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Purification and Characterization of a Novel Fibrinolytic Enzyme by *Candida guilliermondii* Grown on Sunflower Oil Cake

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ABSTRACT

Biochemical and enzymatic properties of a fibrinolytic enzyme purified from *Candida guilliermondii* NRRL Y-2075 cultivated under solid-state conditions using sunflower oil cake (SuOC) as energy source were investigated. The fibrinolytic enzyme produced has been purified 7.46 fold and affected specific activity 8.65 U/mg protein was analyzed by ammonium sulfate precipitation and ion exchange chromatography (DEAE-Sepharose). The enzyme preparation gave a major protein band with molecular weight 72.44 KDa as detected by SDS-polyacrylamide gel electrophoresis. The pure enzyme showed a maximal activity at pH 8.50 and 45 °C. This enzyme was reasonably stable at pH 5.5-8.5 and it has high stability and retains its full activity up to 50 °C for 30 min. The enzyme exhibited a high specificity for the fibrin substrate. The *K*ₘ and *V*ₘₐₓ values were 5 mg and 66.67 µl/ml/min respectively. The fibrinolytic activity was completely inhibited by 1mM Cu²⁺, Hg²⁺ and iodoacetic acid. Also it was strongly inhibited by PMSF and EDTA. So, the fibrinolytic enzyme was identified as a serine metalloprotease. The results pointed out to the potency of the pure enzyme preparation in dissolving blood clot, indicating that *C. guilliermondii* fibrinolytic enzyme is a potent thrombolytic agent.

Key words: Fibrinolytic enzyme, sunflower oil cake, *Candida guilliermondii*, purification, characterization, thrombolytic agent.

Introduction

Accumulation of fibrin in the blood vessels usually results in thrombosis, leading to myocardial infarction and other cardiovascular diseases (Voet and Voet 1990; Kim et al. 1996; Kim and Choi 2000). These diseases are the leading causes of death throughout the world (Mine et al. 2005). Thrombolytic agents convert plasminogen to plasmin, lyse the clot by breaking down the fibrin contained in a clot. Currently, several thrombolytic agents such as streptokinase, urokinase, prourokinase, retelplase (r-PA), alteplase (t-PA), reptilase, brinase and anisoylated purified streptokinase activator complex (APSAC) are available for clinical use (Sumi et al. 1987; Cadroy and Haarkeer 1990; Kim and Choi 2000; Peng and Zhang 2002). Despite their widespread use, all these thrombolytic agents still suffer significant shortcomings, including requirement of large therapeutic dose, short plasma half-life, limited fibrin specificity and excessive bleeding (Reddy 1998; Blann et al. 2002; Wu et al. 2009). Therefore, the search for less expensive and safer thrombolytic agents from various sources is still an urgent issue.

Fibrinolytic enzymes that dissolve blood clots and show promise for thrombosis therapy have been successfully identified from various sources. A wide range of microorganisms has been screened for their fibrinolytic properties (Takeno et al. 1999). Fibrinolytic enzymes have been reported from various bacterial species of *Bacillus* (Yu et al. 2005; Mahmoud et al. 2011), *Staphyloccoccus* (Choi et al. 2009), *Coryneform bacteria* (Egorov et al. 1982), *Pseudomonas* (Imshenetskii et al. 1991) and *Alteromonas* (Demina et al. 1990). Some fungi have also been found to have high fibrinolytic activity, as *Aspergillus ochraceus* 513 (Batomunkueva and Egorov 2001), *Fusarium* sp. (Wu et al. 2009), *Rhizopus chinensis* 12 (Liu et al. 2005) and *Penicillium* sp. (El-Aassar et al. 1990). In addition, fibrinolytic enzymes produced from different species of mushrooms (Kim et al. 2006; Pandee et al. 2008; Cha et al. 2010). The physiochemical properties of these enzymes have been characterized, and their effectiveness in thrombolysis in vivo has been further identified. Therefore, microbial fibrinolytic enzymes, especially those from food grade microorganisms, have the potential to be developed as functional food additives and drugs to prevent or cure. No information about the production of fibrinolytic enzymes from the yeasts.

Oil cakes/oil meals are by-products obtained after oil extraction from the seeds; oil cakes are of two types, edible oil cakes are used as animal feed while non-edible is used as organic nitrogenous fertilizers.
(Ramachandran et al. 2007). Also, they have been widely used for the production of industrial enzymes, antibiotics, bio-pesticides, vitamins, feed supplement and other bio-chemicals (Ramachandran et al. 2007). Sunflower oil cake (SuOC) has been used for some important biotechnological applications including production of α-amylase by B. licheniformis (Haq et al. 2003); cephapycin C and clavulanic acid produced by S. clavuligerus (Sircar et al. 1998; Kota and Sridhar 1999); Mushroom from P. sajor-caju (Shashirekha et al. 2002).

The last decade has witnessed an unprecedented increase in interest in solid state fermentation (SSF) for the development of bioprocesses, such as bioremediation and biodegradation of hazardous compounds, biological detoxification of agro-industrial residues, biotransformation of crops and crop-residues for nutritional enrichment, biopulping, and production of value-added products (Pandey et al. 2000; Jellouli et al. 2011).

In this study, the purification and characterization of a fibrinolytic enzyme from Candida guilliermondii NRRL Y-2075 grown on sunflower oil cake and its effectiveness in thrombolysis were investigated.

Materials And Methods

Experimental organism:

The yeast Candida guilliermondii NRRL Y-2075 strain was obtained from Agricultural Research Service, Peoria, Illinois, USA and maintained on a stock slant medium (Wickerman 1951), then the cultures was stored at 4 °C and sub-cultured monthly.

Fermentation substrate:

Sunflower seeds (Giza 1) were purchased from the local market (Cairo, Egypt). The seeds were pressed with laboratory-type of Carver hydraulic press under 10,000 lb/in² pressure for 1 h at room temperature according to Üstun et al. (1990), then the sunflower oil cake residue (SuOC) was collected, freeze dried and kept at -4°C.

Media and culture conditions:

The inoculum was prepared by transferring a loopful of a stock culture (7 days old) to a certain volume of sterile stock medium (Wickerman 1951), then incubated at 30 °C on a rotary shaker at 200 rpm for 24 h. Aliquots with cell density 1x10⁸ cells/ml of this suspension was inoculated into 250 ml Erlenmeyer flasks containing 10 g of dry SuOC moistened with 15 ml of the nutrient solution (g %) 0.3 beef extract, 0.5 peptone, and 1.0 milk casein with initial pH 7.0 (Kim et al. 1996; Haq et al. 2003). The incubation was carried out at 30 °C for 48 h. The cell free supernatant of fermented culture media was recovered by centrifugation at 10,000 xg for 20 min at 4 °C and considered as a crude enzyme source.

Enzyme activity assays:

Fibrinolytic activity assay:

The fibrinolytic activity was determined according to Moore (1968) by measuring the liberated α-amino nitrogen. Up to 50 µl of enzyme was incubated with 500 µl of 100 mM sodium acetate buffer, pH 4.5, and 100 µl of 3% of the fibrin substrate and adjusted to 1 ml with distilled water. Assays were carried out at 37 °C for 1h and then stopped by the addition of 200 µl of 20% (v/v) trichloroacetic acid. After centrifugation, 0.5 ml of the supernatant was added to 1 ml of ninhydrin reagent (0.5 ml of 1% ninhydrin in 0.5 M citrate buffer, pH 5.5, 0.2 ml of the same buffer, and 1.2 ml glycerol) and boiled for 10 min (Lee and Takahhashi 1966). Four ml distilled water was added to each sample and absorbance at 570 nm was measured and the increase in free amino groups was determined. Isoleucine was used as a standard. One unit of proteolytic activity was defined as 1µmol α-amino acid liberated per hour under standard assay conditions.

Proteolytic activity assay:

The proteolytic activity was measured according to the modified version of Yokosawa et al. (1983) by incubating 1 ml of 1% (w/v) solution of each substrate (casein, azocasein, albumin, collagen, gelatin and hemoglobin) in 0.05 M phosphate buffer pH 7.0 with 0.5 ml enzyme preparation. The reaction was carried out at 40°C and was terminated after 30 min by adding 1 ml of 10% trichloroacetic acid, kept it for another 20 min at the same temperature and then centrifuged at 4,000 xg for 20 min to remove the resulting precipitate. Protease activity was determined as released tyrosine from the supernatant according to the method of Lowry et al.
A standard curve of tyrosine was used for calibration purposes. One unit of enzyme activity was taken as the amount of enzyme which liberated 1 µmole of tyrosine per ml of enzyme per min under the reaction conditions.

Analytical procedures:

Ash, fat and crude fiber contents of SuOC were determined following the methods of A.O.A.C. (1980), while the crude protein contents were determined using micro-Kjeldahl method (Nx 6.25) (Loiseleur 1963). The carbohydrate content was determined by Dubois et al. (1956).

Enzyme purification:

The crude enzyme was partially purified by using ammonium sulfate with different concentrations (0-80%) of saturation according to the method of Green and Hughes (1955). Each fraction was obtained by centrifugation at 13,000 x g, 4 °C for 15 min, re-dissolved in appropriate amounts of distilled water, and then dialyzed exhaustively against distilled water. Enzyme activity and protein content were determined in the fraction. The dialyzed concentrated enzyme was applied on DEAE-sepharose column (18 x 2.0 cm), equilibrated with 0.02 M phosphate buffer, pH 7.0. Elution was carried out using the same buffer, then a linear gradient of NaCl (0.1 and 0.2 M) in the same buffer was eluted. Fractions of 5.0 ml were collected and measured for absorbance at 280 nm. The active fractions were pooled, dialyzed against the elution buffer, 48 h, 4 °C then lyophilized. The eluates were monitored by measuring their absorbance at 280 nm using a Shimadzu UV-2401, UV-VIS recording spectrophotometer (Warburg and Christian 1942). Protein concentration was determined by the Lowry et al. (1951) method using bovin serum albumin as a standard.

Characterization of the purified enzyme:

Molecular weight determination:

The molecular weight of the pure enzyme was determined by using sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (PAGE) according to the method described by Laemmli (1970) with some modifications using an Owl separation system apparatus model P8DS-1-CE. This was performed by using 12.5% acrylamide gel for separation. The samples were electrophoresed at 120 V and 25 mA. For detecting proteins, gels were stained using Coomassie brilliant blue R-250. A broad-range pre-stained marker containing [phosphorylase b (97 KDa), bovine serum albumin (66 KDa), ovalbumin (45 KDa), carbonic anhydrase (30 KDa), trypsin inhibitor (20.1 KDa) and α-lactalbumin (14.4 KDa)] was used as marker for proteins.

Effect of pH on activity and stability of the pure enzyme:

Aliquots of the purified enzyme were assayed with 5 buffering agents, namely citrate (pH 3.0-4.0), acetate (pH 4.5-5.5), phosphate (pH 6.0-7.0) and tris-HCl (pH 7.5-9.0) at 0.05 M for each one, to record pH profiles under the standard assay conditions. To study the effect of pH on the stability, the purified enzyme was pre-incubated for 30 min at 30 °C with the 5 previous buffering systems before testing the enzymatic activities at standard assay conditions, and then residual activity was calculated.

Effect of temperature on activity and stability of the pure enzyme:

The maximum activity of the pure enzyme was determined at different incubation temperatures (30-70 °C). Thermal stability was studied by incubating the purified enzyme at various temperatures (30-70 °C) for 30 min, and the remaining enzyme activity was then assayed using the standard assay conditions.

Determination of the substrate specificity:

The activity of the purified enzyme on several substrates was tested. All substrates were used in concentration of 1%, except fibrin which was used in concentration of 3% (w/v), and all the substrates used were determined with liberated tyrosine (or isoleucine in case of fibrin) as a reference.

Determination of Michaelis constant ($K_m$):

The $K_m$ value was determined according to the method of Lineweaver-Burk (1934) using fibrin as a substrate.
Effect of metal ions and inhibitors on enzyme activity:

The effect of different metal ions and inhibitors (1 mM) on enzyme activity were investigated using BaCl₂, CaCl₂, CdSO₄, CoCl₂, CuSO₄, FeSO₄, HgCl₂, KCl, MgCl₂, MnCl₂, NaCl, Pb(CH₃COO)₂, ethylenediaminetetraacetic acid (EDTA), iodoacetic acid and phenylmethylsulfonyl fluoride (PMSF). The enzyme was pre-incubated with each salt for 30 min at 30 °C, then the residual activity was measured. The activity of the enzyme without any salt was taken as 100%.

Thrombolytic activity of the enzyme:

This assay depends on resolution of blood clot in vitro and was done according to the method of Nakajima et al. (1996).

Results And Discussion

Within the last few years, studies on fibrinolytic enzymes from microorganisms have attracted significant attention because of their potential use in thrombosis therapy. Although many workers have demonstrated fibrinolytic enzyme production from bacteria and fungi, no information from yeast regarding enzyme production is available.

In the present study, we investigated the biochemical and enzymatic properties of the purified fibrinolytic enzyme isolated from *C. guilliermondii* NRRL Y-2075 cultivated in solid-state conditions using sunflower oil cake as the energy source.

The composition and nutritional availability of oil cakes widely vary based on the quality of the seed method of oil extraction, storage parameters, etc. (Ramachandran et al. 2007). The chemical composition of the SuOC was studied. The results revealed that it contains 22.40% and 13.10% of its dry weight carbohydrate and protein respectively. Also it contains 21.80% crude fat, 32.57% fiber and 4.30% ash. SuOC has 3.00% of its weight H₂O.

Enzyme purification:

Fibrinolytic enzyme produced by *C. guilliermondii* was purified by simple method consisting of precipitation and ion exchange chromatography (Table 1). Most of the enzyme activity was achieved at 0-30% saturation ammonium sulfate fraction having 8775 units, in which specific activity was 4.14 U/mg protein with a yield of 53.31% and purification fold 3.57 (Table 1). By ion exchange on DEAE-Sepharose column, one peak having fibrinolytic activity (8652 units), was separated with specific activity 8.65 U/mg protein and 7.46 purification fold with a final yield of 52.60% as shown in Table (1) and Fig. (1). The units of fibrinolytic enzymes activity have been defined differently in many studies, making it difficult to compare absolute values. However, we noticed that the activity of *C. guilliermondii* fibrinolytic enzyme was much higher than that of *Pleurrotus eryngii* fibrinolytic enzyme (280 U) (Cha et al. 2010); and the enzyme from *Bacillus polymaxa* (8123 U) (Mahmoud et al. 2011). Also, *C. guilliermondii* fibrinolytic enzyme had a higher fibrinolytic activity and purification fold than the typical fibrinolytic enzyme from *Staphylococcus* sp. (2012 U, 4.9 fold) (Choi et al. 2009), but these results were lower than that isolated from *Fusarium* sp. (137,000 U, 158.5 fold) (Wu et al. 2009).

<table>
<thead>
<tr>
<th>Purification steps</th>
<th>Activity (Units)</th>
<th>Protein (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Purification Fold</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>16460</td>
<td>14167</td>
<td>1.16</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulfate saturation</td>
<td>8775</td>
<td>2122</td>
<td>4.14</td>
<td>3.57</td>
<td>53.31</td>
</tr>
<tr>
<td><em>0-30%</em></td>
<td>1585</td>
<td>1049</td>
<td>1.51</td>
<td>1.30</td>
<td>9.63</td>
</tr>
<tr>
<td><em>30-60%</em></td>
<td>520</td>
<td>433</td>
<td>1.20</td>
<td>1.03</td>
<td>3.16</td>
</tr>
<tr>
<td>DEAE-Sepharose</td>
<td>8652</td>
<td>1000</td>
<td>8.65</td>
<td>7.46</td>
<td>52.60</td>
</tr>
</tbody>
</table>

* Each value is the average of three to seven experiments for different batches.

Table 1: Typical *C. guilliermondii* fibrinolytic enzyme purification.
Homogeneity and molecular weight determination:

The patterns of protein-bands in the purification steps were examined by SDS-PAGE (Fig. 2). The protein migrated as a single protein band with a molecular weight of about 72.44 KDa (Fig. 2). The results indicated that the preparation obtained in the present study was highly homogeneous and it appears to be a monomeric protein similar to other fibrinolytic enzymes from different microorganisms (Pandee et al. 2008; Wu et al. 2009; Cha et al. 2010). Its molecular weight is nearly the same as those of Schizophyllum commune BL 23 (68 KDa) (Pandee et al. 2008) and higher than of Cordyceps militaris (52 KDa) (Kim et al. 2006), Fusarium sp. (28 KDa) (Wu et al. 2009) but markedly higher than that of Pleurotus eryngii (14 KDa) (Cha et al. 2010).

Enzyme characterization:

Effect of pH on the activity and stability of the C. guilliermondii fibrinolytic enzyme:

The enzyme was active over a wide range of pH values from 4 – 9 and was most active at about pH 8.50 (Fig. 3), identical to that from Bacillus subtilis QK02, Fusarium sp. and Bacillus polymaxa (Ko et al. 2004; Wu et al. 2009; Mahmoud et al. 2011) and near to optimum pH (8.0) for Oidiodendron flavum and Bacillus subtilis.
DC33 (Nagwa and Tharwat 2006; Wang et al. 2006). The stability studies showed that this enzyme is stable at pHs between 5.5-8.5 and lost about 25% only of its activity at pHs 5.0 and 9.0 (Fig. 4).

**Fig. 3:** Effect of pH on the activity of *C. guilliermondii* fibrinolytic enzyme.

**Fig. 4:** Effect of pH on the stability of *C. guilliermondii* fibrinolytic enzyme.

*Effect of temperature on the activity and stability of the *C. guilliermondii* fibrinolytic enzyme:*

The effect of temperature on enzyme activity is shown in Fig. 5. The optimum temperature for activity was approximately 45 °C and the enzyme lost 30% and 50% of its activity at 30 and 70 °C respectively. This optimum temperature was consistence with those mentioned by Liu et al. (2005); Wu et al. (2009) and was higher than those determined (40 °C) by Cha et al. (2010); Mahmoud et al. (2011). The optimum temperature of the fibrinolytic enzyme from *Schizophyllum commune* BL 23 (Pandee et al. 2008) and *Staphylococcus* sp. were recorded 50 and 85 °C respectively (Pandee et al. 2008; Choi et al. 2009).

The thermal stability of *C. guilliermondii* fibrinolytic enzyme was very high. Full activity was retained up to 50 °C for 30 min (Fig. 6). At the same time, the enzyme lost only 22% of its activity at 60 °C, but at 70 °C it rapidly lost 70% of its activity. However, the fibrinolytic enzyme from *C. guilliermondii* was rather stable at high temperatures when compared to others reported previously (Kim et al. 2006; Wu et al. 2009; Cha et al. 2010; Mahmoud et al. 2011). In *Flammulina velutipes* the fibrinolytic activity was completely inactivated at 55 °C within 15 min (Shin and Choi 1998).
Fig. 5: Effect of temperature on the activity of *C. guilliermondii* fibrinolytic enzyme.

Fig. 6: Effect of temperature on the stability of *C. guilliermondii* fibrinolytic enzyme.

**Substrate specificity and kinetic parameters of the purified enzyme:**

For the characterization of *C. guilliermondii* fibrinolytic enzyme regard to substrate specificity a variety of proteins have been tried as substrates (Table 2). The specific protease activity (caseinolytic) of the purified *C. guilliermondii* fibrinolytic enzyme was 0.886 U/mg. The ratio of fibrinolytic activity to caseinolytic activity was 9.76. This relative value was higher than that of other fibrinolytic enzymes, such as AJ (2.80) (Choi *et al.* 2009), Fu-P (1.86) (Wu *et al.* 2009), subtilisin DJ-4 (0.98) (Kim and Chio 2000), CK (0.73) (Kim *et al.* 1996) and subtilisin Carlsberg (0.092) (Kim *et al.*, 1996). The purified *C. guilliermondii* fibrinolytic enzyme had more affinity toward fibrin (100 %), with affinity decreasing in order of fibrin > collagen > azocasein > albumin > casein and did not show activity on both gelatin and hemoglobin. Therefore, *C. guilliermondii* fibrinolytic enzyme has relatively high substrate specificity for fibrin.

The kinetic constants, $K_m$ and $V_{max}$ values for the fibrinolysis of fibrin by the fibrinolytic enzyme from *C. guilliermondii* was determined using Lineweaver–Burk plot. The reaction properly followed Michaelis–Menten Kinetics. The fibrinolytic activity was assayed with various concentrations (2.5–35 mg) of the fibrin substrate in 100 mM acetate buffer (pH 4.5). $K_m$ and $V_{max}$ values of the purified enzyme for fibrin were determined to be 5 mg and 66.67 mM /ml/min, respectively (Fig. 7). So, *C. guilliermondii* fibrinolytic enzyme resemble to a serine protease with a $K_m$ value comparable to those from *Pleurotus eryngii* (Cha *et al.* 2010) and *Bacillus polymaxa* (Mahmoud *et al.* 2011). The present results indicate a relatively high activity of the purified enzyme for fibrin.
hydrolysis. Therefore, the purified *C. guilliermondii* fibrinolytic enzyme might have potential as an effective thrombolytic agent.

**Table 2**: Substrate specificity of the *C. guilliermondii* fibrinolytic enzyme.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrin</td>
<td>100</td>
</tr>
<tr>
<td>Casein</td>
<td>10.90</td>
</tr>
<tr>
<td>Azocasein</td>
<td>24.01</td>
</tr>
<tr>
<td>Gelatin</td>
<td>0.00</td>
</tr>
<tr>
<td>Albumin</td>
<td>20.24</td>
</tr>
<tr>
<td>Collagen</td>
<td>50.77</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>0.00</td>
</tr>
</tbody>
</table>

**Fig. 7**: Lineweaver-Burk plot for fibrin hydrolysis by *C. guilliermondii* fibrinolytic enzyme.

**Effects of metal ions and protease inhibitors on enzyme activity:**

The effects of protease inhibitors on the *C. guilliermondii* fibrinolytic activity were examined and are shown in Table 3. By using the metalloprotease inhibitors, the enzyme was strongly inhibited by EDTA (85%) and completely inhibited by iodoacetic acid. Also fibrinolytic activity was strongly inhibited by the serine protease inhibitor PMSF (97.2%). These results indicated that *C. guilliermondii* fibrinolytic enzyme was a serine metalloprotease. The response of the fibrinolytic enzyme produced by *C. guilliermondii* was similar to those fibrinolytic enzyme purified from *Schizophyllum commune* (Pandee *et al*. 2008) and *Fusarium* sp. (Wu *et al*. 2009). Most known microbial fibrinolytic enzymes are serine proteases and are not inhibited by EDTA. An enzyme from *Staphylococcus* sp. (Choi *et al*. 2009) and *Pleurotus eryngii* (Cha *et al*. 2010) are metalloproteases. The effects of metal ions on enzyme activity were also investigated (Table 3). Among the tested metal ions, the inhibitory effect was detected by different ratios. The enzyme activity was completely inhibited after treatment with Cu$^{2+}$ and Hg$^{2+}$. Based on metal ion interactions with other fibrinolytic enzymes, all reported metallo fibrinolytic enzymes are inhibited by Cu$^{2+}$ and Co$^{2+}$ (Wu *et al*. 2009). Some metallo fibrinolytic enzyme activities were enhanced by Ca$^{2+}$ at 1 mM, such as the enzyme from the thermophilic fungus *Oidiodendron flavum* (Nagwa and Tharwat 2006) and *Fusarium* sp. (Wu *et al*. 2009). The mechanism of metal ions on fibrinolytic activity is not clear and requires further study.

**Thrombolytic activity of C. guilliermondii fibrinolytic enzyme:**

In this experiment, it was thought of importance to investigate the efficiency of the different enzyme preparations from *C. guilliermondii* to resolute in vitro the human blood clot, the time of complete resolution was recorded after incubation at 37°C.

The results in Table 4, indicated that the pure enzyme preparation effected blood clot dissolution in 2.5 h. Although the protein contents of the ammonium sulfate enzyme fraction and the crude preparation were about 13.5 and 35.5 times that of the pure one, respectively, both effected complete clot dissolution in 6 h. These
results pointed out to the potency of the pure enzyme preparation in dissolving blood clot, indicating that *C. guilliermondii* fibrinolytic enzyme is a potent thrombolytic agent.

**Table 3:** Effect of metal ions and inhibitors on the activity of *C. guilliermondii* fibrinolytic enzyme.

<table>
<thead>
<tr>
<th>Metal ions or inhibitors (1mM)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (none)</td>
<td>100.00</td>
</tr>
<tr>
<td>Ba²⁺</td>
<td>30.85</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>80.00</td>
</tr>
<tr>
<td>Cd²⁺</td>
<td>3.85</td>
</tr>
<tr>
<td>Co²⁺</td>
<td>67.80</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>0.00</td>
</tr>
<tr>
<td>Fe²⁺</td>
<td>94.50</td>
</tr>
<tr>
<td>Hg²⁺</td>
<td>0.00</td>
</tr>
<tr>
<td>K⁺</td>
<td>82.00</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>87.06</td>
</tr>
<tr>
<td>Na⁺</td>
<td>70.00</td>
</tr>
<tr>
<td>Pb²⁺</td>
<td>5.00</td>
</tr>
<tr>
<td>EDTA</td>
<td>14.90</td>
</tr>
<tr>
<td>Iodoacetic acid</td>
<td>0.00</td>
</tr>
<tr>
<td>PMSF</td>
<td>2.80</td>
</tr>
</tbody>
</table>

**Table 4:** Blood clot resolution by different *C. guilliermondii* fibrinolytic enzyme preparations.

<table>
<thead>
<tr>
<th>Enzyme sample</th>
<th>Enzyme protein added (mg/ml)</th>
<th>Complete clot resolution time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude enzyme</td>
<td>14.20</td>
<td>6</td>
</tr>
<tr>
<td>0-30% ammonium sulfate saturation</td>
<td>5.4</td>
<td>6</td>
</tr>
<tr>
<td>Pure enzyme</td>
<td>0.40</td>
<td>2.5</td>
</tr>
</tbody>
</table>

**Conclusion:**

In conclusion, the fibrinolytic enzyme obtained from *C. guilliermondii* cultivated under solid-state conditions using sunflower oil cake shows a high degree of specificity toward fibrin. It may become a new source for thrombolytic agent. Hence, this study highlights the potential for *C. guilliermondii* fibrinolytic enzyme as an effective thrombolytic agent. Investigations to further characterize of *C. guilliermondii* fibrinolytic enzyme are underway.

**References**


