Songklanakarin J. Sci. Technol.
30 (4), 447-453, Jul. - Aug. 2008

Songklanakarin Journal of Science and Technology
http://www.sjst.psu.ac.th

Original Article

Production and properties of a fibrinolytic enzyme by *Schizophyllum commune* BL23

Patcharaporn Pandee[1], Aran H-Kittikul[1], Ohsugi, Masahiro[2] and Yaowaluk Dissara[3]*

[1] *Department of Industrial Biotechnology, Faculty of Agro- Industry, Prince of Songkla University, Hat Yai, Songkhla, 90112 Thailand.*

[2] *Department of Food Science and Nutrition, School of Human Environmental Science, Mukogawa Women's University, Nishinomiya 663, Japan*

[3] *Department of Microbiology, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla, 90112 Thailand.*

Received 28 September 2008; Accepted 31 July 2008

Abstract

Schizophyllum commune BL23 was cultivated for the production of a fibrinolytic enzyme under submerged culture conditions. Maximum growth (8.93 g/l) with fibrinolytic enzyme activity (576.73 units) was achieved when *S. commune* BL23 were cultured in a peptone yeast extract dextrose broth with an initial pH of 6.0, a temperature of 35ºC and a shaking speed of 150 rpm for 7 days. The protein fraction precipitated with 80% ammonium sulfate saturation had the highest fibrinolytic activity ($35.12x10^4$ units/mg protein). Ammonium sulfate was found to activate the fibrinolytic activity after dialysis. Fibrinolytic enzyme was partially purified using anion exchange chromatography (DEAE-Sephacel). Purity was increased 86 fold and specific activity of 39.31 $x10^4$ units/mg protein was obtained. A single protein band after native polyacrylamide gel electrophoresis (Native PAGE) exhibited fibrinolytic enzyme activity. The maximum activity of the partially purified enzyme was found at 50ºC. Enzyme was stable in the temperature range of 30-50ºC for 48 h but its activity was progressively lost at 60ºC. Activity was retained by 70% over the pH range of 5.0-11.0 at 28ºC for 20 min. After prolonged incubation (48 h), it was stable only in a narrow pH range (6.0-9.0). At pH 7.0 and 30ºC, its activity was retained for 60 days. The fibrinolytic activity was inhibited by 1,10-phenanthroline and EDTA and was completely inactivated by Hg^{2+}. Increasing concentrations of EDTA progressively decreased the enzyme activity. However, its activity was not affected by PMSF and SBTI. The results indicate that the enzyme was most likely a metalloprotease.

Keywords: *Schizophyllum commune*, mycelial culture, fibrinolytic enzyme

1. Introduction

Hemostasis is a complex process obtained through an optimal balance between bleeding and blood clot formation. In an unbalanced state, fibrin clots may not be lysed resulting in thrombosis. Thrombolytic agents from various sources have been extensively investigated. Enzymes, such as urokinase, streptokinase and tissues plasminogen activators have been widely used in the treatment of thrombosis. However, these enzymes are often expensive, thermolabile and can produce undesirable side effects (Chitte and Dey, 2000). Fibrinolytic enzymes that dissolve blood clots and show promise for thrombosis therapy have been successfully identified from various sources. A wide range of microorganisms has been screened for their fibrinolytic properties (Takeno *et al*., 1999). Mushrooms have long been recognized as good sources of powerful pharmaceutical products (Wasser, 2002).

*Corresponding author.
Email address: yaowaluk.d@psu.ac.th

In Korea, mushrooms have been traditionally used in the treatment and prevention of thrombosis (Kim *et al*., 2006). In recent years, fibrinolytic enzymes produced from different mushrooms including *Pleurotus ostreatus* (Choi and Shin, 1998), *Pleurotus sajor-caju* (Shin and Choi, 1999), *Flammulina velutipes* (Shin and Choi, 1998), *Armillariella mellea* (Kim and Kim, 1999), *Ganoderma lucidum* (Choi and Sa, 2000), *Tricholoma saponaceum* (Kim and Kim, 2001), *Formitella fraxinea* (Lee *et al*., 2006) and *Cordyceps militaris* (Kim *et al*., 2006) have been successfully purified and characterized.

Schizophyllum commune, a wood-rotting basidiomycetes, is distributed world wide and is easily recognized by its fan-shaped basidiocarp with a split gill on the underside (Alexopoulos and Blackwell, 1996). Fruiting bodies of *S. commune* collected from the wild have been used as food-stuff, especially in the southern part of Thailand (Petcharat, 1995). *S. commune* has been found to produce fibrinolytic enzymes (Takeno *et al*., 1999). Moreover, its metabolites, polysaccharides and many enzymes, have been utilized for many products.

Several workers have obtained fibrinolytic enzymes by extraction from mushroom fruiting bodies but very few attempts have been reported on their extraction from mycelial cultures. This paper describes the production, purification and characterization of a fibrinolytic enzyme activity from *S. commune* BL23 grown with submerged culture conditions.

2. Materials and Methods

2.1 Microorganism

S. commune BL23 was isolated from a fresh basidiocarp collected from Bala-Hala Wildlife Sanctuary, Narathiwat, Thailand. The stock culture was maintained on potato dextrose agar (PDA) slants containing (g/l): potato, 200; dextrose, 20; and agar, 15 and subcultured monthly.

2.2 Cultivation conditions

The inoculum was prepared by transferred the mycelium of *S. commune* BL23 from an agar slant onto the centre of a PDA plate and incubated at 30°C for 5-6 days. Five pieces of agar mycelium discs (7 mm in diameter), taken from the edge of the fungal colony were transferred to 100 ml peptone yeast extract glucose medium (PYGM) containing (g/l): peptone, 5: yeast extract, 20; glucose, 10; KH_2PO_4, 1 and $MgSO_4.7H_2O$, 0.5 with an initial pH 6.0 in a 250 ml flask. Unless otherwise specified, incubation was carried out on a shaking incubator at 150 rpm, and 30°C. All experiments were duplicated. The whole contents of each flask were collected at various time intervals and centrifuged at 9,000 rpm, 4°C for 20 min. The mycelium pellets were subjected to dry cell weight measurements and the supernatants were assayed for fibrinolytic enzyme activity.

2.3 Effect of cultural conditions

S. commune was cultivated in PYGM as mentioned above. The optimum incubation time was evaluated by varying sampling times (3, 7, 10, 12 and 14 days). The influence of the initial pH (5, 6 and 7) of the PYGM medium was studied. The effect of incubation temperature was determined by incubating under different temperatures (25, 30 and 35°C). The effect of agitation speeds of 150, 200 and 250 rpm was also determined.

2.4 Enzyme purification

All purification steps were carried out at 4°C. Clear culture supernatants obtained by centrifugation (14,000g, 20 min, RC5C Sorvall) were precipitated with 80% saturation of ammonium sulfate and centrifuged at 13,000 x g for 20 min. The pellet was dissolved in 20 mM Tris-HCl buffer, pH 7.0 and dialyzed in the same buffer. The crude enzyme was applied onto a (2.5 cm x 15 cm) DEAE Sephacel (Pharmacia) column pre-equilibrated with 20 mM Tris-HCl buffer, pH 7.0 at the flow rate of 24 ml/h. The column was washed with the same buffer initially and eluted with a linear 0-0.5 M NaCl gradient. Fractions (3 ml) containing fibrinolytic enzyme were pooled and concentrated by lyophilization. One gram of lyophilized pellet was dissolved in 3 ml Tris-HCl buffer (20 mM, pH 7.0) and dialyzed again in the same buffer. Dialyzed enzyme, with 4-fold dilution, was subjected to native polyacrylamide gel electrophoresis (Native PAGE) (Davis, 1964).

2.5 Enzyme assay

The fibrin plate method modified from Astrup and Mullertz (1952) was used to assay for fibrinolytic enzyme activity. Four ml of bovine fibrinogen solution (0.8% w/v bovine fibrinogen dissolved in 0.23% NaCl in 0.18 M boric acid buffer, pH 7.7) were gently mixed with 2 ml of thrombin solution (10 unit/ml in 0.1% $CaCl_2$ in 0.18 M boric acid buffer, pH 7.7) in a 9 cm diameter Petri dish and left undisturbed for 30 min at ambient temperature (28°C) to form fibrin clots. For the enzyme assay, 30 ml of enzyme solution was carefully dropped onto the surface of a fibrin plate in triplicate. Plates were incubated at 35°C for 18 h and the area of lysed zone was calculated. The mean value of the three fibrinolytic zones was used to represent the enzyme activity. A control was made for each experiment using sterilized medium instead of the enzyme solution. In this study, one unit of the enzyme activity was defined as the amount of enzyme in 30 microliters of enzyme solution that produced a clear zone of 1 mm^2 at pH 7.7 and 35°C for 18 h.

2.6 Characterization of partially purified enzyme

Native polyacrylamide gel electrophoresis (Native PAGE) as described by Davis (1964) was carried out with a

4% stacking gel and 5-15% gradient separating gel without sodium dodecylsulfate (SDS). Ovalbumin (45 KDa), serum albumin (66.2 KDa), phosphorylase B (97.4 KDa), β-galactosidase (116.25 KDa) and myosin (200 KDa) were used as protein markers. The enzyme solution was applied into 2 wells. After electrophoresis, the standard protein lane and one lane of the sample were stained by silver staining. The sample on the other lane was unstained and cut into 2 mm pieces. Each piece was put onto a fibrin plate for determining its fibrinolytic enzyme activity.

2.6.1 Effect of temperature on enzyme activity and stability

The effect of temperature on the enzyme activity was studied using the fibrin plate assay. The enzyme solution was dropped onto a fibrin plate and incubated for 18 h at different temperatures starting from 25°C to 60°C. Maximum activity was expressed at 100% and others were compared to the maximum activity. To study the enzyme stability, the enzyme was incubated (under pH 7.0) at 40, 50 and 60°C for 48 h and at 30°C from 5 up to 60 days. The enzyme activity was determined by the fibrin plate assay.

2.6.2 Effect of pH on enzyme stability

The enzyme was incubated with buffer in the ratio of 1:1. The buffers and pH range were 0.2 M citrate buffer (pH 3.0-6.0), 0.2 M Tris-HCl buffer (pH 7.0-8.0), and 0.2 M glycine NaOH buffer (pH 9.0-11.0). The control was the enzyme at pH 7.0. All experiments were incubated at ambient temperature for both 20 min and 48 h. The enzyme activity was measured by the fibrin plate assay.

2.6.3 Effect of metal ions and chemical reagents on enzyme activity

The concentration of metal ions used was 0.2 mM of $CaCl_2$, $CoCl_2$, NaCl, $CuSO_4$, $FeCl_3$, $HgCl_2$, KCl and $ZnCl_2$. The solutions of ethylenediaminetetra acetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF), 1,10-phenanthroline and soybean trypsin inhibitor (SBTI) were prepared separately. After mixing the enzyme and inhibitors in the ratio 1:1 at ambient temperature (28°C), the enzyme activity was measured by the fibrin plate assay.

2.7 Analytical methods

2.7.1 Soluble protein was measured by the method of Lowry *et al.* (1951) or by the modified method of Bradford (1976).

2.7.2 The dry weight (g/l) of the mycelium was measured after repeated washing of the mycelium pellets with sterile distilled water and drying at 90°C to a constant weight.

3. Results and Discussion

3.1 Effect of cultural conditions

Although many workers have demonstrated fibrinolytic enzyme production from bacteria and fungi, very little information from mushrooms regarding the factors affecting mycelial growth and enzyme production is available. According to our previous study, *S. commune* BL23 preferred PYGM as the medium for fibrinolytic enzyme production. In the present work, we aimed to study optimal conditions for this enzyme production and the results are shown in Table 1. Estimation of fibrinolytic enzyme production during the growth period showed that production began during the log phase of growth (3 days) and continued for 7 days. Thereafter, significant decrease was observed, while the fungal biomass remained nearly constant. Therefore, the optimum incubation time for fibrinolytic enzyme production in PYGM was 7 days (506.91 units). Choi and Shin (1998) demonstrated nearly the same pattern of growth and fibrinolytic enzyme production during mycelium culture of *Pleurotus ostreatus*. In this study maximum enzyme activity (489.46 units) was obtained from PYGM with an initial pH of 6.0 ($p<0.05$). Hirasawa, *et al*. (1997) reported that the optimum initial pH of the medium for fibrinolytic enzyme production was between 5.5-6.5. The initial medium pH has also been shown to affect the production of other enzymes by *S. commune* (Fang *et al*., 1999). *S. commune* BL23 grew over a wide range of temperatures (25-35°C), but the maximum activity was obtained at 35°C. The optimum incubation temperature for fibrinolytic enzyme production varies from strain to strain (Hirasawa *et al.*, 1997; Chitte and Dey, 2000). From this result, the incubation temperature used for further study was 35°C. The enzyme activity was relatively high when incubation was carried out on a shaking incubator of 150 rpm (492.06 units). Although higher speeds enhanced mixing efficiency, higher shear stress had an adverse effect on mycelial growth and metabolite production. A similar result has also been found on polysaccharide production by *Ganoderma lucidum* in submerged culture (Srinath *et al*., 1998). The time course of mycelial growth and fibrinolytic enzyme production obtained under optimum conditions is shown in Figure 1. The enzyme activity reached 576.73 units after 7 days of incubation when the maximum cell dry weight of 8.93 g/l was obtained. The profile of enzyme production indicated that the production of enzyme was a growth associated process. The pH (Figure 1) of the culture was constant for 5 days before gradually increasing to pH 8 on day 12^{th}.

3.2 Enzyme purification

The 7 day culture supernatant of *S. commune* BL23 exhibited a high level of fibrinolytic enzyme activity. The

Table 1. Effect of incubation time, initial pH of the medium, incubation temperature and agitation speed on dry cell weight and fibrinolytic enzyme activity of *S. commune* BL23 in PYGM medium.

Factors	Conditions	Dry cell weight (g/l)	Fibrinolytic activity * (unit)
Incubation time (day)	3	3.94	450.79 [b]**
(pH 6.0, 30°C, 150 rpm)	7	7.14	506.91 [a]
	10	7.62	443.80 [b]
	12	7.56	344.73 [c]
	14	7.49	206.59 [d]
Initial pH	5	7.61	434.13 [b]
(7 days incubation 30°C, 150 rpm)	6	7.51	489.46 [a]
	7	9.00	441.95 [b]
Temperature (°C)	25	9.70	343.99 [c]
(7 days incubation, pH 6.0, 150 rpm)	30	8.79	441.75 [b]
	35	9.74	575.22 [a]
Agitation (rpm)	150	8.62	492.06 [a]
(7 days incubation pH 6.0, 35°C)	200	9.79	259.04 [c]
	250	7.65	290.03 [b]

purification steps are summarized in Table 2. Marked enzyme activity lost after dialysis might be due to an ammonium sulfate effect. Therefore, other ammonium salts and sulfate salts ($NH_4H_2PO_4$, $MgSO_4.7H_2O$ and NH_4Cl) were applied into the culture broth and examined for their effects on fibrinolytic enzyme activity after incubation at 4°C for 4 h. It was found that all salts enhanced enzyme activity about 30-60%. When the dialysate was passed through a DEAE Sephacel column, other proteins were eliminated as shown in Figure 2. The enzyme was purified 86 fold with a yield of about 36.47%. The fractions with fibrinolytic enzyme activity were pooled and lyophilized. After non-denaturing polyacrylamide gel electrophoresis, a broad band of protein was detected (Figure 3). This might result from the number of enzyme fractions collected. However, the gels containing the protein directly hydrolyzed fibrin on the fibrin plate (Figure 3).

3.3 Effect of temperature on enzyme activity and stability

The influence of temperature on enzyme activity was studied from 25°C to 60°C. The enzyme activity increased as the temperature increased up to 50°C, and rapidly decreased at 60°C when only 30% of the maximum activity was maintained (Figure 4). The optimum temperature for the fibrin-

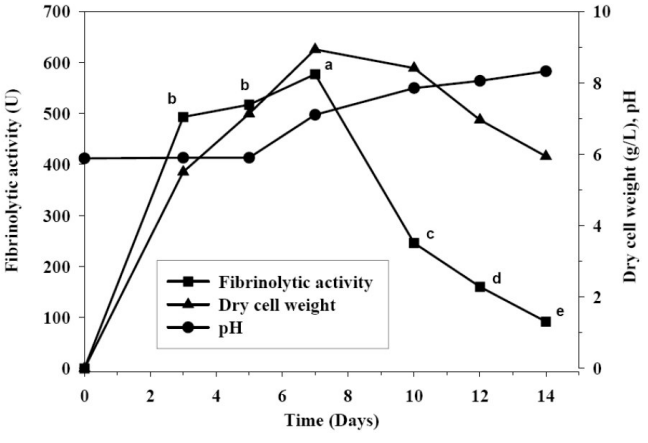

Figure 1. Time course of growth and fibrinolytic enzyme production by *S. commune* BL23 cultivated in PYGM with initial pH of 6.0 at 35°C and shaking speed of 150 rpm. Only fibrinolytic activity in different incubation period were determined for significant comparison. The same letter indicates non-significant differences (p>0.05).

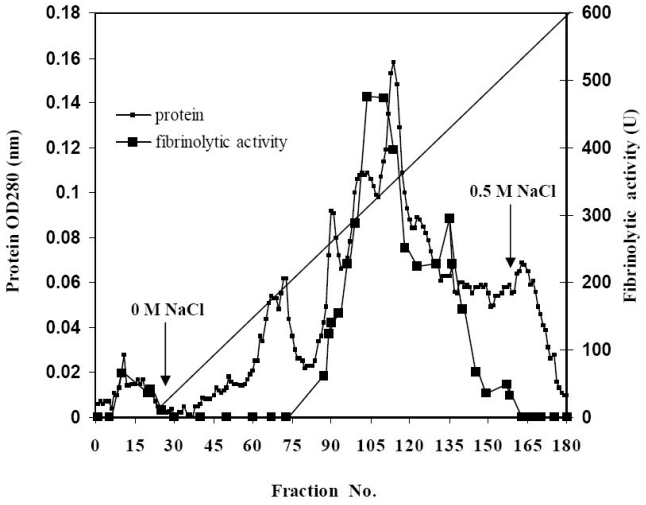

Figure 2. Anion exchange column chromatography on DEAE-Sephacel of a fibrinolytic enzyme of *S. commune* BL23. The sample applied onto the column (2.5x15 cm) was washed with a linear gradient of 0-0.5 M NaCl in 20 mM Tris-HCl, pH 7.0, at the flow rate of 0.4 ml/min.

Table 2. Purification steps of fibrinolytic enzyme of *S. commune* BL23.

Purification steps	Protein concentration (mg/ml)	Activity (units/ml)	Specific activity (units/mg)	Yield (%) (fold)	Purity
Culture filtrate	4.21	19,302	$4.59x10^3$	100	1
80% sat. $(NH_4)_2SO_4$	4.73	1,660,934	$35.12x10^4$	94.65	77
Dialysis	2.52	323,847	$12.85x10^4$	24.33	28
DEAE Sephacel	0.03	11,794	$39.31x10^4$	36.47	86

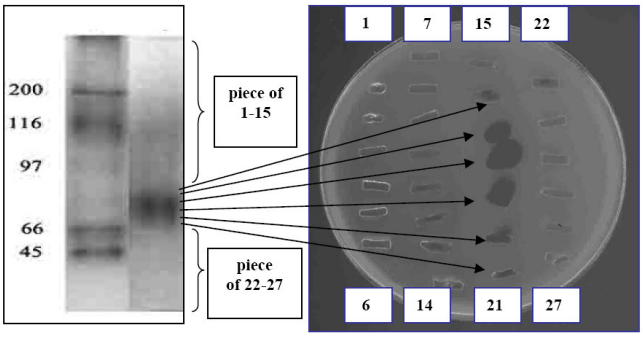

Figure 3. Purified enzyme on native PAGE was cut into 2 mm/ piece and put onto a fibrin plate to show fibrinolytic activity.

olytic enzyme activity derived from *Tricholoma saponaceum* and *Armillariella mellea* was 55°C. This is higher than that found for *Cordycep militaris* (Kim and Kim, 2001; Kim and Kim, 1999; Kim *et al*., 2006). The thermal stability of the enzyme was measured for 48 h at pH 7.0. The enzyme was stable in the temperature range of 40-50°C (relative activity >80%) and a rapid decrease in activity was observed at temperature above 50°C (Figure 5). However, the fibrinolytic enzyme from *S. commune* BL23 was rather stable at high temperatures when compared to others reported previously (Kim and Kim, 1999, Kim and Kim, 2001; Kim *et al*., 2006). In *Flammulina velutipes* the fibrinolytic activity was completely inactivated at 55°C within 15 min (Shin and Choi, 1998). The enzyme from *S. commune* BL23 was stable at 30°C, as 90% of the activity remained, at pH 7.0 for 60 days.

3.4 Effect of pH on enzyme stability

The effect of pH on the stability of the enzyme was determined using buffers at various pH values. Fibrinolytic enzyme from *S. commune* BL23 was stable over a broad pH range of 5.0-11.0 at ambient temperature (28°C) for 20 min. After 48 h incubation, the enzyme was stable only between pH of 6.0-9.0, in which >70% of its activity was retained (Table 3). The enzyme was unstable in very acidic and alkaline conditions and was denatured at pH 3.0 and 11.0. Fibrinolytic enzymes from other mushrooms were stable in pH range of 7.0-9.0 (Choi and Shin, 1998; Kim and Kim, 2001; Kim *et al*., 2006).

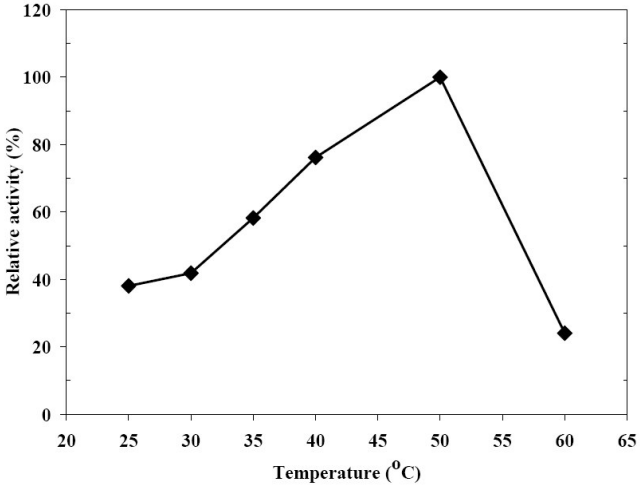

Figure 4. Effect of temperature on fibrinolytic enzyme activity, the enzyme solution was incubated at various temperatures for 18 h before testing.

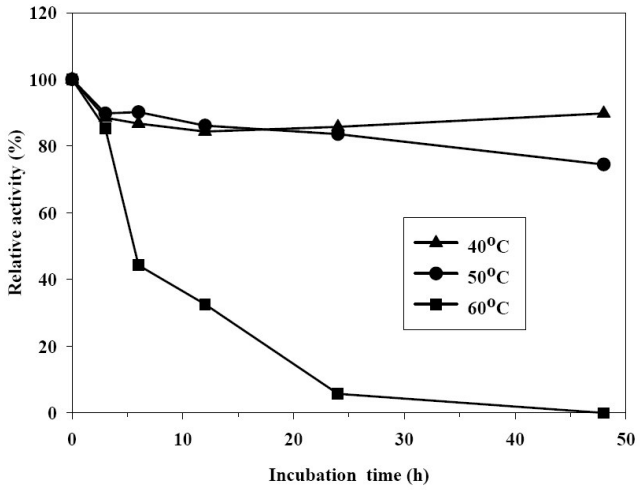

Figure 5. Effect of temperature on enzyme stability. The enzyme solution was mixed with buffer pH 7.0 and incubated for 48 h.

3.5 Effect of metal ions and chemical reagents

The fibrinolytic activity in the presence of 0.2mM metal ions and other reagents is summarized in Table 4. The enzyme activity was strongly inhibited by EDTA, and 1,10

Table 3. Effect of pH on enzyme stability, after incubation at 28°C for 20 min and 48 h.

pH[a]	Relative activity (%)	
	20 min	48 h
3.0	31	0
4.0	62	43
5.0	71	55
6.0	88	70
7.0	92	93
8.0	86	89
9.0	92	77
10.0	95	49
11.0	72	6
control[b]	100	100

[a] Buffers used were 0.2 M citrate buffer (pH 3.0-6.0), 0.2 M Tris-HCl. buffer (pH 7.0-8.0) and 0.2 M glycine-NaOH buffer (pH 9.0-11.0).

[b] The enzyme activity was examined immediately before incubation.

Table 4. Effect of metal ions and chemical reagents on fibrinolytic enzyme activity.

Reagents	Final concentration (mM)	Relative activity (%)
$HgCl_2$	1	0
$ZnCl_2$	1	48
$CuSO_4$	1	71
$FeCl_3$	1	82
NaCl	1	85
KCl	1	86
$CaCl_2$	1	96
$CoCl_2$	1	110
EDTA	1	68
EDTA	10	22
1,10 Phenanthroline	1	23
PMSF	1	121
SBTI	10 µg/ml	127
SBTI	100 µg/ml	114
control	-	100

Control = The enzyme activity that examined after one aliquot of the enzyme solution was mixed with one aliquot of the buffer solution (pH 7.0) without inhibitor.
PMSF = phenylmethylsulfonyl fluoride.
SBTI = soybean trypsin inhibitor.

Phenanthroline but it was unaffected by protease inhibitors, PMSF and SBTI. Therefore, this enzyme from *S. commune* BL23 might be a metalloprotease. Among the tested metal ions, 30% and 50% inhibition was found in the presence of Cu^{2+} and Zn^{2+}, respectively. The enzyme activity was completely inhibited after treatment with Hg^{2+} but Co^{2+} stimulated the enzyme activity. The response of the fibrinolytic enzyme produced by *S. commune* BL23 was similar to those fibrinolytic enzymes purified from mushroom fruiting bodies that were characterized as metalloproteases (Choi and Shin, 1998; Shin and Choi, 1998; Kim and Kim, 1999; Kim and Kim, 2001).

Mushrooms have long been known to contain many biological active substances. Several workers have focused on their anti-tumor and immunostimulating activities. The results of this study show that they may also be a good source of fibrinolytic enzymes. Further characterization of the enzymes is necessary.

Acknowledgement

The Graduate School, Prince of Songkla University, with an Outstanding Scholastic Achievement Awards, supported this work. The authors would like to thank Dr Brian Hogson for editing the manuscript.

References

Alexopoulos, C.J., Mins, C.W. and Blackwell, M. 1996. Introductory Mycology, John Wiley&Son, New York, U.S.A., pp.585-586.

Astrup, T. and Mullertz, S. 1952. The fibrin plate method for estimating fibrinolytic activity. Archives of Biochemistry and Biophysics. 40, 346-351.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry. 72, 248-254.

Chitte, R.R. and Dey, S. 2000. Potent fibrinolytic enzyme from a thermophilic *Streptomyces megasporus* strain SD 5. Letters in Applied Microbiology. 31, 405-410.

Choi, H.S. and Shin, H.H. 1998. Purification and partial characterization of fibrinolytic protease in *Pleurotus ostreatus*. Mycologia. 90, 674-679.

Choi, H.S. and Sa, Y.S. 2000. Fibrinolytic and antithrombotic protease from *Ganoderma lucidum*. Mycologia. 92, 545-552.

Davis, B.J. 1964. Disc electrophoresis-II. Method and application to human serum proteins. Annals of The New York Academy of Sciences. 121, 404–427.

Fang, J., Huang, F. and Gao, P. 1999. Optimization of cellubiose dehydrogenase production by *Schizophyllum commune* and effect of the enzyme on kraft pulp bleching by ligninases. Process Biochemistry. 34, 957-961.

Hirasawa, R., Goto, I., Okamura, T., Horie, N., Kiyohara, T. and Ohsugi, M. 1997. Cultivation condition for production of fibrinolytic active substance by Basidiomycetes. The Bulletin of Mukogawa Women's University Natural of Science. 45, 21-24.

Kim, J.H. and Kim, Y.S. 1999. A fibrinolytic metalloprotease from the fruiting bodies of an edible mushroom, *Armillariella mellea*. Bioscience Biotechnology and Biochemistry. 63, 2130-2136.

Kim, J.H. and Kim, Y.S. 2001. Characterization of a metalloprotease from a wild mushroom, *Tricholoma saponaceum*. Bioscience Biotechnology and Biochemistry. 65, 356-362.

Kim, J.S., Sapkota, K., Park, S.E., Choi, B.S., Kim, S., Hiep, N.T., Kim, C.S., Choi, H.S., Kim, M.K., Chun, H.S., Park, Y. and Kim, S.J. 2006. A fibrinolytic enzyme from the medicinal mushroom *Cordyceps militaris*. Journal of Microbiology. 44, 622-631.

Lee, J.S., Baik, H.S. and Park, S.S. 2006. Purification and characterization of two novel fibrinolytic proteases from mushroom, *Fomitella fraxinea*. Journal of Microbiology and Biotechnology. 16, 264-271.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. 1951. Protein measurement with the Folin Phenol Reagent. Journal of Biological Chemistry. 193, 265-275.

Petcharat, V. 1995. Cultivation of wild mushroom: IV Hed Khlang (*Shizophyllum commune* Fr.). Songklanakarin Journal of Science Technology. 17, 261-269.

Shin, H.H. and Choi, H.S. 1998. Purification and partial characterization of a metalloprotease in *Flammulina velutipes*. Journal of Microbiology. 36, 20-25.

Shin, H.H. and Choi, H.S. 1999. Purification and partial characterization of metalloproteases from *Pleurotus sajor-caju*. Journal of Microbiology Biotechnology. 9, 675-678.

Srinath, P., Diwan, P.V., Kamperdick C., Phuong, N.M., Van Sung, T., Adam, G., Yang, F.C. and Lieu, C.B. 1998. The influence of environmental conditions on polysaccharide formation by *Ganoderma lucidum* in submerged cultures. Process Biochemistry. 33, 547-553.

Takeno, T., Okamura, T., Sera, M., Takana, M., Fukuda, S. and Ohsugi, M. 1999. Screening of fibrinolytic enzymes of microorganisms. The Bulletin of Mukogawa's University Natural of Science. 47, 67-72.

Wasser, S.P. 2002. Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. Applied Microbiology and Biotechnology. 60, 258-274.