

Chapter 2

Materials and Methods

Materials

1. Microorganism

S. commune BL 23 used in this study was kindly provided from Assoc. Prof. Dr. Souwalak Phongpaichit, Faculty of Science, PSU. It was isolated from fresh basidiocarp found in Bala-Hala Wildlife Sanctuary, Narathiwat, Southern Thailand. The culture was maintained on potato dextrose agar slant and subcultured fortnightly.

2. Media (Appendix 1)

- Potato dextrose agar (PDA)
- Potato dextrose broth (PDB)
- Sabraud dextrose broth (SDB)
- Malt yeast extract broth (MY)
- Minimal salt medium (MM)
- Peptone yeast extract glucose medium (PYGM)

3. Chemicals

Analytical grade chemicals were used for assay of fibrinolytic enzyme activity, protein concentration, purification, native gel electrophoresis and enzyme characterization (Appendix 2 and 3)

Instruments

- Autoclave Model SS-320 Tomy Seiko Co.Ltd, Japan.
- Automatic pipette (Eppendorf, Germany)
- Column
- Gradient markers Model Hoefer SG30, Hoefer scientific instruments San Francisco
- Incubator shaker Model INNOVA 4000 (New Brunswick Scientific, U.S.A)
- pH meter Model 713 (Metrohm, Switzerland)
- UV-VIS spectrophotometer Model Lambda25 (Perkin Elmer, U.S.A)
- Water bath (Mettler, Germany)
- Refrigerated centrifuge Model Sorvall RC5C (Dupont, Korea)
- Electrophoresis (Bio-Rad, U.S.A.)
- Fraction collector, Bio-RAD Laboratories
- Hot air oven, Heraeus GmbH, Germany
- Peristaltic pump, Bio-RAD Laboratories
- Power supply (Bio-Rad, U.S.A)

Analytical methods

1. Soluble protein was measured by the method of Lowry *et al.* (1951) or by the modified method of Bradford (1976) (Appendix 3)

2. Dry biomass

The culture was centrifuged at 4 °C and 9,000 rpm for 20 min. The supernatant was kept for fibrinolytic enzyme assay. The pellets were suspended in distilled water and recentrifuged, then dried at 90 °C in hot air oven for 24 h and then weighed to constant weight after cooling in dessicator.

3. Fibrinolytic enzyme activity (modified from Astrup and Mullertz's method, 1952)

3.1 Fibrin plate preparation

The fibrin plate was prepared by gently mixing 4 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) and 2 ml of thrombin solution (10 U/ml in 0.1% CaCl₂ 0.18 M in boric acid buffer, pH 7.7) in a 9.0 cm diameter petri dish and left for 30 min at room temperature to allow fibrin solidification.

3.2 Enzyme assay

Thirty microliters of clear supernatant was dropped onto a fibrin plate and the supernatant was carried out in triplicate on each plate. After incubation at 35°C for 18 h, a clear transparent region, where fibrin was hydrolyzed was observed (Figure 4). Its diameter which proportional to the potency of fibrinolytic activity was measured and then the area of lysed zone was calculated. The mean value of the three determination of lytic zone represented the enzyme activity was presented. Control was made for each experiment using sterilized medium instead of the culture supernatant.

In this study, one unit of the enzyme activity defined as the amount of enzyme in 30 microliters of enzyme solution which produced a clear zone of 1 mm² at pH 7.7, 35°C for 18 h.

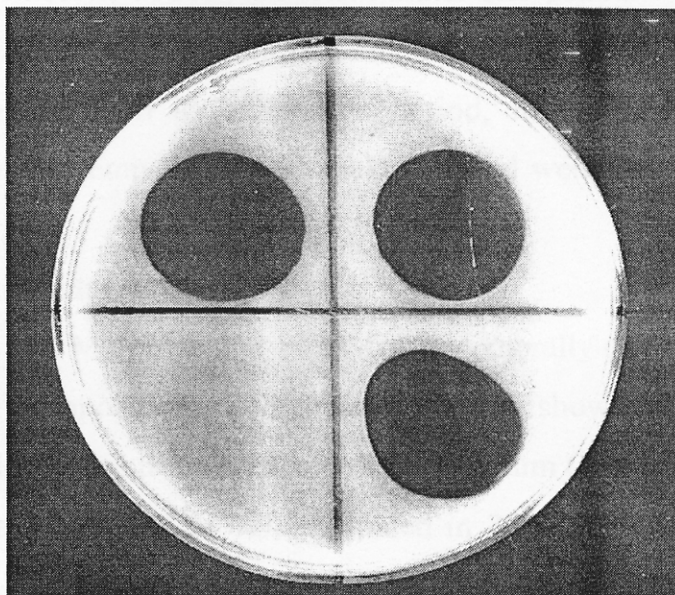


Figure 4 Fibrinolytic activity on fibrin plate

Methods

1. Effect of cultural medium and environmental conditions on growth and fibrinolytic enzyme production

Inoculum preparation

The mycelium of *Schizophyllum commune* BL 23 was transferred from slant and inoculated onto a potato dextrose agar plate. The culture was used as an inoculum after incubation at 30°C for 5-6 days.

Cultivation conditions

S. commune BL 23 was transferred from slant and inoculated onto a potato dextrose agar plate after incubation at 30°C for 5 days. An actively growing mycelium on PDA was cut by using a cork borer (7 mm diameter) about 5 mm from colony edge for using as an inoculum. Five pieces of mycelial disc were transferred to 100 ml of each medium (initial pH = 6) in 250 ml flask and the experiment was duplicated. Unless otherwise specified, the culture was incubated at 30°C for 7 days. After incubation, the culture was centrifuged at 9,000 rpm, 4°C for 20 min, and the supernatant was used for

determining fibrinolytic activity on fibrin plate. The pellets were determined for dry cell weight. Other factors that involving growth and production of fibrinolytic enzyme such as cultivation period, initial pH of the culture medium, cultivation temperature and shaking speed were also investigated in this study.

1.1 Effect of cultural medium

PDB, SDB, MY, MM and PYGM were generally used to cultivate the fungus and the composition of these medium was shown in Table 2. Five pieces of inoculum were transferred to 100 ml medium (PDB, SDB, MY, MM and PYGM with initial pH of 6.0) contained in 250 ml flask. After shaking continuously on orbital shaker at 150 rpm and 30°C for 7 days, the supernatant was assayed fibrinolytic activity on fibrin plate, and the pellet was determined for dry cell weight.

Table 2 Composition of medium for cultivation of *S. commune* BL 23

Ingredients added (g/l)	Media				
	PYGM	MY	SDB	PDB	MM
Malt extract	-	3	-	-	-
Peptone	5	5	10	-	-
Glucose	10	10	20	20	20
Yeast extract	20	3	-	-	1
NH ₄ NO ₃	-	-	-	-	3
KH ₂ PO ₄	1	-	-	-	0.5
NaH ₂ PO ₄	-	-	-	-	0.5
MgSO ₄ .7H ₂ O	0.5	-	-	-	0.5
CaCl ₂	-	-	-	-	0.5
Potato	-	-	-	200	-

1.2 Effect of incubation periods

Optimal medium (from section 1.1) was used to cultivate fungal mycelium. The inoculum was transferred into the optimal medium (100 ml) in a 250 ml flask and cultivated at the same conditions as above. Samples were taken at 3, 7, 10, 12 and 14 days.

1.3 Effect of pHs

The experiment was performed in the suitable culture media (from section 1.1) with varying initial pH of 5.0, 6.0 and 7.0. After the fungus was cultivated at 150 rpm and 30°C in the optimal time (from section 1.2).

1.4 Effect of temperatures

S. commune BL 23 was cultivated under optimal pH (from section 1.3) with varying temperature at 25, 30 and 35°C.

1.5 Effect of shaking speed

S. commune BL 23 was cultivated under optimal cultivation conditions (from section 1.4) with varying shaking speed of 150, 200 and 250 rpm.

1.6 Time course of growth and fibrinolytic enzyme production under optimal conditions

S. commune was cultured in the optimal cultivation (from section 1.1-1.5) for 3, 5, 7, 10, 12 and 14 days. pH of culture broth was measured and the supernatant was assayed for fibrinolytic activity. Cell pellets were used for determining of dry cell weight.

2. Purification of fibrinolytic enzyme

2.1 Ammonium sulfate precipitation

Cultural supernatant was obtained by centrifugation (9,000 rpm, 20 min, 4°C) to remove the cells. The crude enzyme was precipitated by salt fractionation using 20-40, 40-60 and 60-80% saturation of ammonium sulfate. In each fractionation, certain amount of (NH₂)SO₄ was added gradually in the

supernatant (Appendix 3) and stirred well by magnetic stirrer. After each fractionation, the solution was centrifuged at 13,000 \times g (9,000 rpm) for 20 min at 4°C. The supernatant was then treated with next ammonium sulfate saturation and the precipitate was dissolved in 20 mM Tris-HCl buffer, pH 7.0. The determination of fibrinolytic activity and protein content were conducted.

2.2 Dialysis

The enzyme solutions was then dialyzed (using dialysis bag with the molecular weight cut off at 7,000 daltons) in 20 mM Tris-HCl buffer, pH 7.0 in the ratio of 1: 100 (enzyme solution : buffer solution) at 4°C for 24 h and continuously stirred by magnetic stirrer. The buffer was changed after 2 and 4 h then left overnight. The supernatant was kept at 4°C for further purification. The fibrinolytic enzyme activity and specific activity were assayed.

2.3 Anion-exchange column chromatography

DEAE-Sephacel was applied into a 2.5x15 cm column, then equilibrated with 20 mM Tris-HCl buffer, pH 7.0 for 2 bed volumns at the flow rate of 0.4 ml per minute. The dialyzed enzyme was passed through the column and eluted with the same buffer at the same flow rate, then eluted with a linear gradient of 0-0.5 M NaCl in the same buffer. Four ml per fraction was collected by a fraction collector. Each fraction was then measured for protein absorbance at 280 nm, and was assayed for fibrinolytic activity. The active fractions were pooled and concentrated by lyophilization. One gram of the lyophilized pellet was dissolved in 3 ml of 20 mM Tris-HCl buffer, pH 7.0. The reconstituted enzyme was dialysed in the same buffer at 4°C for 24 h, and was subjected to native gel electrophoresis. Three milliliters of dialysed enzyme were diluted to 12 ml and was used for characterization.

2.4 Gel electrophoresis

The enzymes were examined for purity and molecular weights by electrophoresis (Native polyacrylamide gel electrophoresis, Native PAGE). Native- PAGE was carried out as described by Davis (1964). The slab gel with gradient of 5-15% separating gel (7x7x0.1cm) and 4% stacking gel (2x7x0.1cm) were used (Appendix 3).

The enzyme was mixed with 0.5 M Tris-HCl buffer, pH 6.8, 40% glycerol, 0.5% bromophenol blue in the ratio of 3:1 v/v. Standard proteins consisted of ovalbumin (45,000 daltons), serum albumin (66,200 daltons), phosphorylase b (974,00 daltons), β -galactosidase (116,250 daltons) and myosin (200,000 daltons) were prepared the same as above. The sample in each step of purified enzyme and standard proteins were then applied into each well on the stacking gel and the partially purified enzyme was applied into 2 wells on this stacking gel. Electrophoresis was carried out at room temperature using 0.025 M Tris-glycine buffer, pH 8.3 as an electrode (running) buffer. The constant voltage of 250 volts and current of 15 mA were supplied until the tracking dye approached the bottom of the gel and the gel was stained by silver staining (Appendix 3). The standard proteins and the sample were stained by silver staining for determining of the molecular weight size. The sample in one well of separating gel was cut off and not stained. This sample in the separating gel was cut into pieces about 2 millimeters/piece. Each piece of gel was put onto fibrin plate for determining fibrinolytic activity. In this experiment, the sample was about 1-2 μ g protein was loaded into each well.

3. Characterization of the partially purified fibrinolytic enzyme

3.1 Effect of temperature on fibrinolytic activity

The partially purified enzyme solution was dropped onto fibrin plate and incubated at 25, 30, 35, 40, 50 and 60°C in the incubator for 18 h. The diameter of clear zone measured, then calculated the area of lysed zone. The

temperature that gave the highest area of clear zone was selected and used as a standard protocol. The activity of enzyme was presented in term of relative activity compared to the maximum activity that was defined as 100% relative activity.

3.2 Effect of pH on enzyme stability

The partially purified enzyme solution was kept in the following buffer solution (0.2 M citrate buffer for pH 3.0, 4.0, 5.0 and 6.0; 0.2 M Tris-HCl buffer for pH 7.0 and 8.0 and 0.2 M glycine-NaOH buffer for pH 9.0, 10.0 and 11.0, in the ratio of 1:1) for 20 min and 48 h at room temperature (28°C). The partially purified enzyme solution was diluted with 0.2 M Tris-HCl buffer (pH 7.0) in the ratio of 1:1, which was used as a standard protocol. The activity of enzyme was presented in term of relative activity compared to a standard protocol that was defined as 100% relative activity.

3.3 Effect of temperature on enzyme stability

The partially purified enzyme solution was incubated at 40, 50 and 60°C for 48 h. The partially purified enzyme solution was kept at 4°C was used as standard protocol. The activity of enzyme was presented in term of relative activity compared to a standard protocol that was defined as 100% relative activity.

3.4 Effect of metal ions and chemical reagents on enzyme activity

The effects of metal ions were investigated using 2 mM of CaCl₂, CoCl₂, NaCl, CuSO₄, FeCl₃, HgCl₂, KCl and ZnCl₂. In addition, the effects of protease inhibitors were studied using 2 mM and 20 mM ethylenediamine tetra acetic acid (EDTA), 2 mM phenylmethyl sulfonylfluoride (PMSF), 2 mM 1,10-phenanthroline and 1 µg/ml and 100 µg/ml of soybean trypsin inhibitor (SBTI). The partially purified enzyme solution was mixed with each reagent in the ratio of 1:1, then it was incubated at room temperature (28°C). The partially purified enzyme solution in the buffer solution (pH 7) was used as the standard protocol. Fibrinolytic activity was presented as relative activity

compared to standard protocol that was defined as 100% relative activity. All chemical reagents was dissolved with 20 mM Tris-HCl, pH 7.0 except EDTA and 1,10-phenanthroline was dissolved with distilled water.

3.5 Enzyme stability after prolonged incubation

The partially purified enzyme solution was kept at 30°C for 5, 15, 25, 40 and 60 days and the enzyme activity was then investigated. The enzyme activity was presented as relative activity compared to the enzyme activity was kept at 4°C that was defined as 100% relative activity.