

## PURIFICATION AND CHARACTERISATION OF FIBRINOLYTIC ENZYMES FROM ENDOPHYTIC FUNGI AND *LIGNOSUS RHINOCERUS*

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### Article history

Received

21 August 2015

Received in revised form

11 December 2015

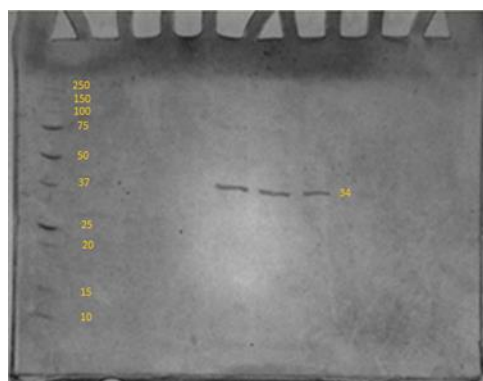
Accepted

13 January 2016

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### GRAPHICAL ABSTRACT



### Abstract

Three enzymes FH3, S13 and LR1 from three different sources showed fibrinolytic activities. Two were from endophytic fungal cultures and one from the sclerotium of *Lignosus rhinocerus* mushroom (LR1). FH3, S13 cultures and LR1, the crude extract of the sclerotium were concentrated and purified by ammonium sulphate precipitation, ion-exchange chromatography and gel-filtration. The molecular weights of the FH3, S13 and LR1 purified enzymes were estimated to be approximately 34kDa, 34kDa and 10kDa, respectively. Maximum fibrinolytic activities were observed for FH3 at pH 7 and 30°C, S13 at pH 8 and 40°C and LR1 at pH 6 and 40°C. In our earlier paper we identified FH3 as *Fusarium sp.* and S13 as *Penicillium citrinum*.

Keywords: Fibrinolytic enzyme, endophytic fungi, *Lignosus rhinoceros*

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## 1.0 INTRODUCTION

Endophytic fungi are among the many groups of special microorganisms, which are well known in producing biologically active and structurally novel metabolites [1]. Several bioactive compounds such as antidiabetic, antitumor, antibiotics and fibrinolytic agents have also been isolated from endophytes [2]. Protease is a well-known integral part for industrial and commercial purposes [3] which can be grouped into metalloprotease, alkaline protease, acid protease, serine protease, aspartic protease and cystein protease [4]. Cardiovascular diseases (CVDs) are one of the most important causes of death in the modern world. It is said to be due to fibrin aggregation in the blood vessel or thrombosis. Treatment of thrombosis is by using fibrinolytic agents

and as a therapeutic treatment [5]. Fibrinolytic agents can be divided into plasminogen activators (Urokinase, Streptokinase) and plasmin-like proteins (Lumbrokinase, Serine Protease) which are classified according to their mode of fibrin degradation. Plasminogen activators have several drawbacks like antigenic reaction, limited fibrin specificity, large therapeutic dose and a short half life span. Newer plasmin-like protein types will be a solution to some of these problems [6]. Studies on fibrinolytic enzymes biosynthesized by endophytic fungi are still too few [2] and this study was done to find new fibrinolytic enzymes which hopefully with less side effects on patients of CVD.

## 2.0 MATERIALS AND METHODS

### 2.1 Materials

*Lignosus rhinocerus* wild type was bought from an Orang Asli of Sg. Perak, who collected them from the forests of Perak. Endophytic fungi were isolated from hibiscus leaves [7,8]. Coomassie brilliant blue R-250 (Fluka) from Sigma Aldrich and Mini-PROTEAN® TGX™ Precast Gels from Bio-rad were used for the SDS PAGE. The protease assay and protein concentration analysis made use of the Merck Millipore Protease Assay Kit and Merck Bradford Assay Kit. Protein purification used the HiTrap IEX Selection Kit and Hi-Prep 26/10 Desalting Column from GE Healthcare Bio-Science.

### 2.2 Purification of Fibrinolytic Enzyme from Fungi

All procedures were carried out at 4°C. The cultured broth samples were centrifuged at 8,000 x g for 30 minutes and the centrifuged supernatant were then used for the estimation of the fibrinolytic enzyme activity. The fibrinolytic enzyme was purified by ammonium sulphate precipitation and followed through using Fast Protein Liquid Chromatography (FPLC) equipped with Hi-Prep 26/10 Desalting Column (26cm x 10cm) and Hi-Trap Benzamidine FF (5 x 1mL)/Hi-Trap IEX Selection Kit (5 x 1mL). In the initial purification step, the culture supernatant was subjected to fractionation by increasing the concentration of ammonium sulphate. Ammonium sulphate was added to the supernatant to give a concentration of 30%. The precipitate was removed by centrifugation (8,000 x g for 30 minutes at 4°C). Ammonium sulphate concentration was increased stepwise to 80% saturation, where at every additional 30% of ammonium sulphate, the precipitates were collected by centrifugation (8,000 x g for 30 min at 4°C). Fractions obtained with 0-30%, 30-60%, 60-80% ammonium sulphate were identified respectively and named as L-1, L-2 and L-3 (LR1-Crude); F-1, F-2, and F-3 (FH3 crude); S-1, S-2 and S-3 (S13 Crude). The precipitates showing fibrinolytic activity on fibrin plates were collected by centrifugation and dissolved in 2 mL of Tris-HCl buffer 10 mM (pH 7.4). The enzyme solution went through a gel filtration process (AKTA purifier plus 10, HIR Universiti Malaya) using a Hi-Prep 26/10 Desalting Column (26cm x 10cm) equilibrated with 10mM Tris-HCl buffer at pH 7.4 and at a flow rate of 2.5 mL/min. Further purification was done using either a Hi-Trap Benzamidine FF (5 x 1mL) which was used for FH3 and S13, or a Hi-Trap IEX Selection Kit FF (5 x 1mL) for LR1.

### 2.3 Molecular Weights of Enzymes Determination

The molecular weights of the enzymes were determined by SDS-PAGE and was carried out using 12% Mini-PROTEAN® TGX™ Precast Gels where the staining material was Coomassie brilliant blue R-250 (Fluka).

### 2.4 Protease Assay

Protease activity was determined using a kit that quantifies activity and used a fluorescein thiocarbonyl-casein derivative (FTC-casein); a method modified from Twining (1984). Enzyme sample or column fraction (100 µL) was added to 50 µL of FTC-casein and 50 µL incubation buffer (10 ml of 200 mM Tris-HCl, pH 7.8, 20 mM CaCl<sub>2</sub>, and 0.1% NaN<sub>3</sub>) in a 1.5 mL microfuge tube. The mixture was incubated at 37°C overnight after which 500 µL of 5% trichloroacetic acid (TCA) was added to it to stop the reaction. The tube was vortexed briefly and incubated at 37°C for 10 minutes. The TCA precipitate was pelleted by centrifuging at 12,000 x g for 5 minutes. 400 µL of the supernatant was transferred to a second microfuge tube and 600 µL of the assay buffer (120 mL of 500 mM Tris-HCl, pH 8.8, 0.1% NaN<sub>3</sub>) was added and contents mixed. Results were viewed at absorbance 492 nm and against a reagent blank. Prior to this, a standard curve of plasmin (fibrinolytic enzyme positive control) was plotted using the protease assay kit.

### 2.5 Effects of pH and Temperature on Fibrinolytic Enzyme Activity

The Calbiochem® protease assay kit was used for estimation of the pH effects on fibrinolytic activity of the enzyme. The optimal temperature for enzymatic activity was determined by measuring the residual activity after incubation of 10 µL of fibrinolytic enzyme in 90 µL of 20 mM Tris-HCl (pH 7.5) at different temperatures (20-80°C) for 1 hour. The optimal pH for the fibrinolytic activity of the enzyme was determined within a pH range of 2-10. Ten microliters of the enzyme solution was added to 90 µL of 0.5 M glycine-HCl (pH 2.0-3.0), 0.5 M acetate (pH 4.0-5.0), 0.5 M Tris-HCl (pH 6.0-8.0), and 0.5 M glycine-NaOH (pH 9.0-10.0) buffers. After 1 hour of incubation at room temperature, the remaining protease activity of each enzyme solution was measured using a Calbiochem® protease assay kit.

## 3.0 RESULTS AND DISCUSSION

Purification of fibrinolytic enzymes FH3 (Table 1), S13 (Table 2) and LR1 (Table 3) are shown below.

**Table 1** Purification of fibrinolytic enzyme FH3

Purification stage Purification (Fold)	Volume (mL)	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg of protein)	Yield (%)	Fold
Culture Supernatant	2500	11900	261300	21.96	100	1
Ammonium sulfate precipitation	115	312	78412	251.32	30	11.4
Hi-Prep 26/10 Desalting Column (26cm x 10cm)	24	30.6	19685	643.3	7.5	29.3
Hi-Trap Benzamidine FF (5 x 1 mL)	2	21.4	5284	246.92	2.02	11.2

**Table 2** Purification of fibrinolytic enzyme S13

Purification stage Purification (Fold)	Volume (mL)	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg of protein)	Yield (%)	Fold
Culture Supernatant	4000	9000	184109	20.46	100	1
Ammonium sulfate precipitation	900	201	55232.7	274.79	30	13.4
Hi-Prep 26/10 Desalting Column (26cm x 10cm)	88	21.2	11854	559.15	6.4	27.3
Hi-Trap Benzamidine FF (5 x 1 mL)	4	11.1	2200	198.2	1.2	9.7

**Table 3** Purification of fibrinolytic enzyme LR1

Purification stage Purification (Fold)	Volume (ml)	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg of protein)	Yield (%)	Fold
Culture Supernatant	6000	2016	80.92	0.04	100	1
Ammonium sulfate precipitation	50	411	65.1	0.15	80.4	3.8
Hi-Prep 26/10 Desalting Column (26cm x 10cm)	9	2.6	4.4	1.69	5.4	42.3
Hi-Trap IEX Selection Kit FF (5 x 1 mL)	1	1.9	2.8	1.47	3.5	36.8

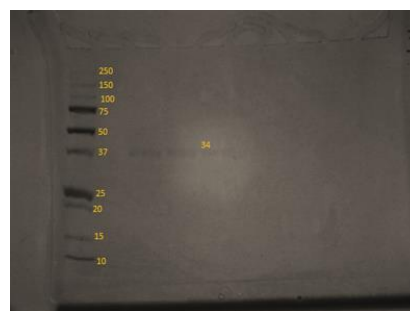
Table 1 summarizes the yield and purity of FH3 and it was about 11.24 fold with a yield of 2.02%. The yield

and purity of the fibrinolytic enzyme S13 at each purification step are summarized in Table 2. The enzyme was purified about 9.69 fold with a yield of 1.19%. LR1 (Table 3) was purified 36.75 fold with a yield of 3.46%. The Hi-Trap column used in this study managed to purify the fibrinolytic enzymes with good yields which were comparable to similar research done [1, 9].

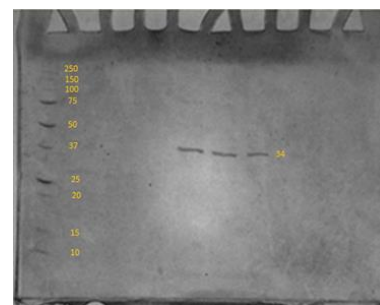
### 3.1 Characteristics Of Fibrinolytic Enzyme From Fungi

#### 3.1.1 Molecular Weights of Fibrinolytic Enzymes and Fibrin Zymography

The molecular weight of S13 was estimated to be 34 kDa by SDS-PAGE (Figure 1). The purified protein was found to show fibrinolytic enzyme activity as shown in the fibrin-zymogram of Figure 3.

**Figure 1** SDS-PAGE of sample S13

Molecular weight of FH3 was estimated to be 34 kDa by SDS-PAGE (Figure 2) and found to show enzyme activity by fibrin-zymography (Figure 4). The molecular weight and fibrin zymogram of LR1 was not included in this paper as it was not ready when this paper was written.

**Figure 2** SDS-PAGE of sample FH3

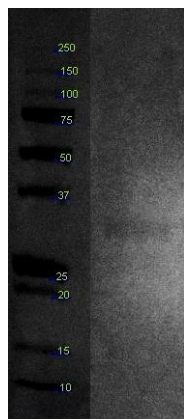


Figure 3 Fibrin zymography of S13



Figure 4 Fibrin zymography of FH3

### 3.1.2 Ph And Temperature Effects of Fibrinolytic Enzymes

Several testing is done to investigate the relative activity of fibrinolytic enzymes. Figure 5 shows the effects of temperature on FH3, S13 and LR1. The highest relative activity is in temperature 40 degree Celsius for LR1 and S13 with almost 1.5%. However, for FH3, the highest relative activity is 30 degree Celsius.

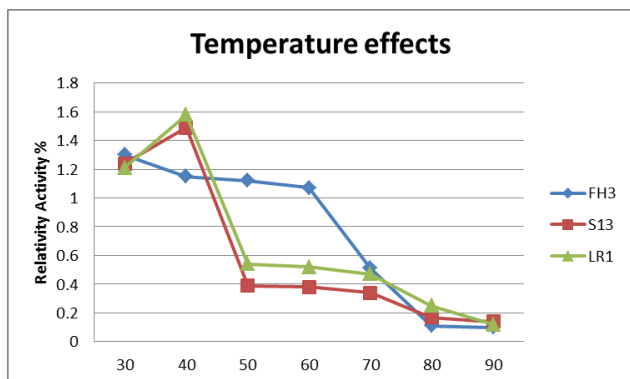


Figure 5 Temperature effects on FH3, S13 and LR1

For pH value, the highest relative activity is S13, 1.2% at 8 pH value compare to the LR1 and FH3 were 1.1% at 6 pH value and 7 pH value respectively. The result of the pH value effect illustrates on Figure 6.

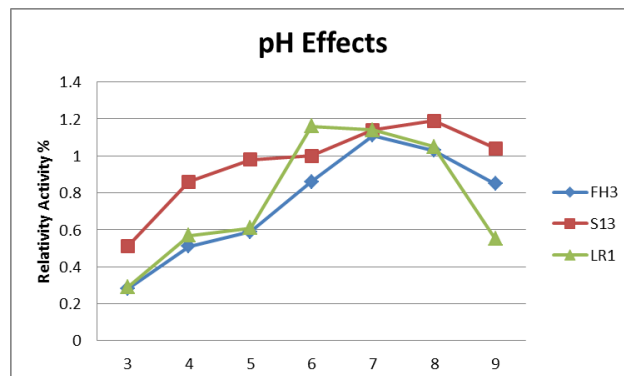


Figure 6 pH effects on FH3, S13 and LR1

## 4.0 CONCLUSION

In conclusion, the three purified enzymes, FH3, S13 and LR1 showed promising results as fibrinolytic enzymes. Further investigation on their amino acid sequences can improve our understanding of their functions at the molecular level which is what is now being done in our ongoing research.

## Acknowledgement

This study is funded by ERGS grant no. 600-RMI/ERGS 5/3 (11/2013), FRGS grant no. 600-RMI/FRGS 5/3 (152/2013), Universiti Teknologi MARA (UiTM) and the Ministry of Education Malaysia (MOE).

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