Production and some Properties of Fibrinolytic enzyme from 
*Rhizomucor miehei* (Cooney & Emerson) Schipper

Usama F. Ali and Z.M. Ibrahim

Department of Biological Sciences, Faculty of Education, 
University of Ain Shams, Cairo, Egypt.

**Abstract:** The fibrinolytic enzyme production of locally isolated fungus *Rhizomucor miehei* (Cooney & Emerson) Schipper and some of its properties were studied. The enzyme was isolated from the grounds of butcheries. Best production of the enzyme was achieved at 35 °C, pH 8.0, for six days using 0.6 (% W/V) bovine blood fibrin and 3.0 (% W/V) glucose. Fibrinolytic enzyme was partially purified according to the following consecutive steps: dialysis, acetone 20% and gel filtration on Sephadex G200. to 13.93 folds. The optimum activity of the enzyme was reached at 40 °C and pH 8.2. the enzyme was stable at pH 8.0 – 8.5 and thermostable at 70 °C for one hour.

**Key words:** Fibrinolytic enzymes, Fungi, *Mucor miehei*, Purification

**INTRODUCTION**

Fibrin is a protein that forms in the blood clots after trauma or injury. This is essential to stop blood loss. There are more than twenty enzymes in the body that assist in clotting of the blood, while only one that can break the clot down. It is an endogenously produced fibrinolytic enzyme called plasmin [26,27,28]. Streptokinase is an extracellular metallo-enzyme produced by beta-haemolytic *Streptococcus* and is used as an effective and cheap clot-dissolving medication in some cases of myocardial infarction (heart attack) and pulmonary embolism. It belongs to a group of medications known as fibrinolytics. It is given intravenously as soon as possible after the onset of a heart attack to dissolve clots in the arteries of the heart wall. This reduces the amount of damage to the heart muscle. It is recommended that this medication should not be used again after four days from the first administration, as it may not be as effective and can also cause an allergic reaction. For this reason, it is usually given only for a person's first heart attack. (Wikipedia, the free encyclopedia.htm)

Fibrinolytic enzymes can be found in a variety of foods, such as Japanese Natto, Tofuyo, Korean Chungkook-Jang soy sauce, and edible honey mushroom [26,27,28,29]. Fibrinolytic enzymes have been purified from these foods, and their physiochemical properties have been characterized. Fermented shrimp paste, a popular Asian seasoning, was shown to have strong fibrinolytic activity. These novel fibrinolytic enzymes derived from traditional Asian foods are useful for thrombolytic therapy. They will provide an adjunct to the costly fibrinolytic enzymes that are currently used in managing heart disease, since large quantities of enzyme can be conveniently and efficiently produced. In addition, these enzymes have significant potential for food fortification and nutraceutical applications, such that their use could effectively prevent cardiovascular diseases [32].

Fibrinolytic enzymes were identified and studied among many organisms including snakes [9,29,2,3], earthworms [12,5,11], bacteria: Streptococcus pyogenes [10], Aeromonas hydrophila [10], Serratia E15 [12,21,13,31,4], B. natto [10,15,14,23], B. amyloliquefaciens [21], Actinomycetes [7], and fungi: *Fusarium oxysporum* [1], *Mucor sp* [16,24], Armillaria mellea [26].

Because of the medical importance of the fibrinolytic enzymes, we studied the fibrinolytic enzyme production and some of its properties from the fungus *Rhizomucor miehei* which is locally isolated from the grounds of butcheries at Zagazig, Egypt.

**MATERIALS AND METHODS**

**Fungi:** Three fungal isolates were chosen for this study among other fungi isolated from the grounds of butcheries at Zagazig, Sharkia Governorate, Egypt. They were identified according to Domsch et al. [6] as *Aspergillus oryzae* (Ahlburg) Cohn, *Aspergillus tamarii* Kita and *Rhizomucor miehei* (Cooney & Emerson) Schipper. These enzymes are used promptly in food industries and and in fermentation processes of Asian foods. After a pilot test the last fungus was studied for the production of fibrinolytic enzyme and some of its properties were elucidated.
Culture Medium and Conditions: The basal medium is modified Czapek medium, consisted of the following ingredients (g/l): casein, 5; sucrose, 30; K2HPO4, 1.0; MgSO4.7H2O, 0.5; KCl, 0.5; FeSO4.7H2O, 0.01 and 1000 ml dist. Water. Agar-agar was added (20 g/l) for solid media and slants preparation. Medium was sterilized by autoclaving at 121 °C for 20 min and cooled to room temperature. One ml of uniformly prepared spore suspension (10⁴ spores ml⁻¹) from 7 day old cultures was used as inoculum, incubated at 35 °C for 7 days and the mycelium was separated by filtration. Fibrinolytic enzyme activity and the protein content in the filtrate were determined. The effect of culture temperature (20-45 °C), pH value (4-9), incubation period (2-7 days), fibrin concentration (0.1 – 1.8 %W/V), different carbon sources (starch, sucrose, glucose, lactose and maltose) and different concentrations of glucose (0.5 – 3.5 % W/V) were investigated.

Assay of Proteolytic Enzymes: Reaction mixtures containing 0.5 ml of 1% of either of casein, haemoglobin or fibrin suspension in 0.1 M tris-HCl (pH 8.0) and 0.5 ml of fungal filtrates were incubated at 37 °C for 30 min. The reaction was terminated by adding 1.0 ml of 0.15 % trichloro acetic acid. Tyrosine was determined in the neat filtrate by measuring the absorbance at 570 nm [7]. One fibrinolytic enzyme Unit is that amount of enzyme which liberates 1µmole of tyrosine in one min. under the assay conditions.

Protein Determination: Protein was estimated by UV absorption at 280 nm [19] using bovine serum albumin as a standard.

Purification of Fibrinolytic Enzyme: Fibrinolytic enzyme was purified from the fungal filtrate after concentration by freeze drying and storing at 4 °C. the enzyme protein was precipitated by acetone (20%) and dissolved in the minimum volume of 0.05 M Tris-HCl buffer (pH 8.0). the enzyme was dialyzed against the same buffer for 24 h at 4°C. the enzyme protein was loaded onto a column chromatogram of Sephadex G-200 (18cm x 2cm), pre-equilibrated with 50 mM Tris-HCl buffer of pH 8.0. the column was eluted with the same buffer at 20 ml h⁻¹ and 5 ml fractions were collected [21]. Fractions were analyzed for protein and fibrinolytic enzyme activity and the most active fractions were pooled, concentrated by freeze-drying and dialyzed as before. High potent fractions were collected and dialyzed once again to remove Na⁺ and Cl⁻ [22]. This enzyme preparation was lyophilized and stored at 0° C for further studies.

Some Properties of the Purified Fibrinolytic Enzyme: Effect of pH & pH stability: The effect of pH on the enzyme activity was assessed by adding 0.5 ml of enzyme to 0.5 ml of 0.1 % fibrin suspension at different pH values (3.6 – 8.5) using 0.05 M Tris buffer and incubating for 30 min. at 37 °C. The tyrosine released was measured as previously described. To determine pH stability, the reaction mixtures were pre-incubated for 24 h at 40 °C, after that the residual activity was measured.

Effect of Temperature and Thermal Stability: This was achieved by incubating the enzyme in 0.05 M Tris (HCl) buffer at various temperatures in the range 30-80°C. To determine thermal stability, the reaction mixtures were pre-incubated for 1 h at 40-70 °C, after that the residual activity was measured at 35 °C.

RESULTS AND DISCUSSION

The fibrinolytic enzymes play an important role in decreasing blood viscosity which in turn, strikes at the root of arteriosclerosis and atherosclerosis as well as by penetration. Thus they are useful mainly in curing cardiovascular diseases such as heart attack, atherosclerosis and stroke [22]. They are obtained from many sources including microorganisms [13,16,5,8,10,12,21,13,33,4,18,24,5,9,29,14,2,3,11]. In a pilot test for our work three fungal isolates were cultured on modified Czapek medium containing casein as nitrogen source at 35 °C. for seven days, the filtrate was tested to break down casein, haemoglobin and fibrin. Results in Table (1) show that Rhizomucor miehei is the most potent in the break down of fibrin (Specific activity 2.30 Units mg⁻¹ protein). Thus this fungus was chosen as our experimental fungus.

It was found that the best production of fibrinolytic enzyme (2.28 Units mg⁻¹ protein) by the experimental fungus was at 35 °C. (Fig. 1). These results were obtained by other investigators: Abdell-Fattah et al., [1] found that fibrinolytic enzyme preparation from Fusarium oxysporum had optimum temperature at 37°C. while, Cheol Yoo et al., found that fibrinolytic enzyme from Armillaria mellea was active at 33 °C and Ming-Zhong Sun et al., [20] found that fibrinolytic enzymes from snake venoms optimally react with fibrin clots at (33 – 41 °C).

The optimum activity as affected by different pH values (Fig 2) was obtained at (pH 7.0) giving (2.29 Units mg⁻¹ protein). This coincides with that obtained by Bello, et al., with a fibrinolytic proteinase from the venom of Bothrops leucurus (white-tailed jararaca). Whereas Sook-Young et al., [21] found the optimum temperature for the Armillaria mellea to be

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**Table 1:** Production of casienolytic, haemolytic and fibrinolytic enzymes by the isolated fungi.

<table>
<thead>
<tr>
<th>Fungi</th>
<th>Protein mg/ml</th>
<th>Casienolytic enzymes (Units/mg protein)</th>
<th>Haemolytic enzymes (Units/mg protein)</th>
<th>Fibrinolytic enzymes (Units/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aspergillus oryzae</strong></td>
<td>2.78</td>
<td>2.78</td>
<td>1.92</td>
<td>1.78</td>
</tr>
<tr>
<td><strong>Aspergillus tamarii</strong></td>
<td>2.55</td>
<td>2.55</td>
<td>1.44</td>
<td>1.87</td>
</tr>
<tr>
<td><strong>Rhizomucor miehei</strong></td>
<td>1.56</td>
<td>1.56</td>
<td>2.21</td>
<td>1.95</td>
</tr>
</tbody>
</table>

**Fig. 1:** Effect of incubation temperature on the production of fibrinolytic enzyme by *Rhizomucor miehei*

**Fig. 2:** Effect of pH value on the production of fibrinolytic enzyme by *Rhizomucor miehei*
pH 6.0 while Ming-Zhong Sun et al.\textsuperscript{[20]}, obtained best fibrinolytic enzymes from snake venoms at pH (7.5-8.3). On the other hand Yong Peng et al.\textsuperscript{[30]} found that the optimal pH and temperature were 9.0 and 48$^\circ$C of the fibrinolytic enzyme of \textit{Bacillus amyloliquefaciens}.

The highest production of the fibrinolytic enzyme by the experimental fungus was obtained after 6 days of incubation giving 2.96 Units mg$^{-1}$ protein (Fig. 3.)

The maximum activity of fibrinolytic enzyme for the experimental fungus was achieved at 0.6 (\%W/V) concentration of fibrin (Fig. 4.) giving 3.1 Units mg$^{-1}$
Table 2: Purification steps of fibrinolytic enzyme of *Rhizomucor miehei*

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg protein)</th>
<th>Recovery Yield (%)</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-free filtrate (CFF)</td>
<td>38.67</td>
<td>244.86</td>
<td>6.33</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Cell-free dialysate (CFD)</td>
<td>23.98</td>
<td>230.77</td>
<td>9.62</td>
<td>94.25</td>
<td>1.52</td>
</tr>
<tr>
<td>Acetone (20%)</td>
<td>7.18</td>
<td>111.96</td>
<td>15.59</td>
<td>45.72</td>
<td>2.46</td>
</tr>
<tr>
<td>Sephadex G-200</td>
<td>0.35</td>
<td>30.87</td>
<td>88.22</td>
<td>12.60</td>
<td>13.93</td>
</tr>
</tbody>
</table>

Fig. 5: Effect of different carbon sources on the production of fibrinolytic enzyme by *Rhizomucor miehei*

Fig. 6: Effect of different concentrations of glucose on the production of fibrinolytic enzyme by *Rhizomucor miehei*
protein, using glucose as carbon source giving 3.16 Units mg⁻¹ protein (Fig. 5) and 3.26 Units mg⁻¹ protein when glucose concentration was 3.0 (% W/V) (Fig. 6).

Results in Table (2) and Fig. (7). showed that fibrinolytic enzyme of the experimental fungus was partially purified by acetone 20% precipitation and gel filtration on Sephadex G-200, up to 13.93 folds. The optimum activity of the enzyme was reached at pH 8.2 and temperature 45 ºC, the enzyme was stable at pH 8.0 – 8.5 and thermostable at 70 ºC for one hour.

Conclusion: To our knowledge it is the first time to isolate a fibrinolytic enzyme from R. miehei, which may be a good candidate in pharmaceutical industry of blood clot busters.

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