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In vitro evaluation of the fibrinolytic and toxicity profile of a protease from Streptomyces parvulus DPUA 1573 isolated from amazonian lichens

Avaliação *in vitro* do perfil fibrinolítico e de toxicidade de uma protease de *Streptomyces parvulus* DPUA 1573 isolada de líquens amazônicos

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ABSTRACT

Introduction: *Thrombosis is one of the main causes of cardiovascular diseases, with blood clots being responsible for events such as acute myocardial infarction and stroke. Fibrinolytic enzymes, particularly those of microbial origin, have shown promise due to their effectiveness in degrading fibrin and their feasibility for large-scale production. Among them, the strain Streptomyces parvulus DPUA 1573, isolated from lichens in the Brazilian Amazon, demonstrated high fibrinolytic potential, drawing interest as a potential antithrombotic agent.* **Objective:** *To obtain and characterise a fibrinolytic enzyme from Streptomyces parvulus DPUA 1573 with potential use as an antithrombotic agent. Methods: The enzyme was produced by submerged fermentation, purified using an aqueous two-phase system, and evaluated through in vitro assays for thrombolytic, fibrinogenolytic, amidolytic, anticoagulant, cytotoxic, and hemolytic activities using human plasma, tumour cells, and erythrocytes.* **Results:** *The enzyme showed preferential partitioning of protease to the PEG-rich top phase. The crude extract and the pre-purified fibrinolytic protease exhibited thrombolytic degradation of 41.47% and 52.98%, respectively. Additionally, the protease displayed amidolytic activity through degradation of the Aα, Bβ, and γ chains of fibrinogen, classifying it as a chymotrypsin-like serine protease. In vitro anticoagulant assays showed a slight prolongation of the prothrombin time and partial thromboplastin time, with no effect on the thrombin time. The enzyme also showed no hemolytic activity in the blood biocompatibility assay. Cytotoxicity testing against MDA-MB-231 and J774.A1 cells demonstrated 80% cell viability.* **Conclusion:** *These findings suggest that the fibrinolytic enzyme from Streptomyces parvulus DPUA 1573 holds potential for the development of a novel antithrombotic drug candidate.*
Keywords*: Proteases; Thrombolytic Therapy; Anticoagulants; Lichens; Biodiversity.*

RESUMO

Introdução: A trombose é uma das principais causas de doenças cardiovasculares, sendo os coágulos sanguíneos responsáveis por eventos como infarto agudo do miocárdio e acidente vascular cerebral. Enzimas fibrinolíticas, particularmente aquelas de origem microbiana, têm se mostrado promissoras devido à sua eficácia na degradação da fibrina e à viabilidade para produção em larga escala. Dentre elas, a cepa *Streptomyces parvulus* DPUA 1573, isolada de líquens da Amazônia brasileira, demonstrou alto potencial fibrinolítico, despertando interesse como potencial agente antitrombótico. **Objetivo:** Obter e caracterizar uma enzima fibrinolítica de *Streptomyces parvulus* DPUA 1573 com potencial uso como agente antitrombótico. **Metodologia:** A enzima foi produzida por fermentação submersa, purificada usando um sistema de duas fases aquosas e avaliada por meio de ensaios *in vitro* para atividades trombolítica, fibrinogenolítica, amidolítica, anticoagulante, citotóxica e hemolítica usando plasma humano, células tumorais e eritrócitos. **Resultados:** A enzima mostrou partição de protease preferencial para a fase superior rica em PEG. O extrato bruto e a protease fibrinolítica pré-purificada exibiram uma degradação trombolítica de 41,47% e 52,98%, respectivamente. Além disso, a protease exibiu atividade amidolítica pela degradação das cadeias Aα, Bβ e γ do fibrinogênio, sendo considerada uma serina protease semelhante à quimotripsina. Em relação à atividade anticoagulante in vitro, a enzima prolongou ligeiramente os tempos de protrombina, ativou parcialmente os tempos de protrombina e sem afetar o tempo de trombina. Além disso, a enzima não apresentou atividade hemolítica no ensaio de biocompatibilidade sanguínea. A atividade citotóxica contra células MDA-MB-231 e J774.A1 demonstrou 80% de viabilidade celular. **Conclusão:** Esses resultados podem indicar que a enzima fibrinolítica de *Streptomyces parvulus* DPUA 1573 tem potencial para o desenvolvimento de um novo candidato a fármaco antitrombótico.
Palavras chaves: Proteases; Terapia Trombolítica; Anticoagulantes; Líquens; Biodiversidade.

INTRODUCTION

Thrombosis is one of the main causes of cardiovascular diseases (CVDs), including acute myocardial infarction, ischemic heart disease, peripheral vascular disease and stroke[1]. Blood clot formation blocking the artery is the leading cause

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of CVDs, so the treatments that eliminate these clots have been shown to reduce morbidity and mortality[2].

Fibrinolytic enzymes degrade fibrin in clots through enzymatic and biochemical reactions. These enzymes can be obtained from several sources, including plants, animals and microorganisms. However, many of these agents could present certain limitations, such as high cost, short half-life, immunogenicity, and the possibility of causing hemorrhagic events[3,4]. Consequently, there is an urgent need to explore new fibrinolytic agents with better specificity and efficacy[5]. Currently, fibrinolytic enzymes from microbial sources play a key role in the management of CVDs, presenting the advantages of being easily produced on a large scale with a short production time and lower cost on a large scale with a short production time and lower cost [6].

In this context, species of the genus Streptomyces stand out as potential sources of new medicinal compounds. The Streptomyces parvulus DPUA 1573 strain, extracted from lichens in the Brazilian Amazon, is already known to produce enzymes with excellent fibrinolytic activity[7,8]. Recently, our research group carried out purification and characterisation processes by using a polyethene glycol-phosphate aqueous two-phase system (ATPS) of fibrinolytic enzymes from Streptomyces parvulus DPUA 1573. The protease activity was enhanced by iron ion[9].

In a continuous effort to develop a new antithrombotic drug, this work describes the in vitro biological profile of the enzyme obtained from Streptomyces parvulus DPUA 1573, isolated from Brazilian Amazon lichens. In this way, we describe the cytotoxicity activity against human mammary gland adenocarcinoma cells, as well as thrombolytic degradation, fibrinogenolytic, fibrin zymogram, and amylolytic activities, and the anticoagulant effect.

MATERIALS AND METHODS

Production, extraction and purification of fibrinolytic protease

Streptomyces parvulus DPUA 1573 was maintained in Hickey-Tresner (HT) medium at 30°C for 48 hours. For the inoculum preparation, the production of the fibrinolytic enzyme by submerged fermentation and the enzyme extraction were performed as described Alencar et al.[9]. The fibrinolytic protease produced in MS-2 medium modified with passion fruit flour at 0.5% (w/w), was initially centrifuged at 4,000 x g for 10 minutes at 20ºC and the supernatant loaded into an ATPS – Polyethylene glycol (PEG)/ Phosphate in the composition of 17.5% (w/w) PEG 8000 g/mol^{-1}, 15% Phosphate (w/w) and pH 8. Then, a PEG-rich phase, recovered from the ATPS, which contains the fibrinolytic protease, was used to perform the subsequent tests.

Fibrinogen obtention

For fibrin clot degradation and fibrinogenolytic activity, fibrinogen was obtained from cryoprecipitate directly from blood plasma. The plasma bag was provided by the Pernambuco Haematology and Hemotherapy Foundation (HEMOPE), and the Research Ethics Committees approved the partnership between UFPE and HEMOPE (Process No. 1,727,579).

In vitro thrombolytic degradation

The test was performed following the methodology of Couto et al.[10]. In glass tubes, 1 mL of human plasma and 200µL of thrombin solution (20 U/mL) (Sigma, St. Louis, MO, USA) were added, being incubated at 37ºC for 10 min; after this time, each fibrin thrombus formed was weighed on an analytical balance. In each glass tube containing the already weighed thrombus, 200 µL of pre-purified fibrinolytic protease (406.8 mg/mL) or the crude extract was added and incubated again at 37 °C for 60 min. The percentage of degradation was calculated according to Da Silva et al.[11]. The entire experiment was performed in triplicate, and the control was performed with 200 µL of 0.15 M saline solution.

Determination of fibrinogenolytic activity

The fibrinogenolytic activity was determined according to Park et al.[12]. The reaction mixtures, one consisting of 100µg of cryoprecipitate (which contains human fibrinogen), were incubated at 37ºC with the addition of 100 µL of pre-purified enzyme (at what concentration), then incubated at 37ºC in 200 µL mM Tris-HCl buffer (pH 7.5). 20 µL of this reagent mixture was withdrawn after 0, 15, 30, 60, and 120 min. The addition of 4 µL of SDS-PAGE sample denaturing buffer stopped the reaction. The resulting products were analysed by 12% SDS-PAGE as described by Chang et al.[13].

Fibrin zymogram

The fibrin zymogram was performed according to Kim et al.[14] with some modifications. Fibrinogen (1.2 mg/ml) (Sigma, St. Louis, MO, USA) and thrombin (1 U/ml) (Sigma, St. Louis, MO, USA) were mixed with 12% polyacrylamide gel solution, and 2 mg of pre-purified enzyme was electrophoresed on the fibrin gel. After electrophoresis, the gel was washed in Triton X-100 2.5% (v/v) for 1 hour, then washed three times with distilled water and incubated in reaction buffer (10 mM Tris-HCl pH 7.5) in a water bath at 37°C for 16 hours. For staining, Coomassie blue was used, similar to the SDS-PAGE staining and discolouration protocol[15].

Determination of amidolytic activity

Amidolytic activity was measured spectrophotometrically using Nα-benzoyl-DL-arginine 4-nitroanilide hydrochloride, N-succinyl-Gly–Gly–Phe-p-nitroanilide and N-succinyl-L-phenylalanine-p-nitroanilide as chromogenic substrates. The reaction mixture (200 µL) containing 30µL of enzyme solution, 30µL of a chromogenic substrate and 140 µL of 20 mM Tris–HCl, pH 7.4, was incubated for 30 min at 37ºC, and the amount of p-nitroaniline was determined by UV-Vis spectrophotometry at 405 nm. A unit of

amidolytic activity (AU) was expressed as the number of micromoles of substrate hydrolysed by the enzyme per minute and per mL, according to Kim et al.[16].

In Vitro Anticoagulant Effect

Prothrombin Time (PT) and Activated Partial Thromboplastin Time (aPTT) were performed using a semiautomatic coagulometer (Maxcoag, Urit Medical Electronic Co., China) and reagents from BIOS Diagnóstica (Sorocaba, Brazil), according to the manufacturer's instructions. A pool of plasma from five healthy volunteers was used as a control for the tests. Activities performed with blood are in accordance with the ethics committee (CAAE: 63547722.6.0000.5208). The controls for the APTT and PT assays were performed with 25 µL of saline solution (NaCl, 0.9%) and 100 µL of the plasma pool.

To evaluate the anticoagulant effect, pre-purified fibrinolytic protease was used at concentrations of 2.5 mg/mL, 5 mg/mL, and 10 mg/mL, with the samples left in contact with the plasma pool for 45 minutes. Thrombin time (TT) evaluation was performed according to the methodology of Wang et al.[17] with some modifications. First, 50µL of fibrinolytic protease (1 mg/mL) were placed in contact with 100 µL of thrombin (20 U/mL) for 45 min, after this period, the solution was transferred to eppendorfs (2 mL) containing a mixture in 400µL of 150mM Tris-HCl-NaCl pH 7.75, 100µL of 245mM phosphate buffer pH 7.0 and 0.72% bovine fibrinogen, being placed in a water bath at 37ºC until the formation of a blood clot is observed. Fibrin.

Cytotoxicity Assay

Human mammary gland adenocarcinoma cells (MDA-MB-231) obtained from the Rio de Janeiro Cell Bank (BCRJ) were used. This strain was maintained in Leibovitz's L-15 and F-12 culture medium (in a 50:50 proportion of each medium) with two mM L-glutamine, without sodium bicarbonate, and fetal bovine serum at a final concentration of 10%, in the absence of CO_2. The J774.A1 strain, also obtained from the Rio de Janeiro Cell Bank (BCRJ), was maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Thermo Fisher Scientific, Massachusetts, USA) supplemented with 10% FBS and 5% CO_2. Cells were counted in a Neubauer chamber to quantify cell density. An MTT (3-4,5-dimethyl-thiazol-2-yl-2,5-diphenyltetrazolium bromide) assay was used to determine the cell viability of the enzyme following the methodology of Mosmann[18]. MDA-MB-231 cells were inoculated in 96-well plates at a density of $1x10^4$ cells/mL, and J774.A1 cells were incubated at a density of $1x10^6$ cells/mL. After 24 hours of incubation, the fibrinolytic enzyme was exposed to final concentrations of 300, 150, 75, 17.5, and 8.75 mg/mL for 24 hours. The enzyme was solubilised in supplemented culture medium. After the treatment period, 25 µL of MTT solution (5 mg/mL) was added, and the plates were incubated for 3 hours. After incubation, the supernatant was removed, and 100 µL of DMSO (Dimethyl Sulfoxide) was added. Absorbance was measured using a microplate reader (BioteK Elx808) at an absorbance wavelength of 630 nm. Cytotoxicity was expressed as cell viability, according to Santos et al.[19]:

$$\frac{\text{Absorbance in the supernatant of the treated cell population}}{\text{Absorbance in the supernatant of the untreated cell population}} X\ 100$$

Hemolytic activity

The fibrinolytic enzyme blood compatibility assay was performed according to Rajendran et al.[20]. To carry out the test, blood from a single healthy volunteer was used. Activities performed with blood are in accordance with the ethics committee (CAAE: 63547722.6.0000.5208). Two millilitres of whole blood were added to 4 millilitres of PBS and centrifuged at 5000 rpm for 5 minutes to isolate red blood cells (RBCs). Red blood cells were washed twice with 10 mL of PBS and then diluted in 20 mL of PBS. Of the final suspension, 0.4 mL was diluted in 1.6 mL of fibrinolytic enzyme at a final concentration of 2.5, 5 and 10 mg/ml. Control groups were divided into three groups: PBS (negative group), distilled water, and Triton-X (positive group). The tubes were kept at 37 °C for 1 hour and then centrifuged for 5 min at 10,000 rpm at 20°C, and the absorbance was measured at 540 nm. The following formula expresses the hemolytic degree:

$$\frac{\text{Absorbance "test" – Absorbance negative}}{\text{Absorbance positive – Absorbance negative}} X\ 100$$

RESULTS

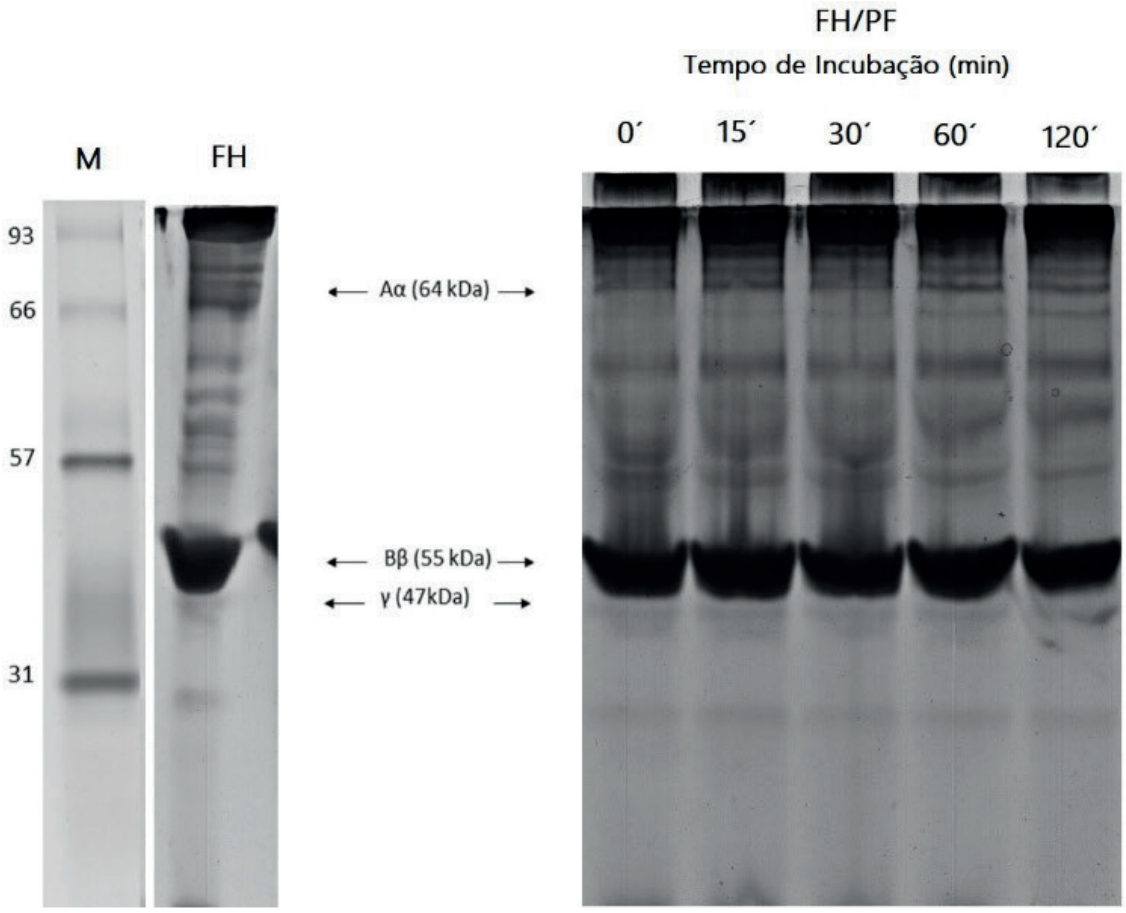
In vitro thrombolytic degradation

After 60 minutes of incubation of the fibrin thrombus with either the crude extract or the ATPS-pre-purified fibrinolytic protease, it was possible to observe the reduction in volume and weight of the fibrin clot. The crude extract and the pre-purified fibrinolytic protease induced thrombus degradation by 41.47% and 52.98%, respectively.

Determination of fibrinogenolytic activity and fibrin zymogram

The fibrinogen degradation products were analysed by SDS-PAGE to elucidate the fibrinogenolytic effect promoted by the fibrinolytic protease from Streptomyces parvulus (FPsp). Cleavage of the Aα chains was detectable within 15 minutes. The γ chains began degrading at 60 minutes, with complete cleavage achieved by 120 minutes (Figure 1). Besides, at 120 minutes, cleavage of the Bβ chains was initiated.

Figure 1 – *Fibrinogenolytic activity of the fibrinolytic protease obtained from Streptomyces parvulus DPUA 1573 on 12% SDS-PAGE. (M) Molecular weight markers; (FH) Control human fibrinogen chains; (FH/PF) FH chains under the action of fibrinolytic protease for 0, 15, 30, 60 and 120 min of incubation.*



A fibrin zymogram was performed to verify the fibrinolytic activity of the protease. The fibrinolytic enzyme appeared as a lighter area in the zymography gel (Figure 2), allowing for the observation of its fibrinolytic activity.

Figure 2 – Fibrin zymogram of the enzyme pre-purified by ATPS.

Amidolytic activity

The amidolytic activity of the pre-purified fibrinolytic enzyme was evaluated using three different types of chromogenic substrates (Table 1).

Table 1 – *Amidolytic activity of the fibrinolytic enzyme from Streptomyces parvulus DPUA 1573 using typical chromogenic substrates with 30 minutes of incubation.*

Chromogenic substrates	***Amidolytic activity (U/mg)***
N-succinil-Gly– Gly–Phe-p-nitroanilida	*1.41*
N -succinil-L-fenilalanina-p-nitroanilida	*0.02*
Nα-benzoil-DL-arginina 4-nitroanilida	*0.00*

U/mg – Unit per milligram

The enzyme demonstrated highest specificity for the chromogenic substrate S-1899 (N-p-tosyl-Gly-Pro-Lys-p-nitroanilide), with a value of 1.41 U/mg, followed by the chromogenic substrate S-2628 (N-succinyl-L-phenylalanine-p-nitroanilide) with an affinity of 0.02 U/mg. No affinity was observed for the chromogenic substrate B-4875 (Nα-benzoyl-DL-arginine 4-nitroanilide), and therefore, the fibrinolytic protease is a serine protease similar to chymotrypsin.

In Vitro Anticoagulant Effect

The anticoagulant activity of FPsp DPUA 1573 in the intrinsic, extrinsic, and common coagulation pathways was investigated using the PT, APTT, and TT assays. As shown in Table 2, there was a weak prolongation of APTT when compared to the control group, with the prolongation increasing with increasing enzyme concentration. Regarding the PT, the prolongation of time was also weak. There was no influence of the enzyme on TT, as clots were formed in less than 1 minute.

Table 2 – *Effect of the fibrinolytic enzyme from Streptomyces parvulus DPUA 1573 on coagulation parameters.*

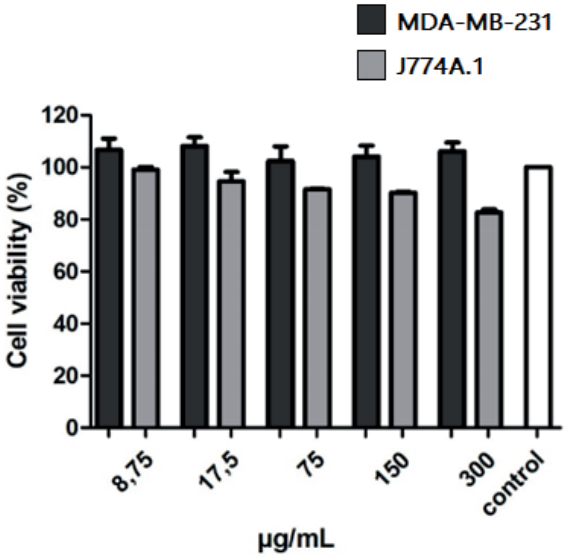
	Enzyme (mg/ml)			
	Control	2,5	5,0	10
APTT	25,5 ± 2,19	26,4 ± 1,55	29,2 ± 0,70	30,7 ± 4,17
PT	12,7 ± 0,14	12,7 ± 0,07	13,5 ± 0,21	13,15 ± 0,07

FPsp modestly prolonged clotting times in the intrinsic (APTT), extrinsic (PT), and common coagulation pathways, suggesting broad-spectrum anticoagulant activity, but does not affect the final stage of coagulation, which is the conversion of fibrinogen into fibrous protein (TT).

Cytotoxicity assay

From the results, it was observed that the FPsp showed no toxicity profile once the cell viability was greater than 80% within 24 hours for both J774.A1 and MDA-MB-231 cells (Figure 3).

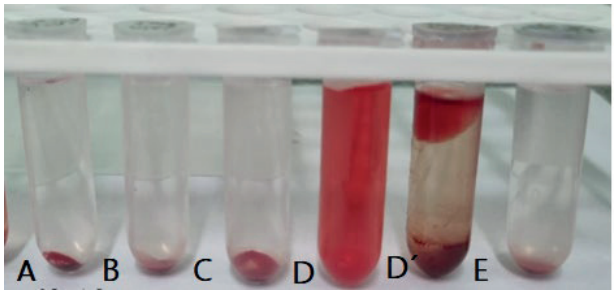
Figure 3 – *Cellular viability of the enzyme in MDA-MB-231 and J774.A1. Statistical differences with control were determined by ANOVA followed by Bonferroni, *P < 0.05 vs Control. Each value determines the mean ± SD of three independent experiments.*



Hemolytic activity

It was possible to verify that the FPsp showed no hemolytic activity at concentrations of 2.5, 5, and 10 mg/mL. At these concentrations, the FPsp promoted 1.05%, 1.16% and 1.27% hemolysis, respectively. The hemolytic profile can be seen in Figure 4.

Figure 4 – *Hemolytic activity of fibrinolytic enzyme produced by Streptomyces parvulus DPUA 1573. (A) Fibrinolytic enzyme 2.5mg/ml; (B) fibrinolytic 5mg/ml; (C) fibrinolytic 10 mg/ml; (D) Positive control – Distilled water; (D´) Positive control – Triton-X; (E) Negative control.*



DISCUSSION

Several studies have confirmed the strong thrombolytic potential of microbial fibrinolytic enzymes. Notably, enzymes obtained from Paenibacillus graminis[10] and Bacillus amyloliquefaciens[21], pre-purified using an ATPS, demonstrated a significant clot degradation capacity, underscoring the therapeutic promise of these biocatalysts. These results highlight not only the relevance of microbial sources in thrombolytic therapy but also the critical role of purification strategies in enhancing enzymatic performance. Together, these findings support the continued exploration of microbial enzymes as effective and innovative alternatives for treating thrombotic disorders.

Fibrinogen is a hexameric glycoprotein and the soluble precursor of fibrin, with a molecular mass of approximately 340 kDa. It is composed of two sets of three polypeptide chains: Aα (63 kDa), Bβ (56 kDa), and γ (47 kDa), linked by disulfide bonds[22]. Based on the observed degradation pattern, the isolated protease can be classified as an αβγ-fibrinogenase, given its ability to cleave all three fibrinogen chains, similarly to the fibrinolytic enzyme obtained from Eupolyphaga sinensis[23]. In contrast, its activity profile differs from that of other known fibrinolytic enzymes, such as the one from Neurospora sitophila, which hydrolyses all three chains indiscriminately[24], and from the enzyme produced by Bacillus subtilis C142, which cleaves only the Aα and Bβ chains, leaving the γ chain intact[25]. It also diverges from the fibrinolytic enzyme from Mucor subtilissimus UCP 1262, which exclusively targets the γ chain[26]. These distinctions highlight the specificity of the studied protease and its potential for targeted fibrinogenolysis.

The ability of a fibrinolytic enzyme to cleave all three fibrin chains, α, β, and γ, appears to be a critical factor in its thrombolytic efficacy. Enzymes capable of degrading the complete fibrin network are generally more efficient in promoting clot dissolution than those with limited specificity for one or two chains[27]. This broader proteolytic

activity facilitates a more effective breakdown of the fibrin mesh, leading to enhanced clot lysis. Therefore, the observed cleavage of all three chains in this study supports the high thrombolytic potential of the enzyme investigated, reinforcing the importance of substrate specificity as a determinant of therapeutic performance.

Several fibrinolytic enzymes exhibiting chymotrypsin-like amidolytic activity have been previously reported from various biological sources, including Mucor subtilissimus[27], Neurospora sitophila[23], Petasites japonicus[28], and Streptomyces sp. CC5[29]. These findings underscore the biochemical diversity among fibrinolytic proteases and reinforce the relevance of exploring novel sources, as differences in catalytic mechanisms and substrate specificity can significantly impact their therapeutic or industrial applicability.

When evaluating fibrinolytic enzymes, it is crucial to assess not only their effect on fibrin degradation but also their potential influence on the enzymes involved in the blood coagulation cascade. The aPTT test measures the activity of the intrinsic and common coagulation pathways, while the PT assesses the extrinsic and common pathways. Additionally, TT reflects the duration required for the conversion of fibrinogen into fibrin strands, a key step in clot formation[30]. These tests help determine whether the enzyme affects the coagulation cascade beyond fibrinolysis, which is critical for understanding its broader impact on hemostasis.

The results obtained in this study are consistent with those reported by Miranda et al.[26] and Zhou et al.[31], who observed similar effects with fibrinolytic enzymes derived from Mucor subtilissimus UCP 1262 and Bacillus velezensis Z01, respectively. In contrast, the findings differ from those of Zhibin Sun et al.[29] and Choi et al.[25], who demonstrated a more pronounced anticoagulant effect with fibrinolytic enzymes obtained from Streptomyces sp. CC5 and Bacillus subtilis C142, respectively. While the observed low or absent anticoagulant effect may seem less significant, it could potentially offer a protective advantage, reducing the risk of adverse hemorrhagic complications associated with more potent anticoagulants.

The absence of significant changes in PT and aPTT after enzymatic treatment suggests that the fibrinolytic agent does not interfere with the serine proteases involved in the coagulation cascade. These results corroborate the substrate specificity of the enzyme, indicating its preferential activity towards fibrin rather than coagulation factors. This characteristic is critical for the therapeutic application of fibrinolytic agents, as nonspecific proteolytic activity can lead to systemic hemorrhagic complications. Similar findings have been reported for fibrinolytic enzymes from Mucor subtilissimus and other microbial sources, which did not significantly prolong PT or aPTT, reinforcing their safety profile in terms of hemostatic balance[26,32]. Furthermore, the preservation of TT in such studies further supports the notion that these enzymes do not interfere with the conversion of fibrinogen to fibrin, an essential step in clot formation. Overall, the unchanged PT/aPTT values observed in this study reinforce the potential of the enzyme as a safe thrombolytic agent with minimal risk of disrupting physiological clotting processes.

The MDA-MB-231 and J774.A1 cell lines are widely used to assess the cytotoxicity profiles of potential drug candidates. These cell lines provide preliminary insights into potential toxicity, particularly concerning the kidneys and immune system, even before in vivo testing is conducted[33]. The results obtained in this study align with those of da Silva et al.[33] and Yeon et al.[34], who found that fibrinolytic proteases produced by Mucor subtilissimus UCP 1262 and Lumbrineris nipponica, respectively, did not induce toxicity in the tested cell lines, which included renal embryonic cells (HEK-293), macrophages (J774.A1), peripheral blood mononuclear cells (PBMCs), Sarcoma (S-180), and human cerebral micro-vessel endothelial cells (hCMEC/D3)[33,34].

The selection of the MDA-MB-231 (human breast carcinoma cells) and J774.A1 (murine macrophages) cell lines was based on their widespread use in cytotoxicity and inflammation models, respectively. MDA-MB-231 cells are frequently used in studies of tumour toxicity and cell proliferation due to their high mitotic rate and relative resistance, while J774.A1 cells provide a relevant model for assessing the immunomodulatory or pro-inflammatory effects of bioactive compounds. However, it is recognised that in vitro results cannot be directly extrapolated to clinical effects in humans due to the complexity of systemic metabolism, bioavailability, and interaction with the immune system in living organisms. Therefore, further studies using animal models are necessary to validate the safety and efficacy of the investigated protease under more physiologically representative conditions.

Regarding the molecular mechanism underlying the observed fibrinolytic activity, the absence of hemolytic activity combined with the ability to degrade fibrin suggests a targeted specificity of the protease. Previous studies on fibrinolytic enzymes of similar origin indicate the presence of catalytic residues typical of serine or metalloproteases, such as histidine, aspartate, and serine in the active sites, as well as structural domains that selectively recognise fibrin. Although detailed structural characterisation of the fibrinolytic protease was not performed in this study, future approaches involving amino acid sequencing, molecular modelling, and site-directed mutagenesis may elucidate the structural and catalytic determinants responsible for the enzyme's specificity. Identifying these elements is essential for the rational development of the enzyme as a potential thrombolytic agent.

Da Costa e Silva et al.[34] demonstrated that the partially purified enzyme from Chlorella vulgaris exhibited minimal hemolytic activity, with less than 4% erythrocyte lysis. Similarly, da Silva et al.[33] found that the purified

enzyme from Mucor subtilissimus showed slight hemolytic activity at a concentration of 10 mg/mL, resulting in only 2.8% hemolysis, with no significant hemolysis observed at lower concentrations.

It is important to consider that this low activity may be associated with reduced in vivo potency, requiring higher concentrations to achieve significant therapeutic effects. This need for higher doses may, in turn, pose pharmacokinetic challenges, such as increased systemic protein load or heightened immunogenicity. The balance between safety and efficacy is a critical consideration in the development of therapeutic agents derived from toxins or bioactive proteins, as enzymes with low toxicity do not always guarantee high pharmacological effectiveness. Thus, although it exhibits a promising safety profile, further studies are necessary to assess its therapeutic window and efficacy in vivo models under more challenging physiological conditions.

CONCLUSION

The enzyme produced by Streptomyces parvulus DPUA 1573, pre-purified by ATPS, promoted 52.98% thrombus degradation, indicating fibrinolytic activity. Also, it was possible to ascertain that the enzyme discretely prolongs the intrinsic, extrinsic and common coagulation pathways, but does not affect the final stage of coagulation. After the fibrinogenolytic activity, the isolated protease can be classified as αβγ-fibrinogenase. In the amidolytic activity, the fibrinolytic protease was a serine protease like chymotrypsin. Concerning the cytotoxicity profile, the enzyme showed no toxicity against MDA-MB-231 and J774.A1 cell lines, nor did it exhibit a hemolytic effect on human red blood cells. Altogether, these results suggest that the enzyme produced by Streptomyces parvulus DPUA 1573 and pre-purified by ATPS could act as a thrombolytic drug candidate.

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