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Isolation and Identification Fibrinolytic Protease Endophytic Fungi from *Hibiscus* Leaves in Shah Alam

Mohd Sidek Ahmad, Zainon Mohd Noor, Zaidah Zainal Ariffin

***Abstract*—**Fibrin degradation is an important part in prevention or treatment of intravascular thrombosis and cardiovascular diseases. Plasmin like fibrinolytic enzymes has given new hope to patient with cardiovascular diseases by treating fibrin aggregation related diseases with traditional plasminogen activator which have many side effects. Various researches involving wide range of sources for production of fibrinolytic proteases, from bacteria, fungi, insects and fermented foods. But few have looked into endophytic fungi as a potential source. Sixteen (16) endophytic fungi were isolated from Hibiscus sp. leaves from six different locations in Shah Alam, Selangor. Only two endophytic fungi, FH3 and S13 showed positive fibrinolytic protease activities. FH3 produced 5.78cm and S13 produced 4.48cm on Skim Milk Agar after 4 days of incubation at 27°C. Fibrinolytic activity was observed; 3.87cm and 1.82cm diameter clear zone on fibrin plate of FH3 and S13 respectively. 18srRNA was done for identification of the isolated fungi with positive fibrinolytic protease. S13 had the highest similarity (100%) to that of Penicillium citrinum strain TG2 and FH3 had the highest similarity (99%) to that of Fusarium sp. FW2PhC1, Fusarium sp. 13002, Fusarium sp. 08006, Fusarium equiseti strain Salicorn 8 and Fungal sp. FCASAn-2. Media composition variation showed the effects of carbon nitrogen on protein concentration, where the decrement of 50% of media composition caused drastic decrease in protease of FH3 from 1.081 to 0.056 and also S13 from 2.946 to 0.198.

***Keywords*—**Isolation, identification, fibrinolytic protease, endophytic fungi, *Hibiscus* leaves.

I. Introduction

ENDOPHYTIC fungi are among many other groups of special microorganisms, which have been well known in producing biologically active and structural novel metabolites [1]. Several bioactive compounds such as antidiabetic, antitumor, antibiotics and fibrinolytic agents have been isolated from endophytes [2]. Proteases are a well-known integral part for industrial and commercial purposes [3]. Proteases are grouped into various classifications such as metalloprotease, alkaline protease, acid protease, serine protease, aspartic protease and cystein protease [4]. Main cause of cardiovascular diseases (CVDs) is due to fibrin aggregation in the blood vessel. Treatment of thrombosis has been done by using fibrinolytic agents on a therapeutic treatment [5]. Fibrinolytic agents are divided into plasminogen activators (Urokinase, Streptokinase) and plasmin like proteins (Lumbrokinase, Serine Protease) depending on the mode of fibrin degradation. But, Plasminogen activators have several drawbacks like antigenic reaction, limited fibrin specificity, large therapeutic dose and short half life span and this problem can be overcome by newer plasmin like protein type [6]. Studies for fibrinolytic enzymes biosynthesized from endophytic fungi are still too few [2].

Mohd Sidek Ahmad and Zaidah Zainal Ariffin are with the Universiti Teknologi Mara Faculty of Applied Sciences 40450 Shah Alam Selangor (phone: +603-55438456; e-mail: sidekahmad77@yahoo.co.uk, drzaidah@salam.uitm.edu.my).

Zainon Mohd Noor was with the Universiti Teknologi Mara Faculty of Applied Sciences 40450 Shah Alam Selangor (phone: +603-55444609; e-mail: dzainonmn@salam.uitm.edu.my).

II. Materials and Methodology

A. Materials

Fibrinogen, Thrombin, Plasmin from Calsbiochem, Germany. Skim milk, Potato dextrose from Oxoid, England. ITS1 and ITS4 primer.

B. Methodology

1. Isolation of Endophytic Fungi from *Hibiscus* Leaves

Fresh *Hibiscus* leaves free from disease symptoms were collected from several places around Shah Alam, Selangor. The leaves were surface sterilised with 70% alcohol and 70% sodium chloride. Hibiscus leaves were cut into small pieces with a sterile scalpel and placed on Potato Dextrose Agar. These plates were incubated for 3 to 5 days at 27°C. Fungi that emerged through the leave pieces were sub cultured onto new Potato Dextrose Agar.

2. Protease Bioassay

Sixteen (16) endophytic fungi collected from the *Hibiscus* leaves were cultured on Skim Milk Agar plates. The sixteen (16) isolated endophytic fungi were inoculated in Potato Dextrose broth with spore suspension of 10^6 spores/ml and incubated for 48 hours in 27°C using an incubator shaker, from the cultivated broth the endophytic fungi were sub cultured onto Skim Milk Agar. Visible clear zones around the fungal colony represented protease activity against protein in the Skim Milk Agar.

3. Fibrinolytic Activity

Fibrinolytic activity was detected by using fibrin plates [7]. Fibrinolytic assay was done using a modified method from Astrup and Mullertz [7]. Five (5) mL fibrinogen solution, 10U thrombin solution and 5 mL 1% agarose were mixed together in a petri dish. The enzyme sample (10µL) was dropped onto the plate and incubated for 18 hours at 37°C. Clear zones on the plates were measured and recorded and these represented fibrinolytic activities of the enzymes.

4. 18srRNA

Fungi isolated were then identified using 18srRNA gene

sequences. DNA extraction was carried out using QIAGEN plant tissue kit. The amplification of 18srRNA was performed using an internal transcriptional spacer (ITS) fragment and was amplified by polymerase chain reaction (PCR) using ITS1 (5'-TCCGTAGGTGAACCTGCG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). The PCR product was examined by electrophoresis, isolated, and then sequenced by MyTACG Sdn Bhd. A sequence similarity search was performed using the National Center for Biotechnology (NCBI) database with Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nih.gov/BLAST/)

5. Media Effects on Protein Concentration

Spore suspension of 10^6 spores/ml endophytes were inoculated in 50ml of modified medium (2g soybean, 1.5g glucose, 0.1g yeast extract, 0.05g K_2HPO_4, and 0.05 g $MgSO_4.7H_20$, pH 7) [8] in 250ml flask at 30°C at 150 rpm. To check whether carbon and nitrogen sources have effects on the protein produced by the endophytic fungi, the media's ingredients were decreased and increased until 50%±. Broth was filtered with PES 0.4µm to remove any fungal spores. Desalting technique of ammonium sulphate was used to remove salt and other large proteins from the crude samples. The sample was then centrifuged at 8,000 g in 4°C for 30 minutes. Precipitate was collected and diluted in 5ml of 10mM Tris-HCl.

III. Results and Discussion

A. Protease Bioassay

Proteolytic activities were observed for 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 and 2). Diameters of these clear zones were tabulated in Table I. Table I showed that the two 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 5.78 cm and S-13 has a slightly smaller clear zone of 4.48 cm.

It can be seen that S13 seemed to produce more proteolytic activity than FH3. This may be due to FH3 has growing hyphae which spread across the plate while S13 does not.

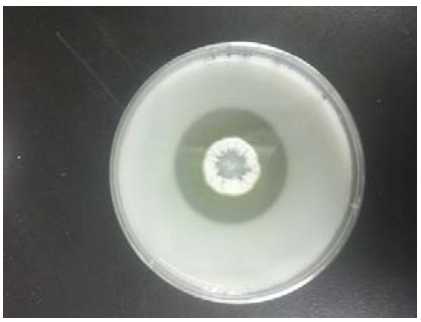
Isolated endophytes showed the ability to produce protease after incubation at 27°C for 96 hours. Results are tabulated in Table I.

(a)

(b)

Fig. 1 Endophyte FH-3showing presence of protease (Skim Milk Agar Test) (Day 2nd (a) 4th (b)

(a)



(b)

Fig. 2 Endophyte S-13; Protease present on Skim Milk Agar day 2nd (a) and 4th (b)

TABLE I
DIAMETER OF CLEAR ZONES OF SAMPLES (FH-3 AND S-13) ON SKIM MILK AGAR

Duration (Hour)	Endophytic fungi	
	FH-3	S-13
24	2.08	1.42
96	5.78	4.48

B. 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. 3 where the black star showing S13, blue star is FH-3 and the white star is the positive control (plasmin). S13 produced 1.82 cm and FH-3 produced 3.87 cm clear zone as shown in Table II. This could

prove that the protease samples from these endophytes have fibrinolytic properties; Serine Protease from *Aspergillus orzyae* KSK-3 and *Fusarium* sp. BLB [9], [8] or Metalloprotease from *Armillaria mellea*) [10].

TABLE II
DIAMETER CLEAR ZONE OF SAMPLES (FH-3 AND S13) ON FIBRIN PLATE

Time (Hour)	Diameter (cm)		
	Positive (Plasmin)	S13	FH3
18	2.96	1.82	3.87

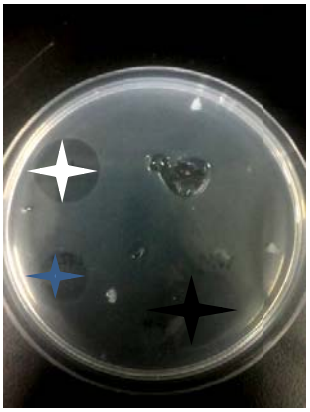


Fig. 3 Endophyte FH3 (Black) and Endophyte S13 (Blue) on a Fibrin plate

C. 18srRNA

The length of the amplicon was approximately 700bp and 600bp for FH3 and S13 respectively.

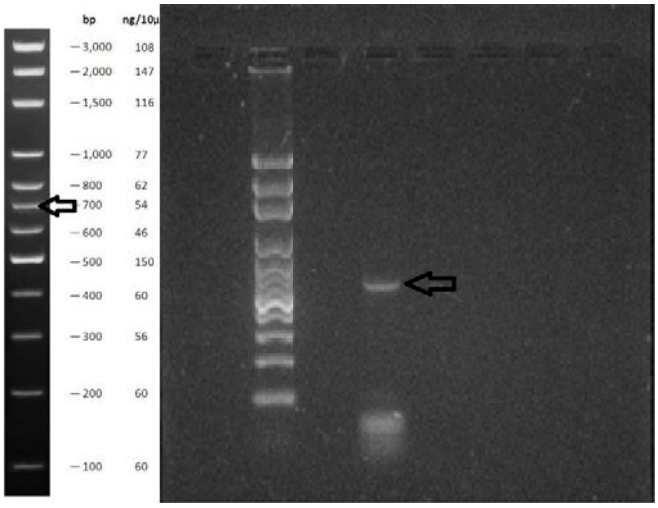


Fig. 4 FH3 PCR gel electrophoresis

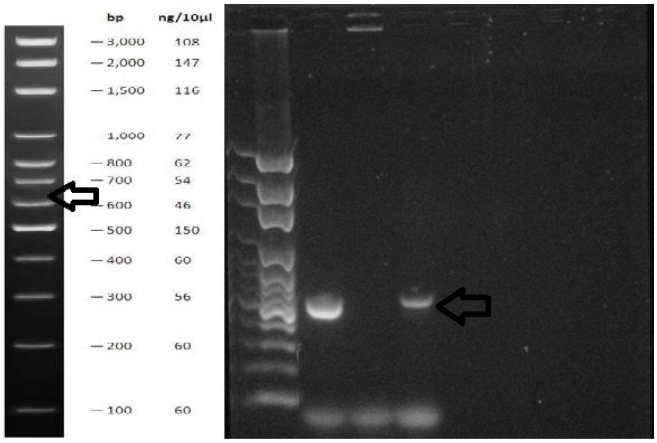


Fig. 5 S13 (utmost right) PCR gel electrophoresis

FH3 gel electrophoresis showed that the band visible were at about 700 base pair, while S13 band were in between 700 and 600 base pair in reference to the ladder provided.

Purified products from PCR were sent to a third party for sequencing, results obtained through Nucleotide Blast from NCBI databank showed that S13 and FH3 were *Penicillium citrinum* and *Fusarium* sp. respectively.

The results showed that the ITS gene sequence of S13 had the highest similarity (100%) to that of *Penicillium citrinum* strain TG2 while ITS gene sequence of FH3 had the highest similarity (99%) to that of *Fusarium* sp. FW2PhC1, *Fusarium sp.* 13002, *Fusarium* sp. 08006, *Fusarium equiseti* strain Salicorn 8 and Fungal sp. FCASAn-2.

D. Media Effects on Protein Concentration

Protein concentration of the media was measured by using Thermoscientific™ Nanodrop 2000 using lid 10, volume of 4µl.

TABLE III
MEDIA EFFECTS ON PROTEIN CONCENTRATION

Media percentage \ Sample	FH3	S13
50%	0.056	0.198
60%	0.112	0.253
70%	0.347	0.498
80%	1.053	1.019
90%	1.074	1.525
100%	1.081	2.946
110%	-	-
120%	-	-
130%	-	-
140%	-	-
150%	-	-

When the media percentage increased greater than 100%, the broth became too thick with mycelium and made it impossible to extract the broth via filtration. It appears that with fixed pH value of 7 and a lower the composition of the media, the least production of protease was detected. With Pekkarinen [11] work on *Fusarium* sp. protease production as guidance, this result proved that these two endophytic fungi,

Fusarium sp. and *Penicillium citrinium* protease production is dependent on the pH and nitrogen as the carbon source.

IV. Conclusion

In conclusion, isolated endophytic fungi of S13 and FH3 have positive protease and fibrinolytic activities. Media composition proved vital in the amount of protein production by the isolated fungi.

Acknowledgment

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