Research Article

DO CULINARY MUSHROOMS HAVE FIBRINOLYTIC ACTIVITIES?

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ABSTRACT

Recently, many culinary mushrooms have become attractive sources of biologically but non-pharmacological activities including immune modulating activities. In this study, the fibrinolytic activities of freeze-dried basidiocarps of ten edible mushrooms were determined using a qualitative fibrin plate assay and a quantitative enzyme assay using spectrophotometric method. Further, the crude extracts of all the ten mushrooms were subjected to an aqueous two-phase system (ATPS) to recover the fibrinolytic enzymes. The crude extracts and ATPS extracts of *Lentinula edodes* showed the highest fibrinolytic activity of 48.06 U/mg and 54.28 U/mg, respectively assessed via the Folin-spectrophotometric method. The recovery of fibrinolytic enzymes from *L. edodes* was the highest and the fibrinolytic enzymes were further analysed through gel electrophoresis study. A 50 kDa sized fibrinolytic enzyme from *L. edodes* was revealed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). **Biomed Rev 2017; 28:91-99.**

Keywords: edible mushroom, fibrinolytic enzyme, *Lentinula edodes*, aqueous two-phase system

INTRODUCTION

Fibrinolytic activity is the process of dissolving the fibrin in blood clots by proteolytic enzymes such as nattokinase, streptokinase or urokinase (1). Fibrin clot formation and fibrinolysis are equilibrium processes in biological systems. However, accumulation of fibrin clot in blood vessels causes thrombosis and leads to myocardial infarction or other heart diseases. These conditions are the prominent causes of death all over the world (2). Plasminogen activators such as a tissue type plasminogen activator, the urokinase type plasminogen activator, and the bacterial plasminogen activator are well-known fibrinolytic agents used for clinical and medicinal applica-

tions. However, excessive use of these agents results in undesirable side effects like internal haemorrhage and allergic reactions, limitations in specificity towards fibrin and also these drugs are costly (3). Hence, search for fibrinolytic agents from another natural source continues. The fibrinolytic agents extracted from microbial sources are more favourable due to the enormous diversity and its ease in industrial production (4). Medicinal and edible/culinary mushrooms may be the alternative sources for novel fibrinolytic enzymes (5). The edible mushrooms constitute part of the human diet for its traditionally recognised valuable sources of nutrients (6-8). The edible mushrooms also play an important role in improving human

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health and strengthen the immune system, which have become significant topics for medical and pharmacological research (9, 10). The fibrinolytic activity of proteolytic enzyme complex from *Flammulina velutipes* by gel chromatography was the first report showing appreciable fibrinolytic activity (11), thus paving the way for exploring new fibrinolytic enzymes from other mushroom species. Several studies have discovered fibrinolytic proteases from the fruiting bodies of edible mushrooms such as *Pleurotus ostreatus* (12), *Pleurotus ferulae* (13), *Cordyceps militaris* (14) and *Auricularia polytricha* (15).

In the present study, ten different edible mushrooms, namely *Lentinula edodes* (Berk.) Pegler, *Pleurotus floridanus* Singer, *Pleurotus pulmonarius* (Fr.) Quél, *Flammulina velutipes* (Curtis) Singer, *Agaricus bisporus* (J.E. Lange) Imbach (white and brown variety), *Pleurotus salmoneostramineus* Lj.N. Vassiljeva, *Hericium erinaceus* (Bull.) Pers., *Pleurotus cystidiosus* O.K. Mill and *Grifola frondosa* (Dicks.) Gray, which are commercially cultivated and used as food have been selected and screened for fibrinolytic activity. The aim of the present study was to assess the fibrinolytic activity of the crude mushroom extracts and aqueous two-phase system (ATPS) recovered samples by fibrin plate assay and a spectrophotometric method. The study further aimed to determine the molecular weight of the recovered protein fractions that showed high fibrinolytic activity.

MATERIALS AND METHODS

Mushroom samples

The fresh basidiocarps of *L. edodes* (Shiitake), *F. velutipes* (Enoki), and *A. bisporus* (white and brown button mushroom) were purchased from the local market. The identity was confirmed by Dr Tan Yee Shin and the herbarium specimens were deposited at Mushroom Research Centre, University of Malaya, Malaysia. *P. floridanus*, *P. pulmonarius*, *P. salmoneostramineus*, *H. erinaceus*, and *P. cystidiosus* were obtained from a local mushroom farm (Ganofarm Sdn Bhd, Tanjung Sepat, Malaysia). The basidiocarps were cut into pieces and freeze-dried using a lyophilizer (Christ Alpha 1-4 LD plus) for 48 h. The freeze-dried Maitake (*G. frondosa*) sample was obtained from Dr Kazuya Hidari, University of Aizu, Japan. All the freeze-dried samples were powdered and stored at $-20 \pm 2^\circ\text{C}$ prior to use.

Preparation of crude extract from basidiocarps

Crude extracts were prepared by suspending the powdered sample in 20 mM Tris-hydrochloride buffer (pH 8.0) in 1:20

ratio. Further, the samples were physically ground for 30 min in an ice bath and centrifuged at 10,000g for 30 min to remove cell debris. The supernatant collected was designated as the crude extract (5).

Recovery of possible fibrinolytic enzymes from crude extracts

Aqueous two-phase system (ATPS) extraction

The crude extracts were further subjected to an aqueous two-phase separation method to recover fibrinolytic enzymes (ATPS recovered samples). The polyethylene glycol (PEG) and the phosphate based ATPS were used based on a previous study (15). The crude extracts (25.0%) were added to 20.0% (w/w) PEG-8000 and 11.6% (w/w) potassium phosphate (pH 7.0). The system was gently mixed and then left until two phases formed. The top layer was carefully pipetted by a micropipette in each system and used as the ATPS recovered samples in the fibrin plate assay and spectrophotometric method to determine its fibrinolytic activity.

The purification fold is the purity of fibrinolytic enzyme prior to the purification process. The purification fold was calculated according to Eq. 1:

$$\text{Top phase purification fold (P\&FT)} = \frac{(\text{Fibrinolytic activity} / \text{Protein concentration}) \text{ in top phase}}{(\text{Fibrinolytic activity} / \text{Protein concentration}) \text{ in crude extract}}$$

Protein precipitation

The precipitation of protein from the top phase of ATPS extraction was carried out according to the method of Wessel and Flügge (16). Four volumes of cold acetone (-20°C) were added to tubes containing the top phase obtained from ATPS extraction. The solutions were mixed and incubated in -20°C for 1 h and then centrifuged for 10 min at 10,000g. The supernatant was removed and the pellets were air-dried and dissolved in glass-distilled water (250 μL) prior to use.

Analytical methods

Fibrin plate method

The fibrinolytic activities in crude extracts and ATPS recovered samples were assessed by fibrin plate assay according to the modified method of Kim and Kim (5). The freeze-dried fibrin powder was prepared and dissolved with distilled water to give a 0.6% w/v solution. The mixture was homogenised at 11,000 g for 30 s followed by centrifugation at 10,000 x g for six minutes. The supernatant denoted as fibrin solution was collected and warmed up to $45 \pm 2^\circ\text{C}$. Subsequently, the agarose was prepared in 2% (w/v) of distilled water and main-

tained at 55 ± 2 °C. Then, the fibrin solution was mixed with 2% agarose in 1:1 ratio and poured onto a petridish. The clot was left for 1 h at room temperature. Then, 20 μ L of samples (crude extracts and ATPS recovered samples) were carefully dropped on the fibrin film in the petri-dish and incubated at 37 ± 2 °C. After one hour, the clear transparent region denoted as lytic zone was measured. Samples with 2.28 mg/mL of plasmin and distilled water (dH_2O) served as positive and negative controls respectively. In this study, a lytic zone was observed on fibrin plate when fibrin was hydrolysed and the diameter measured was directly proportional to the strength of the fibrinolytic activity.

Spectrophotometric method (Quantitative fibrinolytic assay)

The fibrinolytic activities of crude extracts and partially purified crude extracts were determined by a modified spectrophotometric method of Yun et al. (17). The fibrin was dissolved at a concentration of 0.6% (w/v) in 0.1 M McIlvaine buffer (pH 7.0). Further, the solution was homogenized at 11,000g for 30 s followed by centrifugation at 10,000g for 10 min at room temperature. The supernatant was collected as fibrin solution and 0.6 mL of fibrin solution was added to 0.3 mL of crude extracts ATPS recovered samples in separate tubes (50 mg/3 mL). After incubation at 40 ± 2 °C for 10 min, the reaction was terminated by adding 0.6 mL of 0.55 M trichloroacetic acid (TCA) solution. The mixture was left for 10 min before centrifugation and the supernatant was mixed with one mL of 0.4 M sodium carbonate (Na_2CO_3) and 0.2 mL of 1 N Folin–Ciocalteu reagent. A blank was prepared equivalently and crude extracts were replaced with 0.3 mL of McIlvaine buffer (pH 7.0) and ATPS recovered samples with blank ATPS (ATPS without loaded crude extract). The mixture was maintained for 30 min at room temperature and an absorbance was measured at 660 nm. The total activity was measured by comparing the standard curve generated using tyrosine. One unit (U) per mL of enzyme activity was defined as one μ g tyrosine released/mL extract/minute of incubation. Whereas, the specific fibrinolytic activity was expressed as U/mg protein.

Measurement of soluble protein

The amount of soluble protein in crude extracts and ATPS recovered samples were estimated by Bradford method using bovine serum albumin as a standard (18). The crude extracts and ATPS recovered samples (0.2 mL) were mixed thoroughly with 5 mL of Coomassie Brilliant Blue reagent and maintained at room temperature for 15 minutes. The samples were

replaced by 0.2 mL of distilled water and mixed with 5 mL of Coomassie Brilliant Blue reagent for the blank. The amount of protein in the sample was calculated by using the protein calibration plot once the absorbance was taken at 595 nm.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE)

The molecular weight of crude extracts and ATPS recovered samples were determined by SDS–PAGE (19). The samples were mixed with a 10 $\times$ sample buffer (1 M Tris–HCl, 30% glycerol, 0.06% SDS, 16% 2-mercaptoethanol and 0.06% bromophenol blue in 9:1 ratio). 20 μ L of the samples were loaded in each well of an acrylamide gel consisting 12% resolving gel and 4.5% stacking gel. The gel was electrophoresed in Tris–glycine running buffer (30 mM Tris, 144 mM glycine, 0.1% SDS) at 80 V for approximately 120 min. Prestained protein molecular weight markers (10 to 260 kDa) (Thermo Scientific) were used. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 and destained with a destaining buffer of 10% methanol and 10% acetic acid.

Statistical analysis

Data are expressed as mean $\pm$ SEM of at least triplicate individual values. The data for all the parameters obtained were subjected to one-way analysis of variance (ANOVA). Statistical significance was set at $p < 0.05$ using Duncan's multiple range test (DMRT) (20).

RESULTS AND DISCUSSION

Fibrinolytic activities in crude extracts of edible mushrooms

The fibrinolytic activity of all the ten mushroom crude extracts determined by the spectrophotometric method is presented in Fig. 1. Among the ten mushrooms, the highest fibrinolytic activity of 48.06 U/mg was observed in the crude extract of *L. edodes*. Interestingly, the fibrinolytic activity recorded for the crude extract of *L. edodes* was two times higher compared to *A. bisporus* (white), which exhibited the second highest fibrinolytic activity of 24.76 U/mg. The lowest fibrinolytic activity was noted in *P. cystidiosus* with 6.14 U/mg. However, crude extracts of other mushrooms showed only moderate fibrinolytic activity in the range of 9–17 U/mg. According to a previous report, a fibrinolytic activity of 40 U/mg was observed in *P. ostreatus* (enzyme purified through several column chromatography steps) (12), which is lower compared to the fibrinolytic activity of *L. edodes* obtained in

Figure 1. The fibrinolytic activity of crude extracts and ATPS extracts of selected edible mushrooms. Values are mean of triplicate determination $\pm$ S.D. Values with the same alphabet are considered not significant ($p > 0.05$).

this study. The fibrinolytic activity in crude extracts of all edible mushrooms was significantly higher compared to crude extracts of *Armillariella mellea* (5) and *Cordyceps militaris* (21), which showed only 0.17 U/mg and 0.02 U/mg, respectively.

It has been reported that *L. edodes* is rich in selenium (22) and contains sulphur compounds like lenthionine that have the capability to break blood clot (23). Similarly, *A. Bisporus* (white) has also been described as a selenium-riched mushroom (24). Selenium can inhibit platelet aggregation and controls the enzymatic activity of glutathione peroxidase (25). Hence, the selenium component present in both *L. edodes* and *A. bisporus* (white) might be the active constituent responsible for the high fibrinolytic activity.

Recovery of samples by aqueous two-phase system (ATPS)

The ATPS is more suitable for enzyme recovery as well as purification of microbial enzymes. The incompatibility between aqueous solutions of a polymer (PEG) and a salt (phosphate) causes forming of large aggregates once they mixed. Besides that, the aqueous solutions tend to separate into two different phases by steric exclusion (26).

In the present study, the crude extracts of the mushrooms were subjected to ATPS for recovery of possible fibrinolytic

enzymes according to the method adopted in our previous study (15). The fibrinolytic activity of the ATPS recovered samples were determined again by the spectrophotometric method (Fig. 1) and compared with the fibrinolytic activities of the crude extracts. The purification fold of the ATPS recovered samples is presented in Fig. 2.

The highest fibrinolytic activity of 54.28 U/mg, after ATPS recovery was observed in *L. edodes*, which was significantly higher compared to its crude extract. The recovery of fibrinolytic enzymes from *L. edodes* was 1.13-fold. Surprisingly, *P. floridanus* showed the highest recovery by a purification fold of 4.45-fold. Similarly, the recovery of fibrinolytic activity from *P. pulmonarius* (29.37 U/mg) and *P. cystidiosus* (17.70 U/mg) were 3.26-fold and 2.88-folds, respectively. A similar ATPS (PEG/phosphate) showed a fibrinolytic enzyme recovery of 1.51-fold from *Streptomyces* sp (26), which was nearly three times lower than *P. floridanus*, *P. cystidiosus* and *P. pulmonarius* compared in this study.

In contrast, the fibrinolytic activity of *A. bisporus* (white) decreased to 6.84 U/mg compared to its crude extract (24.76 U/mg) after ATPS. Apart from *A. bisporus* (white), the fibrinolytic activity of *A. bisporus* (brown) and *G. frondosa* were lower compared to their crude extracts i.e., 9.16 U/mg

Figure 2. The purification fold of fibrinolytic enzyme in ATPS extracts of edible mushrooms. Values are mean of triplicate determination $\pm$ S.D; Values with the same alphabet are considered not significant ($p > 0.05$).

and 4.63 U/mg, respectively. The presence of high molecular weight PEG in the ATPS created a high hydrophobicity on the top phase and this caused more partitioning of fibrinolytic enzymes to the top phase. In line with our present result, the fibrinolytic enzymes from *Auricularia polytricha* and *Streptomyces* sp were also partitioned in top phase (15,26). It is evident from the present results that the fibrinolytic enzymes of *A. bisporus* (white and brown) and *G. frondosa* may have slightly different characteristics as compared to others. Posttranslational modification of amino acids in protein causes changes in the chemical nature of an amino acid, which also gives characteristics such as polar, non-polar, hydrophobic and hydrophilic (27). These fibrinolytic enzymes have low affinity toward hydrophobic environments that created by top phase in ATPS. Hence, it was inferred that three out of the ten mushrooms, the fibrinolytic components of *A. bisporus* white, *A. bisporus* brown and *G. frondosa* were not suitable for ATPS recovery. Whereas, the recovery of fibrinolytic enzymes of *L. edodes*, *P. pulmonarius*, *P. cystidiosus* and *P. floridanus* by ATPS was 2-4 folds higher when compared to buffer extraction.

Fibrin plate assay

The fibrinolytic activity of all the ten mushroom crude ex-

tracts and ATPS recovered samples were subjected to the fibrin plate assay (Table 1). The crude extracts of *L. edodes*, *P. pulmonaris*, *F. velutipes*, *A. bisporus* (white & brown), and *P. salmoneostramineus* showed strong lytic zones with diameters from 2-3 cm. These were significantly higher compared to the positive control plasmin (1.33 ± 0.12 cm). On the contrary, the crude extract of *P. cystidiosus* did not show any lytic activity.

The ATPS recovered samples of all mushrooms showed higher lytic zones than plasmin and crude extracts. The diameter of lytic zones from ATPS extracts of all mushrooms were between 2.5 and 3 cm. The observed lytic zone of ATPS samples in the present study is higher compared to the purified enzyme lytic zones of *Cordyceps militaris* (21) and *Auricularia polytricha* (15), which were 1.95 cm and 1.03 cm, respectively. The ATPS used in the present study has effectively concentrated the fibrinolytic enzymes, which is evident from the lytic zone of *P. cystidiosus* ATPS purified enzyme (3.17 ± 0.15 cm).

Molecular weight of the fibrinolytic components of *L.edodes* and *P.floridanus*

Based on the prominent activity on fibrinolytic assay and fibrin plate assay after ATPS recovery, *L. edodes* and *P. floridanus* were selected for molecular size determination.

Table 1. The fibrinolytic activity of edible mushrooms on fibrin plate

Mushroom samples	Crude extract	ATPS extract
	Diameter of lytic zone (cm)	Diameter of lytic zone (cm)
dH ₂ O (negative control)	NA	-
Plasmin (positive control)	1.33 ± 0.12	-
<i>L. edodes</i>	1.90 ± 0.10	2.57 ± 0.47
<i>P.floridanus</i>	1.83 ± 0.12	2.70 ± 0.26
<i>P. pulmonarius</i>	2.60 ± 0.10	3.03 ± 0.15
<i>F.velutipes</i>	2.20 ± 0.20	2.83 ± 0.59
<i>A. bisporus</i> (white button)	3.00 ± 0.10	2.90 ± 0.10
<i>A. bisporus</i> (brown button)	2.33 ± 0.12	2.47 ± 0.51
<i>P. salmoneostramineus</i>	2.83 ± 0.06	2.97 ± 0.06
<i>H. erinaceus</i>	1.43 ± 0.15	2.37 ± 0.25
<i>P. cystidiosus</i>	NA	3.17 ± 0.15
<i>G. frondosa</i>	2.33 ± 0.12	2.50 ± 0.10

NA: no activity

The molecular weights of the samples of *L. edodes* and *P. floridanus* were determined by SDS-PAGE (Fig. 3). The crude extract of *L. edodes* showed bands in the range from 9 kDa to 120 kDa. However, the ATPS recovered extract of *L. edodes* from showed a band at 50 kDa. Some bands were also noticeable within the range from 30 kDa to 140 kDa for crude extracts of *P. floridanus*. Nevertheless, the ATPS recovered extracts of *P. floridanus* showed only two bands with molecular weight of 45 kDa and 140 kDa. The band observed at 140 kDa from ATPS recovered extract of *P. floridanus* was very dense and clear compared to the band with 45 kDa (Fig. 3).

The molecular weight of the crude samples and ATPS recovered samples varied in the two mushrooms studied. The molecular weight of fibrinolytic enzymes from mushrooms ranged from 14 to 100 kDa (28). In mushroom basidiocarp, the molecular weight of fibrinolytic enzymes ranged from 24-52 kDa (12,14). In support of the previous reports, the present study the ATPS recovered samples of *L. edodes* showed band with approximately 50 kDa and it was very close to purified extracts of *H. erinaceum* (51 kDa) and *Cordyceps militaris* (52 kDa) based on previous reports (29,30). Besides, the ATPS recovered sample of *P.*

floridanus showed band of 140 kDa, which is larger compared to the range mentioned above. The fibrinolytic enzyme with the largest molecular weight (100 kDa) has been reported from *Ganoderma lucidum* (31). Another band of 45 kDa was observed in the ATPS recovered sample of *P. floridanus*. This band is slightly similar to fibrinolytic enzyme extracted from *Perenniporia fraxinea* (42 kDa) (32).

Mushroom extracts exert their bioactivities by modulation of the immune system; activating macrophages, neutrophils, and lymphocytes. This enhanced immunostimulation by mushroom's polysaccharides has been confirmed to be the mechanism of action for possible anticancer activity (33). However, what about the correlation between the immunomodulating polysaccharides and atherosclerotic vascular diseases? In one study regarding the protection effects of mushrooms against atherosclerotic vascular diseases, *Agaricus blazei* Murrill has been shown to contain thermostable antioxidant substances which comprised of 0.68% β -D-glucan (34). The *A. blazei* preparation which also contained 8% of alcohol showed fibrinolytic activity on bovine thrombus. Overall, the fibrin binding activity of mushroom-derived complex which allows for the activation of the fibrin degradation pathway, needs to be further investigated.

Figure 3. Molecular weight analysis of fibrinolytic enzyme on crude extracts and ATPS extracts of *L. edodes* and *P. floridanus*. The protein marker with molecular weight ranged from 10-260 kDa was used in this analysis.

CONCLUSION

It can be inferred that all the ten-selected edible mushroom crude extracts and ATPS recovered samples showed positive fibrinolytic activities. The crude extracts and ATPS recovered sample of *L. edodes* exhibited the highest fibrinolytic activity, i.e. 48.06 U/mg and 54.28 U/mg respectively. The molecular weight of the fibrinolytic enzyme of *L. edodes* was estimated to be 50 kD by SDS-PAGE.

CONFLICT OF INTEREST STATEMENT

The authors certify that they have no affiliations with or involvement in any organization with any financial interest in the subject matter discussed in this article.

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