

New Thrombolytic Agent from Endophytic Fungi and *Lignosus rhinoceros*

Mohd Sidek Ahmad*, Zainon Mohd Noor and Zaidah Zainal Ariffin

A601, Mycology Lab, Faculty of Applied Sciences, Universiti Teknologi MARA 40450 Shah Alam, Selangor, Malaysia

Abstract: Thrombosis and cardiovascular-related diseases can be caused by fibrin aggregation. Thrombolytic agents have been used to treat thrombolytic problems and cardiovascular-related diseases. There had been searches for new thrombolytic agents from bacteria, fungi, insects and fermented foods. To name a few, recently fungi such as *Aspergillus oryzae* KSK-3, *Fusarium* sp. CICC 480097 and *Fusarium* sp. BLB were found able in producing protease fibrinolytic enzymes. Potential protease fibrinolytic enzymes were observed from *Lignosus rhinoceros* bought from Orang Asli of Sungei Perak. Out of the six (6) sclerotia used, one of them, LR-1, produced the biggest clear zone on Skim Milk Agar with a diameter of 1.31 cm. Fibrin plate assay of LR-1 showed that it produced a clear zone of 0.97 cm when the plate was incubated for 18 hours at 37°C. Leaves of *Hibiscus* sp. were sterilised with 70% alcohol and 70% sodium hypochlorite. The leaves were then cut into pieces of 4 cm and placed on Potato Dextrose Agar plate. Fungi grown from the leaf pieces were sub cultured onto new Potato Dextrose Agar. This then showed that *Lignosus rhinoceros* had protease and fibrinolytic activities. Sixteen (16) endophytic fungi were isolated from *Hibiscus* sp. leaves from six locations in Shah Alam, Selangor. Two endophytic fungi, FH-3 and S-13 showed protease activities; clear zones of 2.89 cm and 2.42 were seen after 4 days incubation at 27°C. FH-3 had fibrinolytic activity which was observed by the 1.98 cm diameter clear zone on fibrin plate.

Keywords: Fibrinolytic enzyme, thrombolytic agent, endophytic fungi, *Lignosus rhinoceros*.

INTRODUCTION

The primary protein component of blood clot is fibrin, formed from fibrinogen by thrombin (EC 3.4.21.5) [1]. When blood vessels get blocked, thrombosis occurs because of fibrin which leads to myocardial infarction and cardiovascular diseases. Cardiovascular diseases, high blood pressure, acute myocardial infarction, ischemic heart disease, valvular heart disease, peripheral vascular disease, arrhythmias, stroke, have been known to be one of the major causes of death throughout the world [2]. Thrombolytic agents have been extensively used in the therapeutic treatment of thrombosis. Thrombolytic agents are differentiated by two different mechanisms. The first one is plasminogen activators, such as tissue-type plasminogen activator (t-PA) [3] and urokinase [4]. This type can activate the endogenous fibrinolytic system and generate plasmin from plasminogen. The second type is plasmin-like proteins, such as lumbrokinase [5], serine protease [6, 7] and metalloprotease [8]. This second type can directly hydrolyze fibrin into fibrin degradation products, thus dissolving the thrombin rapidly and completely [9]. Although widely used, type one thrombolytic agents have undesirable side effects, low specificity, and are relatively expensive. The search for fibrinolytic enzymes from various sources has to go on because of the above reasons [10].

MATERIALS

Fibrinogen, Thrombin, Plasmin from Calsbiochem, Germany. Skim milk, Potato dextrose from Oxoid, England.

METHODOLOGY

Sampling of *Lignosus Rhinoceros*

Sclerotia of *Lignosus rhinoceros* were bought from Orang Asli of Sungei Perak. There were 6 samples and they were labelled as LR-1, LR-2 LR-3 LR-4 LR-5 and LR-6.

Isolation of Endophytic Fungi from *Hibiscus* Leaves

The *Hibiscus* leaves collected from several places around Shah Alam, Selangor were surface sterilised with alcohol and 70% sodium chloride. The leaves were cut into pieces and placed on Potato Dextrose Agar. These plates were incubated for 3 to 5 days at 27°C. Fungi that grew from cut leaves were sub cultured onto new Potato Dextrose Agar.

Protease Bioassay

Lignosus Rhinoceros

Samples were homogenized in liquid nitrogen using a Retsch Mixer mill. The powdered samples were diluted in sterile distilled water. Skim Milk Agar was used to detect the presence of protease. Wells were bored onto the Skim Milk Agar using a cork borer. Twenty (20) µL of the *Lignosus rhinoceros* diluted samples were placed in the wells prepared, then incubated at 37°C for 24 hours. Clear zones around the wells indicated that the protein in the Skim Milk Agar had been degraded by protease in the samples.

*Address correspondence to this author at the A601, Mycology Lab, Faculty of Applied Sciences, Universiti Teknologi MARA 40450 Shah Alam, Selangor, Malaysia; Tel: +603 55438456; E-mail: sidekahmad77@yahoo.co.uk

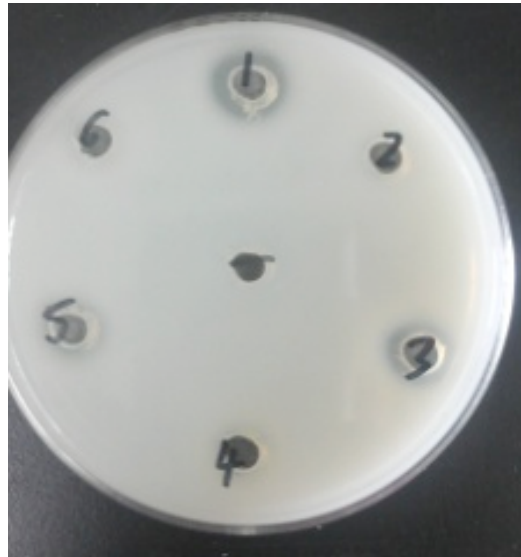


Fig. (1). *Lignosus rhinocerus* Protease present on Skim Milk Agar.

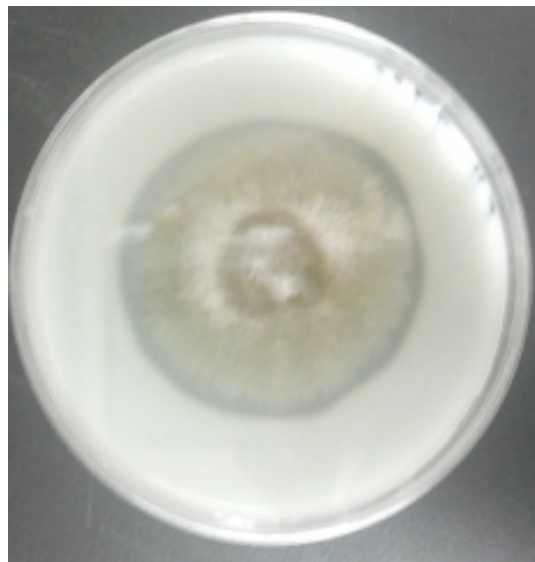


Fig. (2). Endophyte FH-3; Protease present on Skim Milk Agar.

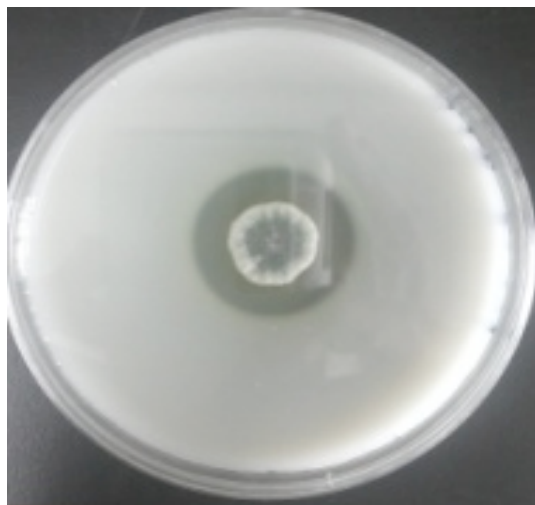


Fig. (3). Endophyte S-13; Protease present on Skim Milk Agar.

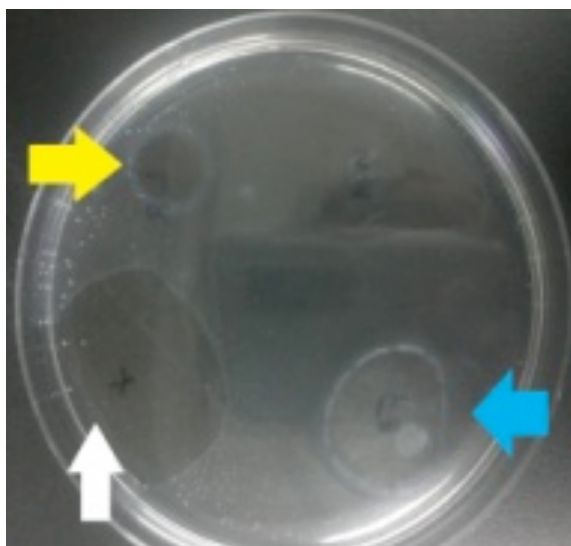


Fig. (4). *Lignosus rhinocerus* (Yellow) and Endophyte FH-3 (Blue) on Fibrin plate.

Table 1. Diameter Clear Zone of Samples (FH-3, S-13, LR-1, LR-3, LR-5) on Skim Milk Agar

Diameter(Cm)\Time(H)	FH-3	S-13	LR-1	LR-3	LR-5
24	1.04	0.71	1.31	0.98	0.47
96	2.42	2.89	-	-	-

Table 2. Diameter Clear Zone of Samples (FH-3 and LR-1) on Fibrin Plate

Diameter(Cm)\Time(H)	Positive (Plasmin)	Negative (DH ₂ O)	LR-1	FH-3
24	3.64	0	0.97	1.98

Endophytic Fungi

Sixteen (16) endophytic fungi collected from the *Hibiscus* leaves were cultured on Skim Milk Agar plates. Endophytic fungi after 48 hours in Potato Dextrose broth were sub cultured onto Skim Milk Agar. As the fungi grew, clear zones became visible around the fungal colony which represented protease activity against protein in the Skim Milk Agar.

Fibrinolytic Activity

Fibrinolytic activity was detected by using fibrin plates [11]. Fibrinolytic assay was done using a modified method from Astrup and Mullertz [11]. Five (5) mL fibrinogen solution, 10U thrombin solution and 5 mL 1% agarose were mixed together in a petri dish. The enzyme sample (10 μ L) was dropped onto the plate and incubated for 18 hours at 37°C. Clear zones on the plates were measured and recorded and represented fibrinolytic activities of the enzymes.

RESULTS AND DISCUSSION

Protease Bioassay

Proteolytic activities were observed in *Lignosus rhinocerus* samples, LR-1 to LR-6 and the two endophytes, FH-3 and S-13. The activity was shown by the formation of clear zones around samples cultured on Skim Milk Agar (Figs. 1, 2 and 3). Diameters of these clear zones were

tabulated in Table 1. Table 2 showed that out of six *Lignosus rhinocerus* samples, only three (3) showed protease activities. LR-1 produced a clear zone of 1.31 cm which is the highest from all three positives for protease activity. The other two LR samples have moderate sized clear zones (LR-3, 0.98 cm and LR-5 0.47 cm). The endophytes, FH3 and S13, showed protease activities of by formation of clear zones around their colonies at 96 hours in 27°C. FH-3 has a clear zone of 2.89 cm and S-13 has a slightly smaller clear zone of 2.42 cm.

It can be seen that the endophytic fungi seemed to produce more proteolytic activity than *Lignosus rhinocerus*. This may be due to the endophytic fungi are growing microorganisms; therefore they are still producing the enzymes. But this is not so for the *Lignosus rhinocerus* as the samples are sclerotia, which are parts of the mushrooms. Unless *Lignosus rhinocerus* are grown then there is a possibility that there will be higher proteolytic activity. Also in comparison, among the 6 *Lignosus rhinocerus* samples, 3 did not show proteolytic activity and between the 3 that produced clear zones, the sizes of clear zones also differ. Their proteolytic potentials may differ due to differences nutrient composition of these mushrooms.

Lignosus rhinocerus samples LR-1, LR-3 and LR-5 showed the presence of protease after 24 hours incubation in 37°C. Isolated endophytes showed the ability to produce

protease after incubation at 27°C for 96 hours. Results are tabulated on Table 1.

Fibrinolytic Activity

Positive samples with protease activities were tested for fibrinolytic activity on Fibrin plates. Fibrinolytic activity was determined by ring formation around the samples indicating degradation of fibrin. This is shown on Fig. (4) where the yellow arrow showing LR-1, blue arrow is FH-3 and the white arrow is the positive control (plasmin). LR-1 produced 0.97 cm and FH-3 produced 1.98 cm clear zone as shown in Table 2. This could prove that the protease samples from these endophytes and mushroom are with fibrinolytic properties; Serine Protease from *Aspergillus oryzae* KSK-3 and *Fusarium* sp. BLB [6, 7] or Metalloprotease from *Armillaria mellea* [8].

CONCLUSION

In conclusion, isolated endophytic fungi and *Lignosus rhinocerus* have positive protease and fibrinolytic activities.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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