

## Characterization and Production of Thermostable and Acid-stable Extracellular Fibrinolytic Enzymes from *Cordyceps militaris*

Seon Ah Kim<sup>1</sup>, Hong Joo Son<sup>1</sup>, Keun Ki Kim<sup>1</sup>, Hyun Chul Park<sup>1</sup>, Sang Mong Lee<sup>1</sup>,  
Byung Wook Cho<sup>2</sup> and Yong Gyun Kim<sup>1,\*</sup>

<sup>1</sup>Department of Life Science & Environmental Biochemistry, Pusan National University, Miryang 627-706, Korea

<sup>2</sup>Department of Animal Science, Pusan National University, Miryang 627-706, Korea

Biochemical and enzymatic characterization for extracellular protease isolated from *Cordyceps militaris* cultivated on rice bran medium was investigated. *C. militaris* produced proteolytic enzymes from 10 days after inoculation, maximum enzyme production was found at 25 days. The optimum temperature and pH of proteases production was at 25°C and pH 7.0, respectively. The protease activity was observed in the four peaks (Pro-I, Pro-II, Pro-III, and Pro-IV) separated through Sephadex G-100 column chromatography. The separated protease was optimally active at 25°C. Optimum pH of the protease was between 7 and 8. Enzyme was also stable over at 30-80°C. The enzyme was highly stable in a pH range of 4-9. Protease activity was found to be slightly decreased by the addition of Mg<sup>2+</sup>, Mn<sup>2+</sup>, Zn<sup>2+</sup>, Fe<sup>2+</sup> and Cu<sup>2+</sup>, whereas inhibited by the addition of Ca<sup>2+</sup> and Co<sup>2+</sup>. Protease activity was inhibited by protease inhibitor PMSF. On the other hand, the partially purified protease was investigated on proteolytic protease activity by zymogram gel electrophoresis using three substances (casein, gelatin and fibrin). Four active bands (F-I, F-II, F-III, and F-IV) of fibrin degradation were revealed on fibrin zymogram gels. Both of F-II and F-III showed caseinolytic, fibrinolytic and gelatinolytic activities in three gels. Thermostability, pH stability, and pH-thermostability of the enzyme determined the residual fibrinolytic activity also displayed on fibrin zymogram gel. The only one enzyme (F-II) displayed over a broad range of temperature at 30-90°C. The F-II displayed fibrinolytic activity in the pH range 3-5,

but was inactivated in the range of pH 6-11. The F-I and F-III showed enzyme activity in the pH range of 6-11. In the pH-thermostability, the F-II only kept fibrinolytic activity after heating at 100°C for 10, 20 and 30 min at pH 3 and pH 7, respectively. On the other hand, the F-II was retained activity until heating for 10 min under pH 11 condition. By using fibrin zymogram gel electrophoresis, extracellular fibrinolytic enzyme F-II from *C. militaris* showed unusual thermostable under acid and neutral conditions.

**Key words:** *Cordyceps militaris*, Fibrinolytic enzyme, Rice bran, Entomopathogenic fungus

### Introduction

*Cordyceps* species are known as entomoparasitism fungi kill their hosts to form a fruiting body, shields with the host defense system (insect cuticle) secrete enzymes that break down to penetrate the host (Leger, 1995). As about 70% of the insect cuticle consists of proteins, in order to begin of insect infection, various proteases play a more important role than other enzymes. Entomopathogenes produce proteases such as serine-like protease, trypsin-like protease or the cuticle degrading enzymes (Clarkson and Charnley, 1996; Paterson *et al.*, 1994; Shen *et al.*, 2007), respectively. *Cordyceps* is one of a growing number of FTCM (fungal traditional Chinese medicine) being considered as cures for modern human diseases. Many commercial products are available in the market. More recently other treatments have been claimed such as for respiratory, renal, liver, nervous system and cardiovascular disease, tumors, aging, hyposexuality and hyperlipidemia. Ascomycete mushroom *C. militaris* also has drawn attention as a nutraceutical and in traditional Chinese medicines due to its various pharmacological activities like other *Cordyceps* species. *C. militaris* is parasitic on

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\*To whom the correspondence addressed

Department of Life Science & Environmental Biochemistry,  
Pusan National University, Miryang 627-706, Korea.

Tel: +82-55-350-5541, Fax: +82-55-350-5549;

E-mail: kygyun@pusan.ac.kr

the larvae and pupae of various lepidopteran insects. *C. militaris* is the typical species of *Cordyceps* (Hypocreales, Ascomycetes), which parasitizes larva or pupa of lepidopteran insects generally and has a worldwide distribution. *C. militaris* is now used as a substitute for *C. sinensis* in traditional Chinese medicine and health food. The extracts of *C. militaris* possess extensive pharmacological properties, such as anti-inflammatory, antibacterial, antifungal, anti-tumor/anti-leukemic, anti-fibrotic, and antioxidant activities (Cui *et al.*, 2008; Li *et al.*, 2007). Fibrin is the primary protein component of a blood clot. When clots are not lysed by fibrinolytic enzymes and plasmin, they are accumulating in blood vessels and cause thrombosis leading to thrombotic disorders. The incidence of thrombotic disorders including cerebral stroke, myocardial infarction, and venous thromboembolism are rapidly increasing worldwide. Fibrinolytic enzymes are agents that dissolve fibrin clots. Based on their different mechanism of action, thrombolytic agents are divided into two types; plasminogen activators and plasmin-like proteins. Plasmin-like protein can directly hydrolyze fibrin into fibrin degradation products, thereby the thrombi are dissolved rapidly and completely. However, despite their widespread use these agents have undesired side effects, are relatively expensive, and exhibit a low specificity for fibrin. Therefore, over the past few decades, thrombolytic agents from animals, plants, and microorganisms have attracted more and more medical interest. Some reports have described fibrinolytic activity occurring in some edible mushroom, including *Pleurotus ostreatus*, *Grifola frondosa*, *Flammulina velutipes* and *Tricholoma saponaceum* (Griesch and Vilicinskis, 1998; Kim, 2000; Shen *et al.*, 2007). In recent years, Maki *et al.* (2005) have reported a trypsin-like protease was purified from the culture supernatant of *C. militaris*. In addition, fibrinolytic enzymes have been purified and characterized from culture supernatant of Chinese *C. militaris* (Li *et al.*, 2007) and fruiting bodies of *C. militaris* (Choi *et al.*, 2010). In this study, we found that *Cordyceps militaris* isolated from silkworm pupa could produce a highly active fibrinolytic enzyme in the culture fluid of the rice bran medium. On the other hand, rice is cereal that produced most in Asia such as Korea. Rice is an important and main crop that sustains approximately 2.5 billion people to world. The rice bran produced after rice process is assumed by about 10% of rice bran. Part among these is used into materials such as rice bran oil production, food for an animal, media for mushroom cultivation, and is usually discarded as most waste (Hernandez *et al.*, 2000; Jiamyanguyen *et al.*, 2005). To our knowledge there is no report about the production of fibrinolytic enzymes from *C. militaris* by using rice bran medium. Firstly, we aimed

to optimize a nutrient medium containing agricultural waste for fibrinolytic enzymes production by *C. militaris*. Therefore, we have attempted to produce fibrinolytic enzymes in *C. militaris* and describe partial purification and characterization of fibrinolytic enzymes from the culture filtrate of rice bran. Secondly, the objective of the study was also to find a use for low-valued or wasted rice bran as a bioresource for the development of some functional food material.

## Materials and Methods

### Materials

Human fibrinogen, thrombin, plasmin, and Aprotinin, PMSF, pepstatin A, E-64, leupeptin, 1,10-phenanthroline, and EDTA were purchased from Sigma (St Louis, MO, USA). Electrophoretic reagents were purchased from Pharmacia Biotech. (Sweden). Other chemicals were of analytical grade.

### Strain and cultural conditions

*Cordyceps militaris* that separating in pupa by wild type used in this study. The strain was cultivated at 25°C for 7 days on PDA (potato dextrose agar) and kept for 4°C. This culture was then used for the seed culture inoculation. Medium for enzyme production is used rice bran that byproduct produced at rice process. Medium puts rice bran 20 g and distilled water 100 ml to 1 L mushroom bottle and sterilizes for 15 minutes at 121°C, the medium is used to enzyme production medium. The strain that cultivated in PDA medium for 7 days was cut to 5 × 5 mm size with sterilized cutter, inoculated to sterilized medium, and incubated at 25°C for 40 days.

### Enzyme preparation

The cultivates were stirred with 500 ml of 50 mM phosphate buffer (pH 7.4) containing 0.15 M NaCl, 10 mM thiourea for 2 hour at 4°C, strained through four layers of cheesecloth, and centrifuged at 10,000 × g for 30 min. The supernatant solution was made 20% saturated with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, the resulting solution was centrifuged to remove a small amount of precipitate. This supernatant solution was adjusted to 70% saturation with solid (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and stirred overnight, and the precipitate was collected by centrifugation, redissolved in 10 ml of distilled water and dialyzed several times in distilled water at 4°C. Crude protein extracts were recovered from *C. militaris* rice bran medium. Crude protein extract was dried with freeze drier and stored at -70°C. The freeze dried protein was resuspended in optimal buffer for Sephadex G-100 gel filtration chromatography. The resuspended crude

protein extract was filtered and then the insoluble material was removed through 0.45  $\mu$ m membrane filter. The Sephadex G-100 was treated with 50 mM sodium phosphate buffer (pH 7.4) for 2 h and degassed under vacuum conditions, and the crude protein then loaded onto the prepared Sephadex G-100 column (1.5  $\times$  10 cm) (Pharmacia Biotech, Sweden) that was pre-equilibrated with 50 mM sodium phosphate buffer (pH 7.4). Proteins were eluted with the same buffer (pH 7.4) at a flow rate 1.0 ml/min at 4°C. The active protein fraction was pooled and concentrated by freeze drying and desalted. The partial purified protein was stored at -70°C and used for the experiments.

### Enzyme activity

Enzyme activity of extracellular protease was assayed using Azocasein as the substrate, according to the method of Braun and Schmiz (1980). The reaction mixture consisted of 0.3 ml of 50 mM phosphate buffer (pH 7.4) containing 1% (w/v) of Azocasein and 0.1 ml of enzyme solution, after incubating at 30°C for 1 h, the reaction was stopped by the 0.6 ml of 10.0% (w/v) TCA, incubating at room temperature for an additional 15 min, and then the precipitate was removed by centrifugation at 13,000  $\times$  g for 5 min. 1.5 ml of 1.0 M NaOH was added to 1.0 ml of the supernatant, and its absorbance was measured at 440 nm. One unit (U) of enzyme activity was defined as the amount of enzyme that increases 0.1 of its absorbance for 1 h under the above conditions. The protein concentrations were determined according to Bradford (1976) method using bovine serum albumin as a standard.

### Effects of temperature and pH on enzyme activity

The optimal temperature for enzyme activity of extracellular proteases was determined by incubating the reaction mixture in 50 mM phosphate buffer (pH 7.0) for 1 h at temperature ranging from 30°C to 100°C. Enzyme activity was assayed by using Azocasein method. The optimal pH for enzyme activity was assayed from pH 3.0 to 12.0. The enzyme in 50 mM sodium phosphate buffer (pH 7.0) was mixed with different buffers. The buffers used and their ranges were 0.1 M citrate-phosphate buffer (pH 2.0-5.0), 50 mM sodium phosphate buffer (pH 6.0-7.0), Tris-Cl buffer (pH 8.0-9.0), and glycine-NaOH buffer (pH 10.0-12.0). The thermostability of the enzyme activity was assayed by measuring the activity remained after incubating the enzyme in 50 mM sodium phosphate buffer (pH 7.0) at temperature ranging from 30°C to 100°C for 1 h. The remaining protease activity of each enzyme solution was measured with Azocasein method and fibrinolytic activity examined by fibrinolytic zymogram gel electrophoresis. The pH stability of the enzyme was assayed from pH 3.0 to 11.0. After incubation for 1 h at

37°C, the remaining protease activity of each enzyme solution was measured with Azocasein method and fibrinolytic activity examined by fibrinolytic zymogram gel electrophoresis.

### Effect of protease inhibitors and metallic ions on activity

The effect of the enzyme was investigated using MgSO<sub>4</sub>, CoCl<sub>2</sub>, MnCl<sub>2</sub>, ZnCl<sub>2</sub>, FeSO<sub>4</sub>, CuSO<sub>4</sub>, and CaCl<sub>2</sub>. The partial purified protein was incubated in both the absence and the presence of metal ions including Mg<sup>2+</sup>, Co<sup>2+</sup>, Mn<sup>2+</sup>, Zn<sup>2+</sup>, Fe<sup>2+</sup>, Cu<sup>2+</sup> and Ca<sup>2+</sup> with a final concentration of 1 mM in Tris buffer (pH 7.4) for 1 h at 37°C and the residual protease activity was assayed using Azocasein method. The influences of the protease inhibitors (Aprotinin, PMSF, pepstatin A, E-64, leupeptin, 1, 10-phenanthroline, and EDTA) were also determined. The protease was pre-incubated with these protease inhibitors for 30 min at 30°C. After incubation, the effects of protease were determined with Azocasein as a substrate.

### PAGE and zymogram gel electrophoresis

SDS-PAGE was performed at room temperature by the method of Laemmli (1970) using 12% separating polyacrylamide gel, and stained with Coomassie Brilliant Blue R 250 and then destained. The gelatin and casein degrading activities were assayed by zymogram polyacrylamide gel electrophoresis as the method described by Heussen and Dowdle (1980) with minor modification. Zymogram gels of gelatin and casein were prepared separating gel solution (12%, w/v) containing 0.1% gelatin and casein as co-polymerized substrate under non-reducing conditions. Fibrin zymogram gel analysis was determined using the method that was slightly modified from a method by Choi and Kim (2000). 12% (w/v) resolving gel solution (without SDS) containing 0.12% (w/v) fibrinogen was prepared in a total volume of 10 ml. Thrombin (10 NIH units/ml) was added to the gel solution in final concentrations of 1 NIH unit/ml and gently stirred, and centrifuged to remove insoluble impurities. The fibrin gel was prepared adding SDS and acrylamide to it. Samples were diluted 2 times with SDS sample buffer without 2-mercaptoethanol. After electrophoresis in a cold room (at 20 mA constantly), the gels were shaken gently at room temperature in 2.5% triton X-100 solution containing 20 mM sodium phosphate buffer (pH 7.0) for 1 h and washed successively with distilled water to remove the triton X-100 for 30 min at room temperature. Finally, the gels incubated with sodium phosphate buffer (pH 7.0) at 30°C for 12 h. The gels were stained with Coomassie brilliant blue R 250 and destained to clear zones of substrate hydrolysis.

### pH-thermostability

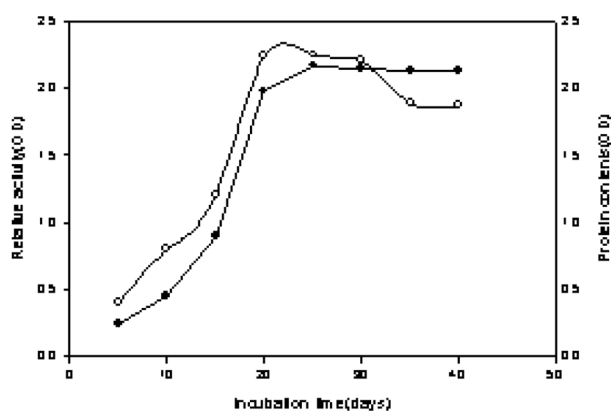
The enzyme in 50 mM phosphate buffer (pH 7.0) that added to 5 vol. of 0.1 M citrate-phosphate buffer (pH 3.0) and glycine-NaOH buffer (pH 11.0) was incubated at 100°C for 10, 20, 30, 40 min, respectively. The effect of pH-thermostability on fibrinolytic activity was assayed by fibrinolytic zymogram gel electrophoresis.

## Results

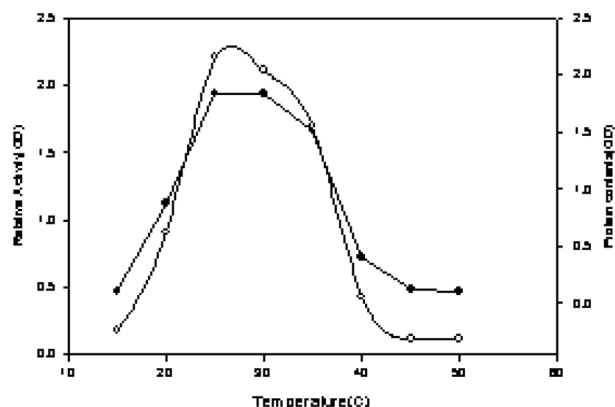
### Protease enzymes production from *C. militaris* in rice bran medium

The effects of time course, incubation temperature, and pH of media on production of proteases from *C. militaris* were determined with rice bran. The extracellular proteases were produced by the procedure described in material and method. *C. militaris* showed an early induction of proteolytic activity by 10 days after inoculation. Maximum activity was found at 25 days after inoculation (Fig. 1). However, a reduction in the index of protease activity was registered by 30 days. On the other hand, casein zymogram gels revealed by three bands were especially clear 15 days after inoculation and onwards (data not shown). The effect of temperature on the extracellular proteases production of *C. militaris* is shown in Fig. 2. Although the production of proteases could be observed in the range of temperature from 20°C to 40°C. The optimum temperature for proteases production was at 25°C. However, when the relative activity of protease compared with 25°C and 30°C it was 98% at 30°C. On the other hand, the relative activities of protease were 82%, and 76% at 20°C and 35°C, respectively.

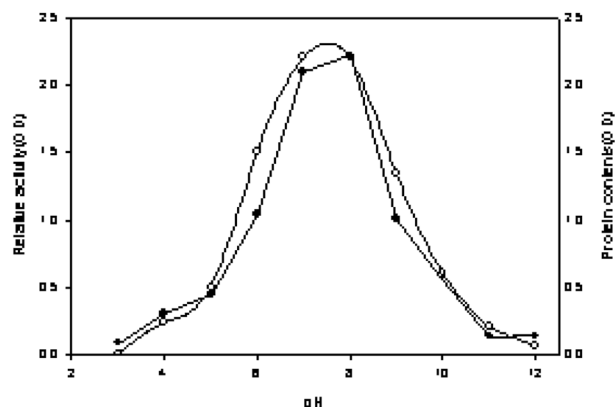
The rice bran contains a lot of nutrition ingredients, mineral and growth factors. pH of medium affects to cell



**Fig. 1.** Effect of incubation time(days) on protease enzymes production in culture media containing rice bran by *C. militaris*. —●—, Protein contents; —○—, Protease activity.



**Fig. 2.** Effect of initial temperature(°C) on protease enzymes production in culture media containing rice bran by *C. militaris*. —●—, Protein contents; —○—, Protease activity.



**Fig. 3.** Effect of initial pH on protease enzymes production in culture media containing rice bran by *C. militaris*. —●—, Protein contents; —○—, Protease activity.

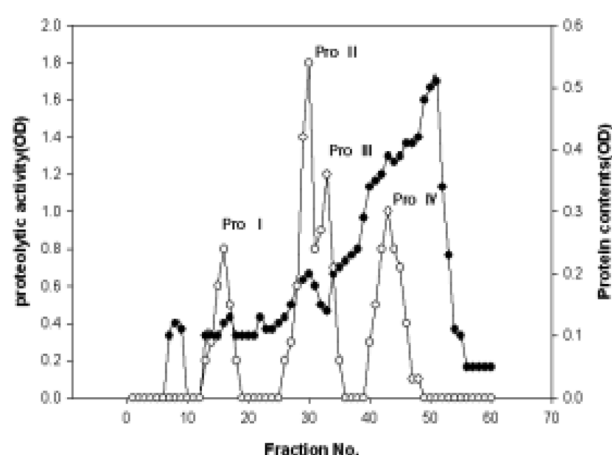
membrane transport rate of these materials that exist in rice bran. It is very important for fungal growth and enzyme production because pH change affects in molecule ionization of components. Therefore, the effect of initial pH on the extracellular proteases production was investigated in the range of pH 3 to 11. As shown in Fig. 3, *C. militaris* produced the extracellular proteases in broad ranges of pH 5 to 11. The optimum pH was at 7-8. The contents of protein was increased in the ranges of pH from 4 to 8, but decreased gradually from pH 9. The highest protein contents were obtained in the pH ranges of 7-8. On the other hand, *C. militaris* was also able to grow in all pH ranges investigated.

### Partial purification of *C. militaris* protease

The pooled culture supernatant was subjected for concentration of protein by ammonium sulfate to 70% saturation

**Table 1.** Comparison of proteolytic activity from culture filtrate of *C. militaris* during purification steps

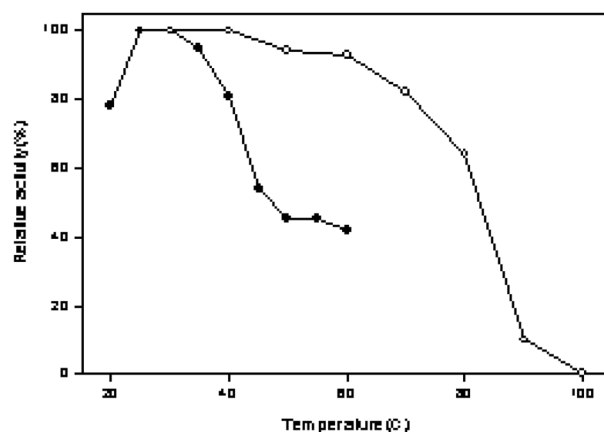
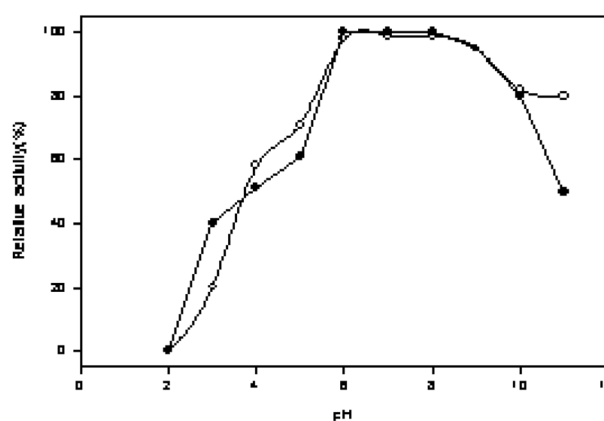
Fraction step	Toatal protein (mg/ml)	Total protease activity (U)	Specific activity (U/mg)	Recovery (%)
Culture supernatant	5820	193.1	0.03	100
Ammonium sulfate	780	145.2	0.19	75
Sephadex G-100	3.2	7.3	2.28	4

**Fig. 4.** Gel filtration chromatography on Sephadex G-100 of protease enzyme in culture filtrate from *C. militaris*. The elution profiles were monitored by spectrophotometry at 280 nm. —●—, Protein contents; —○—, Protease activity.

at 4°C. The separated precipitates were filtrated by Sephadex G-100. Assay of enzyme for proteolytic activity during partial purification protocol were showed in Table 1 and Fig. 4. The results of partial purification are summarized in Table 1. After gel filtration, total protein contents and total protease activity were 3.2 mg/ml and 7.3 units/ml, respectively. Meanwhile, when culture filtrates from *C. militaris* were chromatographed through Sephadex G-100 column, as shown in Fig. 4, major peaks of proteolytic activity were observed in fractions 15-21 (Pro-I), fractions 27-31 (Pro-II), fractions 34-38 (Pro-III) and fractions 44-48 (Pro-IV). This was also confirmed their proteolytic activity on zymogram gels (casein, fibrin and gelatin).

#### Optimum temperature and stability on protease activity

The effect of temperature on the proteolytic activity of the partial purified enzyme was assayed the active between 20 and 60°C (Fig. 5). The protease activity showed that it was

**Fig. 5.** Effect of temperature on protease activity (—●—) and thermostability (—○—) of protease from *C. militaris*.**Fig. 6.** Effect of pH on protease activity (—○—) and pH stability (—●—) of protease from *C. militaris*.

active between 25 and 35°C with an optimum activity at 25°C. We found the enzyme was relatively stable when it was exposed for 1 h to a temperature until 90°C. The enzyme was highly active more than 80% of its activity at a different temperature 30-80°C.

#### Optimum pH and stability on protease activity

The influence of pH on the activity of the partially purified enzyme was determined using buffers at various pH values. The results indicate that the enzyme was active over a wide pH range from 3 to 11, but exhibited maximum activity at pH 7 and 8 (Fig. 6). The enzyme was highly active more than 80% of its activity at a pH range of 5-9. We found the enzyme was highly stable in conditions with a pH range of pH 5-11 at 37°C for 1 h, and more than 80% of its activity was sustained at pH of 10.

#### The effects of metal ions and protease inhibitors

The effects of metal ions  $MgSO_4$ ,  $CoCl_2$ ,  $MnCl_2$ ,  $ZnCl_2$ ,

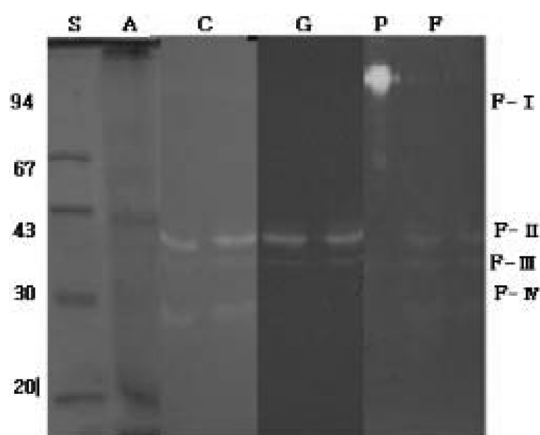
**Table 2.** Effect of protease inhibitors and metal ions on the activity of protease from *C. militaris*

Inhibitors	Concentration (mM)	Relative activity (%)
None	-	100
Pepstatin A	0.5	85
Aprotinin	0.1	73
E-64	1	80
Leupeptin	0.1	88
1,10-phenanthroline	1	68
EDTA	1	91
PMSF	1	53
MgSO <sub>4</sub>	1	88
CoCl <sub>2</sub>	1	53
MnCl <sub>2</sub>	1	89
ZnCl <sub>2</sub>	1	80
FeSO <sub>4</sub>	1	92
CuSO <sub>4</sub>	1	78
CaCl <sub>2</sub>	1	55

FeSO<sub>4</sub>, CuSO<sub>4</sub>, and CaCl<sub>2</sub> on enzyme activity are investigated. The effects of various inhibitors such on the fibrinolytic activity are summarized in Table 2. As shown in Table 2, after incubation of the enzyme with 1 mM of metal ions for 1 h at 37°C, the residual enzyme activity was found to be slightly decreased by Mg<sup>2+</sup>, Mn<sup>2+</sup>, Zn<sup>2+</sup>, Fe<sup>2+</sup> and Cu<sup>2+</sup> but inhibited by the Ca<sup>2+</sup> and Co<sup>2+</sup>. The effects of various inhibitors such as pepstatin A, aprotinin, E-64(N-(trans-epoxysuccinyl)-L-leucine4-guanidinobutylamide), leupeptin, 1,10-phenanthroline, PMSF, and EDTA on the proteolytic activity were also measured. As shown in Table 2, the partial purified fibrinolytic enzyme was inhibited by PMSF which are well-known serine protease inhibitor but the other selected inhibitor reagents did not inhibit proteolytic activity.

#### PAGE and zymography of extracellular protease protein

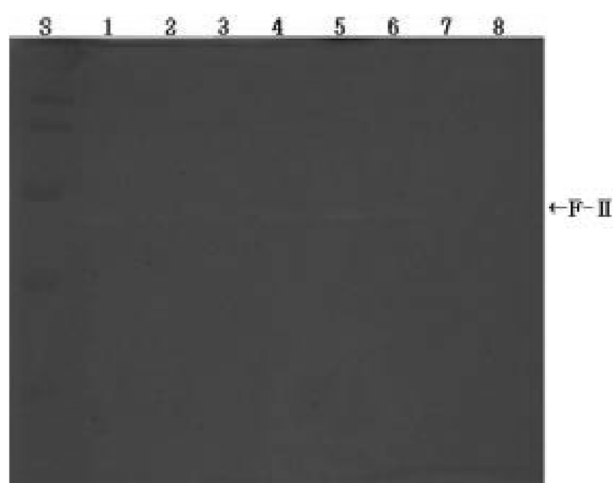
Extracellular proteases produced by *C. militaris* were carried out SDS-PAGE and enzymatic activities investigated on zymogram gel electrophoresis. To confirm the proteolytic activity, three zymographic gels (casein, fibrin and gelatin) were applied to the extracellular proteases from *C. militaris*. Firstly, extracellular proteases extracted by ammonium sulfate were performed on caseinolytic gel. In order to chromatography the protein mixtures, Sephadex G-100 gel chromatography was performed. After fractionation by sodium phosphate buffer (pH 7.4), the fractions of proteolytic activity were pooled and desalted.

**Fig. 7.** Comparison of three substrates casein, gelatin and fibrin in zymographic gels with filtrates from Sephadex G-100 gel chromatography. S: Standard protein. A: SDS-PAGE of Sephadex G-100. C, G, and F are casein, gelatin, and fibrin gels, respectively. P: Plasmin as control in the fibrin gel.

The protein was then performed fibrin, casein, and gelatin zymogram gels (Fig. 7). Zymography of partial purified proteolytic activity enzymes showed several bands of fibrin degradation in electrophoresis gels. Four active bands (F-I, F-II, F-III, and F-IV) of fibrin degradation revealed on fibrin zymogram gels, three active bands (F-II, F-III, and F-IV) also detected on casein zymogram gels. The high molecular range (F-I) showed only clear zone on the fibrin zymogram gel. When the band patterns of proteolytic (fibrinolytic and caseinolytic) activity were compared on zymogram gel, detected active protein bands were located on the same clear bands of the zymogram gels, except for F-I. The molecular weight of these proteins was ranged between approximately 43 kDa and 20 kDa. However, in case of gelatinolytic activity, two active bands (approximately 43 kDa and 30 kDa) were detected. When protease activity was compared with zymography of Sephadex G-100 chromatography filtrates (Fig. 4), the obtained proteins were in the high molecular ranges and low molecular bands that activated to fibrin and casein degradation have not gelatinolytic activity. The resulting of Sephadex G-100 gel chromatography, fibrinolytic activating proteases were released by chromatography. In addition, plasmin (P) used as standard was visualized as clear zones on fibrin zymogram gel.

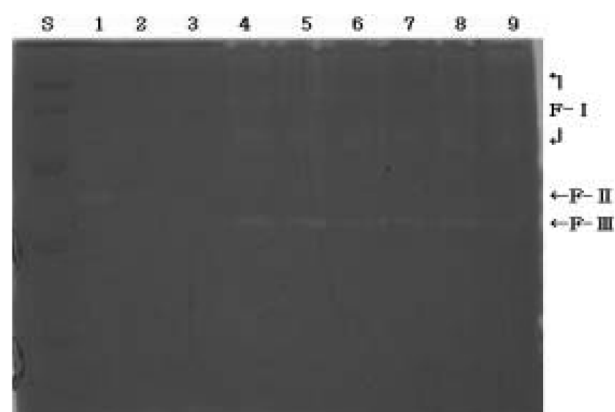
#### Thermostability and pH stability on fibrinolytic activity by fibrin zymogram gel

In order to test the effect of temperature on the stability of fibrinolytic enzymes, the fibrin zymogram gel electrophoresis was used. After the culture supernatant were incubated at each temperature for 1 h under at pH 7 con-

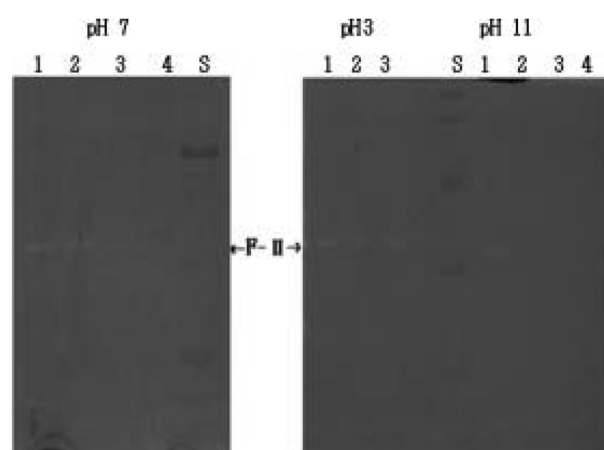


**Fig. 8.** Fibrinolytic activity zymography on stability of extracellular protease from *C. militaris* on the temperature by using fibrin zymogram gel electrophoresis. After incubation of extracellular protease at the indicated temperature for 1 h at 30-100°C, respectively. S: Molecular mass marker. Lanes 1-8: 30, 40, 50, 60, 70, 80, 90, 100°C.

dition, the effect of temperature on the stability of these extracellular fibrinolytic proteases from *C. militaris* was determined on the fibrin zymogram gels. As shown in Fig. 8, thermostability profile of the enzyme showed that the residual fibrinolytic activity was displayed on fibrin zymogram gel by only one enzyme (F-II). Partial purified enzyme preparation showed thermostability over a broad range of temperature at 30-90°C, especially sufficient residual activity was present even when the enzyme was pre-incubated at 80-90°C for 1 h. Meanwhile, in order to test the effect of pH on the stability of fibrinolytic proteases on zymogram gels, we also determined the effect of pH on the stability of extracellular fibrinolytic protease by using fibrin zymography after the culture supernatant were incubated at each pH 3-11 for 1 h under at 37°C condition. As shown in Fig. 9, the pH stability profile of the enzyme showed that the residual fibrinolytic activity was displayed on fibrin zymogram gel by the enzymes. Partial purified enzyme preparation showed pH stability over a broad range of pH 3-11. The one band (F-II) displayed enzyme activity in the pH range 3-5 at 37°C for 1 h incubation. However, the two bands (F-I and F-II) showed enzyme activity in the pH range of 6-11 at 37°C for 1 h incubation. In particular, F-II of pH 3-5 was inactivated in the range of pH 6-11. The high molecular range region (F-I) showed several bands of fibrin degradation on the fibrin zymogram gel. From above result (Fig. 8), although maximum stability of the proteolytic activity was at pH 7 or 8, but the enzymes were found to be thoroughly stable at pH 5-10 where it retained more than 76-80% of the maximum



**Fig. 9.** Fibrinolytic activity zymography on stability of extracellular protease from *C. militaris* on the pH by using fibrin zymogram gel electrophoresis. After incubation of extracellular protease at the indicated pH for 1 h at 30°C, respectively. S: Molecular mass marker. Lanes 1-9: pH 3, 4, 5, 6, 7, 8, 9, 10, 11.



**Fig. 10.** Fibrinolytic activity zymography on stability of extracellular protease from *C. militaris* on the pH-thermostability by using fibrin zymogram gel electrophoresis. After incubation of extracellular protease at the indicated pH 3, 7, and 11 at 100°C for 10-40 min, respectively. S: Molecular mass marker. Lanes 1-4: 10, 20, 30, 40 min.

activity for 1 h incubation. The proteins separated by Sephadex G-100 gel filtration were contained a few fibrinolytic activity enzymes and so we suppose that the property of other proteases containing filtrates have modified by change pH range from acid to alkaline.

#### pH-thermostability of fibrinolytic activity on fibrin zymogram gel

To study pH-thermostability of extracellular fibrinolytic protease under at pH 3, 7, and 11 condition, the enzyme in 50 mM phosphate buffer (pH 7) that added to 5 vol. of 0.1 M citrate-phosphate buffer (pH 3) and glycine-NaOH

buffer (pH 11) was incubated at 100°C for 10, 20, 30, 40 min, respectively. The effect of pH-thermostability on enzyme activity was assayed by fibrinolytic zymogram gel electrophoresis (Fig. 10). The F-II only kept fibrinolytic activity after heating 100°C for 10, 20 and 30 min at pH 3 and pH 7 but the other proteases were not active in these conditions. On the other hand, the F-II had retained activity until heating for 10 min under pH 11 condition, the other proteases (F-I and F-III) showed also no fibrinolytic activity under these conditions.

## Discussion

The main components of insect are proteins and these are the most important barrier to fungal infection (Anderson, 1985). Therefore, proteases secreting fungal pathogens play the most role in cuticle penetration (Clarkson and Charnley, 1996); Griesch and Vilicinskas, 1998). *C. militaris* is a popular Chinese medical mushroom, and an entomogenous fungal species important for the biocontrol of pine moth populations (Alicja, 1998). *C. militaris* known as “Dongchunghwacho” have been reported mainly bioactive constituents from which the stroma and fruit-body grows.

This study investigates characterization and production of extracellular proteases from pupa *C. militaris*. *C. militaris* showed an early induction of proteolytic activity by 10 days after inoculation. Maximum activity was found at 25 days after inoculation (Fig. 1). However, a reduction in the index of activity was registered by 30 days. On the other hand, when early culture filtrates with proteases activity were analyzed with casein zymography, zymograms revealed three bands were especially from 15 days onwards in gels (data not shown). The time-course of proteolytic activity from *P. farinosus* followed a sigmoid curve; the proteolytic activity of culture filtrates was maximum 12-14 days after inoculation (Bidochka and Khachaturians, 1987). Although the production of proteases could be observed at a temperature from 20°C to 40°C, the optimum temperature of proteases production was 25°C (Fig. 2). *C. militaris* produced the extracellular proteases in broad ranges of pH 5 to 11 (Fig. 3). The optimum pH was 7.0. When *C. militaris* culture filtrates were chromatographed through Sephadex G-100 column, four peaks of protease activities were observed in fractions Pro I, Pro II, Pro III, and Pro IV, respectively (Fig. 4). Cui *et al.* (2008) reported that extracellular enzymes from fruiting bodies of *C. militaris* were isolated into 4 peaks of protease activity by using Superdex 75 gel filtration chromatography. The only one peak of all the fractions obtained showed fibrinolytic activity. Maki *et al.* (2005)

reported the trypsin-like proteases from the fruiting body of *C. militaris* were separated into three fractions on the DEAE Bio-Gel Agarose column. However, they did not investigate whether these proteases has fibrinolytic activity or not. A novel protease with fibrinolytic activity was purified by combination of column chromatography steps from the culture supernatant of *C. sinensis* (Zhang *et al.*, 2008). The crude culture filtrates from entomopathogenic fungus *Paecilomyces farinosus* isolated four peaks through gel filtration chromatography (Lopez-Lloca *et al.*, 2002). The optimum temperature (Fig. 5) and pH (Fig. 6) of the partially purified enzyme was measured by varying the reaction temperature and pH, respectively. Thermal and pH stability of the enzyme was also investigated by incubation of the filtrates at different temperature and pH 7.0 for 1 h, and varying pH at 37°C for 1 h, respectively. The protease activity was optimally active at 25°C-30°C. Proteases from *C. militaris* exhibited optimal activity 37-40°C (Kim *et al.*, 2006; Li *et al.*, 2007). Nematode-trapping fungus *Monacrosporium cystosporium* (Yang *et al.*, 2008), *C. militaris* (Li *et al.*, 2007) and *C. sinensis* (Zhang *et al.*, 2008), *A. conoides* (Leger, 1995), *D. shizishanna* (Wang *et al.*, 2006), and *D. varietas* (Yang *et al.*, 2007) were ranged 35-45°C in the optimum temperature. The optimum temperature of proteolytic enzymes were dissimilar to the values of the proteolytic enzyme from these fungi but was very similar to fibrinolytic enzyme from Chinese *C. sinensis* (Zhang *et al.*, (2008). Enzyme was also stable over 20-80°C. Enzyme activity and thermal stability of the enzymes gradually decreased at temperature over 50°C. The proteolytic activity from the culture supernatant *C. sinensis* (Zhang *et al.*, 2008) almost completely was lost when protease was heated to over 60°C. In the case of *C. militaris* (Cui *et al.*, 2008; Maki *et al.*, 2005), the proteolytic activity decreased dramatically when it was exposed for 1 h to a temperature greater than 40°C. The effect of pH on the activity of partial purified proteases was measured using buffers at different pH values (Fig. 6). The enzyme was active over a wide pH range 3-10, but the optimum pH of the enzyme filtrates was between 6 and 8. The enzyme was highly stable in a pH range of 4-9 at 37°C for 1 h, and more than 80% of its activity sustained. These results were quite similar to those of the proteolytic enzyme from *C. militaris* (Choi *et al.*, 2010; Maki *et al.*, 2005), *C. sinensis* (Zhang *et al.*, 2008), nematode-trapping fungi (Wang *et al.*, 2006), and *P. farinosus* (Kim *et al.*, 2008). Proteases of entomopathogenic fungus *P. farinosus* showed an optimum pH of 8.5 using fluorescein isothiocyanate-casein as the substrate. The protease activity of the partial purified protease was inhibited by PMSF which are well-known serine protease inhibitor but the other selected inhibitor reagents did not



highly inhibit protease activity. It was suggested that the protease from *C. militaris* is a serine protease. It is similar to results described by Choi *et al.* (2010), Kim *et al.* (2006), and Maki *et al.* (2005). Proteases of entomopathogenic fungus *Paecilomyces farinosus* were inhibited by PMSF, indicating that it was a serine protease (Lopez-Lloca *et al.*, 2002). In the presence of metal ions such as  $Mg^{2+}$ ,  $Mn^{2+}$ ,  $Zn^{2+}$ ,  $Fe^{2+}$ , and  $Cu^{2+}$ , the residual enzyme activity was found to be slightly decreased, whereas inhibited by the  $Ca^{2+}$  and  $Co^{2+}$ .

On the other hand, we used zymographic techniques to assay fibrinolytic activity following electrophoretic separation in gel (Fig. 7). Three substrates (casein, fibrin and gelatin) were used to zymography technique. Extracellular proteolytic activities in zymogram gels are visualized as clear zones, where proteolytic active proteases have degraded the substrates (casein, fibrin and gelatin). When we compared three zymograms, the same pattern of molecular sizes of three extracellular proteases from *C. militaris* was detected in the three gels. The proteolytic pattern and enzymatic sensitivity of extracellular proteases were quite similar. Molecular weights of these proteolytic enzymes from *C. militaris* were range 67 kDa (F-I), 40 kDa (F-II), 35 kDa (F-III), and 25 kDa (F-IV), respectively. Both of F-II and F-III has presented caseinolytic, fibrinolytic and gelatinolytic activity because they could visualized as clear zones in three gels, but F-IV could not show clear zone in gelatin gel, as well as F-I detected only on fibrin zymogram gel. Lopez-Llorca *et al.* (2002) separated into two fractions and confirmed gelatinolytic activity using gelatin zymography. Proteases of entomopathogenic fungus *P. farinosus* had a molecular mass of 32 kDa calculated by SDS-PAGE. Extracellular serine protease from the nematode-trapping fungus *Monacrosporium cystosporium* (Yang *et al.*, 2008) had molecular mass of 38 kDa that degraded casein, gelatin, BSA, and nematode cuticle. Kim *et al.* (2006) have reported the molecular weight of purified a subtilisin like protease from *C. militaris* mycelia was 52 kDa. The other fibrinolytic enzymes from *C. militaris* fruiting bodies was 34 kDa (Choi *et al.*, 2010) and 27.3 kDa (Li *et al.*, 2007), respectively. Extracellular protease with fibrinolytic activity from the culture supernatant of *Cordyceps sinensis* was 31 kDa (Zhang *et al.*, 2008). The molecular weights were determined for the proteases from *A. oligospora* (35.2 kDa) (Tunlid *et al.*, 1994), *A. frondosa* (20 kDa), *A. mellea* (32 kDa), *D. varietas* (30 kDa) (Yang *et al.*, 2007), *Perenniporia fraxina* mycelia (42 kDa) (Kim *et al.*, 2008) and *Fusarium sp* CPCC480097 (28 kDa) (Wu *et al.*, 2009). As a result, it is obvious that entomopathogenic fungi and nematophagous fungi produce various proteolytic enzymes to degrade insect cuticle. The values of the molecular

weights of fibrinolytic enzymes from basidiomycetes and ascomycetes are quite different. In general, molecular weights of the fibrinolytic enzymes from microbes have been reported to be range from 14 to 52 kDa. The molecular mass of the purified enzyme from *C. militaris* was from 23 kDa to 54 kDa (Kim *et al.*, 2006; Maki *et al.*, 2005). From the above, the fibrinolytic enzymes from culture supernatant of *C. militaris* in this study also were quite similar to those that other proteins reported in the range of molecular mass. Zymography is known as a versatile two-stage technique that involves protein separation by electrophoresis, followed by detection of enzymatic activity in polyacrylamide gels under non-reducing conditions. Through this study, we are found out some aspects. There were significant differences in the pattern of extracellular proteases from *C. militaris* (data not shown). In the case of the three gels, some bands appeared clearly at first, but they disappeared during the destaining process. When their enzymatic sensitivity of the separated protein band compared for between incubation times of gels in the reaction buffer, especially the band that are located in upper clear zone have affected. Therefore, we think that further study will be required to understand the reaction between determined its properties. Meanwhile, thermal stability of fibrinolytic activity was examined by fibrin zymogram gel-PAGE (Fig. 8). Thermostability profile of the enzyme showed that the residual fibrinolytic activity was displayed on fibrin zymogram gel by only one enzyme (F-II). Partial purified enzyme preparation showed thermostability over a broad range of temperature at 30-90°C. In addition, pH stability of fibrinolytic activity was also examined by fibrin zymogram gel-PAGE (Fig. 9). Thermostability profile of the enzyme showed that the residual fibrinolytic activity displayed on fibrin zymogram gel by only one enzyme (Fig. 8). However, the pH stability profile of the enzyme determined that the residual fibrinolytic activity was displayed a different patterns on fibrin zymogram gel by the enzyme (Fig. 9). Partial purified enzyme showed pH stability over a broad range of pH 3-11.0. The one band (F-II) displayed enzyme activity in the pH range 3-5 at 37°C for 1 h incubation. However, the three bands F-I, F-II and F-III showed enzyme activity in the pH range of 6-11 at 37°C for 1 h incubation. In particular, F-II of pH 3-5 was inactivated in the range of pH 6-11. To our knowledge, pH and heat stability of partial purified proteases has not yet been reported for other fibrinolytic enzymes. This is thought to be available to investigate pH and thermal stability of certain proteins and to assay the other characterization before protein purification. The effect of pH-thermostability on enzyme activity was assayed by fibrinolytic zymogram gel electrophoresis (Fig. 10). The F-II only kept fibrinolytic activ-

ity after heating at 100°C for 10, 20 and 30 min at pH 3 and pH 7, respectively, but the other proteases were not activity in these conditions. On the other hand, the F-II was retained activity until heating for 10 min under pH 11 condition, the other proteases were also not activity under these conditions. F-II from extracellular fibrinolytic enzyme of *C. militaris* showed unusual thermostability at acid and neutral condition as shown in Fig. 10. *Staphylococcus* sp. strain AJ (Choi *et al.*, 2009) showed unusual thermostability at acid condition, whereas it was unstable at neutral and alkaline conditions. AJ kept 85% of the initial activity after heating at 100°C for 20 min on the zymogram gel. They reported that this means that SDS and heating may act as a stabilizing factor. Through our experiments, we have known that it will be able to confirm thermostability and pH stability of extracellular fibrinolytic enzymes by zymogram gel before enzyme purification. A combination of pH and heat stability has not yet been reported for other fibrinolytic enzymes from *C. militaris*.

It may be concluded from this work that the organism *C. militaris* has the potential to produce highly thermostable and acid-thermostable extracellular fibrinolytic enzyme which could have potential applications for wide range of industries. However, further work on optimization of rice bran medium for bulk production of enzymes must be initiated; we have to investigate in depth about the molecular basis of high thermostability and acid-thermostability, as well as purification and characterization of fibrinolytic enzyme from *C. militaris*.

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