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Molecular Cloning, Expression and Purification of Fibrinolytic Enzyme from Medicinal Mushroom, *Cordyceps militaris*

朝 鮮 大 學 校 大 學 院

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밀리타리스 동충하초로부터 혈전분해효소 정제 및 특성 분석과 유전자 크로닝

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指導敎授 金 成 俊

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ABBREVIATIONS

ATCC.....American Type Culture Collection
AP..... Alkaline phosphatase
APMSF.....4-amidinophenyl-methane sulfonyl fluoride
A. mellea.....*Armillaria mellea*
BSA .....Bovine serum albumin
CM.....Carboxymethyl
C. militaris.....*Cordyceps militaris*
CVD .....Cardiovascular disease
C. sinensis.....*Cordyceps sinenesis*
DEAE .....Diethylaminoethyl
DEPC..... Diethyl-pyrocarbonate
DIG.....Digoxigenin
E. coli ..... *Escherichia coli*
EDTA ..... Ethylenediamine tetraacetic acid
EtBr ..... Ethidium bromide
FDP.....Fibrin degradation product
Cmfe .....Fibrinolytic enzyme gene
CmFE .....*Cordyceps militaris* fibrinolytic enzyme
FPLC .....Fast protein liquid chromatography
F. velutipes.....*Flammulina velutipes*
G. frondosa .....*Glifora frondosa*
ICH .....Intracranial hemorrhage
IPTG .....Isopropyl-β-D-thiogalactopyranoside
NCBI .....National Center for Biotechnology Information

N.D. .....Not determined
OD ..... Optical density
PA .....Plasminogen activator
PAGE..... Polyacrylamide gel electrophoresis
PCR ..... Polymerase chain reaction
PDA.....Potato dextrose agar
PDB ..... Potato dextrose broth
PMSF.....Phenylmethylsulfonyl fluoride
P. ostreatus .....*Pleurotus ostreatus*
PVDF..... Polyvinylidine difluoride
RACE .....Rapid amplification of cDNA ends
RT-PCR..... Reverse transcriptase-polymerase chain reaction
SDS.....Sodium dodecyl sulfate
SK.....Streptokinase
SP .....Specific primer
TCA..... Trichloroacetic acid
TEMED ..... *N, N, N', N'*-tetramethylethylenediamine
TLCK .....*N*-α-tosyl-l-lysine chloromethyl ketone
tPA .....Tissue-type plasminogen activator
TPCK..... *N*-α-tosyl-l-phenylalanine chloromethyl ketone
T. saponaceum..... *Tricholoma saponaceum*
UK ..... Urokinase
uPA ..... Urokinase-type plasminogen activator
UTR.....Untranslated region
WHO ..... World Health Organization

ABSTRACT

Molecular Cloning, Expression and Purification of Fibrinolytic Enzyme from Medicinal Mushroom, *Cordyceps militaris*

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Blood clots are formed by the conversion of fibrinogen into fibrin via the proteolytic action of thrombin and subsequently, the formation of insoluble fibrin clots. These fibrin clots are dissolved by the hydrolysis of plasmin, which is activated from plasminogen by tissue plasmino gen activator (PA). The hydrolysis of fibrin is also known as fibrinolysis. Fibrin clot formation and fibrinolysis are normally well balanced in biological systems. However, when fibrin is not hydrolyzed due to some disorder, thromboses can occur, leading to serious consequences in human body such as apopelexy, arterio sclerosis, cerebral infraction, cerebral hemorrhage and myocardial infarction.

The fibrinolytic agents available today for clinical use are mostly plasmin ogen activator such as a tissue-type plasminogen activator (tPA), a urokinase -type plasminogen activator (uPA), and the bacterial plasminogen activator

streptokinase (SK). Despite their widespread use, all these agents have unde sired side effects, exhibit low specificity for fibrin, and are also relatively expensive. Therefore, the searches for other fibrinolytic enzymes from vario us sources are being continued. Recently, an investigation was conducted involving the isolation of fibrinolytic enzyme from natural extracts, as the fibrinolytic proteases used in thrombolytic therapy exhibit high specificity for fibrin, and are relatively inexpensive.

Several nontoxic mushrooms contain biologically active substances. There extracts have been reported to exert hematological, antiviral, anti tumorigenic, hypotensive, and hepatoprotective effects. Mushrooms constitute an important source of thrombolytic agents. Korean traditional anecdotes suggest that mushrooms can be, and have been, used in the treatment and prevention of thrombosis. Furthermore, some reports have described fibrinolytic activity occurring in some edible mushrooms, including *Flammulina velutipes*, *Pleurotus ostreatus*, *Grifola frondosa*, *Tricholoma saponaceum*, *C. militaris* and *Armillaria mellea*.

A fibrinolytic enzyme was purified from the *C. militaris* by ion-exchange chromatography followed by gel filtration and fast protein liquid chromato graphy. The purification protocol resulted in a 191.8-fold purification of the enzyme, with a final yield of 12.9%. The apparent molecular mass of the purified enzyme was estimated to be 52 kDa by SDS–PAGE, fibrin-zymo graphy and gel filtration chromatography, which revealed a monomeric form of the enzyme. The optimal reaction pH and temperature were pH 7.4, and 37 °C, respectively. This protease effectively hydrolyzed fibrinogen, preferentially digesting the Aα-chain over the Bβ- and γ-chains. Enzyme

activity was inhibited by Cu^{2+} and Co^{2+}, but enhanced by the addition of Ca^{2+} and Mg^{2+} ions. Furthermore, fibrinolytic enzyme activity was potently inhibited by phenylmethylsulfonyl fluoride (PMSF) and 4-amidinophenyl-methane sulfonyl fluoride (APMSF), and was found to exhibit a higher specificity for the substrate S-2586 for chymotrypsin, indicating that the enzyme is a chymotrypsin-like serine protease. The first 19 amino acid residues of the N-terminal sequence were **ALTTQSN VTHGLATISLRQ**, which is extremely similar to the subtilisin-like serine protease PR1J (NCBI Accession No. CAC95048).

The subtilisin like fibrinolytic enzyme gene of *C. militaris* was cloned and its nucleotide sequence was determined. The nucleotide sequence revealed one large open reading frame, composed of 1,197 base pairs which were translated into 398 amino acids. The deduce amino acid sequence of fibrinolytic enzyme from *C. militaris* is composed of two domains peptidase -S8 and subtilisin-N domain. Peptidase-S8 (Thr_{139}~Asn_{395}) belongs to subtil ase family which is a family of serine proteases. They appear to have indep endently and convergently evolved an Asp/Ser/His catalytic triad, like that found in the trypsin serine proteases. Subtilisin-N domain (Asp_{33}~His_{116}) has subtilisin N-terminal region. This family is found at the N-terminus of a number of subtilisins.

The *Cmfe* was cloned to plasmid pQE30 in the correct reading frame, with an N-terminal 6X His-tag sequence under the control of the T5 promoter and *lac* operator. The *Cmfe* was inserted and orientation in the plasmid pQE+ *Cmfe* was confirmed by double restriction enzyme mapping. The plasmid pQE30+*Cmfe* were transformed into *E. coli* expression host M15. All the

picked clones were expressed the CmFE with a predicted band around 52 kDa. Interestingly all the recombinant protein was found in insoluble pellete which were conformed by the western blot analysis.

In conclusion, the fibrinolytic enzyme purified from *C. militaris* exhibits a profound fibrinolytic activity, and also evidences relatively high substrate specificity to fibrin. Therefore, *C. militaris* may become a new source for thrombolytic agents, and can be used to develop therapeutic agents for the treatment of thrombosis. In addition, the work described here provided a way to obtain a single component with fibrinolytic activity and baseline information for further study on understanding of the structure and function relationship of the enzymes.

I. INTRODUCTION

The maintenance of homeostasis is essential to avoid bleeding and thrombosis. As shown in Fig. 1 and Fig. 2, this is achieved by the sequential and short-lived activation of the coagulation cascade series of enzymes, ultimately resulting in the production of an insoluble fibrin clot (Theopold et al., 2004). Both genetic and environmental factors can cause alterations in the level of activation of these enzymes, and for this reason a number of fail-safe inhibitory mechanisms are required if thrombosis is to be prevented.

These pathways remove any excess procoagulant enzymes and prevent systemic fibrin production (Norris, 2003). As shown in Fig. 3, fibrinolysis inhibitors are also essential to prevent inappropriate breakdown of fibrin and fibrinogen. Both thrombin and fibrin orchestrate their own demise, thrombin by triggering the activation of protein C and fibrin by stimulating plasmin production and fibrinolysis (Fig. 4) (Norris, 2003). Fibrin clot formation and fibrinolysis are normally well balanced in biological systems. However, when fibrin is not hydrolyzed due to some disorder, thromboses can occur. Myocardial infarction is the most common of these thromboses.

According to the World Health Organization (WHO) report, cardio-vascular disease (CVD) is the single biggest cause of mortality worldwide, estimated at 17 million deaths per annum (Longstaff and Thelwell, 2005; WHO, 2002). CVD affects high income nations and increasingly CVD is a problem for low and middle income nations. Developing countries accounted for 60% of the total burden of coronary heart disease in 2002.

But thrombolytic therapy is a cost effective way of improving survival

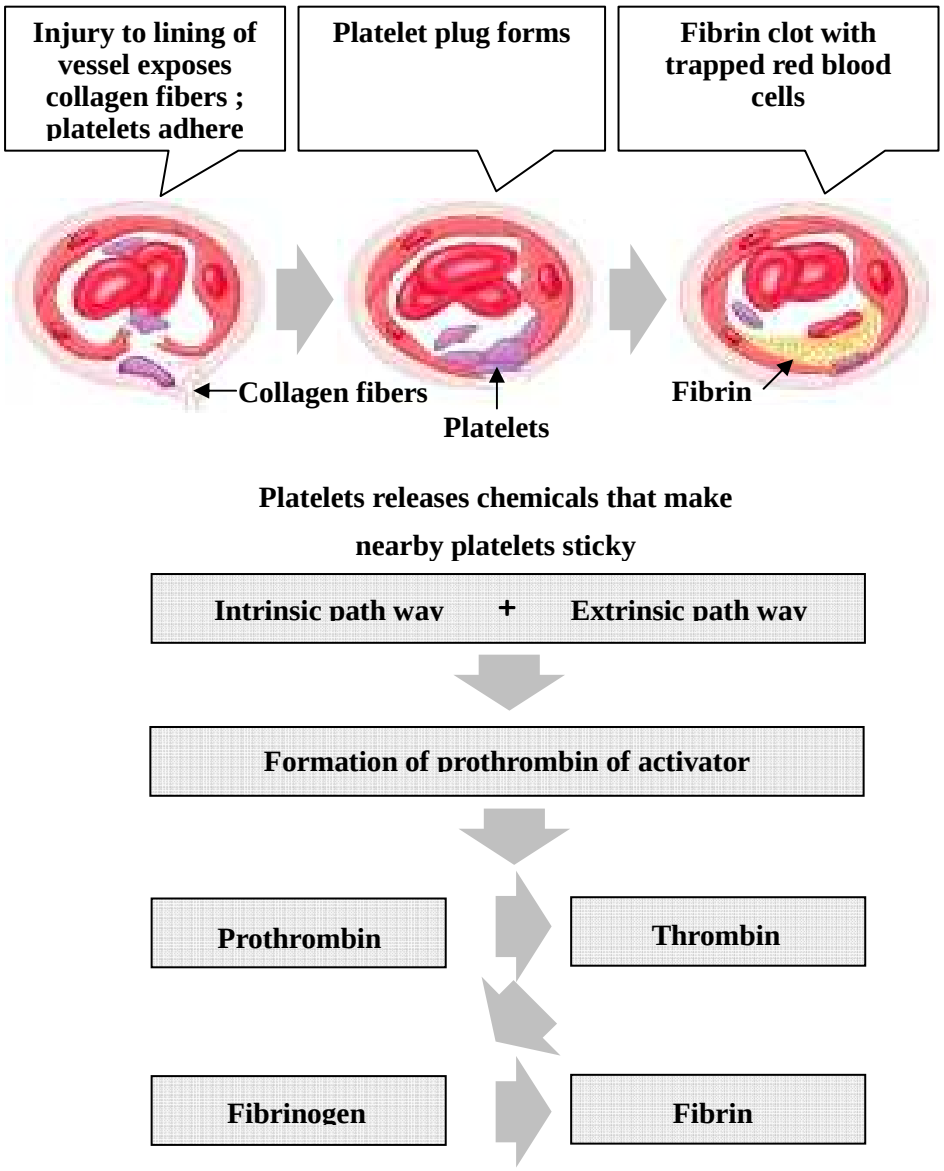


Fig. 1. Schematic diagram of blood clotting.

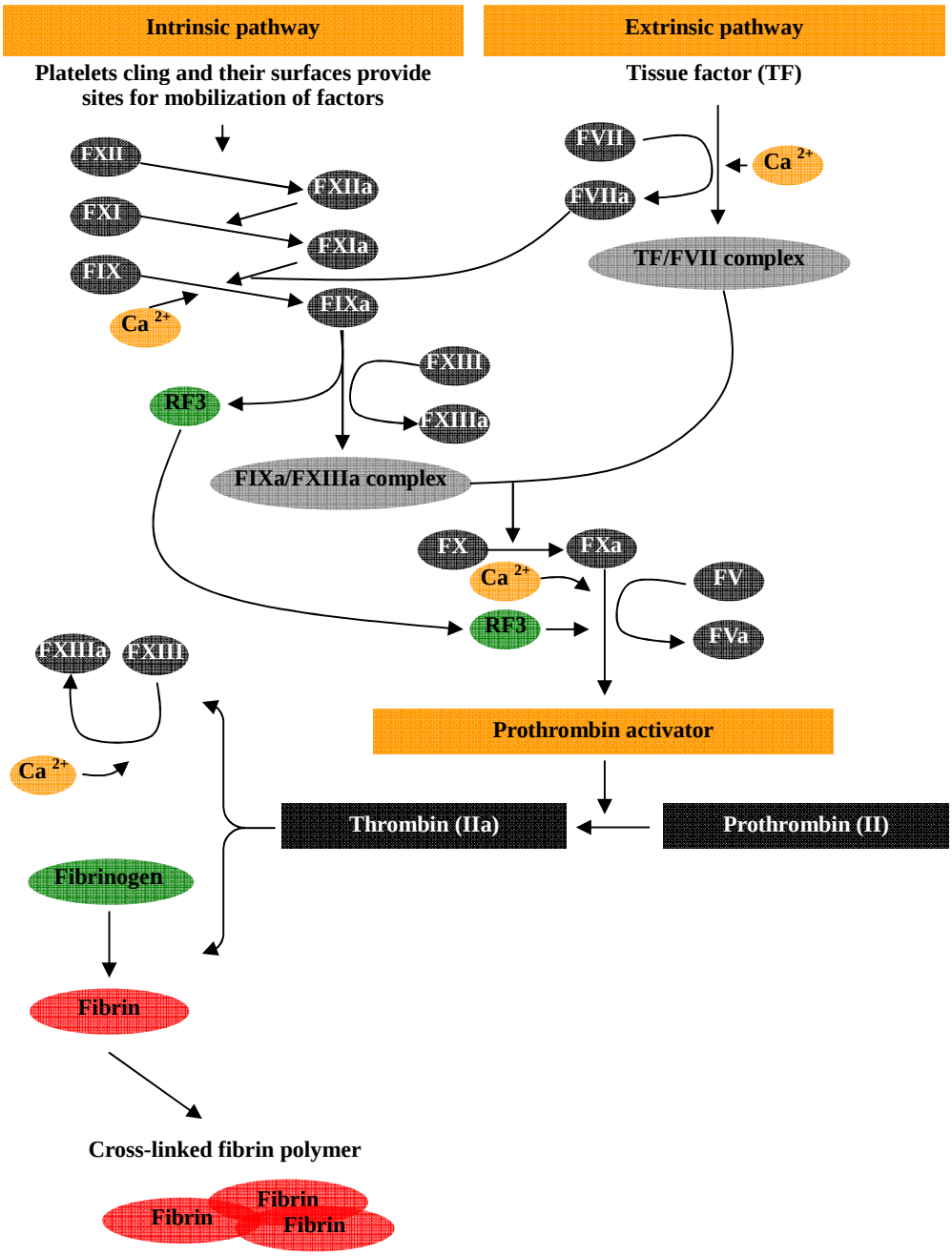


Fig. 2. The pathway of blood clotting by intrinsic and extrinsic pathway.

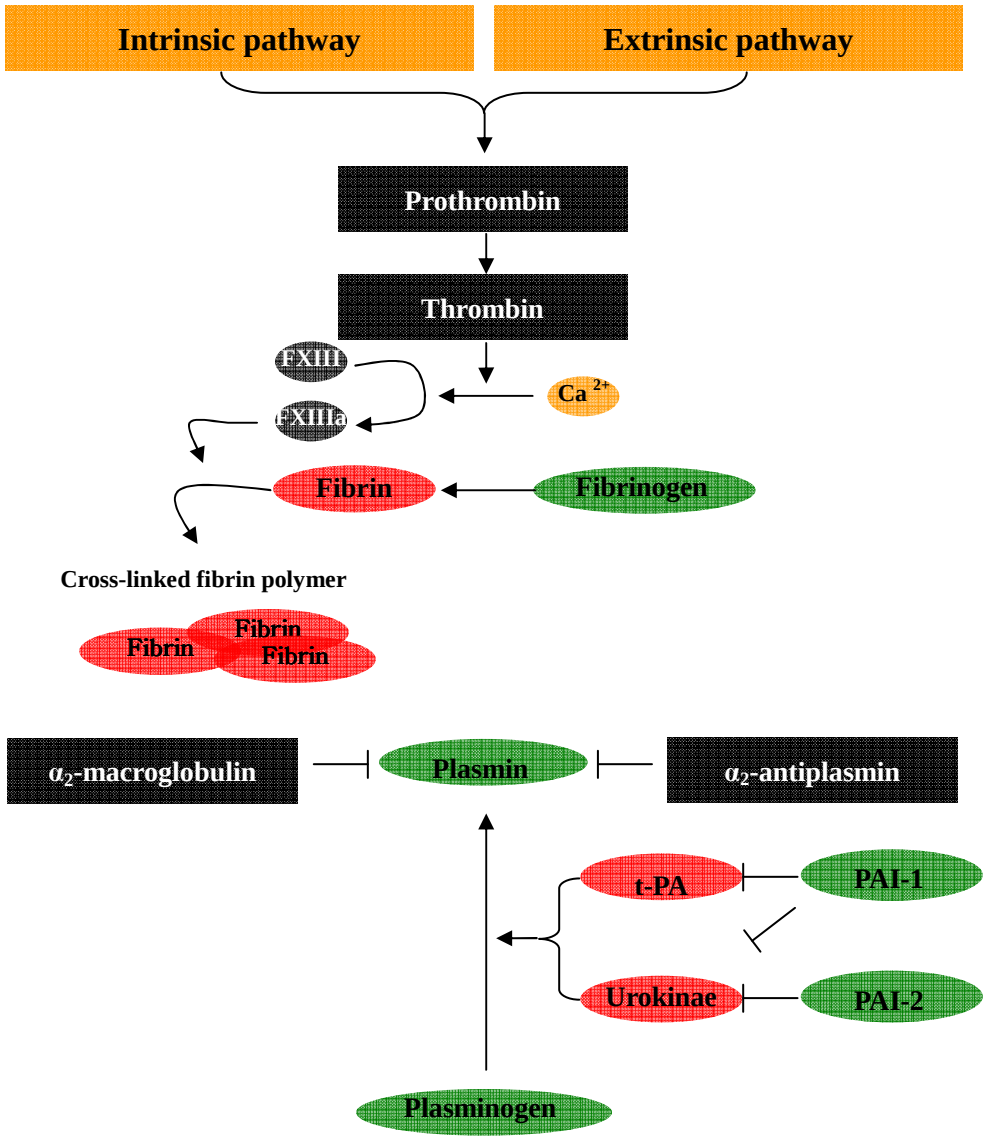


Fig. 3. Schematic diagram of fibrinolytic system.

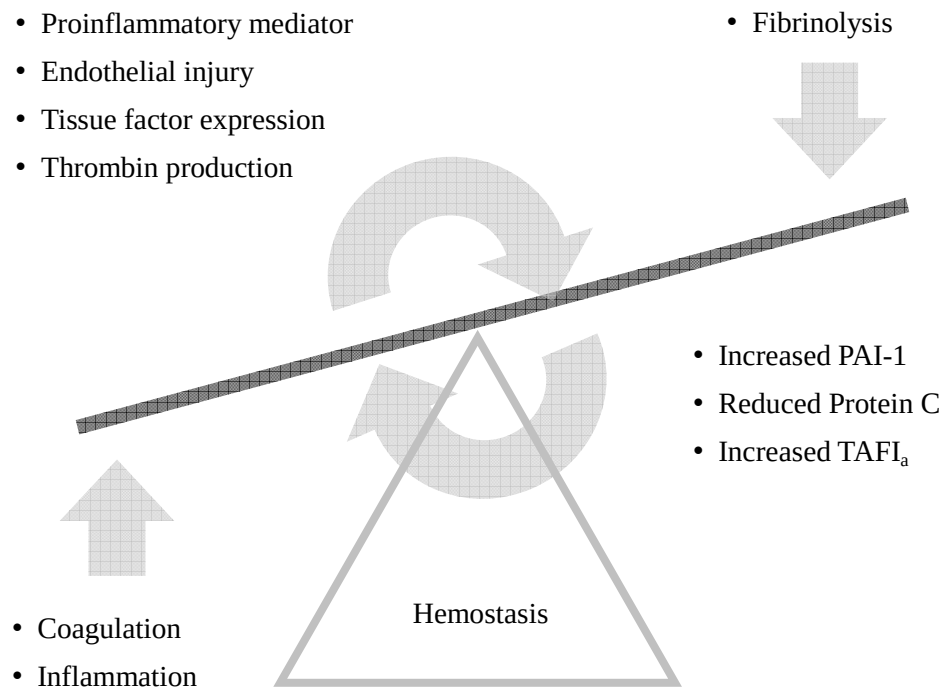


Fig. 4. Hemostasis between fibrinolysis and thrombosis.

from myocardial infarction in developed (Werf et al., 1995) and developing countries (Critchley, 2004). Fibrinolysis is the biochemical process underlying thrombolysis since both processes depend on the degradation of the fibrin network that holds the blood clot together in a coronary or cerebral artery in the case of myocardial infarction (heart attack) or ischaemic stroke, respectively. In a simplified form, fibrinolysis can be represented as a two-step process according to Fig. 2 (Norris, 2003). The two stages are: The first stage is the activation of plasminogen to plasmin by a plasminogen activator such as tissue type plasminogen activator (t-PA), and second stage is the degradation of the structural protein, fibrin, to fibrin degradation products (FDP). The participants in first stage are assembled on the surface of fibrin fibres and the propagation of plasmin action in the circulation is limited by the potent plasma inhibitor system. Thrombolytic therapy is essentially the delivery of PA into the circulation. From the point of view of developing and improving thrombolytic therapy, the characteristics of the PA need to be optimized to promote rapid clot dissolution. The only way of doing this is to maximize the generation of plasmin while minimizing the side effects and this is why the characteristics of PAs are of overriding importance for successful therapy.

Historically, the first generation of thrombolytics (e.g., SK and UK) were systemic activators of plasminogen giving rise to large scale plasmin production in the circulation and concomitant loss of circulating plasminogen, fibrinogen and the plasma inhibitors. SK (and staphylokinase) belong to a group of plasminogen binding proteins secreted by invasive bacteria in order to generate plasmin and facilitate “bacterial metastasis” (Aisina et al., 2005; Bo

karewa et al.,2005 and Dahiya et al.,2005). UK is a naturally occurring protease that can be isolated on industrial scales from human urine, although it is present in circulation as an inactive, single chain pro-enzyme (Degryse et al., 2001; Behrendt and Danø, 1996). Second generation thrombolytics with fibrin binding domains were produced by genetic engineering with the seem ingly rational aim of targeting the PA to a fibrin clot. Thus, the development of tPA, for example, was driven by the theory that since tPA has to bind to fibrin to express significant activity, administered tPA would be targeted at the clot and systemic plasmin generation would be limited (Mölkänen et al., 2002). The idea was that this would be a good thing as systemic loss of plasminogen substrate would be reduced; and possibly bleeding complications (for example intracranial hemorrhage (ICH), which occurs in up to 3% of patients receiving treatment) might be reduced. Following on from tPA, the so-called third generation of thrombolytics were engineered. The goal was improved properties in terms of longer plasma half life, or resistance to natural inhibitors, or improved fibrin binding and "zymogenicity," making them even more fibrin specific (e.g., reviewed (Werf, 1993; and Verstraete, 2000). Many of these variants used the basic tPA template but domains were deleted or modified with the stated aim of improving properties. In spite of the huge and rising global burden of CVD and concomitant massive financial investment by the pharmaceutical industry, protein engineering of second and third generation thrombolytics may be interpreted as a failure. Basic scientific considerations, *in vitro* data and animal studies have not been translated into significant improvements in outcomes in clinical trials (Monrad, 1991 and Collins et al., 1997). This lack of clear improvement and

the much increased cost of second and third generation thrombolytics means that SK is still the most widely used thrombolytic for the treatment of acute myocardial infarction in many parts of the world.

To date no further significant progress has been seen with other tPA-based thrombolytics such as reteplase, monteplase, lanoteplase or pamiteplase for example, or chimeras such as amediplase (the fibrin binding kringle 2 of tPA fused with the serine protease domain of uPA which has better catalytic parameters than tPA), or other molecules such as saruplase (single chain urokinase) or staphylokinase (Verstraete, 2000). Despite all the detailed investigations of protein biochemistry on these molecules the only advantage of the newer thrombolytics is extended plasma half life enabling the delivery of the drugs as a bolus injection rather than the longer infusion of first and second generation treatments. This may be a significant improvement in reducing the lag time from symptoms to treatment since reteplase (Topol, et al., 2000) may be given by paramedics before arrival at hospital in 2 bolus injections and tenectaplase as a single bolus injection (Llevadot, et al., 2001; and Sinnaeve, et al.,2003). Hence, so far the only improvements in thrombolytic therapy relate to the pharmacokinetics of the drugs, not other properties such as fibrin binding or specificity, or inhibitor susceptibility. Our current understanding of PA activity in fibrin has been inadequate to permit the successful rational design of new molecules or optimized treatment regimes.

The research areas of functional foods and nutraceuticals are rapidly expanding throughout the world. Scientists are actively working on the health benefits of foods by identifying the functional constituents, elucidating the biochemical structures, and determining the mechanisms behind the

physiological roles. These research findings contribute to a new nutritional paradigm, in which food constituents go beyond their role as dietary essentials for sustaining life and growth, to one of preventing, managing, or delaying the premature onset of chronic disease later in life (Fitzpatrick, 2000; Kim et al., 2003, and Hasler 1998).

Fibrinolytic enzymes can be widely found in nature (Mine et al., 2005). They have been found in hemorrhagic toxin from snake venom (Nikai, et al., 1984), earthworm secretions (Mihara, et al., 1991 and Jeon, et al 1995), food-grade microorganisms (Choi and Sa, 2000), marine creatures (Sumi et al., 1992), and herbal medicines. In particular, a fibrinolytic protease has been isolated from *Spirodela polyrhiza*, an ingredient of traditional Oriental medicine that has been used for lowering blood pressure and detoxification of snake venom (Nikai, et al., 1984). Also, strong fibrinolytic enzymes are produced by *Bacillus* sp. strains that are used in food fermentation (Sumi et al., 1987; Fujita et al., 1993; Sumi et al., 1995; Kim et al., 1996; Sumi et al., 1990; Chang et al., 2000; Jeong et al., 2001 and Wong et al., 2004), invertebrates like *Stichopous japonicus* (Suzuki et al., 1991; Sugiua et al., 1992; Sumi et al., 1992 and Arocha-Pinango et al., 1999), as well as the seaweed *Codiales codium* (Jeon, et al., 1995).

As shown in edible food source, fibrinolytic enzymes can be found in a variety of foods, such as Japanese *Natto* (Sumi et al., 1987), Korean *Chung kook-Jang* soy sauce (Kim et al., 1996), and edible honey mushroom (Kim and Kim, 1999). Enzymes have been purified from these foods, and their physiochemical properties have been characterized. Among the food sources, fermented food products have been the focus of research. In particular, oral

administration of the fibrinolytic enzyme extracted from Japanese *natto* can enhance fibrinolysis in dogs with experimentally induced thrombosis (Sumi, et al., 1987). Lysis of the thrombus can be observed by angiography. More importantly, fibrinolytic activity, the amounts of t-PA, and fibrin degradation by-product in the plasma are doubled when nattokinase is given to human subjects by oral administration. The underlying mechanism involves the absorption of the administered *natto* enzyme across the intestinal tract, and the release of endogenous PA that induces fibrinolysis in the occluded blood vessel (Sumi, et al., 1987).

Mushrooms are commonly used as food and food flavoring substance and also in traditional oriental medicine. Mushrooms have become attractive as functional foods and as a source of physiologically beneficial substances. In addition, several nontoxic mushrooms contain biologically active substances.

There extracts have been reported to exert hematological, antiviral, antitumorigenic, hypotensive, and hepatoprotective effects (Chang and Miles, 1989; Hobbs, 1995 and Chang, 1996). Mushrooms constitute an important source of thrombolytic agents. Korean traditional anecdotes suggest that mushrooms can be, and have been, used in the treatment and prevention of thrombosis (Kim and Kim, 1995). Furthermore, some reports have described fibrinolytic activity occurring in some edible mushrooms, including *Flammulina velutipes*, *Pleurotus ostreatus*, *Grifola frondosa*, *Tricholoma saponaceum*, and *Armillaria mellea* (Nonaka et al., 1995; Kim et al., 1998; Shin and Choi, 1999; Choi et al., 1999; Kim and Kim, 2001; Joh et al., 2004; and Lee et al., 2005).

The among of medicinal mushrooms, Fungi belonging to the *Cordyceps*

species are especially popular and have long been used as food and herbal medicines in Asia (Huang et al.,2003; Weng et al.,2002). Approximately 400 species of *Cordyceps* are known. They are distinguished from one another and classified according to the color and shape of their fruiting bodies, possession of spores, shape, and host insect species and by other morphological characteristics.

The ascomycetous genus *Cordyceps* is an entomo, pathogenic fungus. Pharmacologically, the immunomodulation and antioxidant activities of *Cordyceps cicadae* (*C. cicadae*) (Weng et al., 2002) have been exploited in addressing hyperglycemia, respiratory disease, liver disease, and renal dysfunction. Both *C. cicadae* and *C. ophioglossoides* exhibit anti tumor activity (Weng et al., 2002**;** Yanada, 1984**)**. Perhaps one of the most fascinating *Cordyceps sp.* is *Cordyceps sinensis* (*C. sinensis*) found in mountains of China (Yalin, et al., 2005**)**. For centuries, *C. sinensis*, a caterpillar fungus, has been used in China as food and herbal medicine for a variety of diseases. *C. sinensis* extract has been used for treatment of hyperglycemia, respiratory and liver diseases, renal dysfunction, and renal failure and as antioxidant (Li et al., 2003; Li et al., 2001(a) and Li et al., 2001(b)). Furthermore it possesses a polysaccharide that protects against free-radical-induced neuronal cell toxicity (Li et al., 2003**)**. *C. pruinosa* has been used to treat stomach disorders, inflammatory disease, and endotoxin shock or sepsis (Fitzpatrick, 1999).

C. militaris belongs to entomogenous fungal species and is important in bio-control of pine moth populations (Alicja, 1998**)**. The entomogenous fungal species invade and proliferate within insect larvae, causing a

systematic infection that eventually kills the hosts (Clarkson and Charnley, 1996). Mechanisms for intracellular survival of *C. militaris* mycelium in caterpillar larvae are unknown, but the fungal pathogens are endowed with mechanisms that actively abrogate the oxidative defense responses of hosts by an array of enzymes. *C. militaris* has been reportedly used in the treatment of geriatric patients with palsy, dizziness, headache, neurasthenia, insomnia, numbness in limbs, and infantile convulsion, and also reportedly exerts neuro-protective effects (Wang, et al., 2005).

There is a renaissance of interest in using fibrinolytic enzymes as targets for developing therapeutic agents. Protease genes from several bacteria, fungi, and viruses have been cloned and sequenced with the prime aims of overproduction of the enzyme by gene amplification, delineation of the role of the enzyme in pathogenecity, and alteration in enzyme properties to suit its commercial application. Protein engineering techniques have been exploited to obtain proteases which show unique specificity and/or enhanced stability at high temperature or pH or in the presence of detergents and to understand the structure-function relationships of the enzyme. Protein sequences of acidic, alkaline, and neutral proteases from diverse origins have been analyzed with the aim of studying their evolutionary relationships. Despite the extensive research on several aspects of proteases, there is a paucity of knowledge about the genetic analysis of fibrinolytic enzymes. Deciphering these secrets would enable us to exploit fibrinolytic proteases for their applications in biotechnology.

Gene cloning is a rapidly progressing technology that has been instrumental in improving our understanding of the structure-function relationship

of genetic systems. It provides an excellent method for the manipulation and control of genes. More than 50% of the industrially important enzymes are now produced from genetically engineered microorganisms (Hodgson, 1994).

Although research has classically focused on the therapeutic effects of *C. militaris*, little information is available in regard to its fibrinolytic activity. In addition, despite the several reports on the cloning and sequencing the protease gene from different sources, there is no information on genetic analysis of fibrinolytic enzymes from *C. militaris.* Therefore, in this paper, we described the purification, characterization, cloning and expression of a fibrinolytic enzyme from the *C. militaris*.

II. MATERIALS AND METHODS

II-A. Materials

For the protein purification and characterization, *C. militaris* was purchased from American Type Culture Collection (ATCC). Bovine fibrinogen, bovine fibrin, human fibrinogen, human fibrin, bovine thrombin (1,000 units), human thrombin (2units/mg), plasmin (10 units), azocasein, sodium doecyl sulfate (SDS), *N*,*N*'-methylenebisacrylamide, glycine, sodium chloride (NaCl), citric acid monohydrate, ethylenediamine tetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF), *N*-α-tosyl-l-lysine chloromethyl ke tone (TLCK), *N*-α-tosyl-l-phenylalanine chloromethyl ketone (TPCK), and 4-amidinophenylmethane sulfonyl fluoride (APMSF) and polyvinylidine difluoride (PVDF) were purchased from the Sigma-aldrich Co. (USA). CM-cellulose, DEAE sephadex A-50, Sephadex G-75 and HiLoad 16/60 Superdex 75 pg were purchased from Pharmacia Biotech (Sweden). Agarose, ProSieve color protein marker and ProSieve protein marker were purchased from Cambrex Co. (USA). Chromogenic subtarates were purchased Chromogenix Co. (Sweden).

For the cloning and cDNA synthesis, pGEM T-easy vector cloning system kit, Universal RiboClone® cDNA Synthesis System, DNA ligase, RNase, DNase and restriction enzyme such as *Eco*RI, *Bam*HI, *Hin*dIII etc. were purchased from the Promega Co.(USA). 5'/3' rapid amplification of cDNA ends (RACE) kit and digoxigenin (DIG) labeling kit were purchased from Roche Diagnostics Co. (Switzerland). Trizol™ was purchased from invitro-

gen Co. (USA) M-MLV, AMV, dNTPs, RNase inhibitor, Taq-polymerase, DNA size marker and synthetic specific primer were purchased from Bioneer Co. (Korea). Ex-taq polymerase was purchased from TAKARA Co. (Japan).

For the expression, pQE expression kit, plasmid DNA isolation kit, Ni-NTA spin kit and DNA gel elution kit were purchased from Qiagen Co. (USA). Isoprophanol, chloroform, ethanol, trizma base, isopropyl-β-D-thiogalactopyranoside (IPTG), ampicillin, tetracycline, kanamycin, diethyl-pyroc arbonate (DEPC) and other chemicals were purchased from Sigma-aldrich Co. (USA). Other reagents were special grade, and were purchased commercially.

II-B. Cultivation of *C. militaris*

The stock culture of *C. militaris* was maintained on PDA slants. The slants were inoculated with mycelia and incubated at 25°C for 7 days, and then used for seed culture inoculation. The mycelia of *C. militaris* were transferred to the seed culture medium by punching out about 5 mm^2 of the slants with a sterilized cutter. The seed were inoculated into 500 ㎖ flasks containing 200 ㎖ synthetic medium (40 g/ℓ glucose, 10 g/ℓ yeast extract, 0.5 g/ℓ KH_2PO_4, 0.5 g/ℓ $K_2HPO_4 \cdot 3H_2O$, and 0.5 g/ℓ $MgSO_4 \cdot 7H_2O$) and incubated at 25℃ on a rotary shaker (110 rpm) for 5 days. To cultivate *C. militaris* fruiting body, 100 g of silk warm larva were packed in the culture bottle and sealed using a polypropylene, sterilized at 121℃ for 90 min. Sterilized bottles contained silk warm larva were inoculated with seed of *C.*

militaris mycelia and incubated for 20 days at 25℃. Soon after the hyphae of *C. militaris* reached the bottom of the bottles, the bottles were moved to a cold room at 16℃ with 95% relative humidity and under incandescent light of 1,000 Lux to induce primordial formation. The incubator containing *C. militaris* was ventilated four times a day to provide fresh air.

II-C. Preparation of crude extracts

All procedures were carried out at 4°C. *C. militaris* fruiting bodies were removed and immediately stored in a -70°C freezer. Frozen mushroom were thawed and homogenized with equal volume of water in a Kenwood blender for 2min at maximum speed. The homogenate was centrifuged at 600 ×*g* and 4°C for 30 min. The crude extract was placed on the ice. An equal volume of pre-chilled ethanol was added, drop-wise, with constant stirring after which the solution was kept stirring for a further 1hr. Precipitated protein was removed by centrifugation at 600 ×*g* for 30 min at 0°C. The clarified ethanol-soluble fraction was returned to the ice. And its ethanol concentration was increased, drop-wise, to 70% with constant mixing. Stirring was continued for 1hr after which precipitated protein was recovered by centrifugation at 600 ×*g* for 30 min and -4°C. Following removal of the supernatant the pellets were dried and the protein was then re-suspended in optimal buffer for ion exchange chromatography. Insoluble material was removed by centrifugation at 10,000 ×*g* for 10 min at 4°C.

II-D. Fibrinolytic activity assay.

II-D-1. Proteolytic activity assay

Protease activity was determined by measuring the release of acid-soluble material from azocasein (B.C.W. Hummel et al., 1965). 50 μℓ of enzyme sample was added to 300 μℓ of 0.1% (w/v) azocasein in 50 mM tris-HCl, pH 7.0. Following 20 min of incubation at 37℃, 600 μℓ of ice-cold 10% (w/v) trichloroacetic acid (TCA) was added, with vortex. The sample was placed on ice for 10 min before centrifugation at 12,000 ×*g* for 15 min. The quantity of acid-soluble material in the supernatant was measured by absorbance at 366 nm. 1.0 unit of protease activity was defined as the amount required to produce enough acid-soluble material from azocasein to yield an absorbance of 0.1 at 366 nm, following 1hr of incubation at 37℃.

II-D-2. Preparation of fibrin plate

Fibrinolytic activity was determined by measuring the halo of lysis region on the fibrin plate (Astrup et al., 1952). 2㎖ of bovine fibrinogen solution (50 ㎎/㎖ in 20 mM tris-HCl buffer, pH 8.0) was completely mixed with 20 ㎖ agar solution (0.75% agar in 20 mM tris-HCl buffer, pH 8.0) at 37°C. The mixed solution was poured into petri-dish (5.5-cm diameter) with 20 μℓ thrombin solution (100 NIH units/㎖ bovine thrombin in 20 mM tris-HCl buffer, pH 8.0). The plate was allowed to stand for 1hr at room temperature to form fibrin clots, and holes were made on the fibrin plate by using a capillary glass tube (3㎜ in diameter).

II-E. Purification of fibrinolytic enzyme

II-E-1. Determination of protein concentration

Protein concentration was determined by sing BCA protein assay reagent kit (Pierce Co., USA). And bovine serum albumin (BSA) was used as a protein standard.

II-E-2. Cation exchange chromatography on CM-cellulose column

CM-cellulose for cation exchange chromatography was swollen at 10 mM citrate-NaOH, pH 6.0. Remove the supernatant and replace with fresh same buffer several times during the swelling periods. The crude extracts were applied to a CM-cellulose column (5×10 cm) pre-equilibrated with the same buffer, and eluted with a linear gradient of 0 to 1.0 M NaCl (pH 6.0) at a flow rate of 0.5 ㎖/min at 4°C. Active fractions were pooled and concentrate-ed by freeze-drying and desalting. Desalted active faction was dissolved 10mM tris-HCl, pH 7.4 for anion exchange chromatography on DEAE sephadex A-50 column.

II-E-3. Anion exchange chromatography on DEAE sephadex A-50 column

DEAE sephadex A-50 was swollen at the 10mM tris-HCl buffer, pH 7.4. For the complete swelling, DEAE sephadex A-50 was boiled for 2 hrs at

90°C and was degassed under vacuum. The active fraction was applied to a prepared DEAE sephadex A-50 column (5×10 cm) pre-equilibrated with the 10 mM tris-HCl buffer (pH 7.4), and eluted with a linear gradient of 0 to 1.0 M NaCl (pH 7.4) at a flow rate of 0.5 ㎖/min at 4°C. Active fraction was pooled and concentrated by freeze-drying and desalting. Desalted active faction was dissolved 10mM tris-HCl, pH 7.4 contained 0.15 M NaCl for gel filtration chromatography on sephadex G-75 column.

II-E-4. Gel filtration chromatography on sephadex G-75 column

In order to further purify the sample, gel filtration was performed with a Sephadex G-75 column (1.5×100 cm) in 10 mM tris-HCl buffer (pH 7.4) containing 0.15 M NaCl at a flow rate of 0.1 ㎖/min. Active fraction was pooled and concentrated by freeze-drying and desalting. Desalted active faction was dissolved in 0.05 M Phosphate buffer contained 0.15 M NaCl (pH 7.4) for FPLC on HiLoad 16/60 Superdex 75 pg column (Amersham Bioscience Co.).

II-E-5. Fast protein liquid chromatography (FPLC) on HiLoad 16/60 Superdex 75 pg column

Further fractionated using a HiLoad 16/60 Superdex 75 pg column equilibrated with 0.05 M phosphate buffer containing 0.15 M NaCl (pH 7.4) at a flow rate of 1.0 ㎖/min. Active fractions were pooled and concentrated by centricon 30,000 (Amicon Co. USA), and analyzed for purity by SDS-

PAGE.

II-F. Molecular weight determination

II-F-1. Size exclusion using FPLC

Size exclusion was performed at room temperature, using a HiLoad 16/60 Superdex 75 pg column (on ÄKTA FPLC, Amersham Bioscience Co.) for the estimation of the molecular weight of the enzyme. The elution buffer was 0.05 M phosphate containing 0.15 M NaCl at a flow rate of 1.0 ㎖/min. A gel filtration protein marker comprising glyceraldehydes-3-phosphate dehydrogenase (35 kDa), carbonic anhydrase (29 kDa), trypsinogen-PMSF (24 kDa) and trypsin-inhibitor (20.1 kDa) was used.

II-F-2. SDS-PAGE

SDS-PAGE was performed in a Bio-Rad Mini protean II electrophoresis cell (Bio-Rad Laboratories) according to the method of Laemmli (1970). Electrophoreses were run on polyacrylamide gels (stacking: 5% and separating: 9%) with a migration buffer consisting of a 0.05 M tris–HCl (pH 8.8), 0.4 M glycine and 0.1% SDS solution. The samples were diluted in a sample buffer consisting of a 60 mM tris–HCl (pH 6.8), 25% glycerol, 14.4mM ß-mercaptoethanol, 0.1% bromophenol blue and 2% SDS solution. The electrophorized gel was stained with a staining solution (0.1% coomassie blue R-250, 45% methanol, 10% glacial acetic acid and 45% distilled water)

and destained with destaining solution (10% methanol, 10% glacial acetic acid and 80% distilled water). Molecular weight was estimated with protein molecular weight marker, prestained ProSieve protein marker (Cambrex co., USA).

II-F-3. Fibrin-zymography

Fibrin-zymography was carried out according to the methods of Kim et al. (1999) and Choi et al. (2000). Resolving gel solution (12 %) containing 0.12 % (w/v) fibrinogen was prepared in a total 10 ㎖ volume, and then centrifuged in order to remove insoluble impurities which were introduced when the SDS stock solution was mixed. Thrombin (1.0 unit/㎖) solution and TEMED (*N, N, N', N'*-tetramethylethylenediamine) were added to the gel solution in final concentrations of 0.1 μunit/㎖ and 0.028 % (v/v), respectively. Purified enzyme was electrophorized on fibrin gel, washed in 2.5% Triton X-100 solution, and incubated in a bath containing reaction buffer (prepared 20 mM tris-HCl, pH 7.5 containing 0.15 M NaCl, 0.02 % NaN_3) at 37°C for 12~15 hrs.

II-G. Determination of N-terminal sequence and structure alignment

The N-terminal amino acid sequence of purified fibrinolytic enzyme was determined using an Applied Biosystems Precise 491 amino acid sequencer at the Korea Basic Science Center in Seoul. Sequenced data and sequence alignment were analyzed using Blast in NCBI protein database and default

parameters.

II-H. Characterization of purified fibrinolytic enzyme

II-H-1. Degradation patterns of fibrin and fibrinogen

Degradation pattern analysis of fibrin and fibrinogen were analyzed by SDS–PAGE according to the method of Laemmli (1970).

Fibrin degradation analysis was performed by a slightly modified method of Datta et al. (1995). In brief, 10 ㎍ of 1.0% human fibrinogen solution (20 mM tris-HCl, pH 7.5, 0.15 M NaCl) was added to human thrombin (0.1 NIH unit), and then allowed to stand for 1hr at room temperature. Formed clots were mixed with 10 ㎍ of a purified enzyme and incubated at 37°C for various time intervals. Plasmin was used as a positive control.

Fibrinogenolytic activity was measured as follows: 10 ㎕ of 1.0% human fibrinogen was incubated with 10 ㎍ of a purified enzyme at 37°C for various time intervals.

II-H-2. Effect of temperature and pH on enzyme activity

The optimal temperature for activity of purified enzyme was determined by measuring residual activity after the incubation of 10 ㎕ of purified enzyme in 90 ㎕ of 20 mM tris-HCl (pH 7.5) at different temperatures (20~80°C) for 1hr. The optimal pH for the fibrinolytic activity of the purified enzyme was determined within a pH range of 2~10. 10 ㎕ of the

enzyme solution was added to 90 $\mu\ell$ of 0.5 M glycine-HCl (pH 2.0~3.0), 0.5 M acetate (pH 4.0~5.0), 0.5 M tris-HCl (pH 6.0~8.0), and 0.5 M glycine–NaOH (pH 9.0~10.0) buffers. After 1hr of incubation at room temperature, the remaining protease activity of each enzyme solution was measured with 0.1% azocasein.

II-H-3. Effect of metal ions and protease inhibitors on the enzyme activity

The effects of metal ions were investigated using $MgCl_2$, $ZnCl_2$, $CoCl_2$, $FeCl_2$, $CaCl_2$, and $CuSO_4$. The purified enzymes were pre-incubated in both the absence and the presence of bivalent cations, including Mg^{2+}, Ca^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, and Fe^{2+}, with a final concentration of 1.0 mM in 10 mM tris-HCl (pH 7.4) for 1hr at 37 ℃. After 1hr of incubation at room temperature, residual protease activity was measured with 0.1% azocasein. The purified fibrinolytic enzyme was incubated with 5.0 mM EDTA, 2.0 mM PMSF, 0.5 mM APMSF, 0.5 mM TPCK, 0.5 mM TLCK, 0.05 mM aprotinin, and 0.5 mM pepstain A at 37°C for 1hr. The residual enzyme activity was determined using azocasein.

II-H-4. Amidolytic activity of the enzyme

Amidolytic activities were measured spectrophotometrically, using synthetic

Table 1. List of synthetic chromogenic substrates

Chromogenic substrate	amino acid sequence	characteristics
S-2222	Bz-Ile-Glu-(OR)-Gly-Arg-pNA	for factor Xa
S-2288	H-D-Ile-Pro-Arg-pNA	for t-PA
S-2238	H-D-Phe-Pip-Arg-pNA	for thrombin
S-2251	H-D-Val-Leu-Lys-pNA	for plasmin and streptokinase-activated plasminogen
S-2444	pyroGlu-Gly-Arg-pNA	for urokinase
S-2586	MeO-Suc-Arg-Pro-Tyr-pNA·HCl	for chymotrypsin
S-2765	Z-D-Arg-Gly-Arg-pNA·2HCl	for factor Xa

chromgenic substrates (Table 1.) such as S-2222 (Bz-Ile-Glu-(OR)-Gly-Arg-pNA for factor Xa), S-2288 (H-D-Ile-Pro-Arg-pNA for tPA), S-2238 (H-D-Phe-Pip-Arg-pNA for thrombin), S-2251 (H-D-Val-Leu-Lys-pNA for plas min and SK-activated plasminogen), S-2444 (pyroGlu-Gly-Arg-pNA for U K), S-2586 (MeO-Suc-Arg-Pro-Tyr-pNA · HCl for chymotrypsin) and S-27 65 (Z-D-Arg-Gly-Arg-pNA · 2HCl for factor Xa). Activities were evaluated by the mixing of the 1 ㎍ of purified enzyme with 300 ㎕ of a 0.5 mM synthetic chromogenic substrate. After continuous measurement for 5min at 37°C with a temperature-regulated spectrophotometer, the amount of released *p*-nitro aniline was determined by measuring the change in 405 nm

II-I. The molecular cloning of fibrinolytic enzyme gene

II-I-1. Total RNA isolation

A piece of *C. militaris* was cut off from the fruiting body and ground to fine powder using a mortar and pestle with liquid nitrogen. Total RNA was extracted from 100 ㎎ of the powder using Trizol™ reagent (Invitrogen Co., USA). Briefly, 1.0 ㎖ of Trizol™ reagent was added to 100 ㎎ of fruiting body powder and mixed thoroughly. After then, 200 ㎕ of chloroform was added to the mixture and centrifuged at 12,000 ×*g* and 4℃ for15 min.

The supernatant was then transferred to a centrifuge tube containing 500 ㎕ of isopropanol, and incubated for 15 min at room temperature. The resulting mixture was then centrifuged at 12,000 ×*g* and 4℃ for 10 min. The visible RNA pellet was washed with 1.0 ㎖ 75% ethanol and resuspended

in diethyl-pyrocarbonate (DEPC) treated water. The concentration and purity of total RNA were measured at 260 nm and 280 nm.

II-I-2. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was reverse-transcribed with an oligo$(dT)_{18}$ and M-MLV reverse transcriptase (Bioneer Co., Korea). For synthesis of cDNA, 1 ㎍ of total RNA with 10 pmol of oligo(dT) was reacted for 10 min at 65℃, followed by addition of supplied mixture, and cDNA synthesis for 1 hour at 42℃. Synthetic cDNA was stored at -20℃.

II-I-3. 5′ and 3′ rapid amplification of cDNA ends (RACE)

To determine complete cDNA of fibrinolytic enzyme, 5′ and 3′ RACE were performed with a 5′/3′ RACE kit (Roche Diagnostics) using several primers based on the sequenced amino acid of purified fibrinolytic enzyme. As shown in Table 2 and Fig. 5, the primer sequences of fibrinolytic enzyme for 5′ RACE were 3’-RCANTGNGTRCCNRANCGNTGNTA-5’ (5’RACE SP-I), 3’-WSNTTRCANTGNGTRCCNRANCGN-5’ (5’RACE SP-II), 3’-CGNRANTGNTGNGTYWSNTTRCANT-5’ (5’RACE SP-III), 5’-GACCACGCGATCGATGTCGACTTTTTTTTTTTTTTTV-3’ (oilgo dT anchor) and 5’-GACCACGCGTATCGATGTCGAC-3’ (PCR anchor). As shown in Fig. 6, the primer sequences of fibrinolytic enzyme for 3′ RACE was 5′-GCNACNATHWSNYTNMGNCAR-3′ (3’RACE SP-IV), 5’-GACCACGCGATCGATGTCGACTTTTTTTTTTTTTTTV-3’ (oilgo dT anchor) and 5’-GACCA

Table 2. Oligodeoxynucleotide primers used for synthesis and amplification of partial, 3′-RACE and 5′-RACE of the *Cmfe* cDNA.

Primer name	Sequence
5’RACE SP-1	3’-RCANTGNGTRCCNRANCGNTGNTA-5’
5’RACE SP-II	3’-WSNTT RCANTGNGTRCCNRANCGN-5’
5’RACE SP-III	3’-CGNRANTGN TGNGTYWSNTTRCANT-5’
oligo d(T)-anchor	5’-GACCACGCGATCGATGTCGACTTTTTTTTTTTTTTTV-3’
PCR-anchor	5’-GACCACGCGTATCGATGTCGAC-3’
3’RACE SP-IV	5′-GCNACNATHWSNYTNMGNCAR-3′
Cmfe-F	5′–ATGTTTTCGTTCAAAAATCTTGCGTCGCTG–3′
Cmfe-R	5′–TTATATGATGCCGTTGTATGCAACCAA–3′
EX-*Cmfe*-F	5’-AAGCTTATG ATGTTTTCGTTCAAAAATCTTGCGTCGCTG -3’
EX-*Cmfe*-R	5’-GGAT CCTTATATGATGCCGTTGTATGCAACCAA -3’

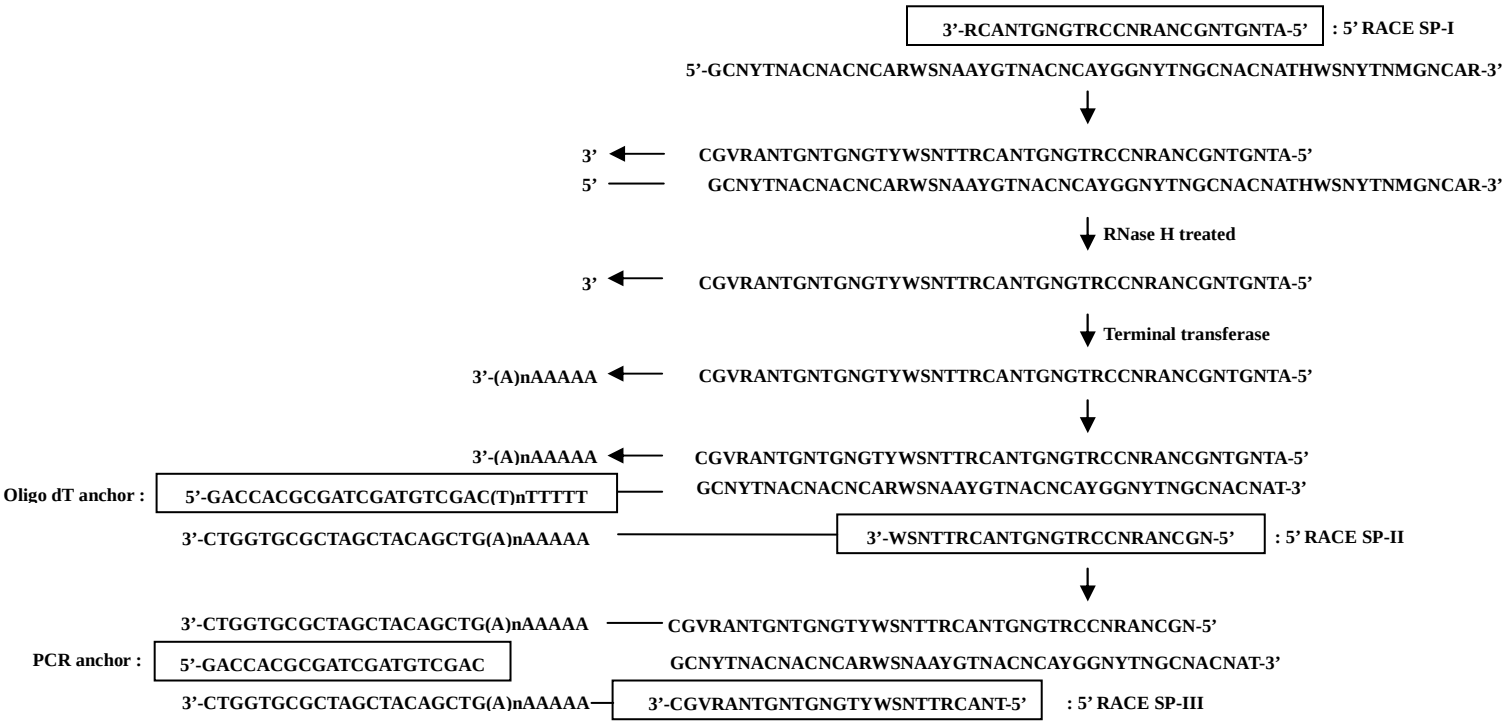


Fig. 5. The strategy of 5’RACE amplification of unknown sequences at the 5’ end of the mRNA.

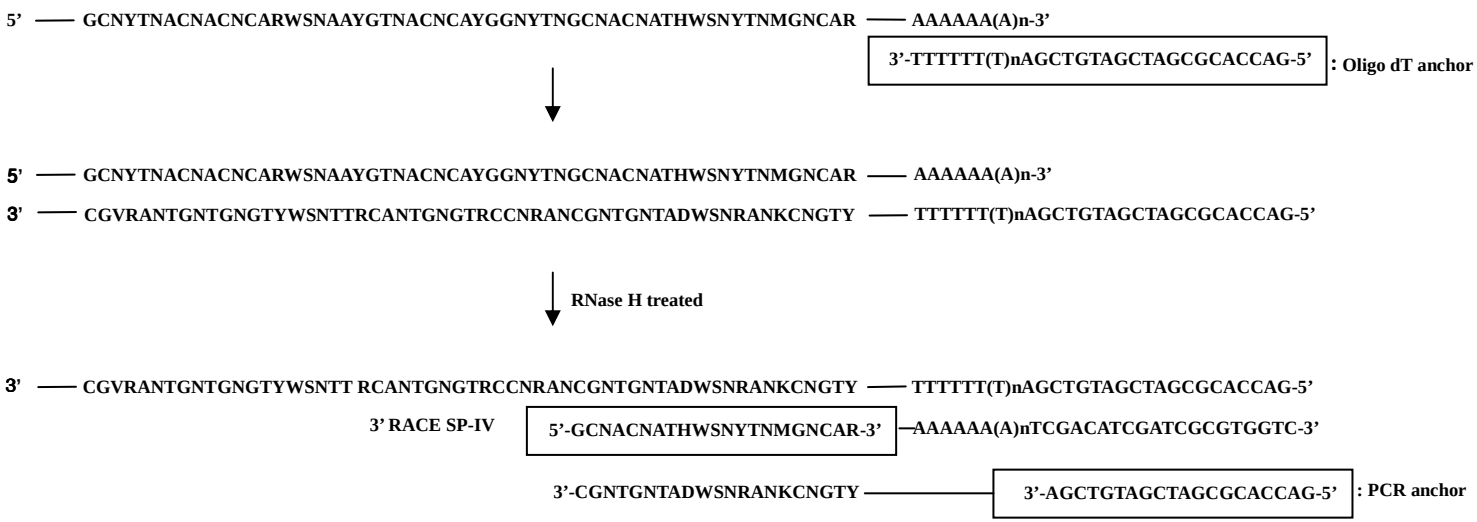


Fig. 6. The strategy of 3’RACE amplification of unknown sequences at the 3’ end of the mRNA.

CGCGTATCGATGTCGAC-3’ (PCR anchor).

For 5′ RACE and 3′ RACE, PCR was performed under the same conditions as those described above except that the annealing temperatures were 55 and 60°C, respectively. The obtained PCR products were then cloned into the pGE M T-easy vector (Promega Co, Korea) and sequenced (Fig. 7).

II-I-4. DNA sequencing and analysis

Plasmid colony was inoculated into LB broth containing 100 ㎍/ ㎖ of ampicillin and was incubated for 12 hrs at 37℃ in shaking incubator. Recombinant DNA was isolated by plasmid DNA isolation kit (Qiagen Co, USA) according to the manufacturer's instructions.

After isolation, plasmids were digested with the restriction endonuclease *Eco*R1, then electrophorized for 1hr at 100 V on a 0.8% agarose gel and stained with ethidium bromide (EtBr) to estimate cDNA insert size.

Isolated pGEM-T easy vector was sequenced by primer walking with an Automatic DNA sequencer (model: ABI PRISM 377, Perkin Elmer, USA). Database searches were performed using the deduced amino acid sequences, from the NCBI (National Center for Biotechnology Information) on-line program and BLAST, for protein sequences in GeneBank (http:// www. ncbi.nlm.nih.gov/ BLAST/).

II-I-5. The cloning of *Cmfe*

By aligning and assembling the sequences of the 3′ RACE and 5′ RACE products, the full-length cDNA of *Cmfe* (*Cordyceps militaris* fibrinolytic enzyme) was amplified by a simple PCR with the cDNA as the template usei-

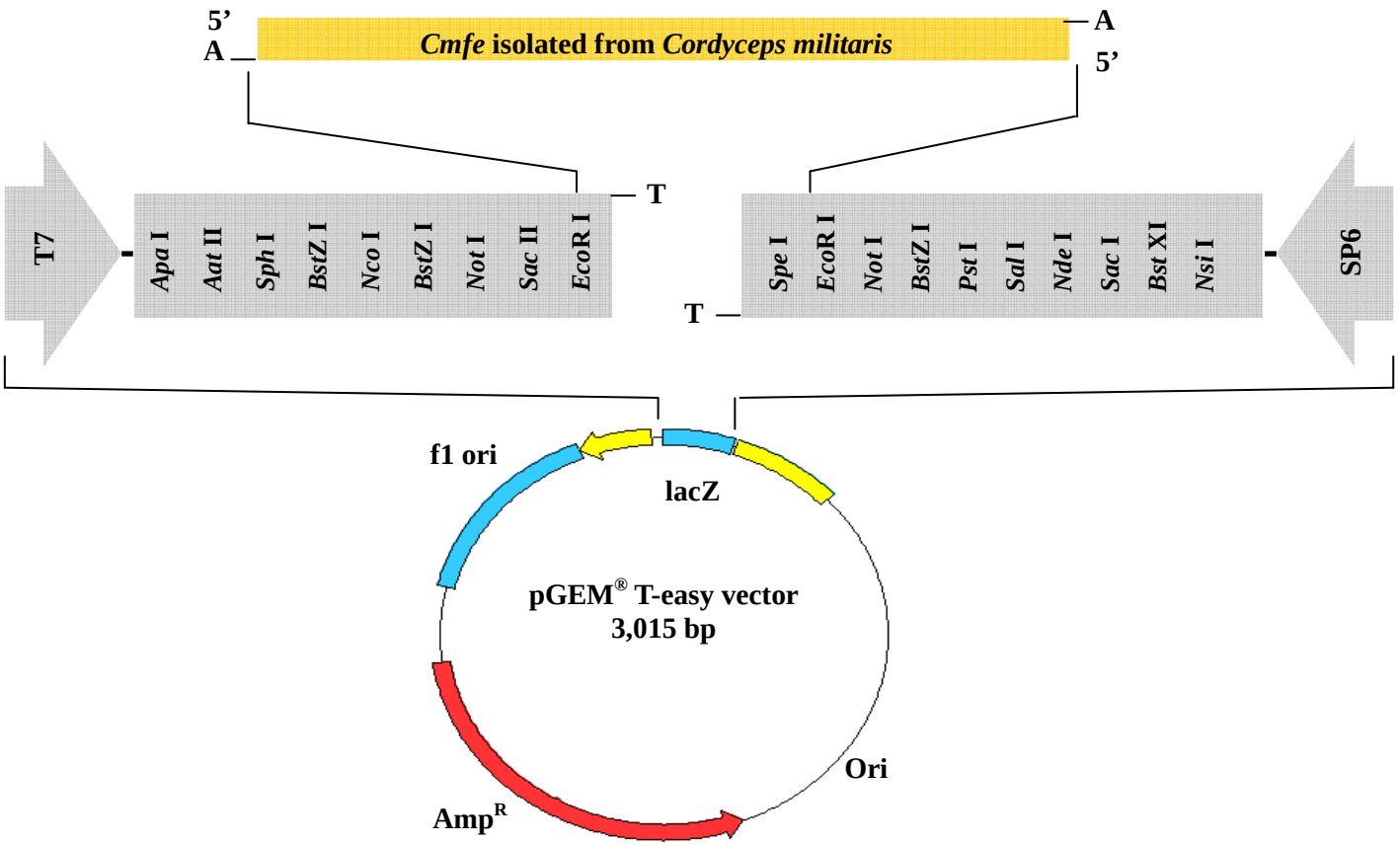


Fig. 7. Construction of *Cmef* cDNA with pGEM T-easy vector.

ng specific primers *Cmfe*-F (5′–ATGTTTTCGTTCAAAAATCTTGCGTC GCTG–3′) and *Cmfe*-R (5′–TTATATGATGCCGTTGTATGCAACCAA–3′). The condition of PCR was performed 35 cycles of amplification: 94 °C for 45 s, 60 °C for 45 s, and 72 °C for 150 s. The amplification and sequencing of the full-length cDNA of *Cmfe* was cloned into the pGEM T-easy vector (Promega Co, Korea) and sequenced (Fig. 7).

II-I-6. Northern hybridization

Isolation of *Cmfe* cDNA clones from *C. militaris* was confirmed by Northern blot analysis. The RNA samples isolated from *C. militaris* were resolved on denaturing formaldehyde/1 % agarose gel and blotted onto nylon membrane. DIG-labelled DNA probes complementary to two isolated cDNA clones were synthesized using the DIG DNA labeling kit (Roche Diagnostics). The membrane was pre-hybridized in prehybridization solution (50 % formamide, 5% blocking reagent, 5X SSC, 0.1 % sarcosyl, 0.1 % SDS) at 52°C for 2hrs, followed by hybridization with DIG-labelled DNA probes overnight. After hybridization, the membrane was washed twice in low stringency wash solution (2X SSC, 0.1 % SDS) at room temperature for 5 min and washed twice in high stringency wash solution (0.1X SSC, 0.1 % SDS) at 52°C for 15 min. The hybridization signals were detected with anti-digoxigenin–alkaline phosphatase conjugated primary antibody (Roche Diagnostics) and visualized with chemiluminescence substrate CDP-Star (Roche Diagnostics).

II-J. Expression of fibrinolytic enzyme gene

II-J-1. Construction of expression vector for recombinant fibrinolytic

enzyme gene

As shown in Fig. 8, the *Cmfe* cDNA isolated from *C. milita ris* was cloned by polymerase chain reaction using a set of primers, EX-*Cmfe* -F primer (5'-AAGCTTATGTTTTCGTTCAAAAATCTTGCGTCGCTG-3') and EX-*Cm fe*-R (5'-GGATCCTTATATGATGCCGTTGTATGCAACCAA-3') from the pGEM T-easy vector containing *Cmfe* gene.

The amplified cDNA of fibrinolytic enzyme gene was transferred into pQE 30 expression vector purchased from Qiagen Co., USA. The sequence and the orientation of the inserted cDNA in the constructed expression vector were confirmed by DNA sequencing.

II-J-2. Expression and purification of recombinant fibrinolytic enzyme

The transformed *E. coli* M15 was inoculated into 10 ㎖ of LB medium containing 100 ㎍/㎖ ampicillin and 25 ㎍/㎖ kanamycin and grow at 37℃ over night. Cultured transformed *E. coli* was diluted 1:60 with fresh LB medium containing the 100 ㎍/㎖ ampicillin and grew at 37℃ with vigorous shaking until the 600 nm reaches 0.6. The fibrinolytic enzyme was induced for 3hrs after the addition of 1.0 mM IPTG (Sigma-aldrich Co., USA). The cells were harvested by centrifugation at 4,000 ×*g* for 15 min. The pellets were suspend ed in 1 ㎖ lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) and added lysozyme to 1.0 ㎎/㎖. The re-suspended pellet was incu bated on ice for 30 min and was disrupted by sonication. The cell lysate was centrifuged at 10,000 ×*g* for 20~30 min at 4℃ and supernatant was collected.

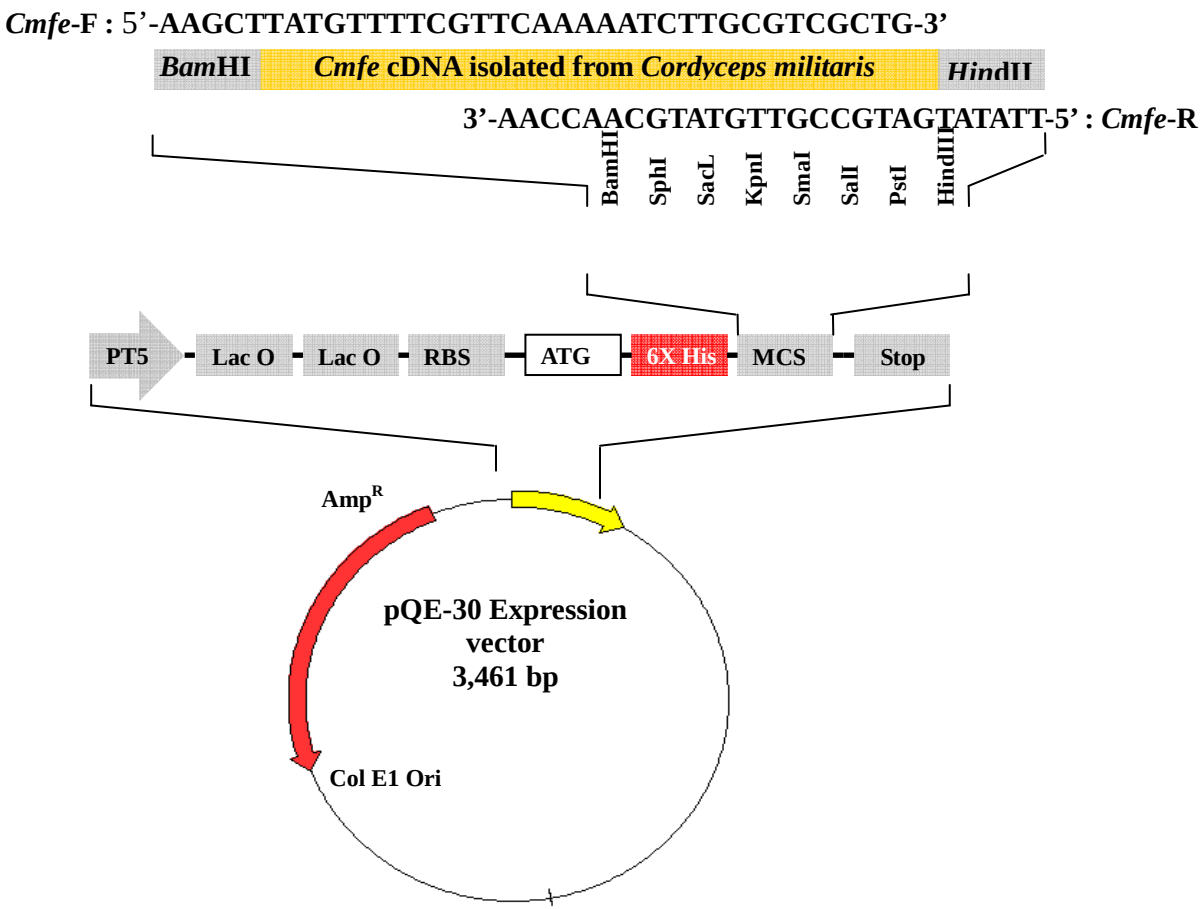


Fig. 8. Construction of expression vector for recombinant fibrinolytic enzyme gene.

The Ni-NTA spin column was equilibrated with 600 $\mu\ell$ lysis buffer and was centrifuged for 2 min at 700 $\times g$. 600 $\mu\ell$ of the cleared lysate contained 6X His-tagged protein was applied on the pre-equilibrated Ni-NTA spin column and was centrifuged for 2 min at 700 $\times g$ and the flow-though was collected. The Ni-NTA spin column attached 6X His-tagged protein was washed the twice with 600 $\mu\ell$ wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazol, pH 8.0) by centrifuge for 2min at 700 $\times g$. The washed Ni-NTA spin column was eluted twice with 200 $\mu\ell$elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazol, pH 8.0) by centrifuge for 2 min at 700 $\times g$ and collected the elute.

II-J-3. Western blot assay

The Purified fibrinolytic enzyme was electrophorized by 10.5% SDS–PAGE carried out according to Laemmli (1970) with a Mini Protean II apparatus (Bio-Rad).

The SDS–PAGE gel was transferred to a PVDF membrane using Mini Trans-Blot cell (Bio-Rad Laboratory) following manufacturer’s instructions. The transblotted membrane was blocked with blocking solution (PBS-T, 3% BSA) overnight at 4 °C. To detect the expressed 6X His tagged fibrinolytic enzyme, the membrane was first incubated for 1hr with anti-His (c-ter) alkaline phosphatase conjugated antibody (Invitrogen) at 1/4000 and visualization was achieved by reaction of the alkaline phosphatase secondary anti bodies with NBT/BCIP stock solution (Roche, Germany).

III. RESULTS

III-A. Purification of fibrinolytic enzyme

The fibrinolytic enzyme was purified by the combination of a variety of chromatographic steps listed in Table 3. The crude extract was first subjected to cation-exchange chromatography on CM-cellulose column (5×10 cm). As shown in Fig. 9, the active fraction was detected from pre-eluted fractions, which indicates that fibrinolytic enzyme from *C. militaris* didn't bind to cation exchanger as flow-through. The active fractions were pooled and concentrated by freeze-drying and desalting. Desalted active faction was dissolved in 10mM tris-HCl, pH 7.4 for anion exchange.

And then, the active fraction was subjected to anion-exchange chromatography on DEAE Sephadex A-50 column (5×10 cm). As shown Fig. 10, the active fractions (fraction No. 120~129) were eluted at 0.3 M NaCl concentration and were concentrated and desalted. Desalted active faction was dissolved in 10mM tris-HCl, pH 7.4 contained 0.15 M NaCl for gel filtration. The active fractions were further separated via gel filtration chromatography on the Sephadex G-75 column (1.5×120 cm). The major fractions (fraction No. 21 ~ 17) with fibrinolytic activity were collected (Fig. 11) and applied onto the HiLoad 16/60 Superdex 75 pg column using ÄCTA fast FPLC, which yielded one major peak (fraction No. 10 ~ 17) showing strong fibrinolytic activity (Fig. 12). As can be seen in Table 3, fibrinolytic enzyme from 100 g of *C. militaris* was obtained finally 0.96 mg. and purified fold and yield were 191.8 and 12.9%, respectively.

III-B. Fibrinolytic activity assay

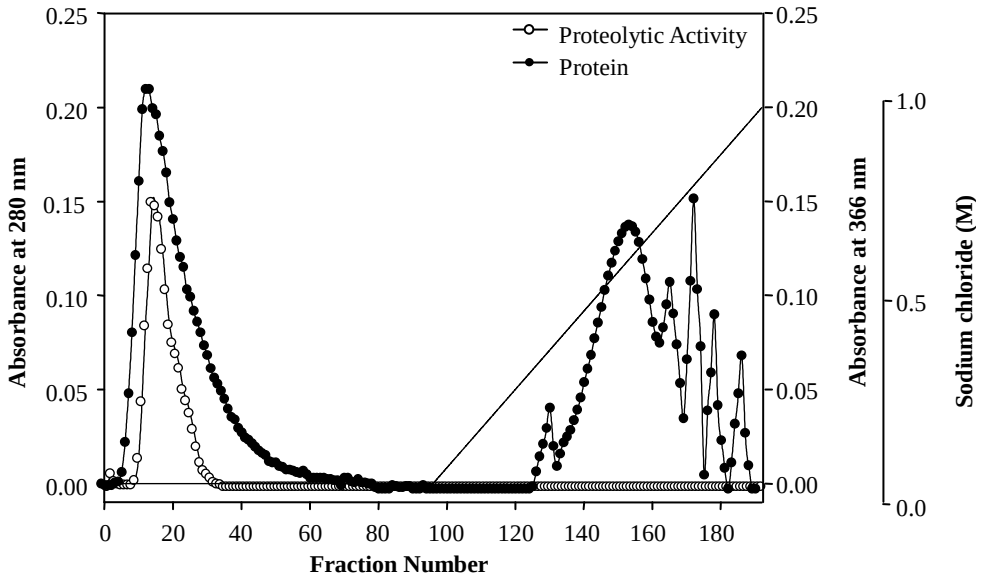


Fig. 9. Purification of fibrinolytic enzyme from *C. militaris* using cation-exchange chromatography on CM-cellulose column. The crude extract was applied to the column (5×10 cm) equilibrated with 10 mM citrate-NaOH buffer (pH 6.0). The protein was eluted with 0 ~ 1.0 M NaCl linear gradient at the flow rate of 0.5 ㎖/min at 4℃.

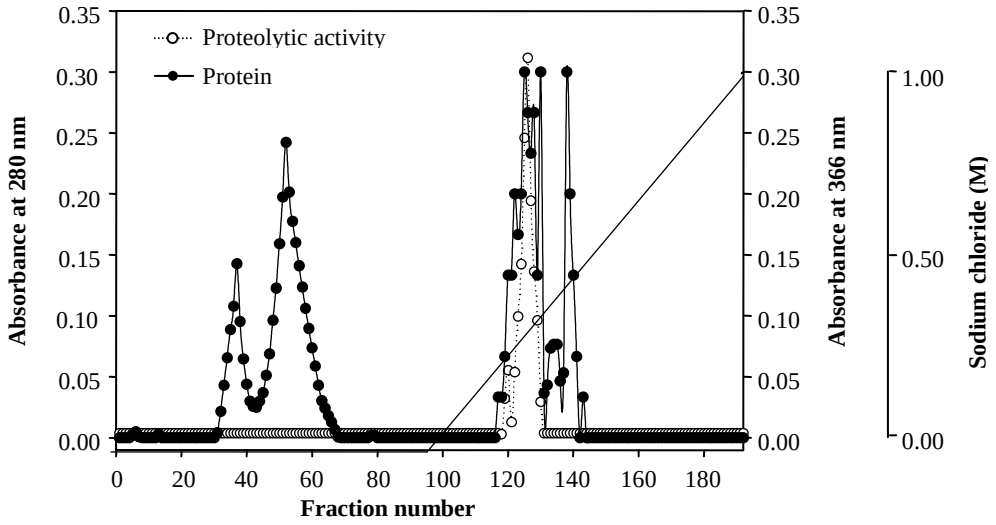


Fig. 10. Purification of fibrinolytic enzyme from *C. militaris* using anion-exchange chromatography on DEAE sephadex A-50 column. The active fraction was applied to the column (5×10 cm) equilibrated with 10 mM tris-HCl buffer (pH 7.4). The protein was eluted with 0 ~ 1.0 M NaCl linear gradient at the flow rate of 0.5 ㎖/min at 4℃.

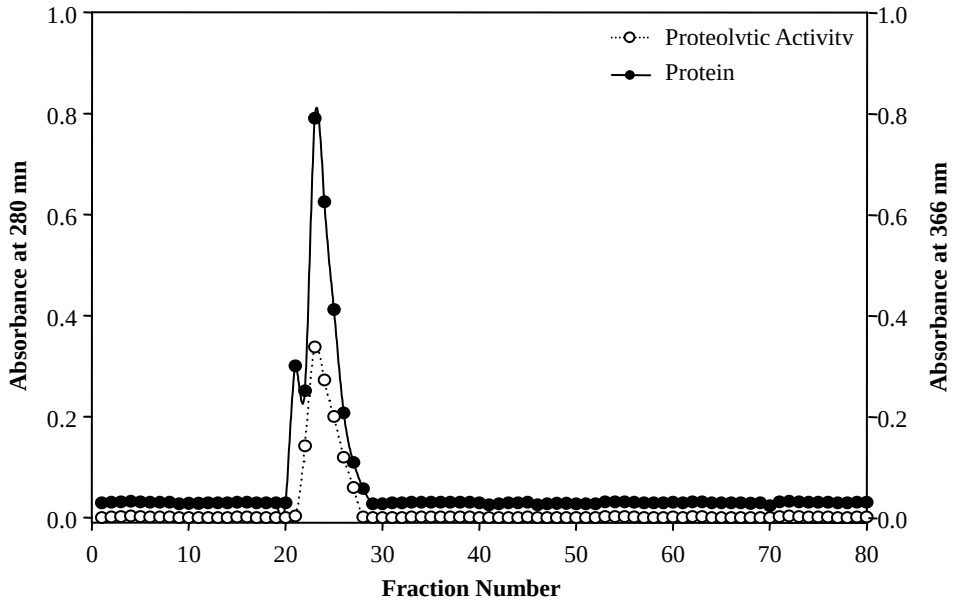


Fig. 11. Purification of fibrinolytic enzyme from *C. militaris* using gel filtration on sephadex G-75 column. The active fraction was applied to the column (1.5×120 cm) equilibrated with 10 mM tris-HCl buffer (pH 7.4) containing 0.15 M NaCl. The protein was eluted at the flow rate of 0.5 ㎖ /min at 4℃.

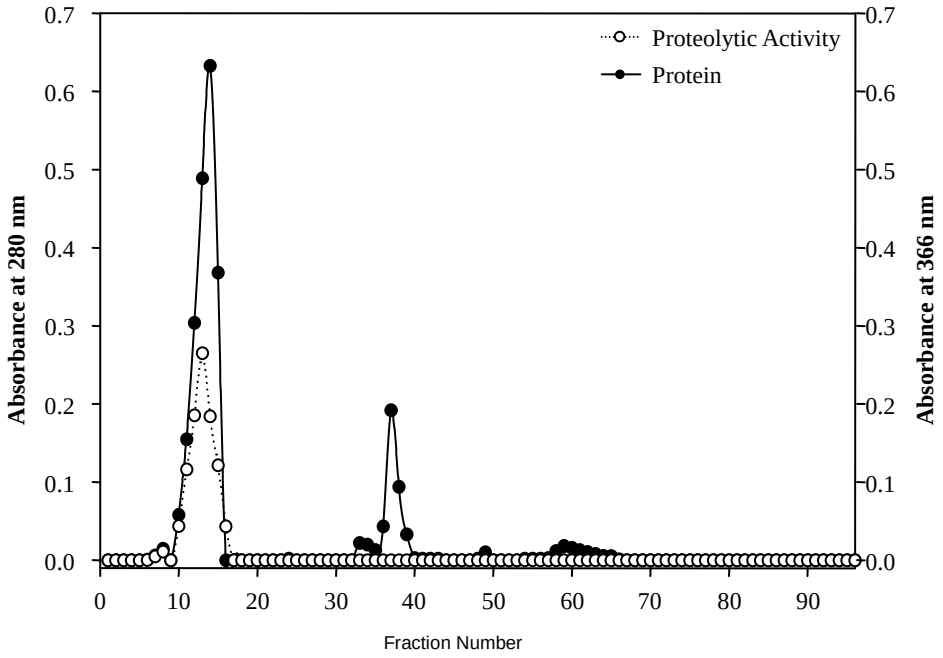


Fig. 12. Purification of fibrinolytic enzyme from *C. militaris* using fast protein liquid chromatography (FPLC) on HiLoad 16/60 Superdex 75 pg column. The active fraction was applied to the HiLoad 16/60 Superdex 75 pg column equilibrated with 10 mM Potassium phosphate buffer (pH 7.2) containing 0.15M NaCl. The protein was eluted at the flow rate of 1.0 ㎖ /min at room temperature.

Table 3. Purified fibrinolytic enzyme activity yields from *C. militaris.*

Purification Step	Volume (㎖)	Protein (㎎)	Proteolytic activity (Unit)	Specific activity (unit/㎎)	Recovery (%)	Fold
Homogenate	250	N.D.	N.D.	-	-	-
Crude extracts	150	1,552	5,122	3.3	(100)	(1)
DEAE Sephadex A-50	30	204	1,848	9.1	36.1	2.8
Shephadex G-75	5	9.2	1,071	116.4	20.9	35.2
HiLoad 16/60 Superdex 75 pg	1.5	0.96	658.5	633	12.9	191.8

Note. N.d., not determined. Protease activity was measured by using the azocasein assay, as described under Materials and methods.

The fibrinolytic activity of the purified enzyme was analyzed using fibrin plates. 20 ㎍ of purified enzyme and 1.0 NIH unit of plasmin were spotted on the fibrin plate and incubated at 37℃ for 6 hrs. Fig.13 shows the lytic area generated by the purified enzyme and plasmin in fibrin plate. The fibrinolytic activity of purified enzyme was about 3.3 unit / 20 ㎍ protein. The negative control had no fibrinolytic activities.

III-C. Determination of molecular weight

The molecular mass of the fibrinolytic enzyme from *C. militaris* was found to be 52 kDa, as estimated by size exclusion on HiLoad 16/60 Superdex 75 pg column, using ÄCTA fast FPLC (Fig. 14). This value is similar to the value estimated by SDS-PAGE and fibrin-zymography (Fig. 15).

III-D. N-terminal amino acid sequence of fibrinolytic enzyme from *C. militaris*

The N-terminal amino acid sequence of the purified fibrinolytic enzyme from *C. militaris* was analyzed via automated Edman method, after SDS-PAGE and electroblotting. The N-terminal sequence of the first 19 residues was ALTTQSNVTHGLATISLRQ, which is very similar to that of the proteolytic enzyme from the subtilisine-like serine protease PR1J (NCBI Accession No. CAC95048) from *Metarhizium anisopliae var. anisopliase* (Bagga et al., 2004). As shown in Fig 16, the N-terminal amino acid sequences of purified enzyme has a homology with subtilisin-like serine protease PR1, purified from *Metarhizium anisopliae var. anisopliase* such as PR1B (NCBI Accession No. CAC95046), PR1G (NCBI Accession No. CAB

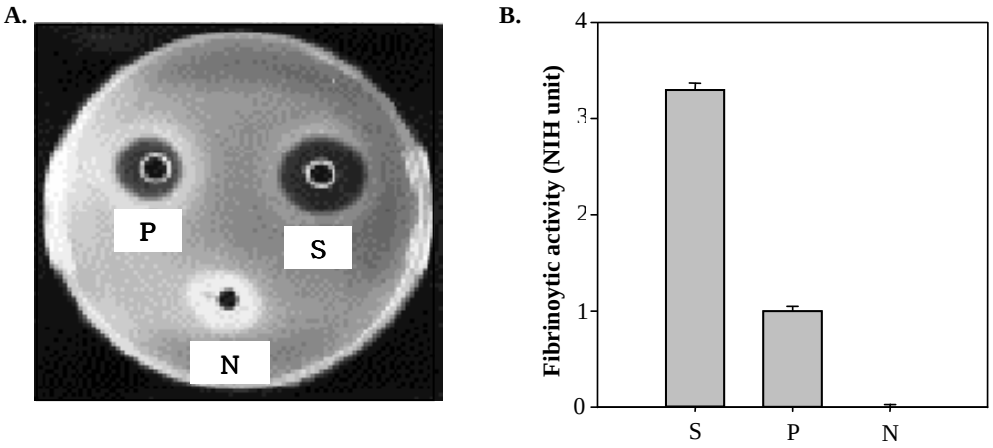


Fig. 13. Fibrin plate assay of purified fibrinolytic enzyme from *C. militaris*. The purified enzyme was spotted onto the fibrin plate and incubated at 37℃ for 6 hrs. A, fibrin plate; B, fibrinolytic activity. S, purified enzyme; P, 1.0 unit plasmin as a positive control; N, 10 mM tris-HCl as a negative control.

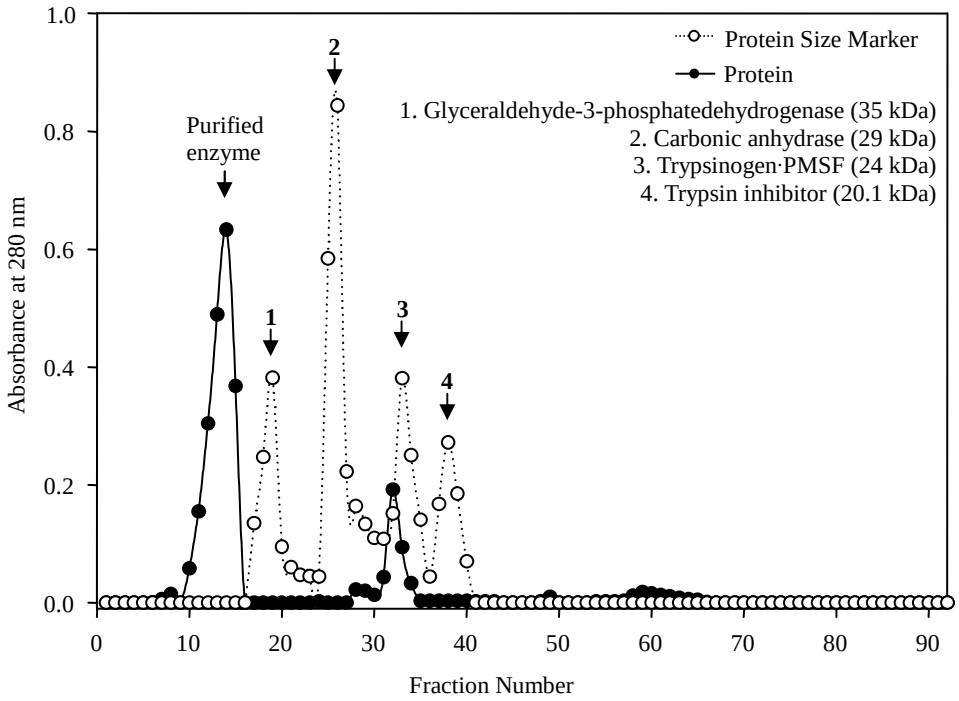


Fig. 14. Molecular weight determination of fibrinolytic enzyme from *C. militaris* using size-exclusion chromatography on HiLoad 16/60 Superdex 75 pg column. The HiLoad 16/60 Superdex 75 pg column was equilibrated with 10 mM potassium phosphate buffer containing 0.15 M NaCl (pH 7.2) at a flow rate of 1.0 ㎖/min.

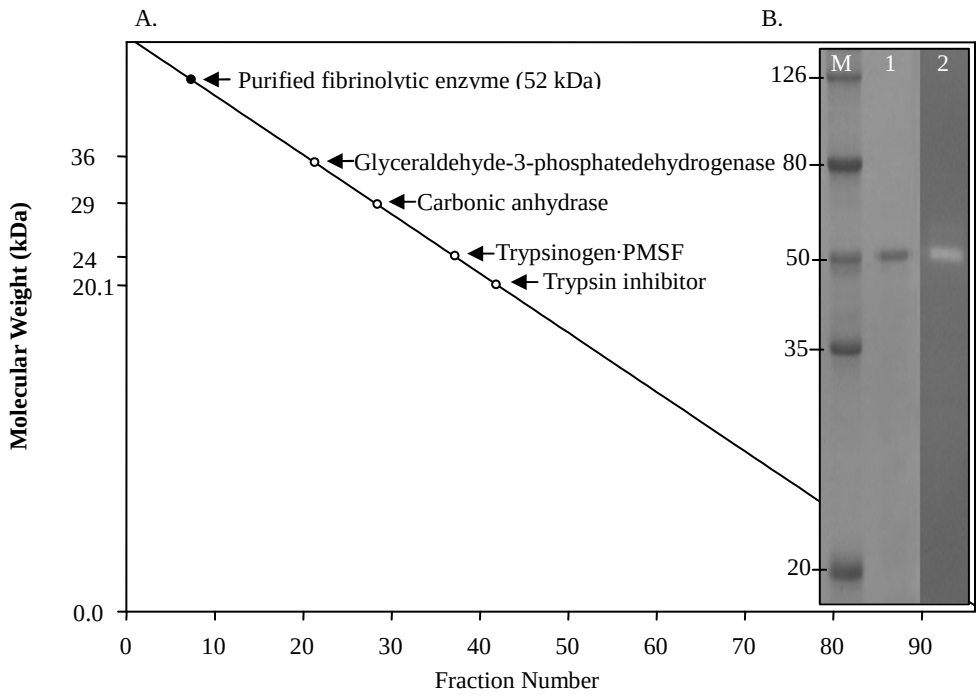


Fig. 15. Molecular weight determination of fibrinolytic enzyme from *C. militaris* using SDS-PAGE and fibrin zymography. A, Semi-logarithmic plot; B, SDS-PAGE and fibrin zymography. Lane 1, protein standard marker; lane 2, purified fibrinolytic enzyme on SDS-PAGE; lane 3, purified fibrinolytic enzyme on fibrin-zymography.

63912), PR1A (NCBI Accession No. CAC95049), PR1K (NCBI Accession No. CAC07219) and PR1I (NCBI Accession No. CAC95043), but dissimilar to other protease purified from mushrooms such as AmMEP (NCBI Accession No. CAB42792) purified from fruiting body and mycelium of *Armillaria mellea* (Healy et al., 1999 and Lee et al., 2005), PoMEP (NCBI Accession No. P80155) of *Pleurotus ostreatus* (Nonaka et al., 1997 and Joh et al., 2004) and GfMEP (NCBI Accession No.BAB32381) of *Glifora frondosa* (Nonaka et al., 1997).

III-E. Effect of pH and temperature on fibrinolytic activity

The effect of pH on the activity of purified enzyme from *C. militaris* was determined using buffers at various pH. As shown Fig. 17, this results indicated that purified enzyme from *C. militaris* was active over a wide pH range (2.0 ~ 10.0), and exhibited maximum activity at pH 7.4. The enzyme was very stable in a pH range of 5.0 ~ 8.0, at 37°C for 1hr, but above pH 8.0, enzyme stability was decreased.

As shown in Fig 18, the effect of temperature on the activity revealed that the enzyme was active between 20 and 50°C. Optimum activity was found to occur at 37°C. However, when exposed for 1hr to a temperature of over 42°C, the activity of purified enzyme decreased dramatically.

III-F. Effect of inhibitors and metal ions on the fibrinolytic activity

The effect of various inhibitors on fibrinolytic activity is summarized in Table 4. The purified fibrinolytic enzyme was inhibited by 2.0 mM PMSF and 0.5 mM APMSF, a well-known serine protease inhibitor, while inhibitors

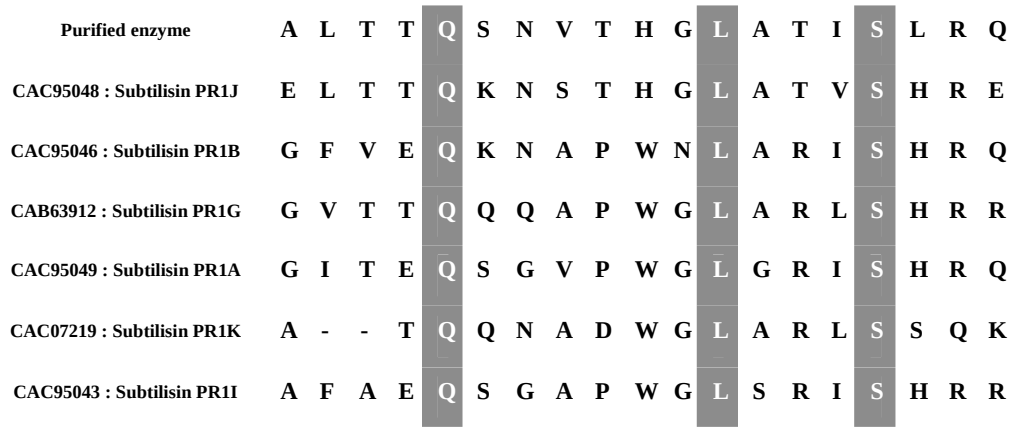


Fig. 16. An aligment of the amino-terminal sequence of purified fibrinolytic enzyme from *C. militaris* with those of the serine protease family from *Metarhizium anisopliae* (PR1J, NCBI Accession No. CAC95048; PR1B, Accession No. CAC95046; PR1G; Accession No. CAB63912, PR1A; Accession No. CAC95049; PR1K, Accession No. CAC07219 and PR1I; Accession No. CA C95043).

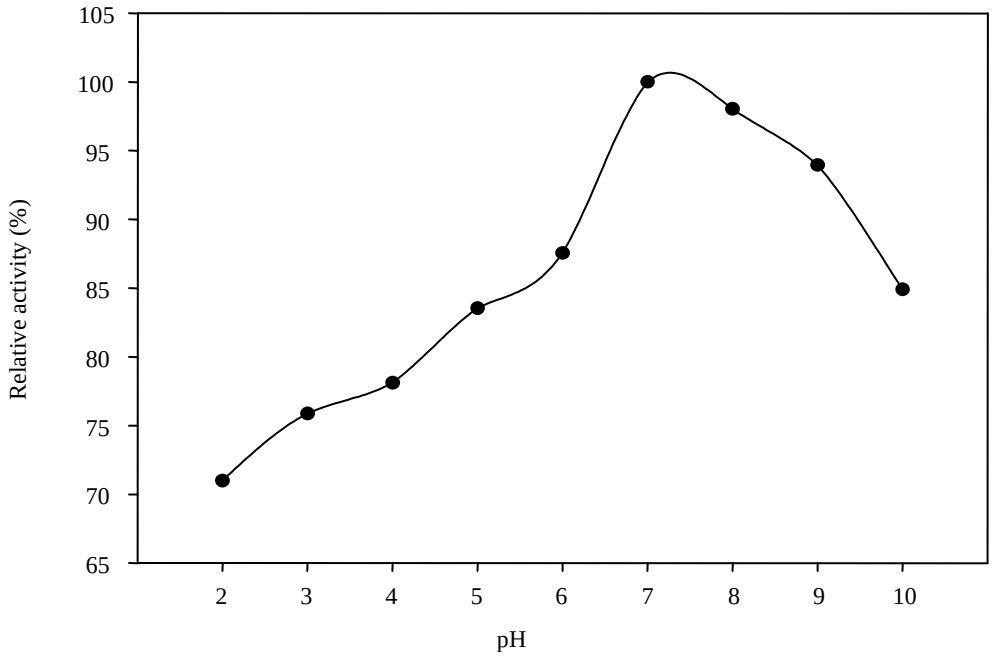


Fig. 17. Effects of various pH on the activity of the fibrinoltyc enzyme from *C. militaris*. Enzyme activity was assayed in the pH range of 2.0 ~ 10.0. 0.5M glycine-HCl (pH 2.0 ~ 3.0), 0.5 M acetate (pH 4.0 ~ 5.0), 0.5 M tris-HCl (pH 6.0 ~ 8.0) and 0.5 M glycine-NaOH (pH 9.0 ~ 10.0) buffer were used with 0.1% azocasein. Enzyme activity was measured by incubation for 1 hr at various pH.

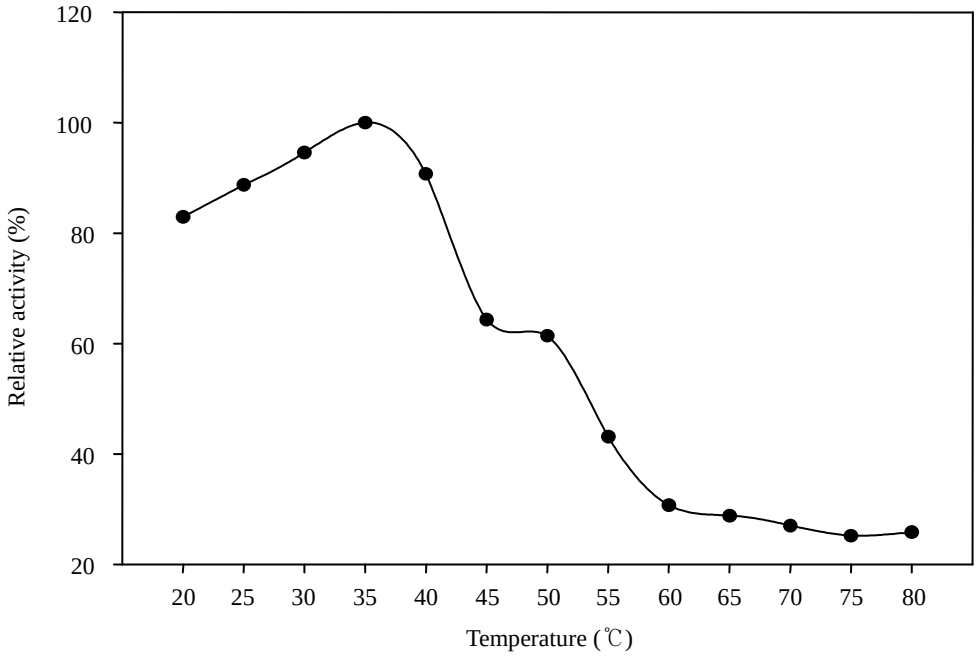


Fig. 18. Effects of various temperature on the activity of the fibrinolytic enzyme from *C. militaris*. The purified enzyme was incubated at temperatures from 20 to 80℃. Fibrinolytic activity was measured by azocasein assay at 366nm.

like TLCK, a trypsin selective reagent and TPCK, a chymotrypsin alkylating agent did not inhibit enzyme activity. As shown in Table 5, the effects of various metal ions on enzyme activity were assessed by assaying residual enzyme activity after the incubation of the enzyme with 1.0 mM of metal ions for 1hr at 37°C. The enzyme activities were found to be slightly enhanced by Ca^{2+} and Mg^{2+}, but were inhibited by the Co^{2+}, Cu^{2+}, Fe^{2+}, and Zn^{2+} ions.

III-G. Analysis of Fibrinolysis and fibrinogenolysis

The hydrolysis of fibrin by purified enzyme was analyzed by SDS–PAGE. As shown in Fig. 19, the purified enzyme rapidly hydrolyzed the α-chain, followed the γ-γ chains and more slowly the β-chain. This hydrolysis pattern of purified enzyme was not identical to that of plasmin, which can rapidly hydrolyzed the α-chain, and more slowly digest β and γ-γ chains. In addition, the substrate specificity of purified enzyme was different from plasmin and other protease purified from natural source such as *Codium* sp. (Matsubara et al., 1998; Matsubara et al., 1999 and Matsubara et al., 2000), mushrooms (Lee et al., 2005) and snake venom (Siigurkey et al., 1989; Koh et al., 2001 and Bello et al., 2006). Also, purified enzyme had fibrinogenolytic activity and the degradation pattern of fibrinogen by purified enzyme was analyzed by SDS–PAGE (Fig. 20). As shown in Fig. 20, purified enzyme rapidly hydrolyzed Aα, Bβ and γ chains. This hydrolysis pattern due to purified enzyme was not identical to that of α-fibibrinogease purified from snake venom which preferentially hydrolyzed Aα chain of fibrinogen rather than Bβ and γ chains (Wei et al., 2004 and Pinto et al., 2004)

Table 4. Effect of various protease inhibitors on the activity of the purified fibrinolytic enzyme from *C. militaris*

Protease inhibitor	Concentration (mM)	Relative activity (100%)
Control	**-**	**100.0 ± 3.5**
PMSF	**2.0**	**53.5 ± 2.1**
APMSF	**0.5**	**51.5 ± 3.2**
TLCK	**0.5**	**73.0 ± 2.5**
TPCK	**0.5**	**77.1 ± 2.7**
EDTA	**5.0**	**98.2 ± 3.1**
Aprotinin	**0.05**	**83.0 ± 2.5**
Pepstain A	**0.5**	**87.3 ± 3.7**

The enzyme was pre-incubated with various 5.0 mM protease inhibitor for 1 hr at 37℃. After incubation, the azocasein assay and fibrin plate (data not shown) was performed. The result was expressed as relative percentage (%) of relative activity. All experiments were performed in triplicate.

Table 5. Effect of metal ions on the activity of the fibrinolytic enzyme from *C. militaris*

Metal ions	Concentration (mM)	Relative activity
Control	**-**	**100.0 ± 3.5**
Cu^{2+}	**1.0**	**77.5 ± 2.2**
Co^{2+}	**1.0**	**79.5 ± 1.3**
Ca^{2+}	**1.0**	**103.6 ± 2.7**
Zn^{2+}	**1.0**	**86.1 ± 2.7**
Fe^{2+}	**1.0**	**88.2 ± 3.4**
Mg^{2+}	**1.0**	**107.1 ± 1.9**

The enzyme was pre-incubated with various 1.0 mM metal ions for 1 hr at 37℃. After incubation, the azocasein assay and fibrin plate (data not shown) was performed. The result was expressed as relative percentage (%) of relative activity. All experiments were performed in triplicate.

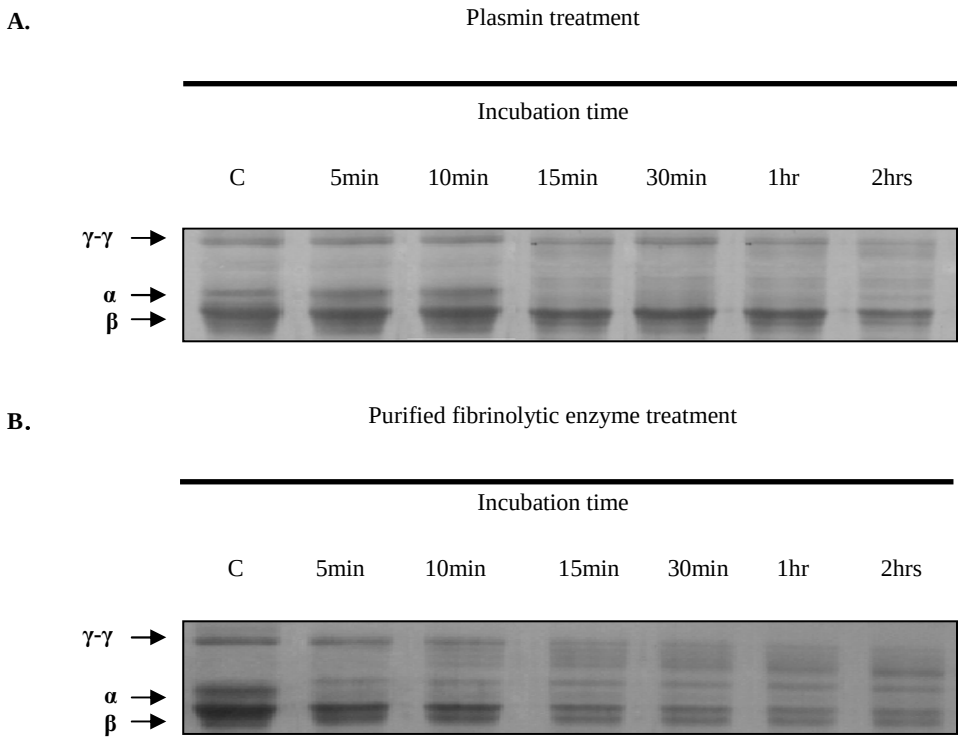


Fig. 19. Analysis of the pattern of fibrinolysis by purified fibrinolytic enzyme. Fibrin was incubated with purified fibrinolytic enzyme for the various times indicated. Plasmin was used as positive control.

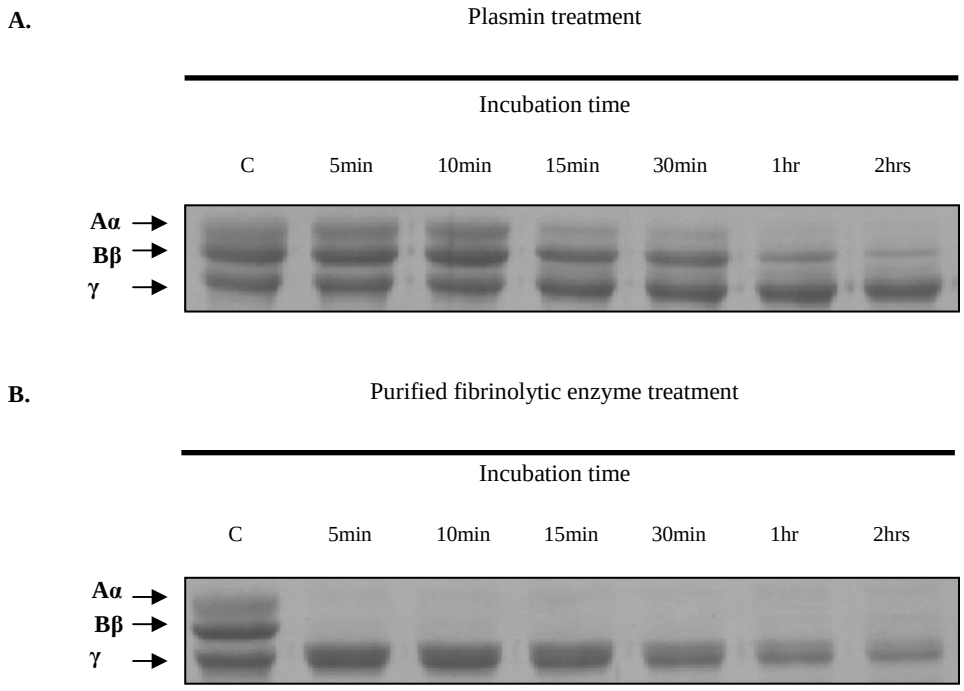


Fig. 20. Analysis of the pattern of fibrinogenolysis by purified fibrinolytic enzyme. Fibrinogen was incubated with purified fibrinolytic enzyme for the various times indicated. Plasmin was used as positive control.

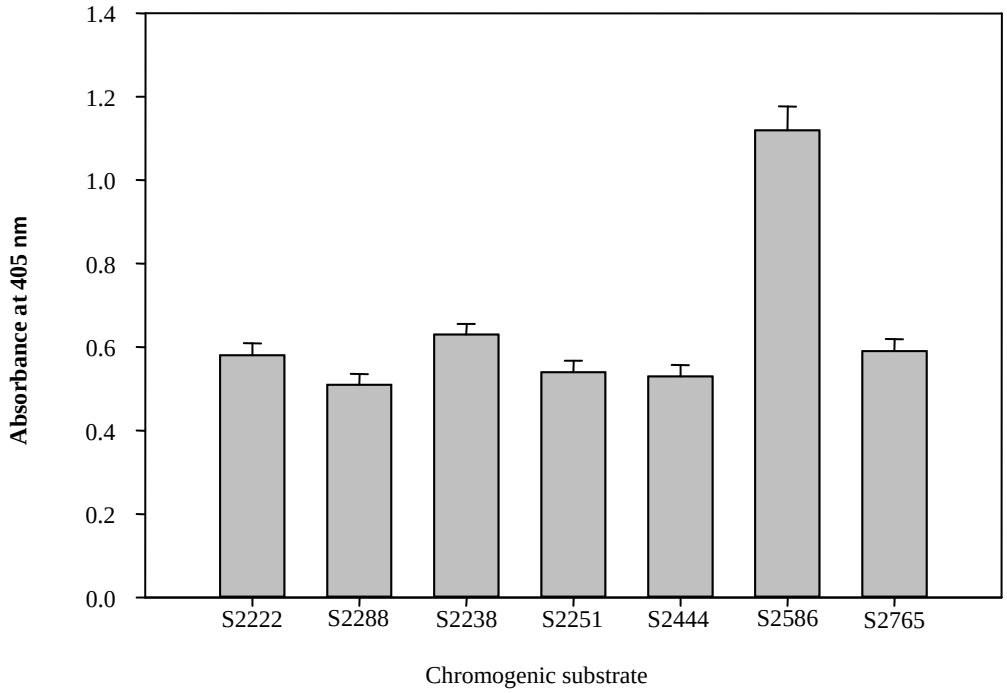


Fig. 21. Amidolytic activity on several chromogenic substrates. Amidolytic activities were measured spectrophotometrically using chromogenic protease substrates, such as S2222, S2288, S2238, S2251, S2444, S2586 and S2765.

III-H. Amidolytic activity of fibrinolytic enzyme from *C. militaris*

The amidolytic activity of purified fibrinolytic enzyme was assessed with several chromogenic substrates. As shown in Fig. 21, the fibrinolytic enzyme exhibited a higher degree of specificity for the substrate s-2586 for chymotrypsin (MeO-Suc-Arg-Pro-Tyr-pNA · HCl).

Therefore, purified enzyme from *C. militaris* was considered to be a chymotrypsin-like serine protease.

III-I. 3'-RACE and 5'-RACE for Molecular cloning of *Cmfe*

To isolate *cmfe* from *C. militaris*, sequenced amino acid was compared with other protease in the GeneBank database using a Blast program. First, we purified a fibrinolytic enzyme from *C. militaris* and found that the sequence of 19 amino acid in N-terminal region was highly similar to that of subtilisin-like serine protease PR1J (NCBI accession No. CAC95048) and subilisin-like serine protease PR1 family purified from *Metarhizium anisopliae var. anisopliae*. Degenerate primers designed from highly conserved region between sequenced fibrinolytic enzyme and subtilisin-like serine protease PR1J were used in rapid amplification of cDNA ends (RACE) PCR reactions to retrieve 5'-end and 3'-end of the *Cmfe* cDNA.

In 3′-RACE procedure, the first strand was amplified dA-tailed cDNA by using the oligo dT-anchor primer (5'-GACCACGCGATCGATGTCGACTT TTTTTTTTTTTTTTV-3') and mRNA template. And the RCR amplification of cDNA (nested RCR) was carried out using the PCR anchor primer (5'-GA CCACGCGTATCGATGTCGAC-3') and 3'RACE-SP IV primer (5'-GCN ACNATHWSNYT NMGNCAR-3').

After the first strand cDNA synthesis and RCR amplification of cDNA, distinct bands of predicted size approximately 900 bp, were shown by agarose gel electrophoresis. After isolation and subcloning of these partial cDNA with pGEM T-easy vector, the nucleotide sequence encoding 3'-ends of *Cmfe* was identified by sequence analysis. The partial cDNA was 855 bp, including an encoding region of 813 bp and a 3′ untranslated region (UTR) of 42 bp, containing a poly A tail. The partial cDNA sequence of *Cmfe* was compared with the published sequences of the subilsin-like serine protease PR1 family, and was found to show 93% identity with subtilisin-like serine protease PR1J (AJ251922). Base on the this result, 3'-end sequence was analyzed 5'-CACAGTCAACTTGGTTGCATACAACGGCATCATATAA-3' using gene runner program and compared with subtilisin-like serine protease PR1J using NCBI Blast.

In 5′-RACE procedure, the first strand was amplified by using the 5'RACE-SP I primer (5'-ATNGTNGCNARNCCRTGNGTNACR-3') and mRNA template. And the synthesized first strand was treated RNase H and digested mRNA strand. After mRNA digested first strand was polymerized dATP by terminal transferase in 3'-end, nested RCR was carried out using the oligo dT anchor primer and 5'RACE-SP II primer (5'-NGCNARNCCRTGN GTNACRTTNSW-3'). After nested PCR carried out, second PCR was carried out using PCR anchor primer and 5'RACE-SP III primer (5'-TNACR TTNSWYTGNGTNGTNARNCG-3'). The finally synthesized PCR product was ligated with pGEM T-easy vector. The nucleotide sequence encoding 5'-ends of *Cmfe* was identified by sequence analysis. The partial encoding 5'-ends cDNA was 511 bp, including an encoding region of 373 bp and a 5′ UTR of 138 bp. The partial encoding 5'-ends cDNA sequence of *Cmfe* was

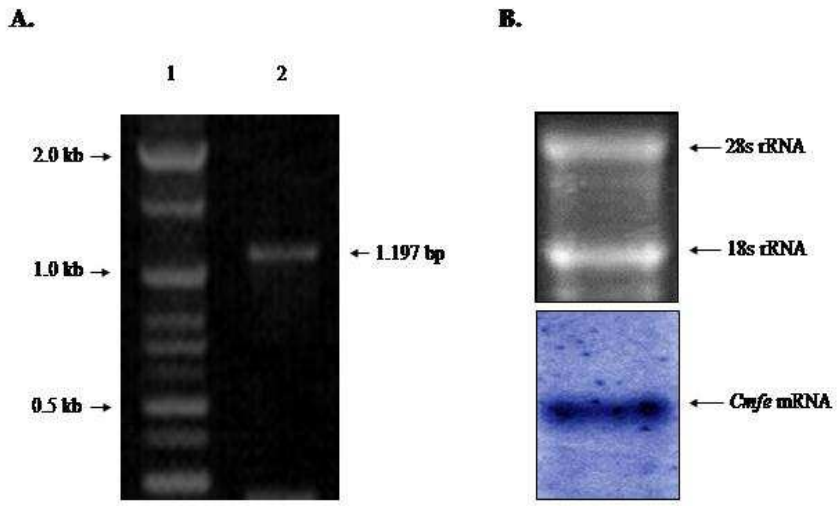


Fig. 22. PCR amplification of *Cmfe* isolated from *C. militaris*. A. PCR product amplified by PCR using *Cmfe*-F (5′– ATGTTTTCGTTCAAAAATC TTGCGTCGCTG –3′) and *Cmfe*-R (5’- TTATATGATGCCGTTGTATGCA ACCAA -3’) primers designed from the 5’/3’ RACE. The resulting PCR products were resolved by electrophoresis on a 2% agarose gel and then visualized by EtBr staining of the gel. Lane 1, 100 bp ladder ; lane 2, *Cmfe* PCR product. B. Northern blot analysis. m DNA (15 µg per lane) was electrophorised and transfer Hybond H^+ membrane, followed by hybridization with the coding sequence of *Cmfe* as probe.

compared with the published sequences of the subilsin-like serine protease PR1 family, and was found to show 89% identity with subtilisin-like serine protease PR1J (AJ251922). Base on the this result, 5'-end sequence was analyzed 5′– ATGTTTTCGTTCAAAAATCTTGCGTCGCTG –3′ using gene runner program and compared with subtilisin-like serine protease PR1J using NCBI Blast.

III-J. Molecular cloning of *Cmfe*

Cmfe-F primer (5′-ATGTTTTCGTTCAAAAATCTTGCGTCGCTG-3′) and *Cmfe*-R (5'-TTATATGATGCCGTTGTATGCAACCAA-3') primers were designed from the 3'-end and 5'-end encoding partial cDNA nucleotide sequence using 3'-RACE and 5'-RACE. The complete *Cmfe* could then be recovered by PCR amplification using *Cmfe*-F and *Cmfe*-R. The 1,197 bp PCR product was purified and confirmed by northern blotting using DIG-labelled probe (Fig. 22). The ligation mixture of *Cmfe* with pGEM T-easy vector was transformed into *E. coli* DH5α (Fig. 23). The recombinant plasmids designated as pGEM T-easy+*Cmfe*, were sequenced by the dideoxy chain termination method, showing that the insert sequences were identical. As shown in Fig. 24 and Fig 25, the recombinant plasmids harbored a 1,197-bp insert, which contained an open reading frame (ORF). Translation of this ORF generated a 398-amino acid polypeptide with a 19-amino acid (Ala_{117}~ Gln_{135}) sequence identical to that of the N-terminal amino acid sequenced of the purified fibrinolytic enzyme from *C. militaris*. The complete sequence of *Cmfe* was compared with the published sequences of the subilsin-like serine protease PR1 family, and was found to show 92% identity with subtilisin-like serine protease PR1J (Fig. 26).

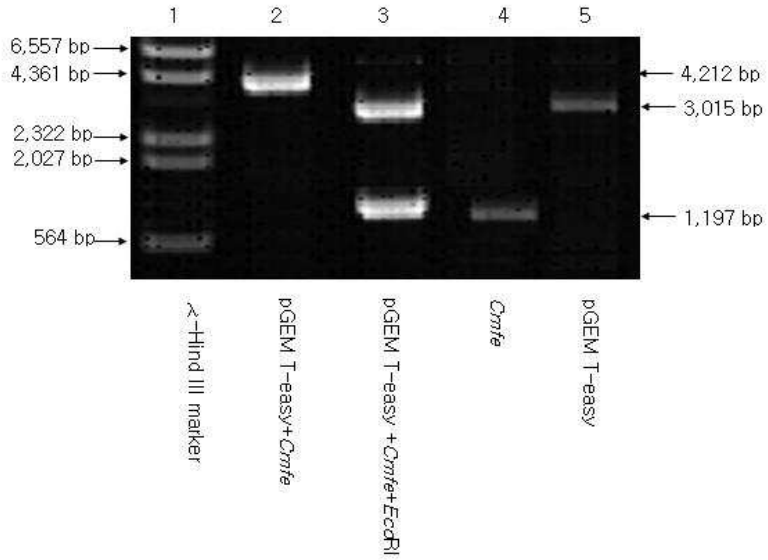


Fig. 23. Construction of cloning vector pGEM T-easy with PCR product of *Cmfe* cDNA. The constructed pGEM T-easy+*Cmfe* were resolved by electr-ophoresis on a 2% agarose gel and then visualized by EtBr staining of the gel. Lane 1, λ *Hin*dIII DNA marker ; lane 2, pGEM T-easy+*Cmfe* PCR product ; lane 3, pGEM T-easy+*Cmfe* PCR product treated *Eco*RI ; lane 4, *Cmfe* cDNA PCR product and lane 5, pGEM T-easy vector.

Fig. 24. Nucleotide sequence of *Cmfe* cDNA isolated from *C. militaris*.

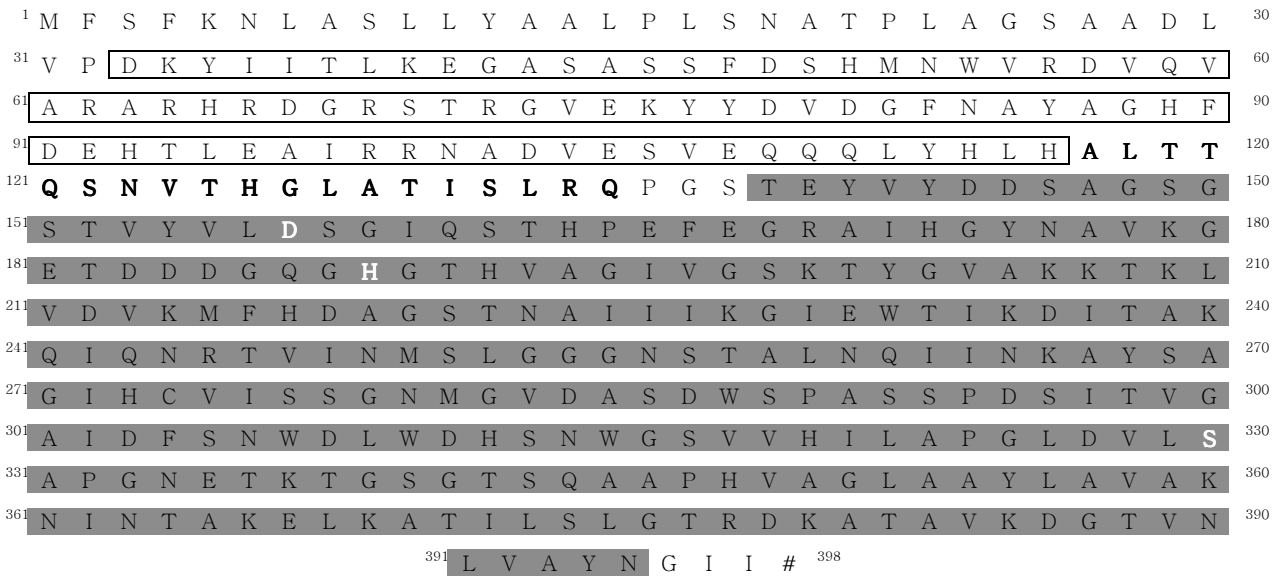


Fig. 25. Deduced amino acid sequence of *Cmfe* cDNA . Isolated Cmfe cDNA was translated into 398 amino acid of fibrinolytic enzyme. Black bold, N-terminal sequenced amino acid; Empty box, subtilisin-N domain (Asp_{33}~His_{116}); Gray box, peptidase-S8 domain (Thr_{139}~Asn_{395}); White bold, Asp/Ser/His catalytic triad.


CmFE 1MFSFKNLASLLYAALPLSNATPLAGSAADLVPDKYIITLKEGASASSFDS 50
CAC95048 1MFSFKNLASLLYAALPLSNATPLAGSAADLVPDKYIITLKDGISASDFNA 50
CAC95046 DSVPGSYIVKFKDGISSSTSFDS 52
CAB63912 32GEAFTLVPDEYIVKLKKGCAKAALDD 59
CAC95049 AESIIADKYIVKFKDDIARIATDD 33
CAC07219 9LVPRGAELIEGKYIIKMKAKAEVSAVSS 36
CAC95043 26PLLVPRGDTIPDKYIVKYKETFDFSTADS 54
CmFE 51HMNWVRDVQVARARHRDGRSTRGVEKYYDVDGFNAYAGHFDEHTLEAIRR 100
CAC95048 51HMNWVRDVQVARARHRRGLNFRGVEKTYGVGNFNAYAGHFDEHTLEAIRR 100
CAC95046 53TLASFTD----------DSHHVYDAVFKGFSATLDSVAIRNRR 85
CAB63912 60AMKIM----------PGDADQVFD-SIFKGFTGRLDSSSLDALRD 94
CAC95049 34TVSAL----------TSKADFVYE-HAFHGFAGSLTKEELKMLRE 67
CAC07219 37AI----------SSIAAEADYTYDTWNGFVATLTPEELQKLTD 69
CAC95043 55---------TIKEYH------AKAEKTYS-HIFNGFAGALNATAIETLRN 89
CmFE 101NADVESVEQQQLYHLHALTTQSNVTHGLATISLRQPGSTEYVYDDSAGSG 150
CAC95048 101NADVESVEQQQLYHLHELTTQKNSTHGLATVSHREPGSTEYVYDSSAGEG 150
CAC95046 86HPDVEFIEQDATFTINGFVEQKNAPWNLARISHRQRGSTSYIYDDSAGEG 135
CAB63912 94NPDVDYVEQNAYYEAYGVTTQQQAPWGLARLSHRRPGASDYIYDESAGEG 143
CAC95049 68HPGVDFIEKFAVMRISGITEQSGVPWGLGRISHRQKGSTTYRYDDSAGEG 117
CAC07219 70DPNVDFIEQDAIMTMYA--TQQNADWGLARLSSQKPGTTTYTYDDSAGEG 117
CAC95043 89HSAVDFIENDATVKISAFAEQSGAPWGLSRISHRRPGGSSYAYDDSAGEG 138
CmFE 151STVYVLDSGIQSTHPEFEGRAIHGYNAVKGETDDDGQGHGTHVAGIVGSK 200
CAC95048 151STVYVLDSGIQLDHPEFEGRAIHGYNAVKGETDDDVQGHGTHVAGIVGSK 200
CAC95046 136TCSYIIDTGIDATHPQFGGRAQNIKSFV--NTATDGNGHGTHLAGVIGSV 183
CAB63912 144TCAYVVDSGLYAAHPEFEGRA-HFLGTFVGDQNDNCL-HGTHVAGTIGGR 191
CAC95049 118TCVYIIDTGIEASHPEFEGRATFLKSFISGQT-SDGHGHGTHVAGTIGSK 166
CAC07219 118TCAFIIDTGIEAGHADFEGRAEFLENFTQDGQNTDGNGHGTHVAGTIGSK 167
CAC95043 139TCAYVIDTGVEASHPEFERRALFLKSFVAGE-NSDRNGHGTHVAGTIGSK 187

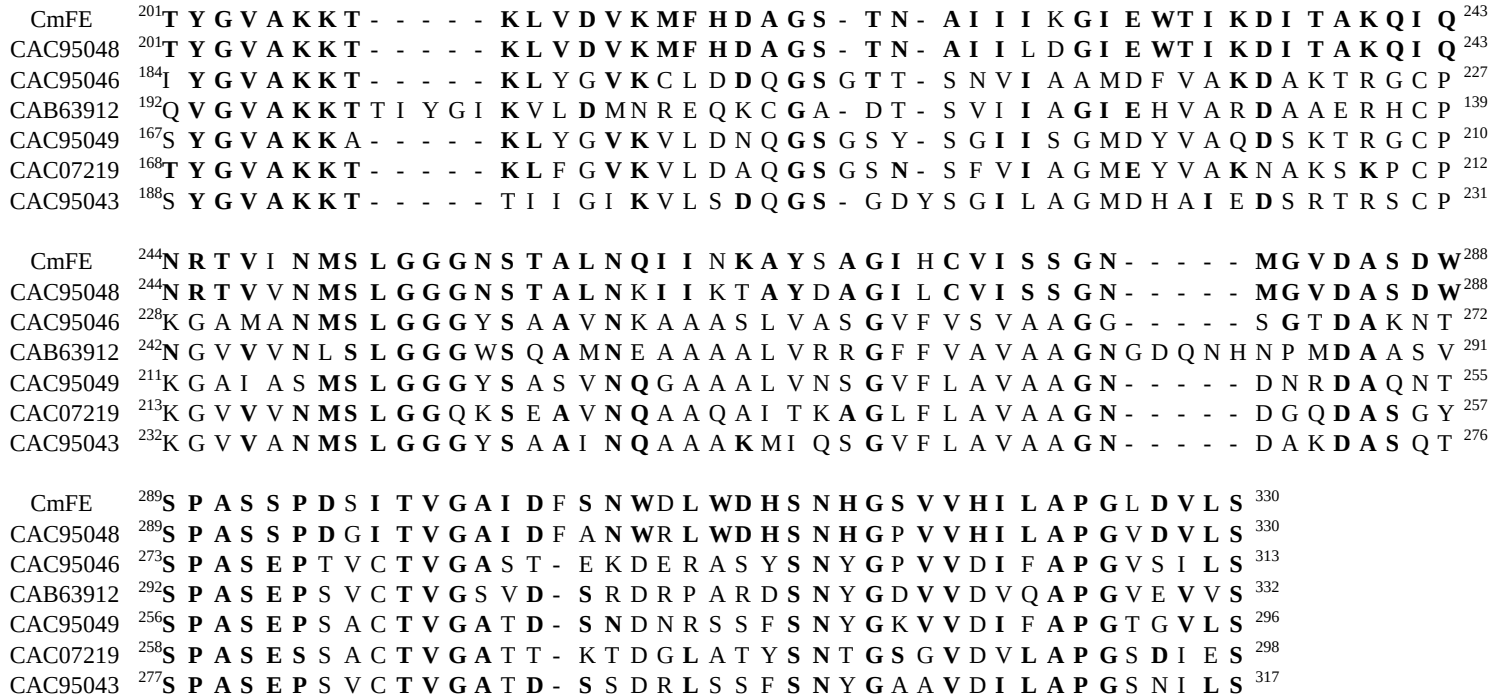
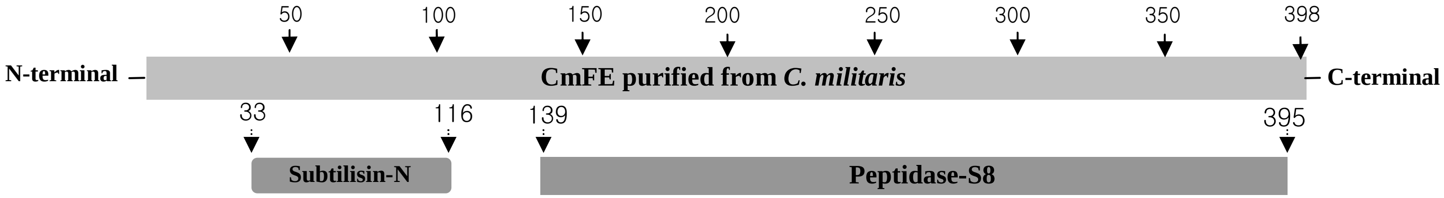


Fig. 26. Alignment of the deduced amino acid sequence of CmFE isolated from *C. militaris* with subtilisin-like serine protease PR1 familiy. subtilisin-like serine protease PR1J (CAC95048); subtilisin-like serine protease PR1B (CAC95046); subtilisin-like serine protease PR1G (CAB63912); subtilisin-like serine protease PR1A (CAC95049); subtilisin-like serine protease PR1K (CAC07219); subtilisin-like serine protease PR1I (CAC95043). The asterisks represent identical amino acids. Conserved positions are molded.



A. Alignment of CmFE with Subtilisin-N domain

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CmFE          334 D K Y I I T L K E G A S A S S F D S H M N W V R D V Q V A R A R H S D G R S T R G V E K Y Y D V D G 82
Subtilisin-N   14 G K Y I V K F K E G A S A A S F D S H H S W V S S I - - L L S A L A S L E S A G G K K V Y S Y K H A 48

CmFE           83 F N A Y A G H F D E H T L E A I R R N A D V E S V E Q Q Q L Y H L H 116
Subtilisin-N   49 F N G F A A K L T E E E A E A L R K H P D V E Y V E P D Q V V H L H 82
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B. Alignment of CmFE with Peptidase-S8

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CmFE          139 T E Y V T D D S L G S G S T V Y V L D S G I Q S T H P E F E G R A I H G Y N - - - - - - - - - - A V K 179
Peptidase-S8    1 P N A W D R G Y T G K G Y T V A V I D T G I D T N H P D L S G N Y I G G K N I S N D D P E G D F N D 58

CmFE          180 G E T D D - - - - D G Q G H G T H V A G I V G S K T - - - - - - Y G V A K K T K L - D V K M F H D A 218
Peptidase-S8   59 N D S D P N P V T D D N G H G T H V A G T I A A V A N N S T G V V G V A P G A K I L A V R V L D G N 108

CmFE          219 G S T - - - - N A I I I K G I E W T I K D I T A K Q I Q N R T V I N M S L G G - - - - - - - - - - - 253
Peptidase-S8  109 G S G T E S L E A S V I R G I D W A V D - - - - - - - N G A D V I N M S L G P G D D S G N T S D G P 151

CmFE          254 G N S T A L N Q I I N K A Y S A G I H C V I S S G N M G Y - - - - D A S D W S P A S S P D S I T V G 299
Peptidase-S8  152 G D S A K L E G A V N Y A V N K G S I F V A A A G N E G S D A C D S C G C D G P A N S I N Y I T V G 272

CmFE          300 A I D F S N W D L W - - D H S N H G S Y V H I L A P G L D V L S A - - - P G N E T K T G S G T S Q A 344
Peptidase-S8  213 A T D L N D T R A S F S E S S N S G L A V D I S A P G Y N I L S D L I P N N G G Y A S H S G T M A A 257

CmFE          345 P H V A G L A A Y L A V A K N I N T A K E L K A T I L S L G T R D K A T A V K D G T V N L V A Y N 255
Peptidase-S8  213 P H V A G V A A L L L S A N P N L T W L E T A A E L R A A L E N T A T S L G G D G I S H L F G Y G 257
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Fig. 27. Domain structure of CmFE purified from *C. militaris*.

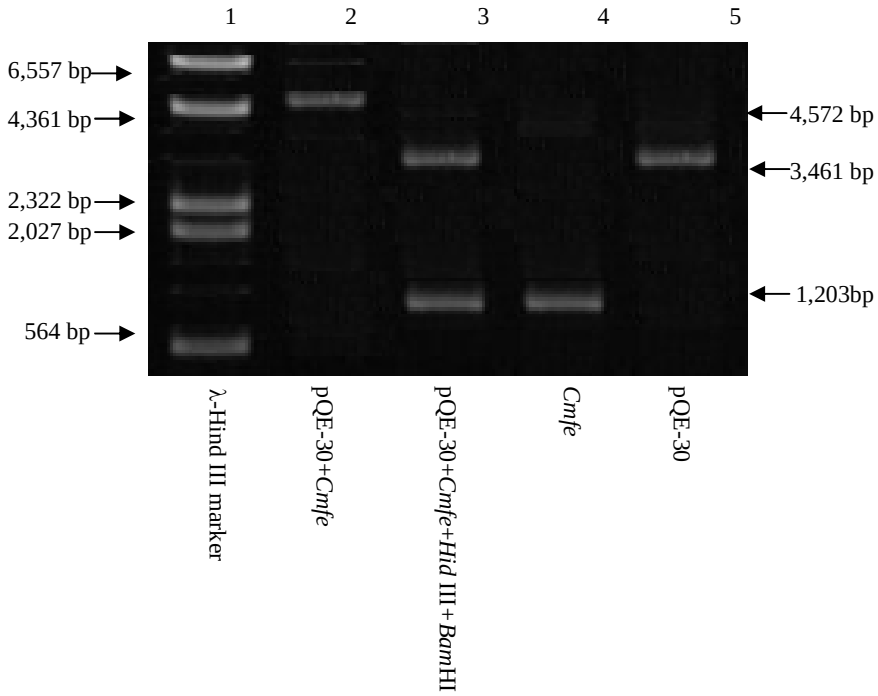

Fig. 28. Construction of expression vector pQE30 with PCR product of *Cmfe* cDNA. The constructed pQE30+*Cmfe* were resolved by electrophoresis on a 2% agarose gel and then visualized by EtBr staining of the gel. Lane 1, λ *Hin*dIII DNA marker; lane 2, pQE30+*Cmfe*; lane 3, pQE30+*Cmfe* digested with *Hin*dIII and *Bam*HI; lane 4, *Cmfe* cDNA PCR product and lane 5, pQE30 vector.

III-K. Analysis of deduce amino acid sequence of CmFE

1,197 base pair were coding sequences that translated into 398 amino acids of the CmFE. The deduce amino acid sequence of CmFE from *C. militaris* is composed of two domains, peptidase-S8 and subtilisin-N domain. Peptidase-S8 (Thr_{139}~Asn_{395}) belongs to subtilase family which is a family of serine proteases. They appear to have independently and convergently evolved an Asp/Ser/His catalytic triad, like that found in the trypsin serine proteases. Subtilisin-N domain (Asp_{33}~His_{116}) has subtilisin N-terminal region. This family is found at the N-terminus of a number of subtilisins. It is cleaved before the activation of the enzyme (Fig. 27).

III-L. Gene expression of *Cmfe* cDNA

The *Cmfe* was cloned into plasmid pQE30 in the correct reading frame under the control of the T5 promoter, with an N-terminal 6X His-tag sequence and *lac* operator. The structures of the expression vector and the *Cmfe* are shown in Fig. 28. The *Cmfe* insert and orientation in the plasmid pQE30+*Cmfe* were confirmed by double restriction enzyme mapping. The plasmid pQE30+*Cmfe* were transformed into *E. coli* expression host M15 strain. All the clones picked were expressing the CmFE with a predicted band around 52 kDa (Fig. 29A). One positive clone was chosen based on the highest relative intensities for the specific band (Fig. 29A). All of the recombinant protein was found in inclusion bodies. Western blot analysis using anti-His AP conjugate confirmed the inclusion body expression of the 6X His tagged fibrinolytic enzyme (Fig. 29B). Although it was confirmed by western blot assay that fibrinolytic enzyme was expressed in prokaryote, but

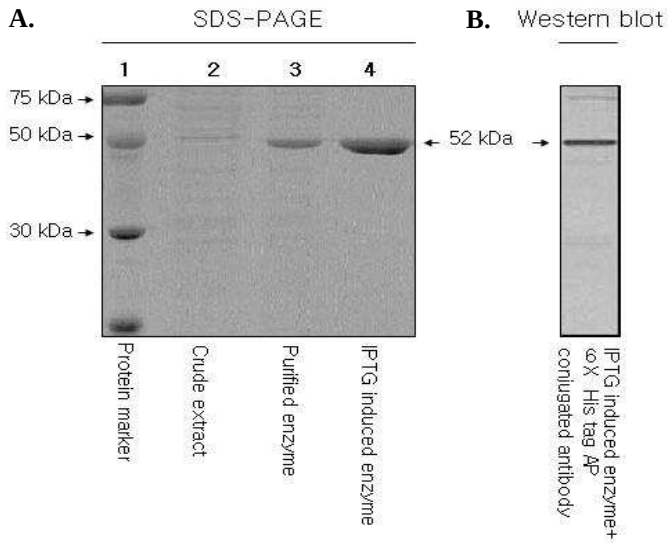

Fig. 29. SDS-PAGE and Western blot analysis of the Ni^{2+} -NTA affinity chromatography of recombinant fibrinolytic enzyme. A, SDS-PAGE; B, Western blot. Lane 1, Protein marker; lane 2, Crude extract; lane 3, Purified enzyme; lane 4, Expressed enzyme.

this enzyme had not fibrolytic activity. When CmFE is expressed in *E. coli*, it is produced as an inclusion body. Therefore, a renaturation procedure is necessary. In addition, because *E. coli* is a prokaryote it is unable to perform many eukaryotic posttranslational modifications such as proteolytic processing, folding, and glycosylation. As a result, the recombinant CmFE produced by *E. coli* has low enzymatic activity after the denaturation and renaturation procedure (Ge et al., 2005). *Pichia pastoris* has been developed into a highly successful system for the expression of heterologous genes. Several types of fibrinolytic enzymes, including streptokinase and urokinase plasminogen activator, have been expressed in *P. pastoris*. (Miele et al., 1997; Wang et al., 2000; Pratap et al., 2000 and Zhang et al., 2002).

IV. DISCUSSION

In recent years, fibrinolytic enzymes have been discovered in a variety of organisms. However, there have been relatively few reports regarding the fibrinolytic enzymes in mushrooms. Recently, mushrooms have begun to elicit increasing attention, as they have been recognized as a nutritious food, with health-stimulating properties and medicinal effects. Here, we have described the purification and characterization of subtilisin like protease from the medicinal mushroom of *C. militaris*. Enzyme was purified 191-fold from 100 g of *C. militaris*, for a total yield of 12.9% (Table 3). The specific protease activity of the purified enzyme was similar to that of the proteinase obtained from of the subtilisine-like serine protease PR1J from *Metarhizium anisopliae var. anisopliase* (NCBI accession CAC95048). The molecular weight of purified enzyme was calculated as 52 kDa by gel filtration, SDS–PAGE and fibrin-zymography, which was different to that determined for the metalloendopeptidases from *G. frondosa* (20 kDa) (Nonaka, et al., 1995), *P. ostreatus* (19 kDa) (Dohmae et al., 1995) and *A. mellea* (32 kDa) (Lee et al., 2005). The optimum temperature of purified enzyme was 37°C, but the enzyme was exposed to temperatures of over 40°C, the fibrinolytic activity of the enzyme degenerated abruptly (Fig. 18). The optimum pH of purified enzyme was 7.0, which is comparable to those of FP I and FP II from *Pleurotus sajor-caju*, and MEF from the egg cases of *Tenodera sinensis* (Hahn et al.,1900; Hahn et al.,2001). Purified enzyme preferentially hydrolyzed the Aα fibrinogen chain. However, all of the three-subunit chains of fibrinogen were completely hydrolyzed. Enzyme activity was inhibited by Cu^{2+} and Co^{2+}, but was enhanced by the addition of Ca^{2+} and Mg^{2+}ions. Furthermore, fibrinolytic activity was potently inhibited by PMSF, and exhibited a higher degree of specificity for the substrate S-2586 for

chymotrypsin. These results indicate that purified enzyme is a serine protease. The N-terminal sequence of the first 19 residues was ALTTQSNV THGLATISLRQ, which is very similar to that of the subtilisin-like serine protease PR1J from *Metarhizium anisopliae* var. anisopliase (NCBI accession CAC95048). The amino acid sequence of purified enzyme was compared with the published sequences of the other subtilisins (Fig. 16), and was found to show 68.4% identity with subtilisin PR1J, low homology with subtilisin PR1B (CAC95046), PR1G (CAB63912), PR1A (CAC95049), PR1K (CAC0 7219) and PR1I (CAC95043) from *Metarhizium anisopliae* (Bagga, et al.,2004**)**. The specific activity of 20 μg of purified fibrinolytic enzyme was 3.3 folds higher than 1.0 unit of plasmin as shown in Fig. 13.

Considering the fibrinolysis pattern it was found that the purified enzyme rapidly hydrolyzed the α-chain, followed the γ-γ chains and more slowly the β-chain. On the otherhand, fibrinogenolysis pattern of purified enzyme revealed that it rapidly hydrolyzed Aα, Bβ and γ chain. Therefore, purified fibrinolytic enzyme from *C. militaris* is a direct-acting fibrinolytic and fibrinogenolytic agent, as it acts via direct cleavage of fibrin and fibrinogen, not by plasmino- gen activator such as SK, UK and tPA as shown in Fig. 19 and Fig 20. These results indicate that purified enzyme is not only can apply on the thrombolytic therapy but also use to prevent formation of blood clotting in vain.

There is a renaissance of interest in using fibrinolytic enzymes as targets for developing therapeutic agents. Protease genes from several bacteria, fungi, and viruses have been cloned and sequenced with the prime aims of overproduction of the enzyme, delineation of the role of the enzyme in pathogenecity, and alteration in enzyme properties to suit its commercial application. Despite the extensive research on several aspects of proteases,

there is a paucity of knowledge about the genetic analysis of fibrinolytic enzymes.

Despite the several reports on the cloning and sequencing the protease gene from different sources, there is a little information on genetic analysis of fibrinolytic enzymes from *C. militaris*. The successful expression of the recombinant CmFE provides a way to obtain a single component with fibrinolytic activity.

A fibrinolytic enzyme from *C. militaris* was purified and found that the sequence of 19 N-terminal amino acids showed a high degree of homology to that of subtilisin-like serine protease PR1J (NCBI accession No. CAC95041) and subilisin-like serine protease PR1 family purified from *Metarhizium anisopliae var. anisopliae.*

At present more than 200 proteases have been assigned to the superfamily of subtilases (subtilisin-like serine proteases), with representatives both in micro-organisms (archaea, bacteria, fungi and yeast) and in higher eukaryotes (Siezen & Leunissen, 1997). All the enzymes belonging to this superfamily have in common a core structure, the catalytic domain, characterized by the presence of 'structurally conserved regions', which correspond to common secondary structure elements. Most of the subtilases characterized so far are extracellular and are subdivided into six families, according to the sequence similarity. The subtilisin family includes true subtilisins as well as minor intracellular proteases (Siezen. & Leunissen, 1997). It is one of the most extensively studied of all proteins, due to its commercial importance (Harwood, 1992,; Wells, 1988). Subtilisin is synthesized as a pre-pro-protein, translocated through the cell membrane via the pre-peptide (or signal peptide) and finally activated by autoproteolytic removal of the pro-peptide (Power et al., 1986) which functions as an intramolecular chaperone (Shinde et al.,

1997). This protease is not essential for either growth or sporulation, but is probably used in nature as a scavenging enzyme.

The subtilisin-like fibrinolytic enzyme gene(*Cmfe*) of *C. militaris* was cloned and its nucleotide sequence was determined. The full length *Cmfe* cDNA was 1,197 nucleotide long. The nucleotide sequence revealed one large open reading frame, composed of 1,197 base pairs which was preceded by 138 bp of 5′-untranslated sequence and followed by 42 bp of 3'-untranslated sequence terminating with a poly (A) tail. By comparing the partial encoding 5'-ends and 3'-ends cDNA sequence of *Cmfe* with the published sequences of the other subilsin-like serine protease family found to show 89% and 93% identity with subtilisin-like serine protease PR1J (AJ251922) respectively. Furthermore the complete sequence of *Cmfe* was compared with the published sequences of the subilsin-like serine protease PR1 family, and was found to show 92% identity with subtilisin-like serine protease PR1J (AJ251922).

1,197 base pair were coding sequences that translated into 399 amino acids of the CmFE. The deduce amino acid sequence of CmFE from *C. militaris* is composed of two domains peptidase-S8 and subtilisin-N domain. Peptidase-S8 (Thr_{139}~Asn_{395}) belongs to Subtilase family which is a family of serine proteases. They appear to have independently and convergently evolved an Asp/Ser/His catalytic triad, like that found in the trypsin serine proteases (see pfam00089). Subtilisin-N domain (Asp_{33}~His_{116}) has subtilisin N-terminal region. This family is found at the N-terminus of a number of subtilisins.

In this report, we described the cloning and expression of sequence of cDNA clone coding for *C. militaris* fibrinolytic enzyme in *E. coli* M15. We selected *E. coli* M15 as an alternative recombinant expression host for recombinant CmFE synthesis for several reasons. *E. coli* M15 as the

advantages of high production yields, the potential to secrete recombinant proteins free into the culture medium, and simple inexpensive culture conditions.The *Cmfe* was cloned to plasmid pQE30 in the correct reading frame, with an N-terminal 6X His-tag sequence under the control of the T5 promoter and *lac* operator. As shown in Fig 28, the *Cmfe* was inserted and orientation in the plasmid pQE30+*Cmfe* was confirmed by double restriction enzyme mapping. The plasmid pQE30+*Cmfe* were transformed into *E. coli* expression host M15. All the picked clones were expressed the CmFE with a predicted band around 52 kDa. It is interesting to note that all of the recombinant protein was found in inclusion bodies which were conformed by the western blot analysis (Fig 29).

Fibrinolytic activity of the expressed protein was analyzed by fibrin plate and found that the recombinant protein showed no fibrinolytic activity. Although, expression of recombinant CmFE was conformed by western blot analysis, the expressed protein was inactive compared to native enzyme. This is, because *E. coli* is a prokaryote it is unable to perform many eukaryotic posttranslational modifications such as proteolytic processing, folding, and glycosylation. As a result, the recombinant CmFE produced by *E. coli* has low or no enzymatic activity after the denaturation and renaturation procedure. However, the increasing popularity of this expression system can be attributed to several factors, most importantly: the simplicity of techniques needed for the molecular genetic manipulation, high production yields, the potential to secrete recombinant proteins free into the culture medium, and simple inexpensive culture conditions. Furthermore, this study provides the significant information on optimum expression condition of the CmFE from *C. militaris.*

In conclusion, the fibrinolytic enzyme obtained from the edible and medicinal mushroom, *C. militaris,* exhibits a profound fibrinolytic activity, and also evidences relatively high substrate specificity to fibrin. Therefore, *C. militaris* may become a new source for thrombolytic agents, and can be used to develop therapeutic agents for the treatment of thrombosis. In addition, the work described here provides a way to obtain a single component with fibrinolytic activity and baseline information for further study on understanding of the structure-function relationship of the enzymes.

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