placed on Petri dish, followed by dropping 0.2µg and 0.5µg fibrinolytic enzyme on clot disks and incubated in 20°C for 8 hours to observe the clot degradation

effects. Phosphate buffer was dropped on clot disk as the control treatment and another clot disk as the blank without any treatment. Figure 6 demonstrated that *S. commune* fibrinolytic enzyme digests the blood clot dominantly (Fig.6).

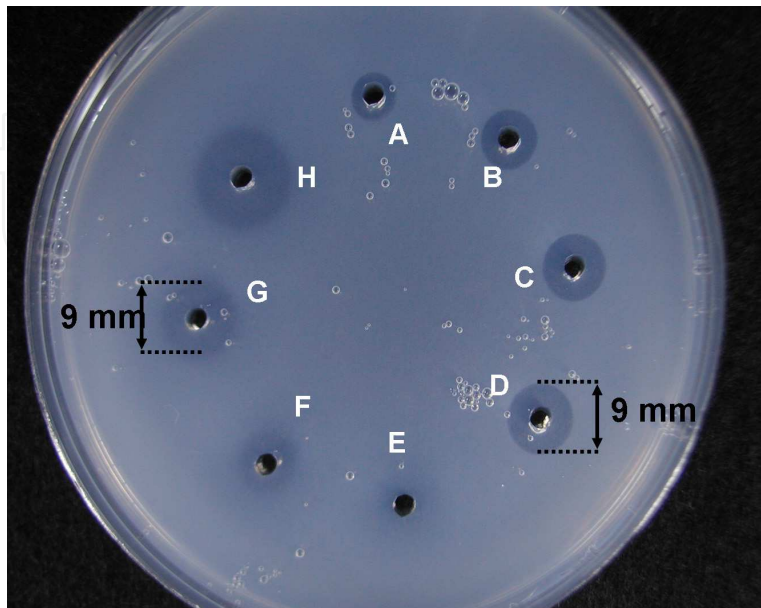


Fig. 5. Fibrinolytic activity of the fibrinolytic enzyme from *S. commune* Fibrin digestion zones A, B, C, and D: 0.2, 0.5, 0.8, and 1.0 μ g of human plasmin, respectively. Fibrin digestion zones E, F, G, and H: 0.1, 0.25, 0.5, and 1 μ g of fibrinolytic enzyme *S. commune*, respectively. Treatment of 1.0 μ g human plasmin (zones D) showed the equal digestion effects to 0.5 μ g fibrinolytic enzyme from *S. commune* (zone G).

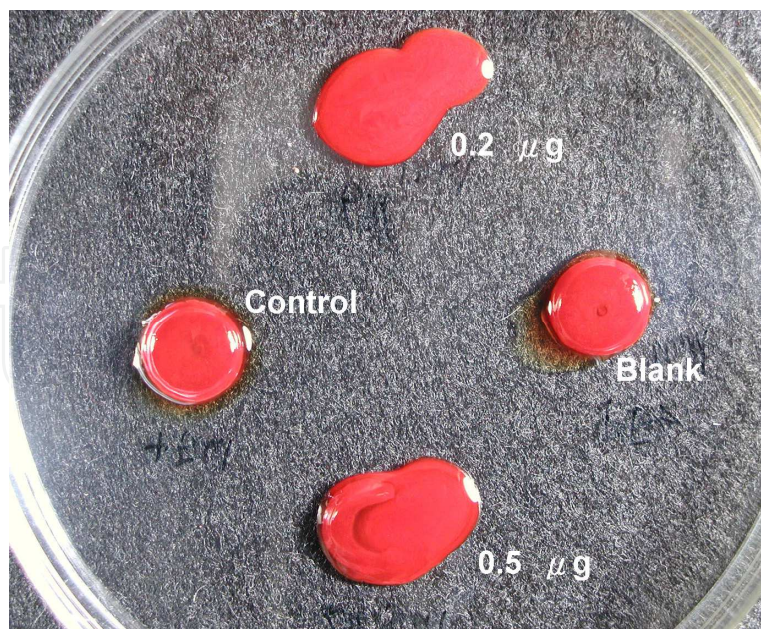


Fig. 6. Clot degradation assay of fibrinolytic enzyme from *S. commune* 0.2 μ g and 0.5 μ g: blood clot disks treated with 0.2 μ g and 0.5 μ g fibrinolytic enzyme; Control: clot disk treated with PBS; Blank: blank blood clot disk. Treatment of 0.2 μ g and 0.5 μ g fibrinolytic enzyme digested the clot disks, but opposite results were observed in control and blank.

6.2 Fibrinogenolytic activity assay

A fibrinogenolytic degradation assay was performed using a 1% fibrinogen solution mixed with the protease from *S. commune* at 37°C. At different time intervals, the reaction mixture was removed and SDS-PAGE electrophoresis was performed. Fibrinogen is composed of peptides α -, β - and γ -chains, and shows three bands on the SDS-PAGE. The fibrinogenolytic activity assay was carried out by the incubation of fibrinolytic enzyme with fibrinogen. Significant degradation of the fibrinogen α -chain and β -chain occurred within 0.5 hour after the reaction. Degradation of the γ -chain occurred after 6 hours of incubation. Most of the γ -chain was digested after 22 hours of incubation. Afterward, the fibrinolytic enzyme completely digested all of peptide chains in 30 hours. Fibrinolytic enzyme from *S. commune* displayed a higher activity in digesting the α -chain and β -chain, and it was less efficient in digesting the γ -chain (Fig. 7).

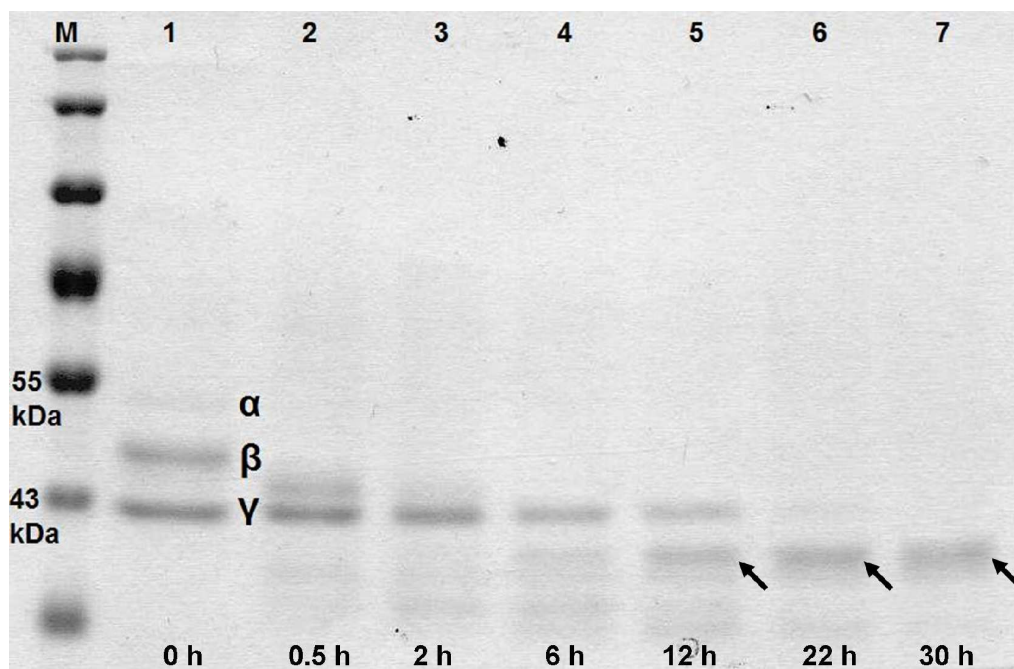


Fig. 7. Fibrinogenolytic assay of the fibrinolytic enzyme from *S. commune* α , β and γ means : α , β , and γ -chains of fibrinogen. M: Protein marker. Lane 1~7: Time course sampling in reaction. Black arrows: Fibrinogen degradation products.

6.3 Plasminogen activation assay

Plasminogen activation assay was performed by incubating 12.5 μ g purified enzyme with plasminogen (0.1UN) and a 10mM plasmin-specific substrate S2251 at 37°C. Substrate S2251 can be cleaved by plasmin and generated measurable p-nitroaniline (p-NA). In this assay, human plasmin and urokinase were used as positive control for S2251 digestion and plasminogen activation. In this assay, fibrinolytic enzyme from *S. commune* was mixed with plasminogen, plasmin substrate S2251 and without fibrin. As the results in Fig. 8, the presence of urokinase, which is known to activate plasminogen, resulted in the digestion of S2251 and an increase in absorbance. The purified fibrinolytic enzyme from *S. commune* failed to digest the S2251 directly and without plasminogen activation activity.

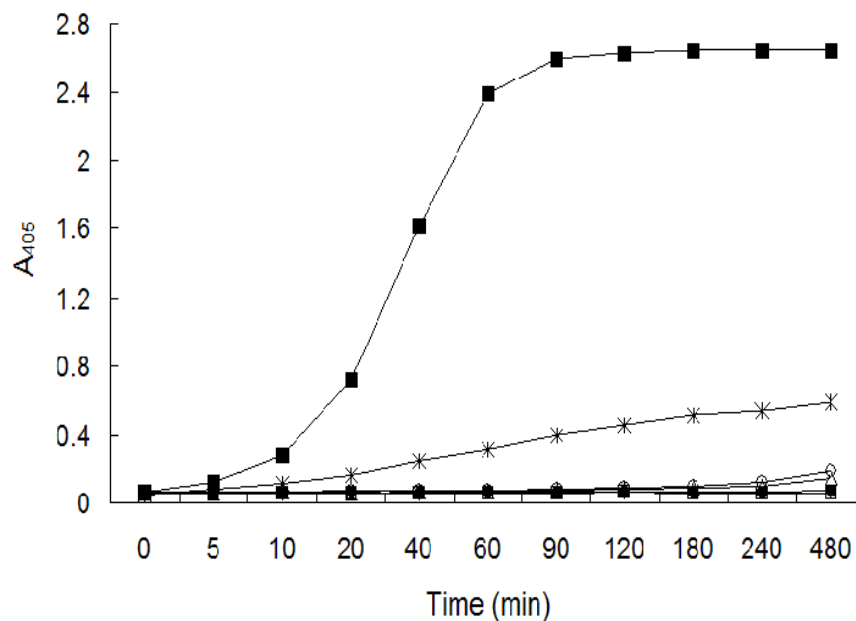


Fig. 8. Plasminogen activation assay of fibrinolytic enzyme from *S. commune* ○ : 1 µg purified enzyme and plasminogen at 0.0001 UN. ■ : 1 µg urokinase and plasminogen at 0.0001 UN. □ : 1 µg purified protease. ∗ : 1 µg human plasmin (≥ 3 units/mg protein). Δ : plasminogen at 0.0001 UN. ● : 20 µg urokinase.

In order to test the plasminogen activation while fibrin present, fibrinolytic enzyme from *S. commune* was applied on a plasminogen-rich fibrin plate containing 0.1UN plasminogen. The protease-digested zone in the plasminogen-rich fibrin plate was then compared to that of the plasminogen-free fibrin plate. In the presence of fibrin, digest representation were equal on the plasminogen-rich and plasminogen-free plates (data not shown). Fibrinolytic enzyme from *S. commune* is not a serine protease for S2251 and does not activate plasminogen *in vitro*, regardless of whether fibrin exists or not.

6.4 Coagulation effects of protease from *S. commune*

In previous study, *G. lucidum* fibrinolytic enzyme was examined in human plasma by activated partial thromboplastin time (APTT) and thrombin time (TT). The anticoagulant activity was performed by inhibition of thrombin and hydrolyzation of fibrin and fibrinogen (Choi & Sa, 2000). In our research, *S. commune* fibrinolytic enzyme applied in rat citrated blood, and the coagulation processes were monitored by thromboelastography (TEG[®]) analysis. Four TEG parameters, including the reaction time for clot initiation (R), the time to reach a 20-mm level of clot formation (K), the slope angle from R to K (α value), and the maximum vertical amplitude of the developed clot (MA) were measured. Fibrinolytic enzyme from *S. commune* was mixed with CaCl₂ and citrated rat blood to initiate recalcification. Fibrinolysis after MA was determined by measuring the loss of clot strength. Urokinase was used as positive treatment to activate plasminogen and resulted in fibrinolysis after reaching of MA (Fig. 9).

The result of TEG reaction time (R) indicated that the protease did not affect the clotting aggregation by the platelets, since clotting initiation completed normally. Fibrinolytic

enzyme of *S. commune* suppressed blood coagulation without excessive fibrinolysis. Clotting time prolongation, clotting velocity depresses and significant decrease of clotting strength were occurred (Table 4). Coagulation suppression effect here we predicate may resulted from fibrinolysis and platelet-mediated clot retraction.

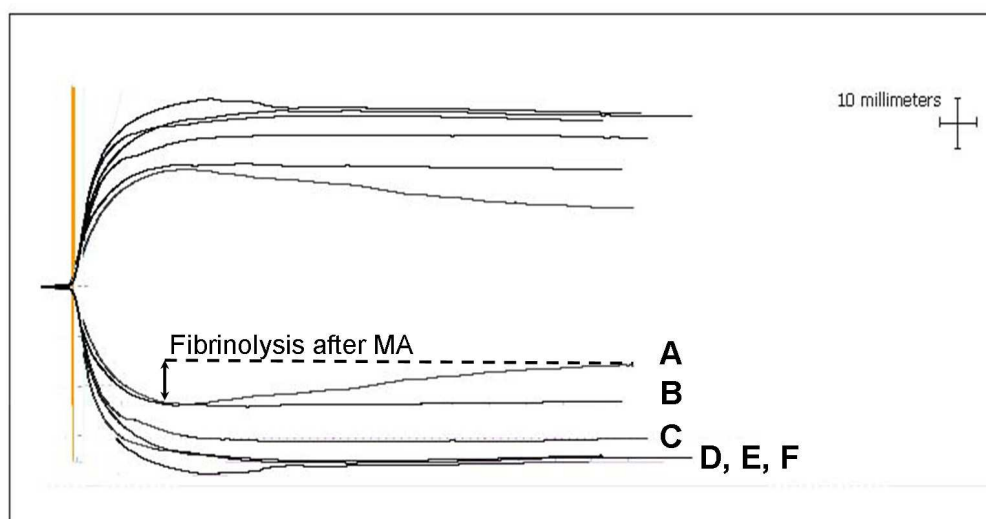


Fig. 9. Thromboelastography tracings of citrated blood added fibrinolytic enzyme from *S. commune* and human urokinase Curve A: Human urokinase 0.5µg added. Curve B, C, D, E and F: Purified protease from *S. commune* 1.5, 1.2, 0.6, 0.3, 0 (control)µg added respectively.

TEG parameters	Range of values	Amount of fibrinolytic enzyme in TEG				
		0 µg	0.3 µg	0.6 µg	1.2 µg	1.5 µg
Reaction time (R)	2.2-4.7 min	100 ^a	92.07	86.43	88.14	81.21
Coagulation time (K)	0.8-3.3 min	100	96.67	97.96	127.22	165.17 ^b
Velocity of clot (α)	50.2-81.1°	100	99.69	101.44	98.76	92.74 ^b
Maximum amplitude (MA)	75.8-24.5 mm	100	94.14	90.00	73.51 ^b	59.07 ^b

^a TEG Parameters with the addition of protease are presented as the relative index obtained by normalized to the values in control sample (0µg) which are set to 100%. (n=8).

^b Statistically significant compared to control samples, $P < 0.05$

Table 4. Effects of various concentrations of fibrinolytic enzyme from *S. commune* to citrated blood by thromboelastography analysis

In an experiment of coexist with Mg^{2+} ion, citrated blood treated by *S. commune* fibrinolytic enzyme and Mg^{2+} ion. The supplementation of Mg^{2+} ion stimulated the depression of blood clot amplitude (Fig. 10). Magnesium therapy in coronary heart disease has recently been proposed and documented in clinical trials (Shechter et al., 1999; Whiss & Andersson, 2002). Additionally, magnesium ion stimulate activity of fibrinolytic enzyme from *S. commune* was proved in study. Thus, a regulatory manipulation of *S. commune* fibrinolytic enzyme activity by magnesium supplementation can be expected in the future. The cost reducing effect by magnesium supplementation is also an innovative ideal for the currently used fibrin-specific antithrombotic agents.

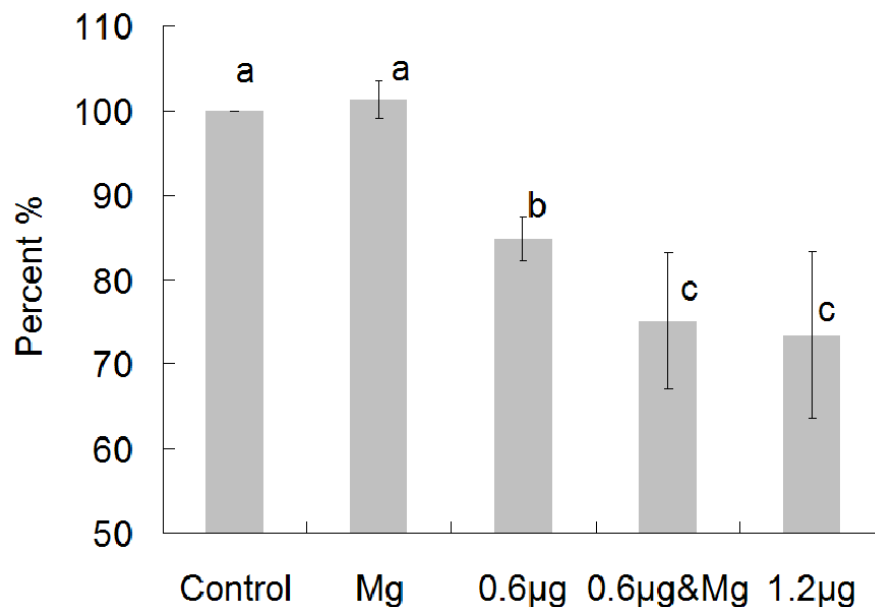


Fig. 10. Blood clot amplitude analysis of fibrinolytic enzyme coexist to Mg^{2+} Control: citrated blood mixed with PBS; Mg: citrated blood mixed with $MgCl_2$ (2.5mM); 0.6 / 1.2µg: citrated blood mixed with 0.6 / 1.2µg fibrinolytic enzyme; 0.6µg&Mg: citrated blood mixed with 0.6µg fibrinolytic enzyme and 2.5mM $MgCl_2$. Alphabetical lowercase means significant variation between other treatments, a difference was considered statistically significant at $p < 0.05$. TEG values were expressed relative to the control sample (at 100%).

7. Conclusion

In recent decades, pharmacologic intervention of an established thrombus has become an ideal therapeutic approach for thrombotic occlusive disease. Clinical application of several plasminogen activators results in activation of circulating plasminogen, which successfully digest abnormal blood clot occluded in arteries or vein; however, risk accompanied with this treatment option may include a life-threatening haemorrhage caused by the systemic activation of fibrinolytic mechanism. While scientists and clinicians are in the search for a better, safer therapeutic agent with fewer side effects, the discovery of the fibrinolytic enzymes from medicinal mushroom may shed the light for the modern thrombolytic therapies.

So far, the methods for purification and characterization of medicinal mushroom fibrinolytic enzyme were carried out by mushroom cultivation, crude protein extraction, protein chromatography, fibrin(ogen)olytic identification and characterization. Purification and characterization of fibrinolytic enzyme from *S. commune* were illustrated in this chapter, and might be a representative model for purification of medicinal mushroom fibrinolytic enzyme.

Fibrin(ogen)olytic enzymes were discovered from mushrooms, such as *P. ostreatus*, *A. mellea*, *T. saponaceum*, *C. militaris*, *G. lucidum*, *P. fraxinea*, *F. fraxinea*, *C. sinensis*, *F. velutipes*, *Fusarium* sp. BLB and *S. commune*. Most of them have been universal dietetic additives and traditional folk medicine for a long time. The low antigenicity and allergic affections of these mushroom fibrinolytic enzymes when human taken orally may be expected definitely. In previous studies, many mushroom fibrinolytic enzymes were investigated which perform superior fibrinolytic activity than known thrombolytic agents and inhibit thrombus

formation. As fibrinolytic effect is not the only solution for thrombus occurrence and decomposition, the therapeutic effect of mushroom fibrinolytic enzyme were further examined in citrated blood on thromboelastography, which monitored the clotting and fibrinolysis action in blood. Fibrinolytic enzymes interfere in antithrombotic interaction may occur amongst plasma proteins, such as platelets, prothrombin, thrombin, plasminogen, plasmin, fibrinogen, and fibrin. As results have shown, using *S. commune* as the model, mushroom fibrinolytic enzyme suppressed blood coagulation without excessive fibrinolysis by prolonging clotting time and decreasing clotting velocity. Consequently, the result is a significant decrease in clot strength. Most notably, the initiation of clotting system and plasminogen activation occurrences were maintained at a normal level. Mushroom fibrinolytic enzyme did not offset haemostasis, but rather reduced the likelihood of thrombus formation without increasing the risk of haemorrhage. These data indicated that the safer antithrombotic effect of mushroom fibrinolytic enzyme and the board application may use as thrombolytic agents clinically.

Metal ion therapy in coronary heart disease has recently been proposed. Although the therapeutic results are still ambivalent, the researches and therapeutic development are never finished to search a more effective therapy for such coronary patients. Review of characters from medicinal mushroom fibrinolytic enzymes, these enzymes revealed significant metal ion dependent activity. The inhibition or the stimulation of enzyme activity by metal ion may provide a pharmaceutical conservation and a critical control manipulation in clinical uses. Although the description in this chapter showed the investigation of mushroom fibrinolytic enzyme is currently at stage of *in vitro* and *ex-vivo* experiment. However, the effectiveness of medicinal mushroom fibrinolytic enzyme in antithrombotic assays indicated the possible clinical application of mushroom fibrinolytic enzyme in the future.

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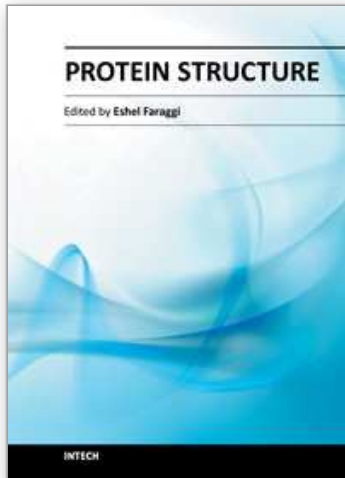
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Since the dawn of recorded history, and probably even before, men and women have been grasping at the mechanisms by which they themselves exist. Only relatively recently, did this grasp yield anything of substance, and only within the last several decades did the proteins play a pivotal role in this existence. In this expose on the topic of protein structure some of the current issues in this scientific field are discussed. The aim is that a non-expert can gain some appreciation for the intricacies involved, and in the current state of affairs. The expert meanwhile, we hope, can gain a deeper understanding of the topic.

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