Chapter 1

Introduction

When a blood vessel is damaged, a hemostatic response occurs in order to stop blood loss. After the hemorrhage reaction, the cross-linked fibrin polymer that is formed from fibrinogen by thrombin is dissolved by endogenous plasmin which is activated from plasminogen by tissue-type plasminogen activator (t-PA). Although fibrin clot formation and fibrinolysis are maintained in balance by the biological system, thrombosis, such as myocardial infarction, occurs when clots are not lysed as a result of unbalance state (Voet and Voet, 1990).

Typical thrombolytic agents used therapeutically are urokinase, streptokinase and tissue plasminogen activator. Urokinase is a serine protease isolated from human urine or kidney tissue culture. Tissue-type plasminogen activator is also a serine protease synthesized in endothelial cells and present in most human tissue. Urokinase and t-PA are very expensive and are produced only on a small scale. Streptokinase, which is the most widely used activator of fibrinolysis, is isolated from the culture broth of β-hemolytic streptococci. Urokinase and streptokinase have a low specificity to fibrin compared to t-PA. (Torchilin, 1988). Since the drawbacks listed earlier, fibrinolytic enzyme from other sources are promising targets.

Fibrinolytic enzyme have been reported from organisms such as earthworm (*Lumbricus rubellus*) (Mihara *et al.*, 1991), marine green algae, *Codium intricatum,* (Matsubara *et al.*, 1998), and *C. latum* (Matsubara *et al.*, 1999) and snake, *Akistrodon contortrix contortrix* (Guan *et al.*, 1991). Great attention has been directed towards a search for thrombolytic agents from microbial sources. Microorganisms as a source of fibrinolytic enzyme have several advantages over animal in low cost of production. Fibrinolytic enzyme

have been found from bacteria yeasts and fungi. Filamentous fungi and mushrooms such as *Scopulariopsis brevicaulis* (Singh and Vezina, 1971), *Penicillium chrysogenum* H9 (El-Aassar *et al.*, 1990), *Fusarium pallidoroseum* (El-Aassar, 1995), *Flammulina velutipes* (Psurtseva and Mnoukhina, 1996), *Lactiporus sulphureus* and *Coriolus* sp. (Hirasawa, 1997b), *F. oxysporum* (Toa *et al.*, 1997; 1998) and *Pleurotus ostreatus* (Choi and Shin, 1998) have been reported to produce fibrinolytic enzyme.

Since mushrooms are easily to cultivate and serve as delicacies for human consumption, fibrinolytic enzyme from mushroom could be another alternative benefit for health. Dissara and Pandee (2000) reported that *Schizophyllum commune* BL 23, the split gill mushroom found in southern part of Thailand produced fibrinolytic enzyme. However, nature and properties of this enzyme are not well defined. Therefore, in this study, condition of fibrinolytic enzyme production, purification and characterization were studied.

Literature Review

1. *Schizophyllum commune*

1.1 General characteristic

Schizophyllum commune is a dikaryotic, wood-rotting basidiomycetes classified in Phylum Basidiomycota. The fruiting body is like the fan-shape (Figure 1). The organism is world wide in distribution and is very easy to recognize. The basidiocarp is usually small (1-4 cm in diameter) and gray. The distinguishing feature of *Schizophylum* is the marginal proliferation of the basidiocarp that from the split gills underside. The fruiting body can dry out and rehydrate many times over the course of a growing season. The fungus have been reported from over 60 substrates including a variety of living plants, decaying logs, branches and sticks. It can be cultivated easily in the laboratory and has been the subject of numerous studies concerning sexuality, genetics, physiology and morphogenesis (Alexopoulos and Blackwell, 1996).

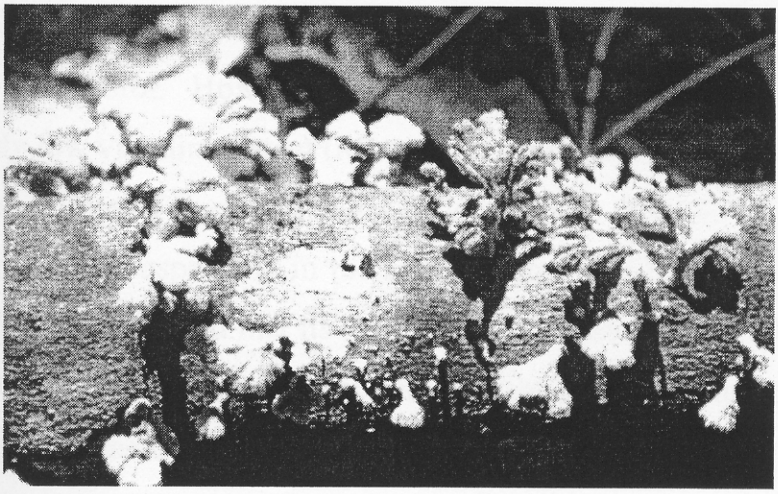

Figure 1 The fruiting body of *Schizophyllum commune*

The life cycle of *S. commune* consists of two distinct stages: a haploid homokaryon and dikaryon consisting of cells in which two haploid nuclei of opposite mating types are maintained per cell. The haploid spores are dispersed from the gills of the mushroom by the wind. The spores germinate on substrate such as rotten wood as haploid homokaryotic (one nucleus) mycelia. The individual cells of the homokaryon are genetically identical, as they are a result of mitotic events originating from a single basidiospore. Two compatible homokaryons fused to form the heterokaryon or secondary mycelium (Alexopoulos and Blackwell, 1996).

1.2 The utilization of *S. commune*

The potential for utilization of *S. commune* was classified here in two prospects, as a foodstuff and as a source of useful metabolites.

1.2.1 As a food source

Filamentous fungi are the source of human foodstuffs known for centuries. They are ideal food because they have a high protein content, typically 20-30% dry matter as crude protein, which contains all of the essential amino acids. Fungal biomass is also a source of dietary fiber, and is nearly free of cholesterol. However, in contrast to yeasts, filamentous fungi have a relatively low vitamin content (Moore and Chiu, 2001).

Fruiting bodies of *S. commune* are collected from the wild and used as foodstuff by the people especially in the southern part of Thailand. A report from Petcharat (1995) claimed that its fruiting bodies costed about 70-80 baht per kilogram. He also reported an appropriate cultivation of this mushroom.

1.2.2 As a source of useful metabolites

Medicinal properties have been attributed to mushrooms for thousand of years. Mushroom extracts are widely sold as nutritional supplements and touted as beneficial for health. In Japan, Russia, China and U.S.A. several different polysaccharide antitumor agents have been developed from the fruiting body, mycelia and culture medium of various medicinal mushrooms

such as *Lentinus edodes, Ganoderma lucidum, Flammulina velutipes and S. commume.* Both cellular components and secondary metabolites of a large number of mushrooms have been shown to effect the immune system of the host and therefore could be used to treat a variety of disease states (Wasser and Weis, 1999). Schizophyllan, an extracellular polysaccharide isolated from *S. commune* is a glucan having repeating units compose of three β-1,3-linked D-glucopyranose residues to one of which a single D-glucopyranose residue is attached by β-1,6-linkage. It has antitumor activity and is used as an adjuvant therapy for the treatment of uterine cervix cancer (Hirata *et al.,* 1994). Moreover, sulfated schizophyllan has been reported to show inhibitory action against HIV but with reduced antitumor effect (Ito *et al.*, 1990).

In Japan, Hirasawa *et al.* (1997b) investigated the fibrinolytic enzymes from basidiomycetes and could be obtained from *S. commune. S. commune* produced a proteolytic enzyme, a small serine protease (ScPrI) had an apparent molecular mass of 22 kDa, and was active against classical substrates for chymotrypsin and subtilisin proteases. The optimal pH for activity was neutral to slightly alkaline and the protein denatured above 50°C. The enzyme was inhibited by phenylmethyl sulfonylfluoride (PMSF), N-toluenesulfonyl-L-phenylalanine chloromethyl ketone (TPCK) and chymostatin and it showed little dependence on metal ions (Johnston *et al.*, 2000). Moreover, *S. commune* produced other useful metabolites such as cellulase (Steiner *et al.,* 1987), ferulic acid esterase (Mackenzie and B ilous, 1988), xylanase (Haltrich *et al.*, 1993), endo-β-1,3-glucanase (Prokop *et al.,* 1994), invertase (Rojo *et al.,* 1994), cellubiose dehydrogenase (Fang *et al.,* 1999) and α-glucuronidase (Tenkanen and Siika-aho, 2000).

2. Fibrinolytic enzyme

2.1 Conversion of fibrinogen to fibrin and and fibrinolysis

Fibrinogen is a large molecule of approximately 340 kDa. It consists of three pairs of nonidentical peptide chain αα', ββ', γγ'structure. The three of chains are linked head to head and side by side by disulfide bonds near the amino terminals to form and enlarged structure called the disulfide knot (Figure 2). The carboxyl terminals of the three pairs of chains are enlarged globular structures. The αα' and the ββ' N-terminal regions, through charge-charge repulsion, play a role in preventing the aggregation of fibrinogen from occurring normally. Fibrin monomers are formed by the cleavage at arginine-glycine peptide bonds of the α and β chains near their amino terminal. Thrombin cleaves these groups and allows the resulting fibrin molecules to aggregate and form the soft clot. This soft clot is stabilized and strengthened by the action of factor XIII, transglutaminase. This enzyme catalyzes a reaction in which an isopeptide linkage is formed by replacing the γ-amide group of glutamine residues of one chain with the ε-amino group of lysine residues of another chain (Pilsum, 1992).

Lysis of the fibrin clot occurs through the action of the enzyme plasmin. The reactions involved in fibrinolysis are shown in Figure 3. When a fibrin clot presented in the blood stream, plaminogen is attracted to the clot. Plasmin exists in the blood in its zymogen form, plasminogen. Plasminogen has a high affinity for the fibrin clot and forms complex with fibrin throughout various regions of the porous fibrin network. Plasminogen is activated to plasmin by the action of another protease, tissue-type plasminogen activator, frequently referred to as t-PA. The t-PA also binds to the fibrin clot and, in the complex, activates plasminogen by cleavage of specific bonds within the zymogen. The clot is then solubilized by the action of plasmin (Smith, 1992).

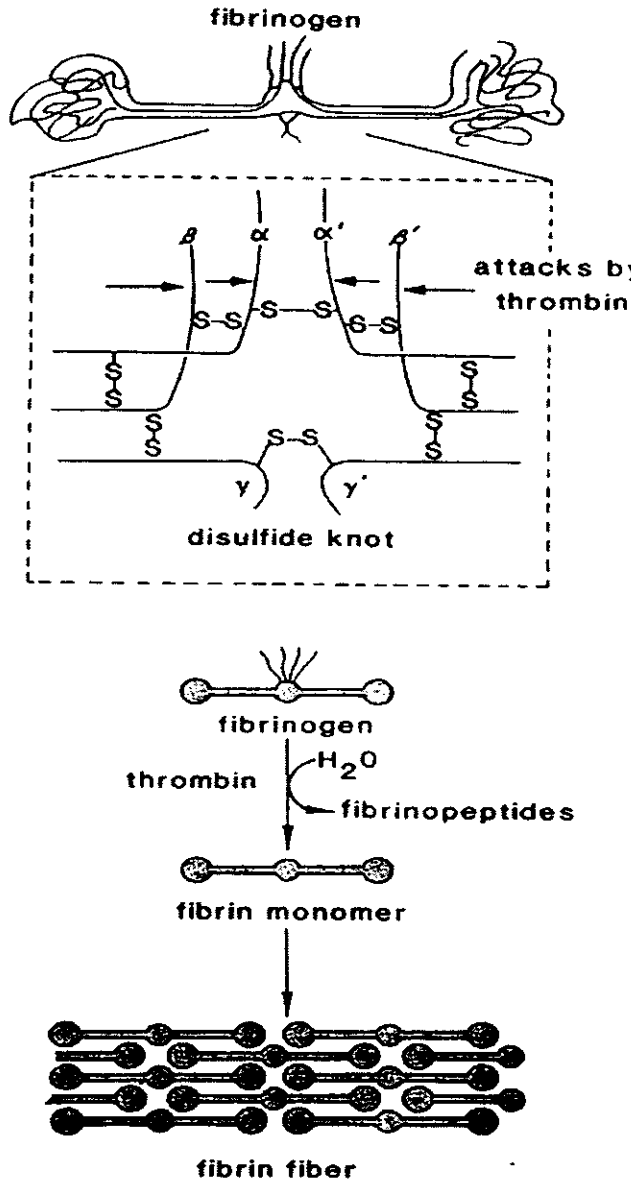

Figure 2 Diagrammatic representation of the fibrinogen molecule and its conversion to the soft clot of fibrin.

Source: Pilsum (1992)

Thrombolytic agents are classified as two types: one is plasminogen activator, such as t-PA and urokinase. Most commonly t-PA convert plasminogen into plasmin. Another is the plasmin-like protein, such as nattokinase (Sumi *et al.*, 1990) and lumbrokinase (Mihara *et al.*, 1991) which can directly degrade the fibrin of blood clot.

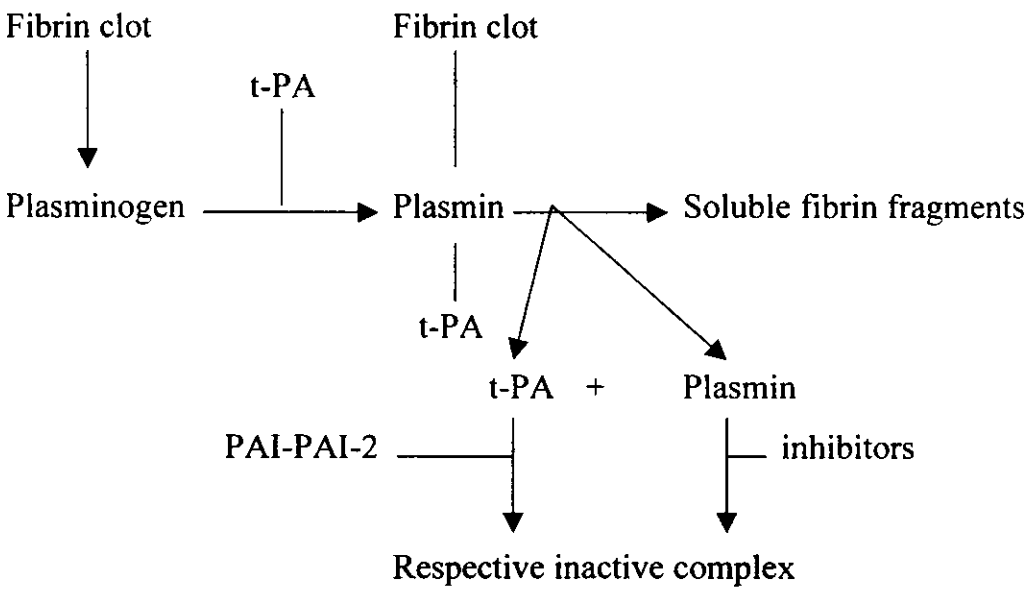

Figure 3 Reactions involved in the dissolution of the clot.

Source: Smith (1992)

2.2 Sources of fibrinolytic enzymes

Fibrinolytic enzymes present in plants, animals and microorganisms. The enzyme were found in snake venom, such as *Agkistrodon halys brevicaudus* (Quin and Ma, 1991), *Vipera lebetina* (Siigur *et al.*, 1996) and *Agkistrodon contortrix contortrix* (Guan *et al.*,1991), *Agkistrodon caliginosus* (Cho *et al.*, 2001).

A strong fibrinolytic enzyme was readily obtained in saline extracts of the earthworm, *Lumbricus rubellus*. It hydrolyzed not only plasminogen-rich fibrin plates, but also plasminogen-free fibrin plates. The average fibrinolytic activity was about 100 CU (plasmin units) or 250 IU (urokinase units)/g wet weight. The molecular weight and isoelectric point were about 20 kDa and 3.4, respectively. The enzyme was heat-stable and displayed a very broad optimal pH range. Diisopropyl fluorophosphate (DFP) and soybean trypsin inhibitor (SBTI) strongly inhibited the enzyme (Mihara *et al.*, 1991). In China, fibrinolytic enzyme was purified from earthworms (*Eisenia fetida*). The enzyme composed of two subunits (26 and 18 kDa) (Yang and Ru, 1997).

The production of fibrinolytic enzymes was investigated from marine green algae, *Codium intricatum,* (Matsubara *et al.*, 1998), *C. latum* (Matsubara *et al.*, 1999) and *C. divaricatum* (Matsubara *et al.*, 2000). In addition, peony roots such as *Paeonia suffruticosa*, *P. lutia*, *P. anomala* and *P. lactiflora* were used to extract fibrinolytic enzymes. The extracts exhibited high anticoagulant properties and the ability to lyse nonstabilised fibrin *in vitro*. Peroral chronic administration of an extract of *P. suffruticosa* increased the fibrinolytic activity of blood and contributed to the prevention of thrombosis in vessels and helped lyse freshly formed thrombi in blood (Lyapina *et al.*, 1995).

Fibrinolytic enzymes found in bacteria, fungi and yeasts were listed in Table 1

Table 1 Fibrinolytic enzymes producing microorganisms

Microbial sources	References
1. Bacteria	
Bacillus sp. strian CK 11-4	Kim *et al.* (1996)
B. natto AKU 0206	Hirasawa *et al.* (1997a)
Bacillus sp. KA38	Kim *et al.* (1997)
B. subtilis IMR-NK1	Chang *et al.* (2000)
Bacillus sp. strain DJ-4	Kim and Choi (2000)
B. amyloliquefaciens DJ-4	Choi and Kim (2001)
B. amyloliquefaciens DC-4	Peng *et al.* (2003)
Nocardia minima	Landau *et al.* (1990)
Streptococci	Tillet and Garner (1933 cited by El-Aassar, 1995)
Streptomyces sp.	Bono *et al.* (1996)
Streptomyces griseus	Hirasawa *et al.* (1997a)
Streptomyces sp. Y405	Wang *et al.* (1998, 1999)
S. megasporus strain SD5	Chitte and Dey (2000)
2. Fungi	
2.1 filamentous fungi	
Aspergillus fumigatus, Humicola grisea, Oidiodendron flavum, S. brevicaulis, and *Thermomyces lanuginosus*	Abdel-Rahman *et al.* (1990)
A. oryzae Y9	Hirasawa *et al.* (1997a)
A. oryzae	Ives and Tosoni (1967 cited by El-Aassar, 1995)
A. flavus, A. ustus, A. terreus, Fusarium oxysporum, Penicillium funiculosum viride and *Trichoderma*	Ismail *et al.* (1990)

Table 1 (continue)

Microbial sources	References
F. oxysporum N.R.C.1	Abdel-Fattah *et al.* (1993)
F. oxysporum	El- Aassar (1995), Tao *et al.* (1997, 1998)
Flammulina velutipes	Iakovlev and Serebryakova (1994) and Psurtseva and Mnoukhina (1996)
P. chrysogenum H9	El-Aassar *et al.* (1990)
Scopulariopsis brevicaulis	Singh and Vezina (1971)
Trichothecium roseum	Sharkova *et al.* (1977 cited by El-Aassar, 1995)
A. oryzae Y9	Hirasawa *et al.* (1997a)
Pleurotus corenucopiae, Laetiporus Sulphureus, S. commune and *Coriolus* sp.	Hirasawa *et al.* (1997b)
Pleurotus ostreatus	Choi and Shin (1998)
2.2 Yeast	
Candida tropicalis	Hirasawa *et al.* (1997a)
Pichia anomala IFO 0568, *Torulopsis aeria, Rhodotorula rubra* IFO 0709, *Saccharomyces rouxii* IFO 0487 and *Hansenula miso* IFO 0146	Takeno *et al.* (1999)

2.3 Factors affecting on fibrinolytic enzymes production

2.3.1 Cultural conditions

The production of fibrinolytic enzymes from microorganisms has been carried out both in solid state cultures and submerged cultures. For solid state cultures, wheat bran, rice bran, soya bean and ground corn were used as the substrate for fibrinolytic enzymes production from *F. pallidoroseum* and the highest fibrinolytic activity was obtained from wheat bran (El-Aassar, 1995). The superiority of solid state cultures for the production of some enzymes by microbial cells may be referred to their growth under conditions closer to their natural habitats (Ramesh and Lonsane, 1991; Ramana Murth *et al.*, 1993 cited by El-Aassar, 1995). Rice chaff was also used as the substrate for production of fibrinolytic enzymes from *F. oxysporum* (Tao *et al.*, 1997; Tao *et al.*, 1998).

In Japan, Hirasawa *et al.* (1997a) produced fibrinolytic enzymes from basidiomycetes such as *P. corenucopiae, L. sulphureus* and *S. commune* in submerged culture. Also, In Russia, Iakovlev and Serebryakova (1994) and Psurtseva and Mnoukhina (1996) studied production of fibrinolytic enzymes from *F. velutipes* under submerged cultivation.

2.3.2 Nutrient composition

Under submerged culture of *F. velutipes*, it was found that organic nitrogen sources were preferable for fibrinolytic enzymes production comparing with inorganic nitrogen (Psurtseva and Mnoukhina, 1996). El-Aassar *et al.* (1990) studied the effect of peptone and yeast extract, casein, corn steep, $NaNO_3$ and $(NH_4)_2SO_4$ on fibrinolytic enzyme production from *P. chrysogenum* H9. Peptone and yeast extract were the best nitrogen source for fibrinolytic enzyme production and the activity of 219.6 μg tyrosine/mg protein was obtained. Moreover, glucose, galactose, fructose, lactose and soluble starch at the concentration of 1% and 0.5% of sucrose and maltose were used as carbon source for the enzyme production from *P. chrysogenum*

H9. The results showed that 1% glucose was the best carbon source for the enzyme production and the activity of 243.8 μg tyrosine/mg protein was obtained.

Hirasawa *et al.* (1997a) cultivated microorganisms in basal medium for fibrinolytic enzyme production. Potato dextrose, malt extract, corn steep liquor, yeast extract, soybean meal, polypeptone and urea was separately added into the basal medium at the concentration of 2%. After cultivation, the cell pellets were separated from supernatant and disrupted by sonication. The undestroyed cells and debris were removed by centrifugation. The supernatant was used for determination of the fibrinolytic activity. The results showed that an appropriated nutrient source for certain microorganism was different. For example, *B. natto* AKU 0206 and *C. tropicalis* preferred soybean meal while corn steep liquor was suitable for *S. commune*, *S. griseus* and *A. oryzae* Y9. Moreover, the effect of carbon source was investigated for fibrinolytic enzyme production from *Coriolus* sp. and *P. corenucopiae.* Corn starch was the most preferable carbon source compared to lactose, glucose, sucrose and glycerol.

2.3.3 Temperature

The optimal temperature on fibrinolytic enzyme production and maximum fibrinolytic enzyme activity from *F. pallidoroseum* was obtained at 25°C (El-Aassar, 1995) while *F. oxysporum* was grown at 28°C for the enzyme production in solid cultures (Tao *et al.*, 1997). Thus, the temperature that affected fungal growth may also affected the enzyme production.

Chitte and Dey (2000) studied the effect of pH and temperature on fibrinolytic enzyme activity from *S. megasporus* SD5. The optimum pH and temperature for production of the enzyme were 8.0 and 55°C, respectively.

2.3.4 Moisture content

The optimal moisture content for fibrinolytic enzymes production from *F. pallidoroseum* was 50% when wheat bran was used as the substrate in solid state culture (El-Aassar, 1995) but the optimal moisture content was 30-50% for enzyme production from *F. oxysporum* when rice chaff was used as the substrate (Tao *et al.*, 1997).

2.3.5 Culture period

The growth of *F. pallidoroseum* and *F. oxysporum* increased with increasing culture time and the optimal fermentation time for enzyme productivity was 4 days in solid state fermentation (El-Aassar, 1995; Tao *et al.*, 1997).

3. Purification of fibrinolytic enzymes

El-Aassar (1995) purified fibrinolytic enzyme from the supernatant of *F. pallidoroseum*. Fractional precipitation of the crude enzyme with ammonium sulfate (25 to 90% saturation) yielded a total recovered protein and activity of 36% and 65%, respectively. The fraction precipitated from 50-65% ammonium sulfate saturation showed the highest specific fibrinolytic activity yielding 37.6% of the original activity. Further purification in Sephadex G-100, indicated that the fibrinolytic enzyme system of *F. pallidoroseum* consisted of three active components. The first major one contained 64% of the activity presented in the applied sample while both other components were minor ones and contained 13.8% and 7.1% only of the original activity. Chromatography on DEAE-cellulose afforded 4 protein peaks. The second peak was a major one recovering about 57% of the applied protein. All the fibrinolytic activity was presented in the fractions of this peak. The pooled fractions of active peak showed 7.5 fold purification. The homogeniety of this active peak was substantiated by polyacrylamide disc electrophoresis, where it moved as a single protein band.

Choi and Shin (1998) detected fibrinolytic activity from crude extract of the fruiting body of *P. ostreatus* by using the fibrin plate method. The supernatant was treated with ammonium sulfate to 80% saturation, the purification steps were followed by Sephadex G-50, hydrophobic phenyl Sepharose, Sephadex G-150 column and anion exchanger Mono Q. Two peaks of activity were found after G-150 gel filtration, a minor fraction at 57 kDa and major fraction at 24 kDa. The major fraction was further purified through phenyl Sepharose and Mono Q FPLC. The results of 52 fold purification with 5% recovery yield were obtained. Its molecular weight was determined to be 12 kDa in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The enzyme cleaved not only fibrin but also B_β, and γ chain of human fibrinogen.

A novel protease with fibrinolytic activity, designated as SW-1 was purified from the culture broth of *Streptomyces* sp. strain Y405 isolated from soil. The purification procedure involved ammonium sulfate fractionation, decolorization on 290 resin, gel filtration on Sephadex G-75, anion-exchange chromatography on DEAE-Sephadex A-25 and affinity chromatography on Lysine Sepharose 4B. About 4.2 mg protein was obtained from a liter of culture broth and the recovery yield was 12.0%. The purified enzyme showed the specific activity of 2952.3 urokinase units/mg which was increased by 230.6 fold over the culture broth. The purity determined by HPLC was 83.5%. The enzyme is a single chain polypeptide with a predicted molecular weight of 30 kDa in SDS-PAGE and an isoelectric point of 8.5. (Wang *et al.*, 1999).

The purified fibrinolytic enzyme of *Bacillus* sp. strain CK 11-4 showed thermophilic, hydrophobic and strong fibrinolytic activity. The enzyme was treated with acetone for precipitation and further purified by consecutive chromatography with carboxymethyl cellulose and Toyo-pearl HW 55. The specific activity of the purified enzyme increased more than 7.5

fold with 95% yield. The purified enzyme migrated as a single protein band and the molecular weight was estimated to be approximately 28,200 daltons by SDS-PAGE. The first 14 amino acids of the N-terminal sequence of the enzyme are Ala-Gln-Thr-Val-Pro-Tyr-Gly-Ile-Lys-Ala-Asp. This sequence is identical to that of subtilisin Carlsberg, however, the enzyme activity was about eight times higher than that of Carlsberg (Kim *et al.*, 1996). Moreover, fibrinolytic enzyme was also found from *Bacillus* sp. KA38, which was screened from a fermented fish known as Joet-Gal in Korea. The enzyme was purified using ammonium sulfate fractionation (80% saturation), DEAE-cellulose column chromatography and Mono Q column chromatography. The enzyme was highly specific toward fibrin clots and directly degraded them. The molecular weight was 41 kDa and the first 10 amino acids of the N-terminal sequence was Val-Tyr-Pro-Phe-Pro-Ile-Pro-Asn. The enzyme was purified 70 fold with 6% yield. (Kim *et al.*, 1997).

A mutant of *B. subtilis* IMR-NK1 produced high fibrinolytic enzyme activity by solid state fermentation using wheat bran as the medium. The enzyme produced was extracted with 1% NaCl, concentrated by ultrafiltration, and dried by lyophilization. The crude enzyme was dissolved in 25 mM imidazole-HCl buffer. The supernatant was fractionated by the addition of ammonium sulfate. The precipitate was formed between 40 and 80% saturation of ammonium sulfate. The purification steps was performed by Sephacryl S-100 HR gel filtration, polybuffer exchange column (PBE 94 chromatofocusing) and Superdex 75 HR gel filtration. At the final step, the enzyme was purified 9.2 fold with yield of 6.5%. The purified enzyme was almost homogeneous, as examined by SDS-PAGE and capillary electrophoresis. The molecular mass estimated by gel filtration was 31.5 kDa and the isoelectric point estimated by isoelectric focusing electrophoresis was 8.3. (Chang *et al.*, 2000).

Kim and Choi (2000) found that *Bacillus* sp. strain DJ-4 isolated from Doen-Jang, a traditional Korean fermented food, secreted four extracellular proteases (80, 53, 38 and 29 kDa) by using SDS-PAGE and fibrin zymography. One of the four proteases (29 kDa) was purified by two successive chromatographic techniques: DEAE-Sepharose CL-6B and gel filtration on Toyopearl HW-55F. The specific activity of the purified enzyme (designated as subtilisin DJ-4) was increased by 6.78 fold and its protein content about 0.72% of the culture supernatant. The purified enzyme migrated as a single band with an apparent molecular weight of 29 kDa on SDS-PAGE (12% gel and fibrin zymography). Lastly, Peng *et al.* (2003) isolated *B. amyloliquefaciens* DC-4 from douchi, traditional Chinese soybean-fermented food, and purified fibrinolytic enzyme from the supernatant. The enzyme (subtilisin DFE) displayed thermophilic, hydrophilic and strong fibrinolytic activity. The enzyme was purified by ammonium sulfate treatment (the precipitated formed in 30-60% saturation of ammonium sulfate), CM-Sepharose FF, DEAE-Sepharose FF, Phenyl Sepharose 6 FF and Sephadex G-50 gel filtration. The fibrinolytic enzyme was purified 11.5 fold with 2.8% yield. It was demonstrated to be homogeneous by SDS-PAGE and isoelectric focusing electrophoresis, and has molecular mass of 28 kDa and an isoelectric point of 8.0.

4. Characterization of fibrinolytic enzymes

Proteolytic enzyme are capable of cleaving proteins into small peptides and amino acids. From an analysis of their *in vitro* properties, proteolytic enzymes have been classified in a number of ways, such as, on the basis of the pH range over which they are active (acid, neutral, or alkaline) and their abilities to hydrolyze specific proteins. The most satisfactory classification is based on the mechanism of action. This classification, which is used by the Enzyme Commission, consists of four groups including serine,

thiol, metal (metal-chelator sensitive), and acid proteases. They are distinguished from each other by their sensitivity to various inhibitors and the primary specificity determined by the amino acid residue (s) near the splitting point (Loffler, 1986). The investigated fibrinolytic enzymes obtained from microorganisms have been characterized and classified into serine protease and metalloprotease.

4.1 Serine proteases

The microbial serine proteases could be sub devided into four group: trypsin-like proteases, alkaline proteases, *Myxobacter* α-lytic proteases and staphylococcal proteases. They are most active at high pH (around 7.0-11.0). and are commonly sensitive to PMSF (phenylmethyl sulfonylfluoride), DFP (diisopropylfluorophosphate), TLCK (N-toluenesulfonyl-L-lysine chloromethyl ketone), TPCK (N-toluenesulfonyl-L-phenylalanine chloromethyl ketone) and SBTI (soybean trypsin inhibitor) (Ward, 1987).

The fibrinolytic enzymes obtained from bacteria were reported as serine proteases. Most of fibrinolytic serine protease were produced by *Bacillus* strains which screened from fermented food such as *Bacillus* sp. CK 11-4 (Kim *et al.*, 1996), *B. subtilis* IMR-NK1 (Chang *et al.*, 2000), *Bacillus* sp. strain DJ-4 (Kim and Choi, 2000), *B. amyloliquefaciens* DJ-4 (Choi and Kim, 2001) and *B. amyloliquefaciens* DC-4 (Peng *et al.*, 2003). In addition, *Streptomyces* sp. Y405 (Wang *et al.*, 1999) and *S. megasporus* SD5 (Chitte and Dey, 2000) could also produced fibrinolytic serine protease. In particular, the enzyme activity was inhibited with PMSF which a well-known inhibitor of serine protease.

A strongly fibrinolytic enzyme was produced by *Bacillus* sp. CK 11-4 which was screened from Chungkook-Jang, traditional Korean fermented-soybean sauce. The enzyme activity had an optimum pH around 10.0-12.0 and its activity decreased rapidly at the pH levels below 6.0. The enzyme was stable in the pH range of 7.0-10.5 at 30°C for 20 h. Above pH 11.0, enzyme

stability abruptly decreased. The optimum temperature for the enzyme activity was 70°C when examined at pH 6.0. The enzyme was very stable at 40°C for 60 min incubation. The enzyme was classified as a thermophilic alkaline protease (Kim *et al.*, 1996). Chang *et al.* (2000) studied fibrinolytic enzyme from *B. subtilis* IMR-NK1 and found that the optimum pH and temperature for fibrin hydrolysis were 7.8 and 55°C, respectively. The range of pH and temperature for the enzyme stability was 5.0-10.0 and 30-40°C, respectively. The optimum conditions for enzyme activity of *B. subtilis* IMR-NK1 were lower than those of the enzyme from *Bacillus* sp. CK 11-4.

Bacillus sp. strain DJ-4 was screened from Doen-Jang, traditional Korean fermented food. It secreted four extracellular protease (80, 53, 38 and 29 kDa). One of the four proteases (29 kDa) was identified as subtilisin DJ-4. The enzyme was stable in the pH range of 4.0-11.0 at 4°C for 48 h and 80% of the enzyme activity remained after incubation at 50°C for 1 h. It was strongly inhibited by the presence of Cu^{2+} or Zn^{2+} ions. Subtilisin DJ-4 was identified to be a plasmin-like serine protease base on amidolytic assay and a fibrin zymography inhibitor assay (Kim and Choi, 2000). Moreover, Choi and Kim (2001) found that two serine type fibrinolytic proteases (29 and 38 kDa) from *B. amyloliquefaciens* DJ-4 showed thermostability from 65 to 70°C for 30 min.

Lastly, *B. amyloliquefaciens* DC-4 was isolated from douchi, a traditional Chinese soybean-fermented food and produced fibrinolytic enzyme (subtilisin DFE). The enzyme activity was obtained at the pH range of 9.0 and the enzyme stability showed in the pH range of 6.0-10.0 at 37°C for 60 min. The enzyme was active between 28 and 60°C. The optimum temperature for the fibrinolytic activity was 48°C when the enzyme was kept in the buffer solution (pH 9.0). After 60 min incubation, subtilisin DFE was very stable at 40°C, but at 70°C for 10 min, the fibrinolytic activity of subtilisin DFE was completely lost after 10 min. The fibrinolytic activity was also severely

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inhibited by serine protease inhibitors (PMSF) and partially inhibited by benzamidine hydrochloride, leupeptin and pepstatin A. It was classified into alkaline protease. (Peng *et al.*, 2003).

Furthermore, *S. megasporus* SD5 could also produced fibrinolytic enzyme. The optimum temperature for the dissolving fibrin clot was 37°C. The enzyme was resistant to a broad pH range of 6.0 to 9.0 and the temperature range of 37-60°C. The enzyme was inhibited by PMSF including other serine protease inhibitors such as chymostatin, pefabloc, aprotinin, antipain. In addition, bestatin, leupeptin and pepstatin partially inhibited the enzyme activity. The characteristics of enzyme was a chymotrypsin-like serine protease (Chitte and Dey, 2000).

From the reviews, it can be concluded that the fibrinolytic enzymes from investgated bacteria were active at high temperature with the optimum temperature around 40-70°C, and active at high pH (around 7.0-11.0). The enzyme activity was inhibited by PMSF.

4.2 Metalloproteases

Metalloproteases can be divided into four groups: neutral and alkaline proteases and *Myxobacter* proteases I and II. The neutral proteases show specificity for hydrophobic or bulky amino acid residues, alkaline proteases exhibit very broad specificity, *Myxobacter* proteases I is specific for small amino acid molecules at either side of the splitting point while proteases II is specific for a lysine residue at the amino side of the splitting point. All metalloprotease are sensitive to chelating agents such as ethylenediamine tetraacetic acid (EDTA) but insensitive to sulfhydryl agents such as ρ-chloromercuribenzoate (ρCMB) and inhibitors such as DFP (Ward, 1987).

Fibrinolytic enzymes which was classified as metalloprotease, were widely distributed in filamentous fungi and found in certain strain of bacteria. Microorganisms could produced fibrinolytic metalloprotease such as

F. oxysporum N.R.C.1, *F. pallidoroseum*, *P. ostreatus*, *Bacillus* sp. KA 38 and *Streptomyces* sp. Y405.

The fibrinolytic enzyme from *F. pallidoroseum* was highly active on human fibrin and showed an optimum reaction temperature of 40°C but it was relatively sensitive to heat treatment at 55°C. Maximum enzyme activity was performed at pH 7.0 and the enzyme was fairly stable in a pH range of 6.5 to 9.0 while it showed less stability in acidic pH values. The enzyme activity was highly stimulated by cobalt ions. Ca^{2+}, Zn^{2+} and Na^{+} ions showed a stabilizing effect on the enzyme and its activity was stable or slightly higher in their presence. EDTA strongly inhibited the enzyme activity but its activity restored when $CoCl_2$ was also presented. These results suggested that fibrinolytic enzyme might be a metalloprotein containing cobalt ion. (El-Aassar, 1995). In addition, Choi and Shin (1998) isolated and characterized a fibrinolytic metalloprotease from the fruiting body of *P. ostreatus*. The enzyme was strongly inhibited with 2 mM of 1, 10 phenanthroline but to a lesser degree with 1 mM EDTA and ethylene glycol-o-o'-bis [2-amino-ethyl]-N-N- N'-N'-tetraacetic acid (EGTA) but the inactivated enzyme activity was restored by addition of Zn^{2+} and Co^{2+}. The optimum pH of the enzyme was in the range of 7.5 to 8.0 and the enzyme was quite stable to heat treatment (50°C). Moreover, the fibrinolytic enzyme from mycelial culture of *P. ostreatus* was also studied and characterized and the specific activity was similar to that of fruiting body.

Fibrinolytic enzyme from *Bacillus* sp. KA38, isolated from the Korean fermented fish (Jeot-Gal) have been characterized. The maximum enzyme activity was observed in the narrow range of 30 to 40°C. The enzyme was stable up to 40°C, but its activity rapidly decreased at higher temperatures. The enzyme was stable at pH 7.0-9.0 but became unstable out of this range. The optimum pH was pH 7.0. The metal chelating agent, EDTA, and two metalloprotease inhibitors (*o*-phenanthroline and 2,2' bipyridine),

highly repressed the enzyme activity. The activity was totally lost when the enzyme was treated with 0.1 mM *o*-phenanthroline but 10% and 14% of its activity still remained in the presence of 2,2' bipyridine and EDTA, respectively. The relative activity of the fibrinolytic enzyme gradually decreased with increasing amounts of EDTA, however, it was alleviated to a large extent by the presence of Zn^{2+}. The enzyme was classified as a neutral metalloprotease with Zn^{2+} on its active site (Kim *et al.*, 1997).

Wang *et al.* (1999) studied the characteristics of a novel fibrinolytic protease (designated as SW-1) from *Streptomyces* sp. Y405 and found that the enzyme was entirely inhibited by PMSF and its activity was also inhibited by EDTA and lysine. Therefore, the enzyme was suggested to be a serine protease and metalloprotease, and that the lysine binding site might play a role in the activity. The fibrinolytic activity of SW-1 was stable between 4-37°C and pH 4.0-9.0 and the optimum pH was 8.0.

Hirasawa *et al.* (1997b) studied the effect of inhibitors on fibrinolytic activity of many fungi by mixing the inhibitors with culture broth. The enzyme activity of *L. sulphureus* was inhibited by sulfhydryl agents (ρCMB and iodoacetic acid) but was unaffected by serine protease inhibitors (PMSF, SBTI, TLCK, TPCK) and EDTA. However, the enzyme activity of *Coriolus* sp. was severely inhibited by serine protease inhibitors including ρ-nitrophenyl-ρ-guadinobenzoate-HCl (NPGB). NPGB and ρCMB completely inhibited the enzyme activity of *S. commune* while its activity was sensitive to PMSF and EDTA. The fibrinolytic activity from *L. sulphureus* and *Coriolus* was resistant at 60°C for 20 min, however, the activity from *S. commune* retained at 50°C during the same period of time. Fibrinolytic enzyme from the supernatant of *L. sulphureus* was stable in the narrow pH range of 4.0-4.5 while it was more broader pH range for stability of the supernatant of *S. commune* (pH 6.5-8.0) and *Coriolus* sp. (pH 3.0-10.0). The fibrinolytic enzyme from *S. commune*, *Coriolus* sp. and *L. sulphureus* was stable at –15°C

for 12 months. At 37°C, fibrinolytic activity from *S. commune* totally lost with in one day but 65% of the enzyme activity from *Coriolus* sp. still retained after 3 days. The culture supernatant from *L. sulphureus* was very resistant. Its activity still retained after 6 months incubation at 37°C.

Objectives :

1. To study factors affecting growth and fibrinolytic enzyme production of *S. commune* BL 23.
2. To partially purify and characterize fibrinolytic enzymes from *S. commune* BL 23