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A novel fibrinolytic serine metalloprotease from the marine *Serratia marcescens* subsp. *sakuensis*: Purification and characterization

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ABSTRACT

This study demonstrates the purification and characterization of a fibrinolytic serine metalloprotease from the marine *Serratia marcescens* subsp. *sakuensis* (KU296189.1). The purified enzyme (1033 U/mg) had a molecular weight of 43 KDa, with optimum pH and temperature being 7 and 55 °C. The *in vitro* half-life of the fibrinolytic enzyme at 37 °C was found to be 19 h. The kinetic constants, K_m and V_{max} of the purified enzyme determined using fibrin as substrate was 0.66 mg/mL and 158.73 U/mL. The K_{cat} and catalytic efficiency of the enzyme was found to be 12.21 min^{-1} and 18.32 mL/(mg min) respectively. The fibrinolytic enzyme did not show any proteolytic activity towards blood plasma proteins like haemoglobin, γ-globulins and transferrin. *In vitro* studies revealed that the fibrinolytic enzyme displayed 38% clot lysis for a period of 3 h which was higher than that displayed by streptokinase and heparin. A total of seven peptide sequences were obtained after the LC-MS/MS-TOF analysis, out of which only four sequences showed 67% homology with the sequences of the other proteases. All these results suggest its novelty and potential application in thrombolytic therapy.

1. Introduction

Under normal physiological conditions, a constant state of dynamic equilibrium is maintained between blood coagulation and fibrinolysis. This equilibrium when disturbed due to tissue injury or genetic variation, leads to thrombosis, thereby restricting the blood circulation (formation of blood clots) in the arteries or veins giving rise to medical conditions such as acute myocardial infarction, stroke, ischemic heart disease and high blood pressure [1]. Two key components involved in maintaining the equilibrium within the human body are enzymes, thrombin (3.4.21.5) and plasmin (3.4.21.7). Thrombin catalyses the conversion of fibrinogen to fibrin, leading to coagulation. This is followed by the action of plasminogen activators that convert the proenzyme plasminogen to active plasmin. Plasmin then degrades the fibrin into soluble fibrin degradation products (FDPs) [2].

Fibrinolytic agents can be defined as drugs that help in the dissolution of blood clot, restoring uniform blood flow within the blood vessels. Fibrinolytic agents used for clinical application fall under three categories based on their mode of action. The first category comprises of plasminogen activators such as tissue-type plasminogen activator (tPA), urokinase-type plasminogen activator (uPA) and bacterial type plasminogen activator like streptokinase (SK). These convert the proenzyme plasminogen to active plasmin and thereby facilitate fibrin clot lysis. The second category of fibrinolytic agents are plasmin like enzymes such as nattokinase, which has a similar role to that of the natural plasmin found within the body and therefore directly lyse the fibrin clots [2]. The third category of fibrinolytic agents consists of oral anticoagulants (OACs) which operate either as vitamin K antagonists or direct thrombin inhibitors and/or bind to factor X. Some of the OACs are heparin, warfarin (Coumadin), dabigatran (Pradaxa) and rivaroxaban (Xarelto) [3,4].

Despite the availability of these varied fibrinolytic agents, not all of them have been put to therapeutic use owing to the drawbacks associated with them. Some of the demerits are inter and intra patient variability of effective dose, food and drug interactions, intra-cranial haemorrhage, short half-lives, lower fibrin specificity, allergic reactions and high production costs. Hence, there arises a need for the development of novel fibrinolytic agents that are safe for therapeutic use with high efficacy and lower cost. Several attempts have been made to produce fibrinolytic enzymes by harnessing the natural resources, such as the microbial sources, medicinal mushrooms, earthworms and snakes [1]. One such potential fibrinolytic enzyme was produced by *Serratia marcescens* subsp. *Sakuensis*, a marine bacterium isolated earlier in our lab from water samples collected from the Surathkal Coast in the Arabian Sea [5]. In the present work, this fibrinolytic enzyme was purified and its physicochemical properties were studied. The proteolytic activity of the fibrinolytic enzyme against several substrates was examined aside from determination of its *in vitro* clot lysing ability and fibrinolytic mechanism. Further, the partial amino acid sequence of the fibrinolytic enzyme was identified and compared with existing database.

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2. Materials and methods

Human fibrin, phenyl-methylsulfonyl fluoride (PMSF), ethylenediamine tetraacetic acid (EDTA), dithiothreitol (DTT), ɣ-globulin, holo-transferrin and serum abumin of bovine origin were purchased from Sigma-Aldrich, India. ENrich SEC 650 column, Standard protein marker and Bovine haemoglobin was procured from BIO RAD India, Merck Millipore India and HiMedia, India respectively. Streptokinase (≥3500 U/mg) and Heparin (140 U/mg) was purchased from Sigma-Aldrich, India. Other chemicals used were of analytical grade.

Serratia marcescens subsp. *sakuensis* (KU296189.1), isolated and studied earlier in our lab was the source of fibrinolytic enzyme for this study. Fibrinolytic enzyme was produced by culturing *Serratia marcescens* subsp. *sakuensis* in the medium comprising yeast extract (12% w/v), soya peptone (1.25% w/v), NH_4Cl (4% w/v) and $CaCO_3$ (0.075% w/v) for 32 h at 37 °C, 150 rpm [5]. This marine isolate has been preserved on starch casein agar slants at 4 °C with periodic subculturing. The fermentation broth was centrifuged at 12000 ×*g* for 15 min to obtain the cell free crude enzyme solution, which was then subjected to ammonium sulphate precipitation. Concentration of ammonium sulphate was varied (40%, 50%, 60%, 70%,and 80% saturation), in order to determine an optimal percentage saturation of the salt required for precipitation of fibrinolytic enzyme. Care was taken to ensure that the desired final concentration of ammonium sulphate was achieved by gradually adding increasing concentrations of the salt at each step, following which the samples were centrifuged. The precipitated proteins were later dialysed against 20 mM sodium phosphate buffer of pH 7.0.

2.1. Enzyme purification

The dialysed enzyme samples were applied to ENrich SEC 650 column in fast protein liquid chromatography (FPLC) system (BIO RAD NGC Chromatography System) of dimensions (10 mm × 300 mm) pre-equilibrated with 20 mM phosphate buffer saline pH 7.0. Flow rate was varied (0.75 mL/min – 1.5 mL/min) to determine a suitable flow rate for maximum separation of desired protein. A constant flow rate of 1 mL/min was maintained with 20 mM phosphate buffer saline of pH 7.0. Active fractions of 1 mL each were collected and pooled together and were lyophilised. These were subjected to standard fibrinolytic assay in addition to total protein estimation.

2.2. Fibrinolytic assay

Fibrinolytic enzyme activity was determined by the method described by Agrebi et al. [6] using fibrin as substrate. Substrate solution was prepared by dissolving 10 mg of fibrin in 100 mL of 0.1 M glycine-NaOH buffer (pH 9.0). 1 mL of enzyme solution was mixed with equal volume of substrate solution, incubated at 55 °C for 15 min. Following which, 1 mL of 0.2 M TCA solution was added and incubated at room temperature for 15 min to stop the reaction. The contents were centrifuged at 10,000 ×*g* for 15 min at 4 °C to separate the precipitate. Absorbance of the supernatant was measured at 280 nm against a suitable blank. A standard graph was constructed using varying concentrations of tyrosine (0–100 μg/mL) and its absorbance being measured at 280 nm. One unit of fibrinolytic enzyme activity was expressed as 1 μg of tyrosine liberated per min under standard assay conditions.

2.3. Protein concentration

Protein concentration in the enzyme solution was determined using standard Lowry's [7] wherein, bovine serum albumin (BSA) was used as the standard and the absorbance was read at 660 nm.

2.4. Molecular mass estimation

The molecular weight of the fibrinolytic enzyme was determined using 12% polyacrylamide in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described previously by Laemmli [8] with a broad range protein ladder. Samples for SDS-PAGE were run under reducing and denaturing conditions and were stained with silver nitrate staining method [9].

2.5. Effect of pH on fibrinolytic enzyme activity and stability

Optimum pH for the enzyme activity was determined using fibrin as substrate. Fibrinolytic activity was assayed over a pH range of 3.0–11.0 at 55 °C. Buffer systems such as acetate buffer solutions (pH 3.0–6.0) and phosphate buffer solutions (pH 7.0–11.0) were used. In order to determine the stability of the purified enzyme at different pH, the purified enzyme was pre-incubated in the respective buffer solutions (pH 3.0 to pH 11.0) at room temperature for 1 h and the residual fibrinolytic enzyme activity of the samples were determined under standard assay conditions. Activity of the unincubated enzyme was taken as 100%.

2.6. Effect of temperature on fibrinolytic enzyme activity, stability and in vitro half-life

The effect of temperature on the fibrinolytic enzyme activity was studied over temperatures ranging from 25 °C to 85 °C during the reaction assay in 20 mM phosphate buffer of pH 7.0, using fibrin as substrate. The thermal stability profile of the purified enzyme was investigated by incubating the enzyme at different temperatures, 25 °C, 30 °C, 37 °C, 45 °C, 55 °C, 65 °C, 75 °C and 85 °C in the 20 mM phosphate buffer solution of pH 7.0, for 2 h. Aliquots were withdrawn at different time intervals and the residual fibrinolytic enzyme activities were determined under standard assay conditions. Activity of non-heated enzyme was considered as 100%.

The *in vitro* half-life of the fibrinolytic enzyme was determined using the thermal stability profile exhibited by the enzyme at both its optimum temperature and at physiological temperature for different time intervals. The half-life for the fibrinolytic enzyme was calculated using a graph that was plotted with relative enzyme activity *versus* time.

2.7. Effect of metal ions and chemical reagents on enzyme activity

The effects of various metal ions (Co^{2+}, Cu^{2+}, Pb^{2+}, Li^{+}, Mn^{2+}, Zn^{2+}, Ca^{2+}, Mg^{2+}, Fe^{3+}, Fe^{2+}, K^{+}, Na^{+}) and chemical reagents (PMSF, EDTA and DTT) at concentrations as mentioned in Table 2 on the fibrinolytic enzyme activity were investigated. The concentration of these metal ions were chosen based on their respective concentration range usually found in human blood. The purified enzyme sample was pre-incubated with these metal ions and chemical reagents at 37 °C for 1 h in 20 mM phosphate buffer (pH 7.0),

2.8. Kinetic studies

Different substrate concentrations (0.1 mg/mL to 5 mg/mL) were used to determine the initial reaction rates. The assay was conducted under optimum conditions (pH 7.0 and 55 °C). The Michaelis-Menten constant (K_m) and rate of reaction (V_{max}) values were determined according to Lineweaver-Burk double reciprocal plot upon plotting the respective $1/[S_0]$ and $1/[V_0]$ values [10]. Subsequently, the K_{cat} and the catalytic efficiency (K_{cat}/K_m) of the enzyme were also calculated.

2.9. Proteolytic activity with different substrates

The proteolytic activity of the fibrinolytic enzyme was measured using fibrinolytic enzyme assay with minor modifications. Fibrin was replaced with other proteins such as haemoglobin, ɣ-globulin, transferrin,

serum albumin and casein as substrates at 1% (w/v) concentration. The purified enzyme sample was incubated with each of these substrates for 15 min at 37 °C and the remaining assay procedure was carried out similar to fibrinolytic enzyme assay. Control was maintained with fibrin as substrate. One unit of proteolytic activity was expressed as 1 μg of tyrosine liberated per min at 37 °C. Enzyme activity was expressed as relative activity (%) while considering that of the control as 100%. In addition, the ratio of fibrinolytic activity/caseinolytic activity (F/C ratio) was calculated.

2.10. In vitro trials with purified enzyme

2.10.1. Percentage clot lysis

The clot lysis activity of the fibrinolytic enzyme was estimated by the method of Prasad et al. [11] with minor modifications. Informed consent was obtained for experimentation with human subjects. Fresh whole blood is collected from healthy volunteers and was immediately citrated using 3.2% (w/v) of 105 mM trisodium citrate (pH 7.4) in the ratio of 9 parts venous blood to 1 part of anticoagulant solution. From this 0.5 mL of blood is added into pre-weighed eppendorf tubes (W1), followed by the addition of 0.2 mL of 2% (w/v) $CaCl_2$ and the mixture is incubated at 37 °C in water bath for an hour to induce clot formation. The tubes are then centrifuged at 5000 rpm at 0 °C for 10 min and the serum was aspirated out. Each of the tubes were weighed again to determine the weight of the clot (W2) following the addition of 0.5 mL of test enzyme to these clots. These tubes were then incubated at 37 °C for a period of 3 h after which the fluid formed due to clot lysis is carefully removed and the tubes with the remnants of the clot are weighed (W3). 0.5 mL of two different concentrations of purified enzyme (29 U and 38 U) was used. 0.5 mL of 0.9% (w/v) saline was used as negative control and 0.5 mL of streptokinase (248 U) and heparin (95 U) each were used as positive control. Percentage clot lysis was expressed as difference in the weight before and after clot lysis, *i.e.*

$$\left\{\frac{W2-W3}{W2-W1}\right\} * 100$$

2.10.2. Determination of *in vitro* clot lysis mechanism of the enzyme

The thrombolytic mechanism of the purified enzyme was determined by the method described by Ju et al. [12] with minor modifications. Informed consent was obtained for experimentation with human subjects. Fresh whole blood is collected from healthy volunteers and is allowed to form a clot upon the addition of $CaCl_2$ followed by removal of serum as described above. Later, one set of tubes containing the clot formed were incubated in water bath at 85 °C for 30 min in order to degrade the plasminogen, while the other set of tubes containing the clot formed were kept unheated. The test enzyme was then added to the heated and unheated blood clots and incubated at 37 °C for a period of 3 h, the clot dissolution rates were evaluated at different time intervals.

2.11. Identification of partial amino acid sequence

The purified enzyme (lyophilised form) was submitted for partial amino acid sequence analysis to National Chemical Laboratories, Pune, India. The sample was subjected to trypsin digestion and sequenced using TripleTOF 5600 (LC-MS/MS-TOF analysis). AB SCIEX Protein pilot software database was used for identification of the peptide sequence. The homology of the peptide sequences were then analysed using NCBI-BLAST database.

3. Results and discussion

3.1. Purification of enzyme

The fibrinolytic enzyme from *Serratia marcescens* subsp. *sakuensis* was purified from the cell free fermentation broth using a three-step purification process. Cell free broth was subjected to ammonium sulphate precipitation (40% saturation) followed by dialysis and the dialysed enzyme solution was then passed through the ENrich SEC 650 column (BIO RAD NGC Chromatography System). With each step of purification, the yield decreases as the impurity proteins get removed resulting in pure protein. The resulting FPLC chromatogram is as shown in Fig. S1. Purified fibrinolytic enzyme showed an increased specific activity (1033 U/mg) by 21.08 fold with a 19.38% recovery (Table 1). The enzyme activity obtained in the present study is significantly higher than the fibrinolytic enzymes from *Pheretima posthumous*, *Brevibacillus brevis* strain FF02B, *Lyophyllum shimeji*, with a maximum specific activity of 18.92 U/mg, 64.9 U/mg and 469.3 U/mg respectively [13–15].

3.2. Molecular mass estimation

SDS-PAGE analysis was performed in order to determine the molecular weight of the purified fibrinolytic enzyme using 12% polyacrylamide gel under reducing and denaturing conditions. As seen in Fig. 1, the purified enzyme resulted in a single band corresponding to a molecular weight of 43 KDa. The fibrinolytic enzyme produced in the present work is a smaller molecule in comparison to streptokinase, fibrinolytic enzyme from *Serratia* sp. RSPB 11 and Serratiopeptidase from *Serratia marcescens* that have a molecular weight of 47 KDa, 50 KDa and 52 KDa respectively [16–18]. As it is widely known, smaller the size of the molecule lesser will be the immunogenicity exhibited by the molecule, thereby rendering it relatively safe for human use.

3.3. Effect of pH on enzyme activity and stability

The ability of an enzyme to function effectively in a broad range of pH is preferred when it has an intended therapeutic use. Thus, it is essential to evaluate the fibrinolytic activity of the enzyme at different pH, in addition to assesing its stability at different pH for a fixed time interval. Relative fibrinolytic activity (%) was calculated for pH 3.0 to pH 11.0 and the maximum activity (at optimum pH) shown by the fibrinolytic enzyme has been considered as 100%. As shown in Fig. 2A, the optimum pH for the fibrinolytic enzyme activity was found to be 7.0, which is close to the human physiological pH, while being active over a wide range of pH between 5.0 and 10.0, exhibiting relative activity of 35% or more. For the pH stability studies, residual fibrinolytic activity (%) was determined and the fibrinolytic activity of the unincubated enzyme was taken as 100%. From the pH stability profile it can be inferred that the enzyme was highly stable over a broad range of pH, maintaining 67% of its original activity at pH values ranging between 3.0 and 11.0 for a period of one hour at room temperature (Fig. 3A). The results

Table 1
Summary of steps involved in purification.

Purification steps	Total protein (mg)[a]	Total activity (U/mL)[b]	Specific activity (U/mg)[c]	Recovery (%)	Purification fold
Crude enzyme	3.26	160	49 ± 7	100	1
Dialysis	0.78	59	76 ± 13	36.87	1.55
FPLC SEC 650	0.03	31	1033 ± 23	19.38	21.08

[a] Protein concentration was estimated by standard Lowry's.
[b] One unit of fibrinolytic enzyme activity was defined as 1 μg of tyrosine liberated per min under the experimental conditions used.
[c] Values represent the mean of three independent experiments and ± standard errors are reported.

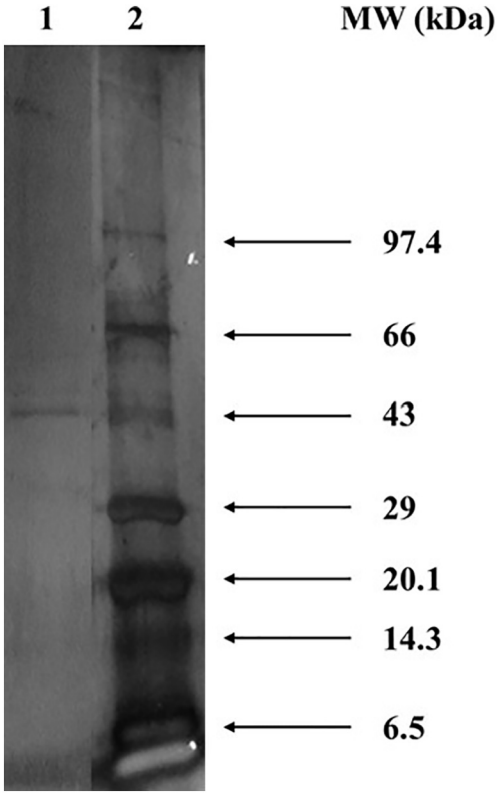


Fig. 1. SDS-PAGE of the purified fibrinolytic enzyme. Lane 1, purified enzyme; Lane 2, broad range protein marker on 12% polyacrylamide gel.

obtained were similar to the pH optimum of fibrinolytic enzyme produced by *Streptomyces* sp. XZNUM 00004, which was found to be 7.8 and displaying stablility between the pH range 5 and 8 [12]. Another such fibrinolytic enzyme produced from a marine *Bacillus subtilis* showed optimum pH at 9 with a broad pH stability between the pH range of 5 and 11 [19].

3.4. Effect of temperature on enzyme activity and stability

Relative fibrinolytic activity (%) was calculated for temperatures 25 °C to 85 °C and the maximum activity (at optimum temperature) shown by the fibrinolytic enzyme has been considered as 100%. The optimum temperature for the fibrinolytic enzyme activity was found to be 55 °C (Fig. 2B), exhibiting >50% of the relative activity between the temperature range of 25–85 °C. In order to determine the thermal stability profile of the fibrinolytic enzyme, residual fibrinolytic activity (%) was determined and the enzyme activity of the non-heated enzyme was taken as 100%. Uptill 65 °C, the enzyme retained >50% of its activity for a period of 2 h, however, at 85 °C, only 13.6% of the activity was retained for a period of 2 h (Fig. 3B). As shown in Fig. 2B and Fig. 3B at physiological temperature of 37 °C, >80% of the enzyme activity was displayed and retained >50% of its activity for 2 h, rendering it favourable for human use. The results obtained differ from that reported by Mahajan et al. [19], where the fibrinolytic enzyme produced by marine *Bacillus subtilis* showed an optimium activity at 50 °C while being stable upto 37 °C and exhibited a complete loss in enzyme activity at 60 °C and 70 °C for an incubation period of 10 min. The fibrinolytic enzyme produced by *Streptomyces* sp. XZNUM 00004 showed 35 °C as its optimum temperature for enzyme activity and remained active at temperatures below 65 °C [12]. The *in vitro* half-life of the fibrinolytic enzyme at physiological temperature (37 °C) and optimum temperature (55 °C) was found to be approximately 19 h and 29 h respectively (Fig. 4). However, further studies need to be carried out to evaluate the same under *in vivo* conditions.

3.5. Effect of metal ions and chemical reagents on enzyme activity

Blood is composed of several metal ions that have a significant role to play and thus, it becomes critical to determine the effect of metal ions on the catalytic activity of the fibrinolytic enzyme activity. Metal ions such as Co^{2+}, Cu^{2+}, Pb^{2+}, Li^{+}, Mn^{2+}, Zn^{2+}, Ca^{2+}, Mg^{2+}, Fe^{3+}, Fe^{2+}, K^{+}, Na^{+} at concentrations as mentioned in Table 2 were studied. As a control, fibrinolytic activity without the addition of any metal ion to the reaction mixture was considered as 100%. The fibrinolytic enzyme activity was enhanced in the presence of divalent cations such as Mn^{2+}, Mg^{2+} and Zn^{2+} ions (Table 2). Similar such observations were reported by Yogesh and Halami [20], wherein the fibrinolytic serine metallo protease showed enhanced activity by more than two folds in the presence of both Mn^{2+} and Mg^{2+}. Peng et al. [21] had suggested that in case of metallo fibrinolytic enzymes, the influence of divalent metal ions on the enzyme activity are a common phenomenon. Fukasawa et al. [22] had reported the metal preferences of the zinc-binding motif of metalloproteases. They inferred that zinc in many of the zinc metalloproeases are substituted by divalent cations such as

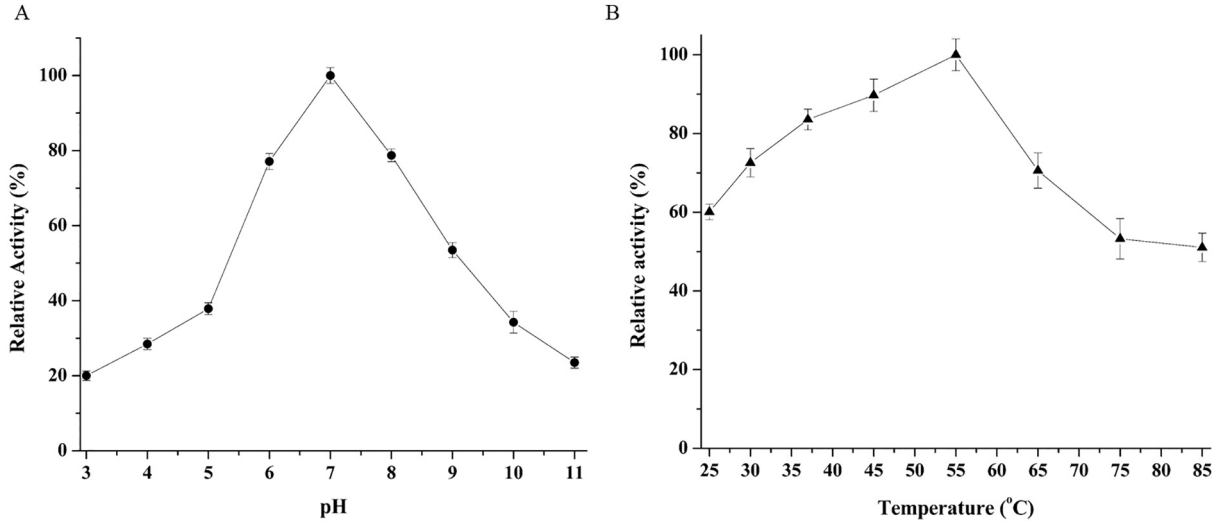


Fig. 2. Evaluation of fibrinolytic activity of purified enzyme (A) Effect of pH on activity of the purified enzyme. (B) Effect of temperature on activity of the purified enzyme. Bars represent standard deviation (n = 3).

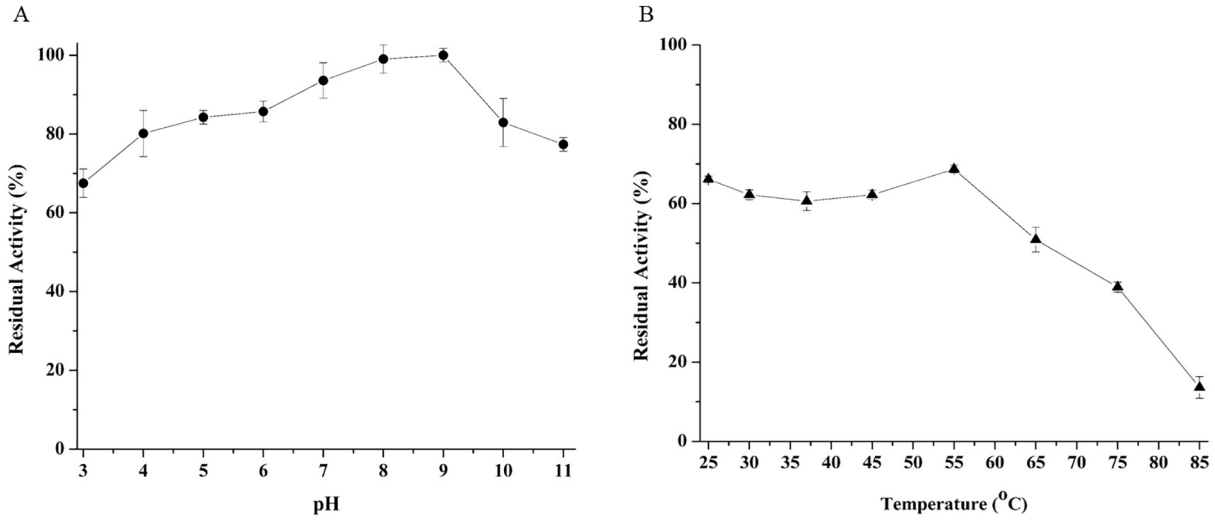


Fig. 3. Evaluation of stability of purified fibrinolytic enzyme (A) Effect of pH on stability of the purified enzyme (B) Effect of temperature on stability of the purified enzyme. Bars represent standard deviation (n = 3).

cobalt (II), Mn (II) and the substituted enzymes show nearly restored catalytic activity or even enhanced activity from apoenzyme due to the flexible metal coordination geometries of Zn (II) and Mn (II) [22]. Among the chemical reagents, both the protease inhibitors, PMSF and EDTA inhibited the fibrinolytic enzyme activity as compared to the control, indicating the serine metalloprotease nature of the enzyme. These findings were comparable to the other reports by Wu et al. [23], Simkhada et al. [24], Mander et al. [25], Choi et al. [26], Moon et al. [15] and Yogesh and Halami [20]. Reducing agent such as DTT showed an increase of 11% in the enzyme activity, perhaps due to its role in reducing the disulphide bonds in proteins and peptides, thereby preventing the formation of intermolecular and intramolecular disulphide bonds between the cysteine residues of proteins [27]. Perhaps, some of the undesirable disulphide bonds are eliminated due to the action of DTT, which in turn would have given more flexibility to the enzyme macrostructure which is favourable. Similar findings were reported by Bohn et al. [28] where DTT was used to stimulate the enzyme activity.

3.6. Kinetic studies

The kinetic parameters were determined under standard assay conditions (pH 7.0 and 55 °C) using different concentrations of fibrin as substrate. The study revealed that the purified fibrinolytic enzyme obeys Michaelis-Menten kinetics (Fig. 5A). Lineweaver-Burk double reciprocal plot was constructed with $1/[S_0]$ values *versus* $1/[V_0]$ values using Origin Pro 8 (Fig. 5B). The Michaelis-Menten constant (K_m) was found to be 0.66 mg/mL and maximum velocity of the enzyme reaction (V_{max}) was estimated to be 158.73 U/mL respectively. The turnover number (K_{cat}) and catalytic efficiency of the fibrinolytic enzyme were found to be 12.21 min^{-1} and 18.32 mL/(mg min), respectively. Most of the literature available on kinetic studies of fibrinolytic enzymes

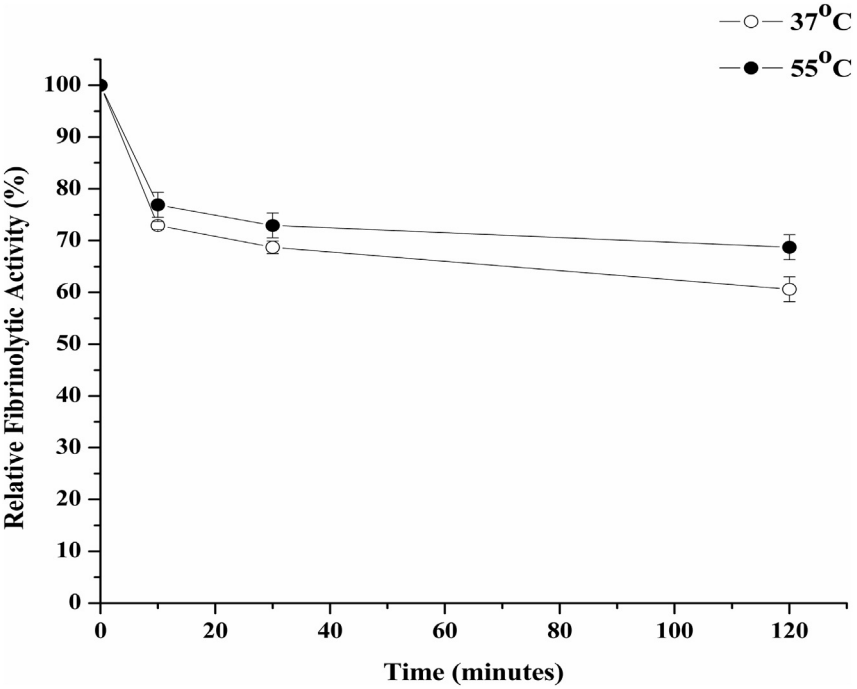


Fig. 4. Comparitive *in vitro* half-life of the fibrinolytic enzyme at 37 °C (colourless circle) and 55 °C (black colour circle).

Table 2
Effect of metal ions and chemical reagents on the fibrinolytic activity.

Chemicals	Concentration (mM/L)	Relative activity* (%)
Control	–	100
Co^{2+}	0.15	67 ± 2.87
Cu^{2+}	0.15	65.96 ± 0.5
Pb^{2+}	0.15	67.76 ± 1.79
Li^{+}	0.15	53.33 ± 0.49
Mn^{2+}	0.15	158.88 ± 4.71
Zn^{2+}	0.15	103.33 ± 1.57
Ca^{2+}	1	64.46 ± 2.86
Mg^{2+}	1	107.77 ± 0
Fe^{3+}	5	89.84 ± 1.43
Fe^{2+}	5	68.78 ± 1.79
K^{+}	50	68.78 ± 1.79
Na^{+}	150	68.07 ± 3.47
PMSF	1	62.6 ± 1.11
EDTA	1	65.15 ± 0.45
DTT	1	111 ± 2.59

* Data represents mean ± SD (n = 3).

reported the use of substrates such as casein and azocasein. The K_m values reported by Simkhada et al. [24], Ju et al. [12], Bhargavi and Prakasham [17], Verma and Pulicherla [13] were 4.2 mg/mL, 0.96 mg/mL, 1.216 mg/mL, and 0.09–0.4 g/mL respectively, using casein/azocasein as substrate. On the contrary, kinetic parameters determined with casein/azocasein not necessarily reflect higher affinity for the substrate fibrin. K_m value of the enzyme studied in the present work was 0.66 mg/mL, which is less than most of the enzymes reported in literature, therefore, indicating higher affinity for the substrate used. As the substrate used in the present work was fibrin, the result becomes even more significant.

3.7. Enzyme activity using several protein substrates

As the fibrinolytic enzyme would be delivered into the circulatory system, it is important that the enzyme does not breakdown any protein other than the protein of interest present in the blood, that is a part of the coagulation and thrombolysis. Therefore, it is necessary to assess the proteolytic activity exhibited by the test enzyme. The proteolytic activity of the fibrinolytic enzyme was evaluated using different substrates (1% w/v) such as haemoglobin, ɣ-globulin, transferrin, serum albumin and casein. Relative fibrinolytic activity (%) was calculated and the enzyme activity with fibrin substrate (control) was considered as 100%. From Table 3, it can be observed that the proteolytic activity with casein and serum albumin was 3.2 times and 6.4 times higher,

Table 3
Enzyme activity against several protein substrates.

Protein substrates	Relative activity* (%)
Control (Fibrin)	100
Caesin	321.76 ± 0
Serum Albumin	644.11 ± 0
γ-Globulins	N.A
Haemoglobin	N.A
Transferrin	N.A

* Data represents mean ± SD (n = 2).

respectively in comparison to the control. The Fibrinolytic activity/Caseinolytic activity ratio (F/C ratio) for the enzyme was found to be 0.31 (Table 4). The F/C ratio obtained in the present work was closely similar to the F/C ratio of the commercially prepared protease Subtilisin BPN and was higher than that of proteases purified from *Streptomyces griseus*, *Aspergillus oryzae*, *Tritirachium album* and trypsin from bovine pancreas [29], while human plasmin shows the maximum F/C ratio [30] (Table 4). Determination of F/C ratio helps in comparing the rate of reaction with different substrates, which gives an indication about the affinity of the enzyme for fibrin substrate. Since this work was aimed at characterising a fibrinolytic enzyme, it is important to understand the enzyme specificity towards the substrates fibrin and casein. A higher F/C ratio would indicate higher affinity towards fibrin and a lower F/C ratio would indicate higher affinity towards casein. No enzyme activity was evidenced with the plasma proteins such as haemoglobin, ɣ-globulins and transferrin as shown in Table 3. This could be because of the complex nature of the substrates, thereby rendering the enzyme incapable of breaking them down and hence can be considered as a potential therapeutic agent. However, further studies can be performed to improve the enzyme secificity towards fibrin. Similar study on a fibrinolytic enzyme from *Bacillus megaterium* KSK-07 was reported by Kotb [30] wherein, enzyme activity towards blood proteins such as fibrinogen, collagen, mucin, serum albumin, elastin and IgG was evaluated and mild proteolytic activity was observed with haemoglobin, mucin, γ-globulins, elastin and collagen.

3.8. In vitro clot lysis

The clot lysing ability (%) of the fibrinolytic enzyme at different concentrations (29 U and 38 U) was evaluated *in vitro* using fresh whole blood collected from healthy volunteers. Saline (0.9% w/v) was used as the negative control. The *in vitro* clot lysis study of the fibrinolytic enzyme (38 U) showed a maximum of 37.88% clot lysis, which was

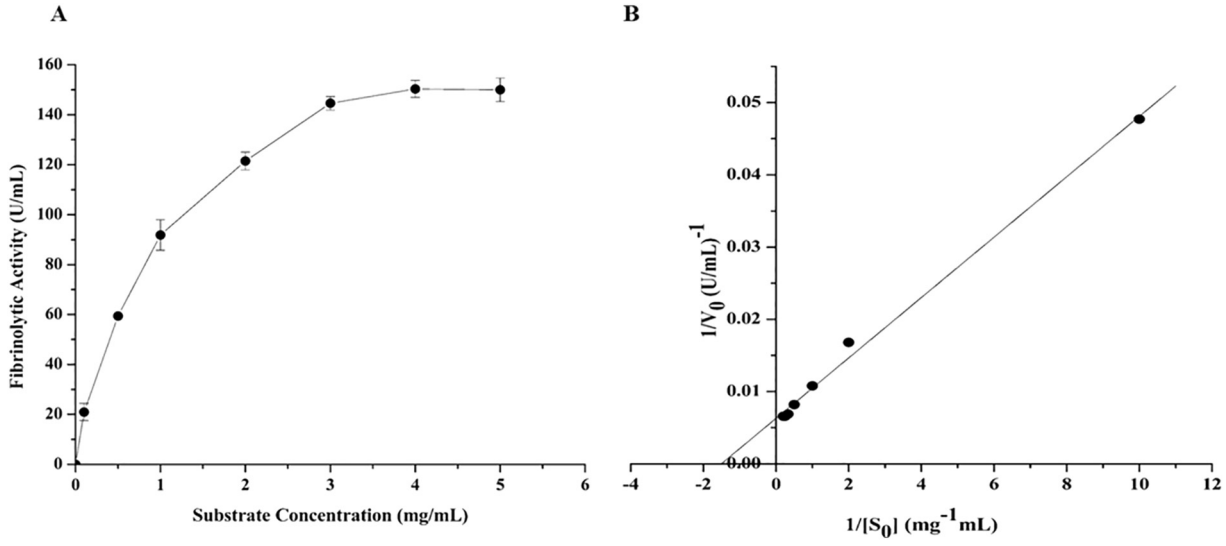


Fig. 5. Enzyme kinetics of the fibrinolytic enzyme. (A) Michaelis-Menten curve for enzyme-substrate reaction. (B) Lineweaver-Burk Plot. Bars represent standard deviation (n = 3).

Table 4
Comparison of the F/C ratios of the fibrinolytic enzyme produced in present study with that of the other proteases.

Protease source	F/C ratio	Reference
Streptomyces griseus (type XXI)	0.20	Kim et al. [29]
Trypsin (bovine pancreases type I)	0.22	Kim et al. [29]
Aspergillus oryzae (type XIII)	0.28	Kim et al. [29]
Tritirachium album (type XXVIII)	0.30	Kim et al. [29]
Serratia marcescens subsp. *sakuensis*	0.31*	Present work
Subtilisin BPN (type XXVII)	0.32	Kim et al. [29]
Plasmin (human plasma)	1.24	Kotb [30]

* Data represents mean ± SD (n = 2).

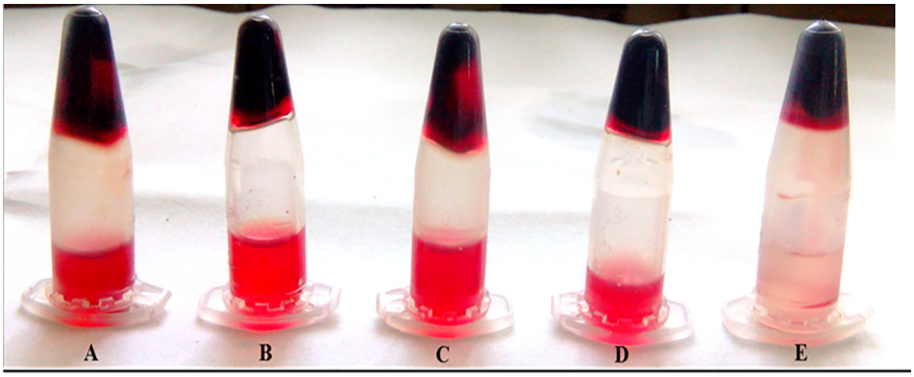
relatively higher than that exibited by heparin and streptokinase for a period of 3 h (Fig. 6). This could be due to its higher affinity towards the fibrin clot as compared to the commercial streptokinase (248 U) and heparin (95 U). Similar results were obtained by Ju et al. [12], where the test enzyme SFE1 (200 IU/mL) showed complete clot lysis as compared to their positive control urokinase (200 IU/mL) and negative control saline. Prasad et al. [11] reported the *in vitro* dissolution of the clots by four different dilutions of streptokinase, achieving a maximum clot lysis of 70.8% with 30,000 IU streptokinase.

Attempt was made to understand the thrombolytic mechanism of the purified fibrinolytic enzyme *in vitro*. Clot lysis was observed under both heated and unheated conditions. It is presumed that plasminogen molecule present in the blood clot gets denatured when the clot is incubated at 85 °C and hence the clot lysis activity observed with heated blood clot would signify plasmin-like activity. However, if clot lysis is observed under unheated condition, the enzyme might either act as plasminogen activator that converts plasminogen to plasmin and thereby lyse the clot or as a plasmin-like enzyme. The percentage clot lysis achieved in case of the unheated blood clots was almost double than that obtained with blood clots that were heated (Table S2). Thus, the results obtained indicate that the fibrinolytic enzyme lysed the blood clot by both direct, indirect clot lysis mechanism and might perhaps function better as a plasminogen activator. These results differ with the only existing report which had made a similar attempt to determine the *in vitro* thrombolytic mechanism of fibrinolytic enzyme from *Streptomyces* sp. XZNUM 00004 [12]. However, further studies are required to elucidate precise mechanism of clot lysis exhibited by the enzyme in question.

3.9. Identification of partial amino acid sequence

A total of seven peptide sequences were obtained as shown in the MS-TOF Chromatogram (Fig. 7). The amino acid sequences of the identified peptide fragments were FSAEQQQQAK, GIDKIDLSFFNK, INLNEK, SFSDVGGLK, DQSYNGFTINAK, SLGTDGAVNTSSFK, YGNWTQNER. Further, these peptides were analysed using the NCBI BLAST database for sequence similarity with the previously reported similar enzymes (Table 5). The four former peptide sequences showed a high degree of homology with peptidase M10 (also known as metalloprotease) from *Yersinia frederiksenii*, serine 3-dehydrogenase from *Serratia grimesii* and serralysin from *Serratia* (Table 5). These represent the conserved regions of the protein sequence. However, the three latter peptide sequences did not show any homology with the sequences available on the NCBI-BLAST database, thus, indicating that the fibrinolytic enzyme produced in the present study could be a unique enzyme.

From the results obtained, it can be inferred that the fibrinolytic enzyme produced in our study has several favourable/ideal characteristics for human use, as the enzyme exhibits significant activity at the physiological pH and temperature, which is important if it has to be used for therapeutic purposes. The half-life of drug becomes a critical parameter that needs to be assessed in order to know the time required for its disintegration inside the human body so as to exhibit maximum efficacy. In the present study, the half-life of the enzyme was determined *in vitro* and found to be 19 h at physiological temperature (37 °C). Although, *in vivo* studies would provide a conclusive information on the half-life of the enzyme, the results obtained *in vitro* would be another step closer in understanding the pharmacokinetics of the enzyme. The kinetic parameters were determined using fibrin as substrate and thus the results obtained are more reliable and specific as compared to that reported in the literature. The fibrinolytic enzyme displayed proteolytic activity with casein and serum albumin but not with the proteins such as haemoglobin, γ-globulins and transferrin, that are majorly present in blood. Although albumin concentration is higher than the other plasma proteins, as compared to haemoglobin in the whole blood, it is a minor component. Majority of the proteolytic enzymes in their native



	Clot lysing agents	% Clot lysis*
A	Positive Control (Heparin 95 U)	14.2±2.27
B	Positive Control (Streptokinase 248 U)	13.17±0.58
C	Test Enzyme (38 U)	37.88±4.16
D	Test Enzyme (29 U)	15.57±1.17
E	Negative Control	1.55±0.48

Fig. 6. Percentage clot lysis observed with various fibrinolytic agents. Data represents mean ± SD (n = 2).

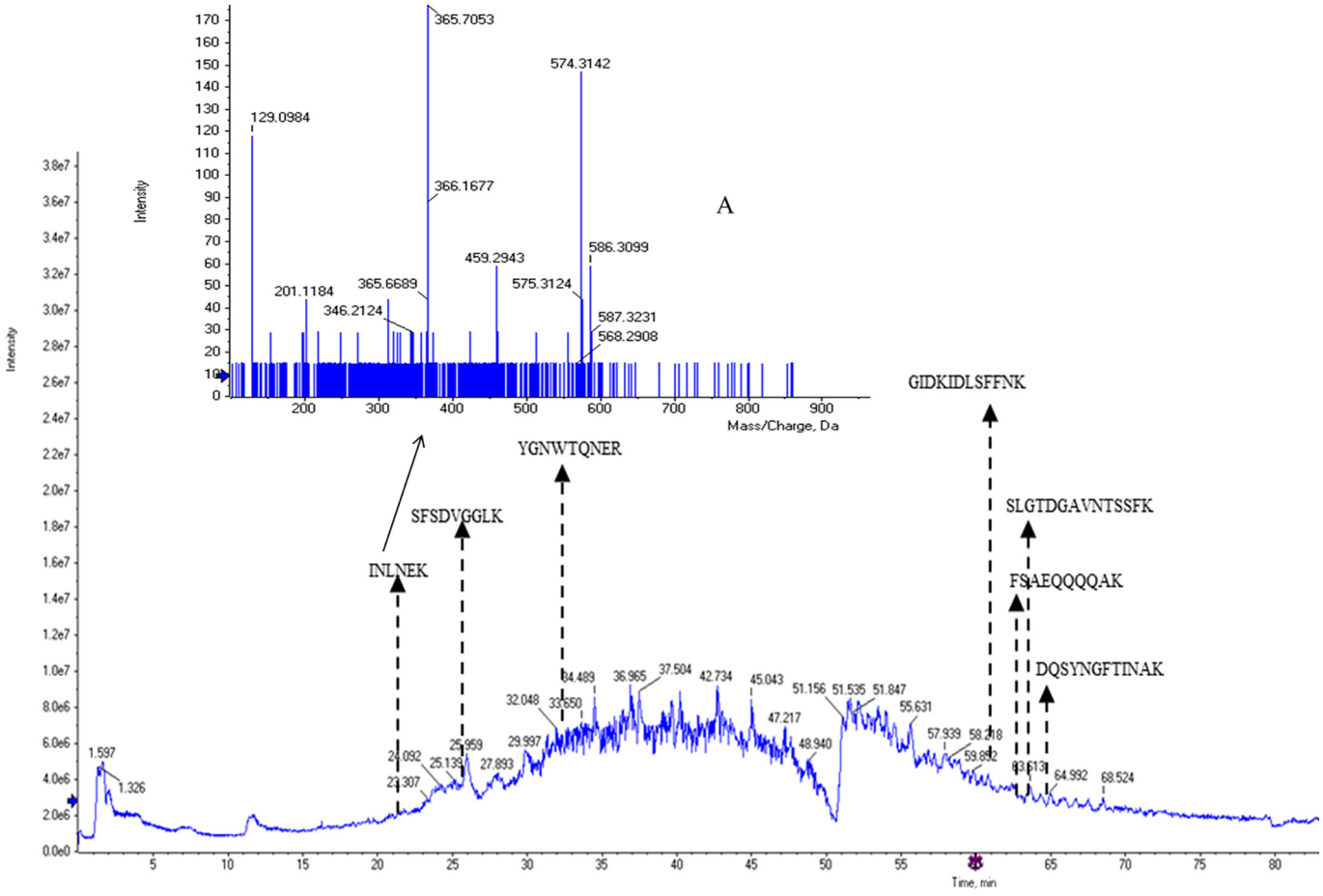


Fig. 7. MS-TOF Chromatogram showing the identified peptide sequences with their retention time, (A) MS spectrum of the peptide sequence INLNEK.

form do not show absolute specificity towards a single protein. Thus, finding a fibrinolytic enzyme showing absolute specificity to fibrin is a distant dream. Under these circumstances, alternative approaches such as chemical modification or genetic modification of the native enzyme can be explored to improve/enhance its specificity towards the target protein. Studies on the *in vitro* clot lysis potential of the enzyme revealed that the purified enzyme resulted in 38% clot lysis, which was significantly more than that shown by streptokinase and heparin. In addition, the partial amino acid sequence analysis gave rise to a total of seven amino acid sequences, out of which three do not share

Table 5
Comparison of the peptide sequences of the fibrinolytic enzyme from *Serratia marcescens* subsp. *sakuensis* with earlier reported similar enzymes.

Name of the source	Sequence[a]		Identity (%)	Positives (%)	Accession number
Peptidase M10 *Yersinia frederiksenii*	**Peptide 1:** FSAEQQQQAK AG	**Peptide 2:** GIDKIDLSFFNK GTDTFDFSGYSQNQK	67%	72%	WP 50099498.1
	Peptide 3: INLNEK INLNEK	**Peptide 4:** SFSDVGGLK SFSDVGGLKG			
Serine 3-dehydrogenase *Serratia grimesii*	**Peptide 1:** FSAEQQQQAK AG	**Peptide 2:** GIDKIDLSFFNK GNDTFDFSGFSQNQR	67%	72%	WP 037422473.1
	Peptide 3: INLNEK INLNEK	**Peptide 4:** SFSDVGGLK SFSDVGGLK			
Serralysin *Serratia*	**Peptide 1:** FSAEQQQQAK AG	**Peptide 2:** GIDKIDLSFFNK GNDTFDFSGYTANQR	67%	72%	CUW14522.1
	Peptide 3: INLNEK INLNEK	**Peptide 4:** SFSDVGGLK SFSDVGGLK			

[a] Identical amino acid sequences are underlined, peptide sequence of the fibrinolytic enzyme (present work) is in red colour.

any homology with several other amino acid sequences present in the database, indicating that this enzyme could be novel and can be explored further for its therapeutic applications.

4. Conclusion

In the current study, we have purified and biochemically characterised a fibrinolytic enzyme from the marine *Serratia marcescens* subsp. *sakuensis* (KU296189.1). Characterization study revealed that it is a serine metalloprotease. Kinetic parameters, *in vitro* clot lysis assay and its proteolytic activity against several blood proteins shows its potential in the treatment of thrombolysis. It appears to exhibit fibrinolysis by both direct and indirect mechanisms. Further, the LC-MS/MS-TOF analysis of the purified protein resulted in a total of 7 peptide sequences, of which three peptide fragments failed to show any homology with any of the protein sequences present in the database, indicating the novelty of the fibrinolytic enzyme produced in the present work.

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Conflict of interest

The authors declare that they have no conflicts of interest with the current work or its publication.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2018.01.129.

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