Serine metalloprotease with plasmin-like fibrinolytic activity of Serratia marcescens isolated from the Amazon basin

Serina metaloprotease com atividade fibrinolítica semelhante à plasmina de Serratia marcescens isolada da bacia Amazônica

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ABSTRACT

Research on new fibrinolytic agents has been oriented towards the discovery of microbial proteases with plasmin-like activity, as alternative thrombolytic agents for thrombosis treatment. This study reports the characterization of an extracellular serine metalloprotease of approximately 56 kDa, with fibrinolytic and fibrinogenolytic activity, isolated from a *S. marcescens* strain obtained from the Amazon (CBAM 519). The enzyme showed optimum activity at pH 9 and temperature of 37 °C. Activity was reduced in the presence of Na⁺, Cu²⁺ and Fe²⁺ ions, and increased with Mn²⁺. The enzyme did not show hemolytic activity, nor did it activate plasminogen, but it effectively hydrolyzed fibrin and fibrinogen, rapidly degrading all fibrinogen chains Aα, Bβ and γ. Therefore, fibrinolysis was promoted only by the direct route, with a fast acting, potent fibrinolytic activity. Based on this, we expect the enzyme to be a protease of the plasmin type that directly degrades fibrin. These characteristics demonstrate great potential for its application in the treatment of thrombosis.

Keywords: fibrinolytic enzyme, serine metalloprotease, thrombosis.

RESUMO

A investigação de novos agentes fibrinolíticos tem sido orientada para a descoberta de proteases microbianas com atividade semelhante à plasmina, como agentes trombolíticos alternativos para o tratamento da trombose. Este estudo relata a caracterização de uma serina metaloprotease extracelular de aproximadamente 56 kDa, com atividade fibrinolítica e fibrinogenolítica, isolada de uma cepa de *S. marcescens* obtida da Amazônia (CBAM 519). A enzima apresentou atividade ótima em pH 9 e temperatura de 37 °C. A atividade foi reduzida na presença de ions Na+, Cu2+ e Fe2+, e aumentada com Mn2+. A enzima não apresentou atividade hemolítica, nem ativou o plasminogênio, mas hidrolisou efetivamente a fibrina e o fibrinogênio, degradando rapidamente todas as cadeias de fibrinogênio Aα, Bβ e γ. Portanto, a fibrinólise foi promovida apenas pela via direta, com uma atividade fibrinolítica potente e de ação rápida. Com base nisso, esperamos que a enzima seja uma protease do tipo plasmina que degrada diretamente a fibrina. Estas características demonstram um grande potencial para a sua aplicação no tratamento da trombose.

Palavras-chave: enzima fibrinolítica, serina metaloprotease, trombose.

1 INTRODUCTION

Thrombosis is characterized by the formation of a blood clot, composed of fibrin, responsible for causing obstruction and inflammation in the vessel wall. Thromboembolic events can be venous or arterial. Both can be life-threatening and sometimes fatal. Venous
thromboembolic events are manifested as deep venous thrombosis (DVT), pulmonary embolism and thrombosis in several blood vessels. Arterial thromboembolic events can include stroke and myocardial infarction [1].

Several risk factors are described for thrombosis development: age over 40, obesity, pregnancy, postpartum, stroke, and cardiovascular diseases (CVDs) such as hypertension, peripheral arterial disease, heart disease and heart failure [2]. Cardiovascular diseases (CVD), such as acute myocardial infarction and stroke, are the leading cause of death and premature disability in many countries, including Brazil [3,4].

In the event of thrombosis development, physiological fibrinolytic activity aims to dissolve the thrombus. The proenzyme plasminogen is activated to form plasmin, which is responsible for degrading many blood plasma proteins, and lysing blood clots. As a pharmacological aid, fibrinolytic agents stand out among the most important biopharmaceuticals available for clinical use [5] and can be classified into three groups, based on their mode of action. The first group consists of thrombolytic plasminogen activating agents, such as tissue plasminogen activator (tPA), urokinase type plasminogen activator (uPA) and streptokinase type bacterial plasminogen activator (SK) [6]. The second category is composed of plasmin-like enzymes, such as nattokinase, which have a role similar to that of natural plasmin found in the human body, directly lysing fibrin clots [7,8]. The third group of fibrinolytic agents consists of oral anticoagulants (OACs) that function as vitamin K antagonists or direct thrombin inhibitors and/or factor X binding proteins. Examples of OACs are heparin, warfarin, dabigatran and rivaroxaban [9].

Despite the availability of these diverse fibrinolytic agents and their widespread use, the compounds have some disadvantages, such as high production costs, low specificity for fibrin, risk of intracranial hemorrhage development, allergic reactions, drug and food interactions and risk of bleeding in the intestinal tract when orally administered [10,6].

To overcome these drawbacks, research for new fibrinolytic agents has been oriented towards the discovery of microbial proteases with activity similar to that of plasmin, proposing cheaper, more specific and low toxicity alternatives to current thrombolytic agents, with less side effects, and improving the quality of the patient’s life [11].

Some studies have reported characterization and purification of fibrinolytic proteases produced by bacteria, such as Bacillus subtilis natto (nattokinase) [8]. B.
In view of the increasing interest in microbial enzymes as biopharmaceuticals, the objective of this work was to characterize a fibrinolytic enzyme of a *Serratia marcescens* strain (CBAM 519) isolated from the Amazon region.

### 2 MATERIALS AND METHODS

#### 2.1 MICROORGANISM AND PROTEASE PRODUCTION

*S. marcescens* (CBAM 519) was obtained from the Amazon Bacterial Collection (CBAM / FIOCRUZ-Amazonas, Brazil). This culture was selected after screening for fibrinolytic enzyme activity of 150 strains isolated from soil and water in the Amazon region (SisGen: AD34ED). The strain was grown in Nutrient Agar in petri dishes at 37 °C for 24 hours and then inoculated (OD$_{600nm}$~0.2) in 250-mL erlenmeyer flasks containing 100 mL of Manachini solution [15] consisting of (w/v): 0.2% KH$_2$PO$_4$; 0.1% (NH$_4$)$_2$SO$_4$; 0.1% MgSO$_4$.7H$_2$O; 0.09% NaH$_2$PO$_4$.H$_2$O; 0.1% yeast extract and 0.5% gelatin. The pH was adjusted to 6.9. The samples were cultivated for 24 h at 37°C on a rotary shaker at 150 rpm. The culture was then centrifuged at 8.000 xg for 10 min/4°C and the supernatant filtered through a 0.22 μm membrane. This cell-free supernatant was used for enzyme characterization.

#### 2.2 BACTERIAL GROWTH CURVE AND ENZYME SECRETION PROFILE

Bacteria were cultivated in 250-mL erlenmeyer flasks containing 50 mL of Manachini solution, as described above. Culture was started at an OD$_{600nm}$ of 0.2 and bacterial growth was monitored for 24 hours, at 2h intervals. Samples were centrifuged at 8.000 xg for 10 min and the proteolytic activity was determined in the supernatant, while the temporal profile of protease secretion was evaluated after protein precipitation with trichloroacetic acid (TCA; 17% final concentration), followed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and fibrin zymography.

#### 2.3 AMMONIUM SULFATE PRECIPITATION

For enzyme characterization, 100 mL of culture supernatant obtained from bacterial grown as described above was subjected to ammonium sulfate fractionation at different saturation ranges (20, 40, 60 and 80%) and precipitates were collected by centrifugation at 15.000 xg for 10 min at 4°C. The 60% saturation fraction, which
contained the highest fibrinolytic activity, was resuspended in 100 mM sodium phosphate buffer, pH 7.0. Enzyme was assessed by SDS-PAGE and specific activity by a fibrinolytic assay, as described below.

2.4 PROTEIN CONCENTRATION MEASUREMENT

Protein concentration was estimated with the Bradford method [16] using bovine serum albumin as a standard. Each experiment was performed in triplicate.

2.5 SDS-PAGE AND FIBRIN ZYMOGRAPHY

SDS-PAGE was carried out on 12% polyacrylamide gels according to the Laemmli method [17]. Protein bands were detected by staining with Coomassie Brilliant Blue R-250. Fibrinolytic activity was analyzed on a fibrin zymography gel as described by Kim et al [18]. Briefly, fibrinogen (0.12% w/v) and thrombin (1U/mL, both from Sigma) were mixed with a 12% polyacrylamide gel solution, and electrophoresis of the protease solution was carried out. The gel was then washed with 2.5% Triton X-100 for 1 h, rinsed twice with distilled water, and incubated in the reaction buffer (0.1 M glycine, pH 8.4) at 37°C for 18 h. The gel was stained with Coomassie blue for 1 h and then destained. The digested bands were visualized as the nonstained regions of the fibrin gel. The molecular mass was calibrated using a Broad Range protein marker (BioRad).

2.6 PROTEOLYTIC ACTIVITY ASSAY

The proteolytic activity was determined according to Leighton et al [19]. For the crude extract, 0.25 ml of the substrate (azocasein, Sigma) in 0.1 M Tris-HCl buffer, pH 7.6, and 0.15 ml of the enzymatic extract were used. The reaction mixture was incubated at 25 °C for 1 h. The reaction was stopped by adding 1.2 ml of 10% (w/v) trichloroacetic acid. The samples were centrifuged at 8000 xg for 10 minutes, at 4 °C, and 0.8 mL aliquots were transferred to test tubes containing 1.4 mL of 1 M sodium hydroxide prior to absorbance measurement at 440nm. One unit of proteolytic enzyme was defined as the amount of enzyme that produces a 0.01 OD increase of absorbance in 1 h at 440 nm. All samples were prepared in triplicate.

2.7 FIBRINOLYTIC ASSAYS

Fibrinolytic activity was determined by both the plasminogen-free and the plasminogen-rich fibrin plate method, with minor modifications [20]. Plasminogen-free
Fibrinogenolytic activity was measured as follows: 150 μl of a 1.0% human fibrinogen solution was incubated with 50 μl of the enzyme at 37°C for different times [14]. The reaction was stopped by the addition of denaturing buffer. The digested products were analyzed by 12% SDS-PAGE according to the method of Laemmli [17].

Biochemical characterization of fibrinolytic protease

The enzyme produced by *S. marcescens* (CBAM 519) was characterized using 1% (w/v) azocasein [19]. All the experiments were done in triplicate.

2.9 EFFECT OF PH ON PROTEASE ACTIVITY AND STABILITY

To determine the effect of pH on the proteolytic activity, the reaction system and the blank system were prepared using azocasein as substrate in the following buffer solutions: 0.1 M citrate (pH 4-6), 0.1 M Tris-HCl (pH 7-8) and 0.1 M sodium carbonate/bicarbonate (pH 9) [21]. Reactions were incubated for 1 hour at 25°C and proteolytic activity was determined by the method described above. For evaluation of the enzyme stability, the enzymatic extract was dispersed (1:1) in the buffers mentioned above and maintained at 25°C for 24 hours. After that, residual proteolytic activity was determined under standard assay conditions.

2.10 EFFECT OF TEMPERATURE ON PROTEASE ACTIVITY AND STABILITY

Optimum temperature was determined by incubating the enzymatic extract at different temperatures ranging from 25 to 80°C for 1h, at optimal pH, and assaying the activity. Relative activities were determined according to the optimal conditions of pH and temperature [22].
2.11 EFFECT OF INHIBITORS ON PROTEASE ACTIVITY

To evaluate the effect of inhibitors on enzyme activity, the enzymatic extract was exposed to the following inhibitors: 10.0 mM methylphenylsulfonyl fluoride (PMSF), 10.0 mM 2-mercaptoethanol, 10.0 mM ethylenediaminetetraacetic acid (EDTA) and 1.0 mM pepstatin A [23].

2.12 EFFECT OF METAL IONS ON PROTEASE ACTIVITY

The protease activity was evaluated in the presence of the following metal ions described as inhibitors or activators of protease activity, at concentrations of 1.0 mM and 10 mM: zinc (Zn\(^{2+}\)), magnesium (Mg\(^{2+}\)), copper (Cu\(^{2+}\)), ferrous (Fe\(^{2+}\)), calcium (Ca\(^{2+}\)), manganese (Mn\(^{2+}\)), sodium (Na\(^{+}\)), potassium (K\(^{+}\)), and incubated at 37˚C for 60 min [23].

2.13 HEMOLYSIS ASSAY IN VITRO

Hemolysis assay was performed according to Huang et al [24], with some modifications. Blood agar plates composed of blood agar base and fresh defibrinated sheep blood were prepared for the assay. Then, 20 ul of enzyme (25 ug/ul) were added to slots punched in the blood agar (Ø = 5 mm). After incubation at 37°C for 3d, the plate was examined for the presence of translucent halo. This experiment was performed in triplicate.

3 RESULTS AND DISCUSSION

3.1 ENZYME SECRETION PROFILE

*S. marcescens* (CBAM 519), when grown in Manachini's solution supplemented with 0.5% gelatin, initiates detectable production of a secreted fibrinolytic protease after 4 hours of growth. The peak of activity was observed after 24 hours, yielding 187 U / mL in the culture medium (Fig. 1a), still in the log growth phase. The increase in proteolytic activity was proportional to the increase in cell density. After 24h, proteolytic activity decreased, as the stationary growth phase set in. This is similar to what occurred with nattokinase produced by *Pseudomonas* sp. TKU015 [25] and in the submerged production of proteases from *Bacillus licheniformis*, where this kind of fermentation is often the best way to produce enzymes due to the control of physical-chemical parameters and easier scale-up to an industrial level [26, 27].

The protein secretion profile during the growth of *S. marcescens* (CBAM 519) was evaluated in the culture filtrate, after protein precipitation, through polyacrylamide
gel electrophoresis (SDS-PAGE) and fibrin zymography (Fig. 1b, c). Gelatin (inducing substrate) was not consumed by the bacteria in the first 2 hours. Immediately after 4h, gelatin started to be metabolized and a single protein band persisted on the gel until 24h. In addition, as observed by fibrin zymography, fibrinolytic protease activity can be detected in samples obtained after 4 hours of bacterial growth, corroborating with results in Fig.1a. The fibrinolytic activity was most intense close to 55 kDa, a size compatible with the band visible on the SDS-PAGE gel. It is worth mentioning that the enzyme remained active even after precipitation with 17% TCA.

3.2 PARTIAL PURIFICATION BY AMMONIUM SULFATE FRACTIONATION

The fractions obtained after ammonium sulfate precipitation were analyzed by SDS-PAGE (Fig. 2A) and 60% saturation was selected, as it showed a more intense band after staining with CBB-R250. The single band observed in SDS-PAGE in the 60% saturation fraction, suggested that the fibrinolytic enzyme has a molecular mass of approximately a 56 kDa (Fig. 2A). The fibrin zymography (Fig. 2B) showed a clear hydrolysis zone in this same position. The observed molecular weight of the enzyme in the present study is in accordance with reports for extracellular fibrinolytic proteases of *S. marcescens* RSPB11 (50 kDa) [14] and *Serratia* sp. KG-2-1 (52 kDa) [28], while *S. marcescens* subsp. *sakuensis* presented 43 kDa [6].

3.3 ENZYME CHARACTERIZATION

The degree of purification provided by 60% ammonium sulphate precipitation followed by buffer exchange yielded sufficient material for the next steps in enzyme characterization.

3.4 EFFECT OF PH ON PROTEASE ACTIVITY AND STABILITY

The relative fibrinolytic activity (%) was measured for the pH range 4.0 to 9.0, setting the maximum activity in the curve as 100%. As shown in Fig. 3, fibrinolytic activity reached a maximum at pH 9.0. In addition, the enzyme maintained more than 55% activity in the pH range 7.0 and 8.0. For pH stability analysis, the residual fibrinolytic activity (%) was determined, where the proteolytic activity of the unincubated enzyme was considered as 100%. The enzyme is highly stable at room temperature over a wide pH range, maintaining more than 80% of its activity in the pH range 5.0 to 9.0 for a 24 hour period. The alkaline pH was also reported to provide better results for the
fibrinolytic enzymes of *Streptomyces parvulus* [29], *S. marcescens* RSPB11 [14] and *Bacillus subtilis* ICTF-1 [30] which showed optimal activity at pH 9. Another fibrinolytic enzyme produced by *Bacillus subtilis*, showed greater activity at pH 7.0, and was relatively stable between pH 6.0-10.0 [31]. Alkaline proteases obtained from microorganisms have attracted great attention from industry due to their pH stability, temperature optimum at 37°C, high catalytic activity, and the high degree of specificity to the substrate [32].

### 3.5 EFFECT OF TEMPERATURE ON PROTEASE ACTIVITY AND STABILITY

The relative fibrinolytic activity (%) was evaluated for temperatures between 25 °C and 80 °C and the maximum activity shown by the enzyme was considered as 100%. The fibrinolytic enzyme of *S. marcescens* (CBAM 519) showed its highest activity at 37 °C (Fig. 4a). For comparison, the optimal temperatures for proteases of *S. marcescens* RSPB11 [14], *S. marcescens* [33], *S. marcescens* ATCC 25419 [34], *S. marcescens* subsp. *sakuensis* TKU019 [35] and *S. marcescens* subsp. *sakuensis* (KU296189.1) [6] have been reported to be at 37 °C, 40 °C, 45 °C, 50 °C and 55 °C, respectively.

Residual enzyme activity at different temperatures after 1 hour of incubation is shown in Fig. 4b. The enzymatic activity before incubation was considered as 100%. The enzyme retained more than 80% of activity after 1 hour of incubation in the temperature range of 25 to 40 °C which is encouraging, as thermostability and resistance to denaturing agents increase the scope of useful applications [36]. Likewise, fibrinolytic proteases from *S. marcescens* RSPB11 [14] demonstrated thermal stability at physiological temperature, maintaining more than 80% of the enzymatic activity, an important characteristic for potential biopharmaceutical applications.

On the other hand, the enzymatic activity decreased dramatically at 70 °C resulting in less than 5% residual activity. These thermostability results were similar to those reported for fibrinolytic enzymes produced by *Bacillus subtilis* ICTF-1 [30] and DC27 [31] which remained stable between 25-37 °C and 37-50 °C, respectively, and also exhibited complete loss of enzymatic activity above 70 °C.

### 3.6 EFFECT OF METAL IONS AND INHIBITORS ON PROTEASE ACTIVITY

To determine the class of the *S. marcescens* (CBAM 519) fibrinolytic enzyme, assays were performed with different protease inhibitors and expressed as relative (%) activity compared to control without additives. As can be seen in Fig. 5, the enzyme
activity was strongly inhibited by EDTA and PMSF, reducing the activity to 52% and 34%, respectively. In the presence of other inhibitors, the enzyme activity remained above 70%. These results suggest that the fibrinolytic enzyme has features of a serine metalloprotease. PMSF blocks the active site of some proteases by sulfonation of the essential serine residue, resulting in the inhibition of protease activity [11]. Most of the metalloproteases are enzymes containing the His-Glu-Xaa-Xaa-His (HEXXH) motif, which has been shown by X-ray crystallography to form part of the site for binding of the metal, usually zinc [33]. Serine metalloproteases have also been found in studies with fibrinolytic proteases from Serratia marcescens subsp. sakuensis [6], and B. circulans [37]. According to Wu et al [38], most fibrinolytic enzymes derived from microorganisms are serine proteases.

Normal human blood contains several metal ions that have a significant role in the body physiology and, therefore, it is essential to determine the effect of metal ions on the catalytic activity of the fibrinolytic enzyme. The protease produced by S. marcescens (CBAM 519), showed a reduced activity in the presence of K⁺, Cu²⁺ (10 mM) and Fe²⁺ (1mM and 10 mM). On the other hand, Mn²⁺ at 10mM enhanced the proteolytic activity, providing a 527% increase (Table 1). This suggests that this cation plays a vital role in maintaining the active site and is thought to contribute to the enzyme's thermostability [39].

Our results differ from those obtained by Hu et al [31]. The B. subtilis serine protease showed higher activity in the presence of Ca²⁺ and K⁺ ions, while Cu²⁺ and Fe²⁺ inhibited the purified fibrinolytic enzyme. Similar results were obtained for the B. circulans strain serine metalloprotease [37]. In this study, the addition of Cu²⁺ reduced the activity of the enzyme by more than 50%, while Mn²⁺ at 10 mM showed a positive effect on the catalytic activity. Likewise, Mn²⁺ has also been shown to potentiate the activity of the fibrinolytic enzyme of S. marcescens subsp. sakuensis [6], which might be explained by a better substrate binding in the presence of such metal ions, or even by changes in the folding of the enzyme arising from electrostatic changes [40].

3.7 FIBRIN AND FIBRINOGENEOLYTIC ACTIVITY

The enzyme of S. marcescens (CBAM 519) formed a zone of lysis of similar size in both plasminogen-rich and plasminogen-free plates (Fig. 6A and B), indicating that the enzyme in question is not capable of activating plasminogen. Based on this, it is suggested that the enzyme is a protease of the plasmin type that directly degrades fibrin.
To elucidate the effect on fibrinogen, the enzyme was incubated with human fibrinogen and the reaction mode was analyzed by SDS-PAGE showing that the fibrinogen bands Aα and Bβ disappeared first, after 1 min, followed by the γ chain band (Fig. 6B). Therefore, all fibrinogen chains, Aα, Bβ and γ, were susceptible to fibrinolytic enzyme cleavage. The results showed that the fibrin chain was completely digested in 30 minutes.

This hydrolytic pattern was similar to that reported for other purified fibrinolytic proteases from Fusarium sp. CPCC 480097 [41], Codium fragile [41] and S. marcescens RSPB11 [14], but it was different from the enzymes of B. subtilis HQS-33 [24] and B. circulans [37] in which the γ chain resisted degradation.

The enzyme of S. marcescens reported here exhibited fibrinogenolytic activity, after rapidly hydrolyzing the Aα, Bβ bands and then the γ band. Thus, this enzyme has fibrinogenase activity. In addition, it appears to be a fibrinolytic agent of direct action, as it acts via fibrin and fibrinogen cleavage and not through plasminogen activators such as streptokinase, urokinase type plasminogen activator (uPA) and tissue plasminogen activator (tPA). Fibrinogen is the precursor of fibrin, which is the main protein component of blood clots. Fibrinogen, when hydrolyzed by thrombin, loses fibrinopeptides A and B and becomes fibrin. Thus, it is the final molecule in the coagulation cascade before fibrin deposition, being involved in primary hemostasis, platelet aggregation and as a major determinant of plasma viscosity [40]. A fibrinolytic enzyme of direct action has an advantage over plasminogen activators used clinically, since side effects, such as platelet activation, related to plasmin formation, can be avoided [38]. A reduction in the level of fibrinogen decreases the incidence of thrombosis. Therefore, this protease could be a candidate for use in thrombolytic therapy and in preventing the formation of blood clots.

3.8 HEMOLYSIS

The effects of the crude extract and the enzyme obtained after precipitation with ammonium sulfate, on blood were studied to determine whether the enzyme activity resulted in hemolysis. As shown in Fig. 7, only the crude extract caused hemolysis in blood agar plates, similar to the study by Huang et al [24], in which the fibrinolytic enzyme of B. subtilis did not cause hemolysis in vitro, being considered as a potential thrombolytic agent for safe therapy. Hemorrhage is the most common complication due to thrombolytic agents, and can cause serious adverse effects, so the use of fibrinolytic
agents associated with a lower risk of bleeding is strongly recommended for improved patient survival [42].

4 CONCLUSIONS

In this study, we identified a potent fibrinolytic enzyme from *S. marcescens* (CBAM 519) isolated in the Brazilian Amazon region. *S. marcescens* (CBAM 519) showed rapid and high production of this fibrinolytic enzyme. Based on the biochemical properties analyzed, the enzyme demonstrated favorable characteristics for biopharmaceutical use, since its activity remained stable at physiological pH and temperature, and it lacked hemolytic action. The enzyme was classified as a serine metalloprotease of approximately 56 kDa. Fibrinolysis was promoted only by the direct route, without activating plasminogen, with a fast and potent fibrinolytic action. It can thus be considered a plasmin-like fibrinolytic enzyme. All these characteristics combined point to the conclusion that the fibrinolytic protease of *S. marcescens* (CBAM 519) shows potential as a therapeutic agent in the treatment and prevention of thrombosis. Cloning and expression of this enzyme in recombinant form is ongoing. Further studies should be directed to analyze the effects of its thrombolytic activity *in vivo*. In addition, this study demonstrates the great biotechnological potential present in samples from the Amazonian biodiversity, with priceless value for research and development of alternative biomolecules with therapeutic action.

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REFERENCES


ANNEXES

Figures and tables

Figure 1. Temporal profile of protease secretion. A. Growth (•) and proteolytic activity (-) of *S. marcescens* (CBAM 519) in Manachini’s solution. B. SDS-PAGE of culture filtrates recovered at time 0 and after 2h, 4h, 6h, 8h and 24h growth, as indicated. Molecular weight markers indicated to the left, in kDa (BioRad, Broad Range). c. Fibrin zymography of the culture filtrates recovered after 4h, 6h, 8h and 24h of growth. Proteins precipitated from 0.8 mL of supernatant were applied to each lane in gels b and c. Data points in panel A represent average from 2 replicates.
Figure 2. Precipitation of the *S. marcescens* CBAM 519 enzyme. 12% SDS-PAGE stained with CBB-R250 showing fractions obtained after precipitation with ammonium sulphate at 20, 40, 60 and 80% saturation (panel A, lines 1-4, respectively) and corresponding fibrin zymography (panel B). Molecular weight markers indicated to the left in kDa (M).

Figure 3. Effect of pH on the enzymatic activity of *S. marcescens* (CBAM 519). (A) Enzymatic activity in the pH range 3 to 9, expressed as % of maximum activity. (B): stability of the enzymatic activity over a 24h period at room temperature at the indicated pH, expressed as % of maximum activity at the end of the period. Buffers used were citrate buffer (pH 4-6), Tris-HCl buffer (pH 7-8) and sodium carbonate-bicarbonate buffer (pH 9.0). All buffer concentrations were 0.1 M. Assays were performed at 25°C. Data represent average and SD from three independent experiments (n=3).
Figure 4. Effect of temperature on the enzymatic activity of *S. marcescens* (CBAM 519). (A) optimal temperature: the maximum activity shown by the enzyme was set as 100% of the relative activity. (B) enzyme stability: the effect of temperature on enzyme stability was measured after 1h of incubation at different temperatures and expressed as percentage of residual activity compared to that obtained at 37 °C. The tests were performed at the optimum pH (9.0) determined for the enzyme. Data represent average and SD from three independent experiments (n=3).

![Figure 4](image)

Figure 5. Effect of inhibitors on the fibrinolytic activity. The level of inhibition was expressed as a percentage of the remaining activity compared to activity before addition of inhibitor (control). Data represent mean ± SD from three independent experiments (n=3).

![Figure 5](image)
Figure 6. Analysis of plasminogen activation by the fibrinolytic enzyme on plasminogen-free (A) and plasminogen-rich (B) fibrin plates. C. Fibrinogenolytic activity of the enzyme analyzed by SDS-PAGE at the incubation times (1 – 120 min) indicated above lanes. C: Fibrinogen control (before enzyme addition); E: protease (20ug), M: protein MW marker (kDa).

Figure 7. In vitro hemolysis assay. (A) 500 ug of S. marcescens crude extract and (B) 500 ug of enzyme were added to slots on blood agar plates and incubated at 37 °C for 3d.
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<sup>a</sup>Data represents mean ± SD (n=3).