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Purification and Characterization of Fibrinolytic Enzyme from *Pseudoalteromonas* sp., IND11 and its *in vitro* Activity on Blood Clot

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ABSTRACT

Fibrinolytic enzymes are agents that dissolve fibrin clots. These fibrinolytic agents have potential use to treat cardiovascular diseases, such as heart attack and stroke. The aim of the study was to purify fibrinolytic enzyme from the marine isolate, *Pseudoalteromonas* sp., IND11. Enzyme was purified to electrophoretic homogeneity using ammonium sulphate precipitation, ion exchange and affinity chromatography. The SDS-PAGE showed that it was a monomeric protein with an apparent molecular weight of 64 kDa. The purified enzyme was active at pH 6.0-9.0 with an optimum pH of 8.0. It was stable upto 50°C, exhibiting maximum activity between 30 and 60°C. Among the ions, Na^+ and Ca^{2+} activated enzyme activity. The Fe^{2+} did not obviously activate or inhibit the enzyme activity. The ions such as Cu^{2+}, Hg^{2+} and Zn^{2+} strongly affected enzyme activity. This enzyme activated plasminogen and also had direct clot lytic activity. It digested the fibrin net of blood clot, suggests its potential as an effective thrombolytic agent. This study explores new sources of fibrinolytic enzymes to treat and prevent CVDs.

Key words: Fibrinolytic enzyme, thrombolytic agent, bacteria, cardiovascular diseases

INTRODUCTION

Cardiovascular diseases (CVDs) are the one of the leading cause of death worldwide and an estimated 17.3 million people died of CVDs in 2008 which representing 30% of all global deaths. The number of death may increase to 23.3 million by 2030 (WHO., 2011). A common cause of CVD is the formation of fibrin clot in the unbroken blood vessel walls or accumulation of fibrin in the blood vessels. Fibrin is formed by the enzymatic action of thrombin through fibrinogen during the blood clotting process (Wolberg, 2007). The accumulation of fibrin in blood vessels increases thrombosis which is dangerous and can lead to strokes or heart attacks (Collen and Lijnen, 1991). Plasminogen activators and plasmin like proteins are mainly used to treat CVDs (Collen and Lijnen, 2004). Plasminogen activators activate plasminogen into active plasmin to degrade fibrin whereas plasmin-like proteins (Sumi *et al*., 1987) directly degrade fibrin. Disadvantages of using these thrombolytic agents include hemorrhagic side effects, short half-life in the blood circulation and are expensive. Therefore, a search for new thrombolytic agents from various sources still continues. Previously the production of thrombolytic agents were carried out from many organisms,

like, animals (Mihara *et al*., 1991; Zhang *et al*., 1995; Ahn *et al*., 2003), plants (Maurer, 2001) and microorganisms (Fujita *et al*., 1993; Kim *et al*., 1996; Chitte and Dey, 2000; Peng *et al*., 2003). Among these sources, microorganisms are identified as the potent sources of thrombolytic agents, due to the large quantity of production using fermentation methods (Sumi *et al*., 1987).

The diverse marine microbes are exposed to extremes in pressure, temperature, availability of nutrients and salinity (Kennedy *et al*., 2008). Hence, the enzymes produced by marine microbes can provide wide range of environmental condition (Rasmussen and Morrissey, 2007). The microorganisms from the marine environment provide novel metabolites, in particular enzymes that could lower side effects and toxicity when used as thrombolytic agent (Sabu, 2003). In recent years, many potential fibrinolytic enzymes have been isolated and characterized from various marine microorganisms, such as *Bacillus subtilis* and *B. subtilis* ICTF-1 (Agrebi *et al*., 2009; Mahajan *et al*., 2012). However, only a small proportion of fibrinolytic enzymes were isolated from marine microorganisms. Therefore, much work needs to be done to utilize these organisms for the characterization of these fibrinolytic enzymes. The main objective of the study was to purify and characterize fibrinolytic enzyme from *Pseudoalteromonas* sp., IND11.

MATERIALS AND METHODS

Microorganisms and culture condition: Previously the isolate, *Pseudoalteromonas* sp., IND11 was subjected for the optimized production of fibrinolytic enzyme in Solid State Fermentation (SSF) using cow dung as the solid substrate (Vijayaraghavan and Vincent, 2014a). About 5.0 g (w/w) of the substrate was moistened with buffer (Tris-HCl, pH 8.0, 50 mM) at 120% (v/w) level and was supplemented with 1% (w/w) maltose and 0.1% sodium dihydrogen phosphate. The contents were mixed, sterilized and inoculated with 0.5 mL of 18 h grown (1.245 OD at 600 nm) culture broth under sterile conditions. The culture flasks were incubated at 37°C for 72 h and enzyme was extracted by filtration, followed by centrifugation (10,000 rpm, 10 min, 4°C). This cell free extract was used as the source of crude enzyme.

Purification of fibrinolytic enzyme from *Pseudoalteromonas* sp., IND11: Ammonium sulphate precipitation: The crude enzyme of the bacterial isolate was precipitated with ammonium sulphate (saturation). The mixture was allowed to stand at 4°C for 1 h followed by centrifugation (30-70% saturation) (10,000×g for 10 min). These precipitates were suspended in 5.0 mL double distilled water. All collected precipitates were subjected to protein profile analysis and fibrinolytic enzyme assay. The highly active fractions were dialyzed against water (two changes) and buffer and used for chromatographic methods of enzyme purification.

Isolation of fibrinolytic enzyme from *Pseudoalteromonas* sp., IND11 using column chromatography: The ammonium sulphate precipitated fraction was subjected to ion exchange chromatography and casein-agarose affinity chromatography. The dialyzed sample was loaded on DEAE-cellulose column which was equilibrated previously with sodium phosphate buffer (pH 7.4, 50 mM). The column was washed with the 10 bed volume of sodium phosphate buffer (pH 7.4, 50 mM) and the enzyme was eluted with sodium phosphate buffer (pH 7.4, 50 mM) containing NaCl (0-1000 mM). The active fractions from the DEAE-cellulose chromatography were combined and dialyzed against Phosphate Buffered Saline (PBS) (pH 7.4, 25 mM) and loaded on casein-agarose affinity column. This column was previously equilibrated with 25 mM PBS (pH 7.4)

and the bounded proteins were eluted with PBS containing 100-1000 mM NaCl. The extinction of all fractions was measured at 280 nm and all collected fractions were subjected to fibrinolytic enzyme assay.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and zymography: Molecular mass and purity analysis of fibrinolytic enzyme were performed by SDS-PAGE according to Laemmli (1970). Ten microgram of enzyme was loaded into the 12% SDS-PAGE. The molecular weight of the fibrinolytic enzyme was estimated with phosphorylase b (97.4 kDa), Bovine Serum Albumin (66 kDa), Ovalbumin (43 kDa), Carbonic Anhydrase (29 kDa), Soyabean Trypsin Inhibitor (20.1 kDa) and Lysozyme (14.3 kDa) molecular markers. Fibrin zymography was carried out as described by Kim and Choi (2000).

Effect of pH and temperature on enzyme activity and stability: The optimum pH for the activity of the enzyme was determined using the following buffers (100 mM): Citrate buffer (pH 3.0-4.0), succinate buffer (pH 5.0), sodium phosphate buffer (pH 6.0 and 7.0), tris-HCl buffer (pH 8.0) and glycine-NaOH buffer (pH 9.0 and 10.0). The stability of fibrinolytic enzyme in response to pH was evaluated by incubating 100 μL enzyme with 0.1 mL of the above buffers at 37°C for 1 h. Then enzyme assay was carried out with substrate. The effect of temperature on the purified fibrinolytic enzyme was determined by assaying the reactions at various temperatures (30-70°C). To determine the thermal stability, the enzyme was incubated (without substrate) at increasing temperatures (30-70°C) for 1 h. Then enzyme assay was carried out.

Effect of ions on enzyme activity: The effect of ions on enzyme activity was evaluated. The enzyme sample was incubated with 10 mM of Ca^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Hg^{2+}, Fe^{2+}, Na^{+} and Zn^{2+} ions for 1 h. The enzyme activity was determined as described earlier and the relative enzyme activity was calculated.

Effect of fibrinolytic enzyme on plasminogen-rich and plasminogen-free fibrin plate: The plasminogen-rich and plasminogen-free plates were prepared to evaluate the plasminogen activator activity and direct fibrin clot lytic activity of enzyme. The fibrin plate composed of sodium phosphate buffer (100 mM, pH 7.4), 1% (w/v) agarose, 1.2% (v/v) fibrinogen and thrombin (100 NIH U mL^{-1}) (Astrup and Mullertz, 1952). This plate was allowed to stand for 60 min at 30°C to form a fibrin clot layer. In an another experiment, the fibrin plate was heated at 80°C for 30 min to inactivate plasminogen present with the fibrinogen and used as plasminogen-free plate. About 20 μL of purified enzyme was dropped into well sand incubated at 37°C for 5 h. A clear zone around the sample well indicate the fibrinolytic activity.

Fibrinolytic activity of enzyme on human blood clot: Artificial blood clot was made by coagulation in the micro-centrifuge vials. Then, different doses of the purified fibrinolytic enzyme of *Pseudoalteromonas* sp., IND11 were added with the clot. Streptokinase was used as the positive control and buffered saline solution as negative control for this experiment. These vials were incubated at 30°C for 60 min and blood clot lytic activity of fibrinolytic enzyme was analyzed.

RESULTS AND DISCUSSION

Purification of fibrinolytic enzyme from *Pseudoalteromonas* sp., IND11: The crude fibrinolytic enzyme of *Pseudoalteromonas* sp., IND11 was purified for homogeneity by the combination of ammonium sulphate precipitation, DEAE-cellulose ion exchange chromatography

and casein-agarose affinity column chromatography. Precipitation is generally used for the separation of protein from the crude mixtures (Bell *et al*., 1983). The next step of enzyme purification can be achieved by chromatography methods of enzyme purification. The matrix such as cationic exchanger is helpful for the separation of proteases by pH gradient or increasing salt concentration (Tsuchiya *et al*., 1992). From the earlier studies the fibrinolytic enzymes purified from the earthworm *Lumbricus rubellus* (Mihara *et al*., 1991); *Streptococcus aureus* (Avilan *et al*., 1997) and from the fruiting body of mushrooms, namely *Armillaria mellea, Tricholoma saponaceum* and *Cardyceps militaris* (Kim and Kim, 1999, 2001; Kim *et al*., 2006).

In the present study, the ammonium sulphate fraction (70% saturation) showed 8027 units of total enzyme activity with 53% yield and 2.77 fold purification was achieved. The DEAE-cellulose ion exchange column chromatography showed three minor peaks and a major peak (Fraction 8-13). The major peak contained fibrinolytic activity (Fig. 1a). The enzyme yield and purification were 20% and 6.75 fold, respectively. These active fractions were combined and dialyzed against 50 mM sodium phosphate buffer (pH 7.4) and passed through casein-agarose affinity column for further purification. This chromatography fractions showed a peak and possessed fibrinolytic activity (Fig. 1b). The specific activity of the purified enzyme was 579 U mg^{-1} protein with 8.5% yield. This yield was higher than the reported fibrinolytic enzyme from *Fusarium* sp., BLB (Ueda *et al*., 2007) and *A. mellea* mycelium (Lee *et al*., 2005).

The purification procedure of fibrinolytic enzyme was summarized in Table 1. The SDS-PAGE showed a single band with an apparent molecular mass of 64 kDa and it indicated the homogenity of the fibrinolytic enzyme. Also, the fibrin zymography showed enzyme activity (Fig. 2). The

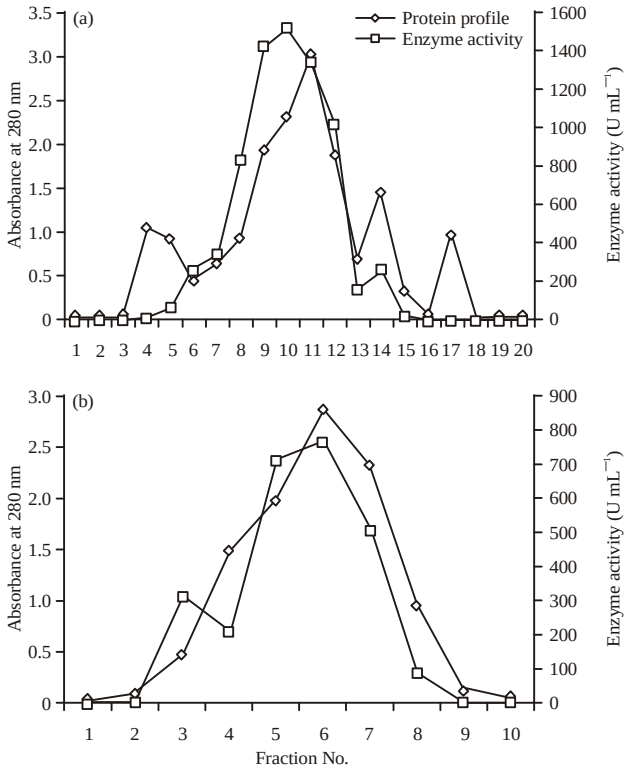

Fig. 1(a-b): Elution profile of *Pseudoalteromonas* sp., IND11 fibrinolytic enzyme from a (a) DEAE-cellulose column and (b) Casein-agarose affinity column

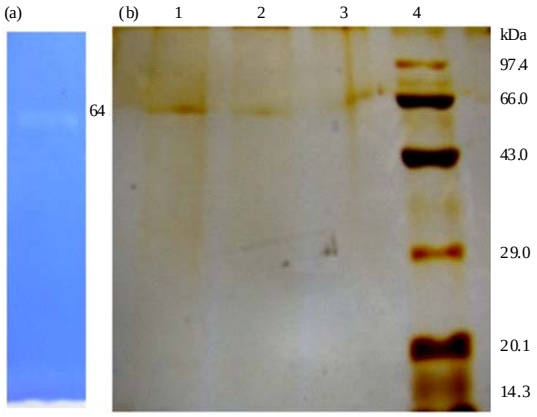

Fig. 2(a-b): Sodium dodecyl sulphate polyacrylamide gel electrophoresis (12%) of the purified fibrinolytic enzyme from *Pseudoalteromonas* sp. IND11 (a) Fibrinolytic activity appeared as colourless band after stained with CBB and (b) 1 and 2-purified enzyme, 4-relative molecular mass standards

Table 1: Summary of purification of fibrinolytic enzyme from *Pseudoalteromonas* sp., IND11

Procedures	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Purification (fold)	Yield (%)
Crude enzyme	15100	520.00	29	1.00	100.0
Ammonium sulphate fraction	8027	184.00	43	2.77	53.0
DEAE-cellulose	3048	15.50	196	6.75	20.0
Casein-agarose	1289	2.48	579	17.90	8.5

molecular weight of *Pseudoalteromonas* fibrinolytic enzyme was not previously reported. The molecular weight of reported protease from Pseudoalteromonas sp., NJ276 (Wang *et al*., 2008) was lower than that of fibrinolytic enzyme from *Pseudoalteromonas* sp. The alkaline serine protease derived from the metagenome of *Shewanella* sp., was almost similar to the enzyme in the present study (Pushpam *et al*., 2011).

Effect of temperature and pH: The fibrinolytic enzyme from the marine isolate, *Pseudoalteromonas* sp., IND11 was highly active at 40°C. This result was similar to that of proteases from *Bacillus pseudofirmus* SVB1 (Sen *et al*., 2011). As the temperature was increased to 50°C, the fibrinolytic enzyme activity declined sharply (Fig. 3a). Earlier reports showed that the fibrinolytic enzyme stability was less than 50°C (Sumi *et al*., 1987; Fujita *et al*., 1993). Also Chang *et al*. (2000) reported the thermostability of fibrinolytic enzyme was lower than 40°C. The fibrinolytic enzyme from *Pseudoalteromonas* sp., IND11 had more stability than in earlier reports. Fibrinolytic enzyme activity of *Pseudoalteromonas* sp., IND11 was high at pH 8.0 (Fig. 3b). This pH optimum was similar to that of fibrinolytic enzyme from *Streptomyces* sp. and *B. subtilis* HQS-3 (Wang *et al*., 1998; Huang *et al*., 2013). The optimum pH of the isolated fibrinolytic enzymes was higher than that of some of the microbial fibrinolytic enzyme from *Bacillus* sp., KA38 (Kim *et al*., 1997). Previous study demonstrated that the amino acid composition of fibrinolytic enzyme plays an important role in its optimal reaction pH value (Wong and Mine, 2004). Therefore, it is speculated that in the present study, the *Pseudoalteromonas* sp., IND11 enzyme may contain more basic amino acids than acidic amino acids.

Effect of ions on enzyme activity: In *Pseudoalteromonas* sp., IND11, the fibrinolytic enzyme activity was inhibited by Cu^{2+}, Hg^{2+} and Zn^{2+} ions (Fig. 4). In *Pseudoalteromonas* sp., CF4-3, the

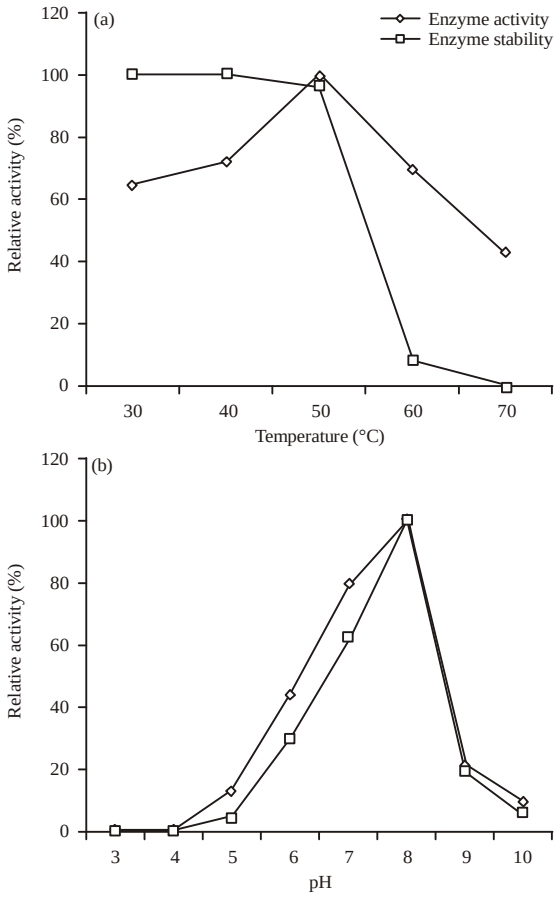

Fig. 3(a-b): Effect of (a) Temperature and (b) pH on *Pseudoalteromonas* sp., IND11 fibrinolytic enzyme activity and stability

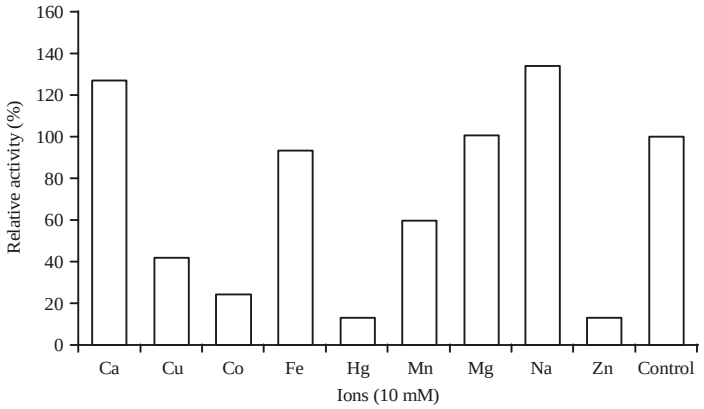

Fig. 4: Effect of ions on *Pseudoalteromonas* sp., IND11 fibrinolytic enzyme activity

protease activity was inhibited by Zn^{2+}, Ni^{+}, Co^{2+}, Sr^{2+} and Fe^{2+} ions (Zhou *et al*., 2009) which was comparable to that of the present study. In this study, Fe^{2+} had little or no effect on enzyme

activity. This property was different from the fibrinolytic enzyme of *Pseudomonas* sp., TKU015 (Wang *et al*., 2009). In *B. amyloliquefaciens* CH86-1, the enzyme activity was inhibited by Co^{2+}, Ca^{2+}, Ag^{+}, K^{+}, Cu^{2+} and Zn^{2+} and Mg^{2+} had not inhibited enzyme activity (Lee *et al*., 2010).

Plasminogen activator and direct plasmin like activity of fibrinolytic enzyme: The fibrinolytic enzyme from *Pseudoalteromonas* sp., IND11 has attracted interest as thrombolytic agents because of its efficiency in the fibrinolytic process including the activation of plasmin. The larger zone observed shows that the purified enzyme was able to convert plasminogen to plasmin. According to the present study, the transparent clear zone of plasminogen-rich plate was larger in size than that of plasminogen-free plate (Fig. 5). This suggests that, the fibrinolytic enzyme have plasminogen-activator activity and also plasmin like activity. Similar result was also reported with other bacterial species previously (Jeong *et al*., 2001; Wang *et al*., 2006).

***In vitro* blood clot lytic activity of fibrinolytic enzyme:** The blood clot lytic effect of fibrinolytic enzyme was studied in *in vitro* condition. The fibrinolytic enzyme digested the blood clot within 60 min of incubation at room temperature (30°C). Clot lysis was not observed in the control vial. The fibrinolytic enzyme digested blood clot effectively at higher doses (Fig. 6) and are dose dependent. The fibrinolytic enzyme activity of *B. subtilis* LD-8547 and *B. cereus* IND1 was dose dependent (Yuan *et al*., 2012; Vijayaraghavan and Vincent, 2014b).

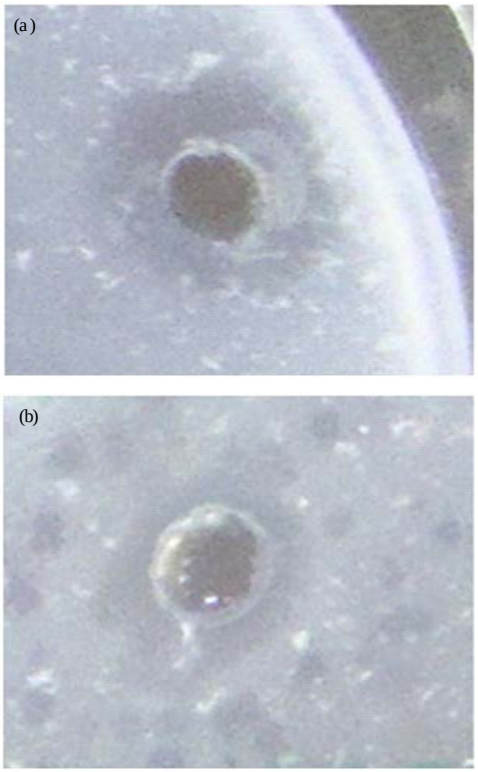

Fig. 5(a-b): Analysis of fibrinolysis by fibrinolytic enzyme on (a) Plasminogen-rich fibrin and (b) Plasminogen-free plate

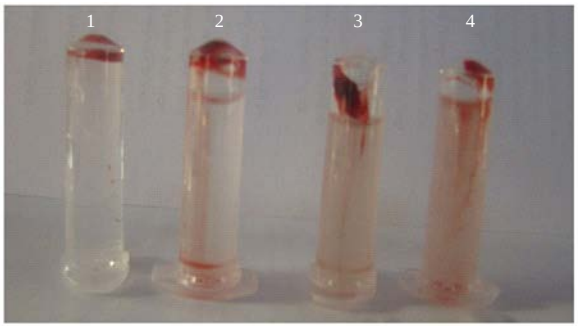

Fig. 6: Effect of *Pseudoalteromonas* sp., IND11 fibrinolytic enzyme on human blood clot (1: Control, 2: 200 units enzyme, 3: 300 units enzyme, 4: 250 units streptokinase)

CONCLUSION

A potent fibrinolytic enzyme was isolated from *Pseudoalteromonas* sp. The purified enzyme was active at higher pH ranges and temperature. It digested fibrin net of human blood clot *in vitro*. This study explores new sources of fibrinolytic enzyme to treat and prevent CVDs.

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