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MICROBIOLOGY

Production and biochemical and biophysical characterization of fibrinolytic protease of a *Mucor subtilissimus* strain isolated from the caatinga biome

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Abstract: Cardiovascular diseases, resulting from the deposition of clots in blood vessels, are the leading cause of death worldwide. Fibrinolytic enzymatic activity can catalyze blood clot degradation. Findings show that 36 fungal isolates recovered from Caatinga soils have the potential to produce fibrinolytic protease under submerged conditions. About 58 % of the isolates displayed fibrinolytic activity above 100 U/mL, with *Mucor subtilissimus* UCP 1262 being the most active. The protease was biochemically and biophysically characterized, showing that the enzyme had a high affinity for *SAApNA* substrate and was significantly inhibited by fluoride methyl phenyl sulfonyl-$C_7H_7FO_2S$, suggesting that it is a chymotrypsin-like serine protease. The highest enzyme activity was detected at pH 5.0 and 28 °C. This fibrinolytic protease's far-UV circular dichroism (CD) showed that its secondary structure was primarily α-helical. The purified fibrinolytic enzyme may represent a novel therapeutic agent for treating thrombosis. At temperatures above 65 °C, the enzyme lost all its secondary structure. Its melting temperature was 58.1 °C, the denaturation enthalpy 85.1 kcal/mol, and the denaturation entropy 0.26 kcal/K·mol.

Key words: circular dichroism, fibrinolysis, fibrinolytic enzyme, *Mucor,* protease, submerged fermentation.

INTRODUCTION

Fibrin is the main protein constituent of blood clots and thrombus and is formed from fibrinogen by thrombin action (Deng et al. 2018, Katrolia et al. 2020, Sharma et al. 2021). The insoluble fibrin fibers are hydrolyzed into fibrin degradation products by plasmin, generated from plasminogen activators, such as tissue-type plasminogen activators (tPA). Some proteins can also hydrolyze fibrin (Liu et al. 2016). Hemostasis is a complex process achieved through an optimal balance between bleeding and blood clot formation, but fibrin clots may not be lysed in an unbalanced state, resulting in thrombosis (Deng et al. 2018, LaPelusa & Dave 2023).

Most of the fibrinolytic agents currently available for the treatment of thrombosis are plasminogen activators such as tPA and urokinase-type plasminogen activators, and all these agents exhibit adverse side effects (Deng et al. 2018, Vijayaraghavan et al. 2019). Cardiovascular disorder is a wide-reaching primary cause of morbidity and mortality, and most of the fibrinolytic agents available for

clinical treatment are barely satisfactory. Therefore, the search for fibrinolytic enzymes from diverse sources as new therapeutic agents for treating thrombotic disorders is needed, as these enzymes are seen as a great approach to therapeutic thrombolysis (Patel et al. 2012, Chandramohan et al. 2019, Liu et al. 2016).

Several microbial serine proteases possessing fibrinolytic activity have been considered treatments for thrombosis, and fibrinolytic enzymes have been characterized to improve superior thrombolytic drugs (Deng et al. 2018, Mukherjee et al. 2012, Moula & Bavisetty 2020). Microbial enzyme production requires investigating the parameters affecting enzyme yield, optimizing the production parameters, and using effective downstream processing techniques. Microbial fibrinolytic enzymes have received attention for their potential medicinal use for thrombotic disease (Deng et al. 2018, Moula & Bavisetty 2020, Bi et al. 2013), with a growing interest in fungal enzymes (Raina et al. 2022).

Numerous nutrients are required during cell growth and enzyme production, and some low-cost nutrient-rich feedstocks, such as soybean flour, can be a great alternative to synthetic nutrient sources (Silva et al. 2015). The implementation of screening methods is needed to find the crucial components and get their corresponding proportions. Traditional optimization methods, such as the single-factor method, would significantly increase the workload, and they typically could not be finished under laboratory conditions. Factorial design is an experimental method used to accurately estimate the primary influence of various factors with less experimental time (Porto et al. 2007). It is a significant approach to enhancing fibrinolytic enzyme production (Sharma et al. 2021).

In this work, fungal strains from the Caatinga soil produced a fibrinolytic enzyme, and the highest-producing strain was selected. The best medium conditions for production were also studied. The produced fibrinolytic enzyme was biochemically and biophysically characterized, which allowed the discovery of its optimum temperature and pH and its behavior in the presence of different metal ions and potential inhibitors. It was also possible to identify the type of protease through substrate specificity, verify the fibrinolytic activity on blood clots, and partially determine the secondary structure of the enzyme through circular dichroism.

MATERIALS AND METHODS

Screening of the fungal strains

The Culture Bank of Microorganisms from the Universidade Católica de Pernambuco (UCP) provided the fungal species (SISGEN AA30B0B) isolated from Caatinga soil (Northeast region of Brazil). These isolates were allowed to grow at 30 °C for seven days and then 10^4 spores/mL were transferred to the MS-2 medium described by Porto et al. (1996). The flasks were incubated in an orbital shaker at 30 °C, pH 7.0, and 120 rpm. After 96 h of fermentation, cultures were centrifuged for 15 min at 10000g and 4 °C. The clear supernatant was used to determine enzymatic activity.

Fermentation for fibrinolytic enzyme production

The medium for fibrinolytic protease production (Porto et al. 1996) was adjusted for the *M. subtilissimus* UCP 1262 needs, following the determination of the best conditions was performed according to a 2^3 factorial design. The independent variables were the nitrogen source: Soybean (S) and Wheat Bran (WB) at different concentrations (%) and Calcium Chloride Concentration (%), whose levels are

described in Table I. The response variables were Fibrinolytic Activity (U/mL), Specific Activity (U/mg), and Protease Activity (U/mL). The influences were evaluated by an analysis of variance (ANOVA) with a 95% significance level. Statistical analysis of the experimental design was performed using the software Statistic 8.0 (Statsoft Inc., USA).

Determination of fibrinolytic activity, protease activity, and total protein

Fibrinolytic activity was measured using a fibrin degradation assay. For this determination, 0.4 mL of 0.072 g fibrinogen/L was placed in a test tube with 0.1 mL of 0.245 M phosphate buffer (pH 7.0) and incubated at 37 °C for 5 min. Then, 0.1 mL of a 20 U/mL thrombin solution was added. The solution was incubated at 37 °C for 10 min, 0.1 mL of diluted (1:10 v/v) clarified culture medium was added, and incubation continued at 37 °C. At 60 min 0.7 mL of 0.2 M TCA (trichloroacetic acid) was added and mixed. The reaction mixture was centrifuged at 15000 g for 10 min. After that, 1 mL of the supernatant was collected, and the absorbance at 275 nm was measured. Each experiment was performed in triplicate, and the average value was then calculated after correcting the corresponding blank. In this assay, 1 U (fibrin degradation unit) of enzyme activity is defined as a 0.01 per minute increase in absorbance at 275 nm of the reaction solution (Wang et al. 2011).

Total extracellular protease was assayed at 25 °C as described by Ginther (1979) in the culture media previously clarified by centrifugation (12000 g). Then, 0.1 g azocasein/L in 0.2M Tris-HCl, pH 7.2, which contained 0.001M $CaC1_2$, was utilized as substrate. One unit of activity was determined as the amount of enzyme that increases the optical density of 0.1 in 1 h at 440 nm.

The protein content was determined by the method described by Bradford (1976) using BSA (bovine serum albumin) as a standard. Each experiment was performed in triplicate.

Biochemical characterization of the enzyme

The biochemical characterization was carried out by measuring the protease activity once the fibrin, the fibrinolytic activity substrate, only formed in physiological conditions. The optimum pH and temperature for fibrinolytic activity in the culture medium, as well as the dependence of this activity on pH, temperature, and the presence of metal ions, enzyme inhibitors, and surfactants, were evaluated. To study the effect of pH on enzyme stability, the crude extract was mixed with different buffers: sodium acetate (pH 3.0 to 5.0), citrate phosphate (pH 5.0 to 7.0), Tris-HCl (pH 7.0 to 8.5), and glycine-NaOH (pH 8.5 to 11.0), and incubated at 37 °C for 60 min. The effect of temperature on protease activity was determined by setting the crude extract at temperatures ranging between 4 °C

Table I. Levels of the independent variables of the complete factorial design 2^3 for the production process of the fibrinolytic protease from *Mucor subtilissimus* UCP 1262.

Variable	Level		
	(-1)	(0)	(+1)
NS	S	S+WB	WB
[NS] (%)	1	2	3
[$CaCl_2$] (%)	0	0.5	1

NS: Nitrogen Source, [NS]: Nitrogen Source Concentration, [$CaCl_2$]: Calcium Chloride Concentration, S: Soybean, WB: Wheat Bran, S+WB: Soybean and Wheat bran.

and 85 °C for 30 min. In both assays, aliquots were withdrawn every 15 min for 60 min, and protease activity in these aliquots was measured.

The total protease activity of the crude extract was also evaluated in the presence of several ions that have been described as inhibitors or activators of protease activity. The crude extract was exposed to the following ions at 5 mM concentrations: zinc sulfate [($ZnSO_4$) .7 H_2O], magnesium sulfate [$MgSO_4$], copper sulfate [$CuSO_4$], ferrous sulfate [$FeSO_4$], cobalt chloride [($CoCl_2$).2H_2O] and incubated at room temperature for 60 min. The ions were dissolved in 0.010 M Tris-HCl, pH 7.75, with 0.150 M NaCl.

The influence of surfactants - tween-20, tween-80, triton X-100, and dodecyl sodium sulfate (SDS) - was studied at surfactant concentrations of 0.5, 0.1, and 1.5 %. The enzyme was incubated with surfactants in 0.010 M Tris-HCl, pH 7.75, with 0.150 M NaCl for 30 min at room temperature before the residual protease activity was measured.

For the evaluation of the influence of inhibitors on enzyme activity, the crude extract was exposed to the following inhibitors at five mM concentrations: PMSF (fluoride methyl phenyl sulfonyl-$C_7H_7FO_2S$), mercuric chloride ($HgCl_2$), 2-mercaptoethanol (2-hydroxy-1-ethanethiol-C_2H_6SO), and EDTA (Ethylenediaminetetraacetic acid-$C_{10}H_{16}N_2O_8$) and was incubated at room temperature for 60 min in 0.010 M Tris-HCl pH 7.75 with 0.150 M NaCl. The residual protease activity was measured.

Substrate specificity

Amidolytic activity was measured spectrophotometrically using the specific substrates: N-Succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide (*SAApNA*) – a chymotrypsin substrate – and Gly-Arg-*p*-nitroanilide dihydrochloride – a urokinase and plasmin substrate. The mixture (0.8 mL) contained 30 µL of crude extract solution, 30 µL chromogenic substrate (0.5 mM), and 140 µL of 0.020 M Tris-HCl (pH 7.4). After incubation for 15 min at 37 °C, the amount of free *p*NA (*p*-nitroaniline) was calculated by spectrophotometric absorption at 405 nm. One unit of amidolytic activity was expressed as micromoles of substrate hydrolyzed per minute and milliliter by the enzyme (Kim et al. 1996).

Circular dichroism (CD)

Circular dichroism spectroscopy evaluates the secondary structure of proteins and peptides (Gopal et al. 2012). The enzyme was purified as described by Sales et al. (2015). Far-UV CD (195–260 nm) spectra of proteins were measured using a JASCO J-815 spectropolarimeter at room temperature. A solution of protein (110 µL, 0.1 mg/mL) was placed into a 0.2 mm pathlength cell, and the CD spectra were acquired with 20 nm/min scan speed at 0.2 nm step size and 1.0 nm bandwidth under constant purging with nitrogen. Three spectra were accumulated and averaged for the sample. The decay curve was fitted by the Boltzmann function using the following decay model:

$$y = A2 + \frac{A1 - A2}{1 + e^{\frac{(X - Xo)}{dX}}} \quad (1)$$

A_1 and A_2 are the fit parameters corresponding to initial and final fluorescence intensity, X_0 is the central point and dX is the time constant. The Boltzmann equation was also used to calculate the melting temperature (T_m) of the enzyme through a derivative, which was also obtained directly from the spectropolarimeter, and the results were compared. Thermodynamic parameters for denaturation were also obtained from the equipment.

RESULTS AND DISCUSSION

Screening of fungal strains

36 fungi cultures of different genera (Figure 1) were isolated from soil samples in the Caatinga Biome (Northeast region of Brazil). The species were incorporated into the Culture Collection UCP (Universidade Católica de Pernambuco), Recife, PE-Brazil. All microorganisms studied showed fibrinolytic activity, demonstrating the biotechnological potential of species isolated from Caatinga Biome soils. Several reports describe the efficient protease biosynthesis by the fungi belonging to the genera *Aspergillus* (Shirasaka et al. 2012), Neurospora (Liu et al. 2016, Deng et al. 2018) and *Rhizopus* (Xiao-Lan et al. 2005).

The isolates were screened in the MS-2 medium described by Porto et al. (1996). Among the 36 isolates studied, 58% showed fibrinolytic activity above 100 U/mL (Figure 1). The microorganism with the highest fibrinolytic activity was *M. subtilissimus* UCP 1262 characterized by the fibrinolytic activity of 415 U/mL at 96 h of fermentation. Initially, the protein present in the culture media was 0.498 mg/mL. After 72 h of fermentation, the protein levels decreased to 0.026 mg/mL, showing the protein degradation by the microorganism.

In general, fungi were shown to be a rich source of enzymes. For example, two closely related species of zygomycetes, *Mucor pusillus* and *Mucor miehei*, secrete aspartic proteases, also known as *Mucor* rennin, into the medium (Abdelouahab et al. 2015, Araújo et al. 2015). Milk-clotting protease was produced by *Mucor mucedo* DSM 809 in submerged fermentation and the cultivation profile was reported (Yegin et al. 2010).

Production of fibrinolytic protease

The main components of the medium for the production of fibrinolytic proteases by submerged fermentation were studied through a 2^3-factorial design. The matrix of the design variables and the results for the response variables – Fibrinolytic Activity (U/mL) and protease activity (U/mL) – are shown in (Table II).

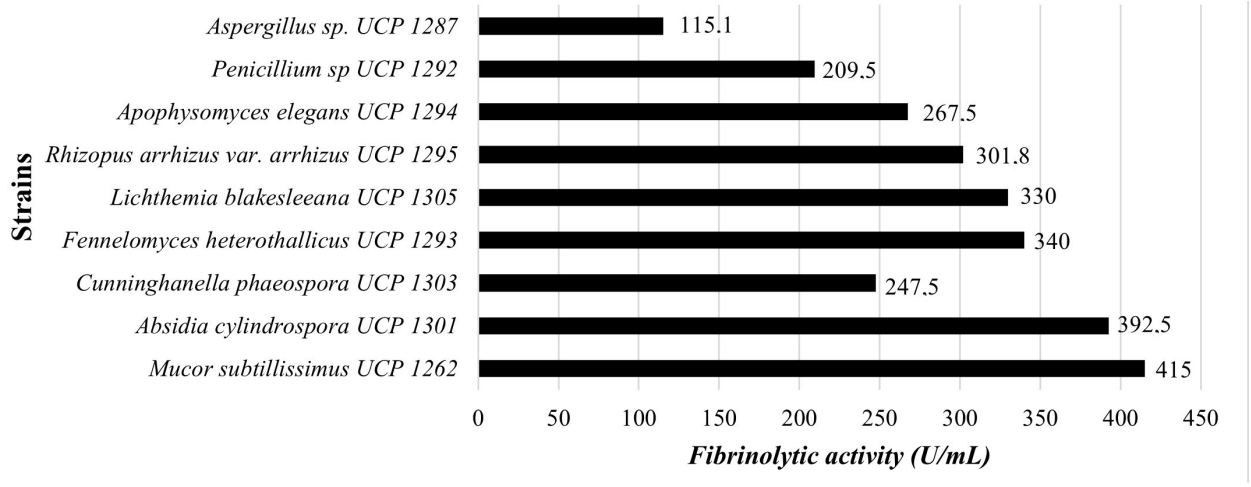

Figure 1. Average of the fibrinolytic activity (FA U/mL) of the fungal species. The representative for the best fibrinolytic activity.

As can be seen in Table II, it was possible to reach up to 1075 U/mL of fibrinolytic activity by using 1% (w/v) wheat bran as the nitrogen source and $CaCl_2$ at 1% (w/v). For protease activity, the best condition was 3% (w/v) of soybean flour as the nitrogen source and 1% of $CaCl_2$. The actual influence of each variable can be seen in Table III, which shows their individual effects and interactions over the fibrinolytic and proteolytic activities.

The statistical analysis showed that the variable Nitrogen Source (NS) has a positive statistically significant effect on producing the fibrinolytic enzyme, while protease production had a negative impact. As can also be seen in Table II, wheat bran is the best nitrogen source to enhance the production of the fibrinolytic enzyme, and soybean flour is the best source for producing protease. Considering that this work aimed to produce fibrinolytic enzymes for further studies and production, the best option would be to use wheat bran. The interaction between the nitrogen source and its concentration was also significant for fibrinolytic enzyme production. As the effect is negative, the interaction is antagonistic, meaning that, to improve the response, one of the variables needs to be increased and the other one needs to be decreased. For protease activity, the nitrogen source concentration was also significant, regardless of the source used.

The fungal biomass was evaluated throughout the fermentation. Figure 2 shows the activity levels obtained during the fermentation for 4 days. Using an organic nitrogen source supported the organism's growth adequately, and the average yield of dry mycelium at 72-120 hours was 0.261 mg/mL. These results further confirm the potential of fungi in the production of enzymes secreted into the medium (Alves et al. 2002). Apparent growth inhibition at the end of fermentation was probably caused by the low pH values (pH 5.8 to 6.1) that developed in the media. In all trials, mainly in assay 6, the pH drifted towards the acidic side, probably due to the accumulation of residual anions. Somkuti & Babel (1968) observed the same pH drift, for protease production using *Mucor pusillus*. The fibrinolytic protease activity reached a maximum level (1075 U/mL) on the fourth day of the fermentation and the biomass development was parallel to enzyme production (Figure 2), as expected for a primary product of metabolism.

The same behavior was observed for milk-clotting protease production by *Mucor mucedo* DSM 809 (Yegin et al. 2010). At the start of cultivation for the enzyme production, the pH values decreased

Table II. Matrix decoded and results of full factorial design 2^3 for the fibrinolytic protease production.

Trial	NS	[NS](%)	[$CaCl_2$](%)	FA(U/mL)	PA(U/mL)
1	S	1	0	950.0	198.3
2	WB	1	0	1025.0	141.7
3	S	3	0	1002.5	274.0
4	WB	3	0	1027.5	192.0
5	S	1	1	965.0	165.7
6	WB	1	1	1075.0	56.0
7	S	3	1	987.5	290.1
8	WB	3	1	1007.5	91.0
9 (C)	S+WB	2	0.5	1035.0	223.7
10 (C)	S+WB	2	0.5	1022.5	190.0
11 (C)	S+WB	2	0.5	1012.5	209.7
12 (C)	S+WB	2	0.5	1025.0	244.0

NS: Nitrogen Source, [NS]: Nitrogen Source Concentration, [$CaCl_2$]: Calcium Chloride Concentration, FA: Fibrinolytic Activity, PA: Protease Activity, S: Soybean, WB: Wheat Bran, S+WB: Soybean and Wheat bran, (C): Central points.

and remained constant up to the end of fermentation. Andrade et al. (2002) obtained similar results using *Mucor circinelloides* for protease production. The optimal initial pH of the medium for protease production may vary depending on the culture medium and microbial organism under study. The initial pH of the cultivation media is a parameter affecting both maximum enzyme production levels and the properties of the crude extract (Yegin et al. 2010).

Biochemical characterization: optimum temperature and pH

The optimum temperature of the protease was at room temperature (28 °C ± 3). The crude extract showed 70 % of protease activity at 37 °C, 77 % at 10 °C, and only 48 % of activity at 45 °C. Enzymatic activity was completely lost at 75 °C. These results differed from those obtained for a fibrinolytic protease from *Aspergillus oryzae* KSK-3, whose optimum temperature was 50 °C and was completely inactivated at 60 °C (Shirasaka et al. 2012). Similarly, the optimum temperature of the fibrinolytic enzyme from *Cordyceps militaris* was 25 °C (Choi et al. 2011).

Table III. Estimated effects of each variable from the factorial design and of their interactions over the fibrinolytic and proteolytic activities.

Variable/ Interaction	Estimated Effects	
	FA (U/mL)	PA (U/mL)
NS	8.8*	-6.9*
[NS](%)	0.4	4.4*
[CaCl2](%)	1.1	-3.1
1x2	-5.4*	-1.8
1x3	1.1	-2.6
2x3	-3.8*	0.5
1x2x3	-1.5	-1.0

*** Significative effect over the response.**

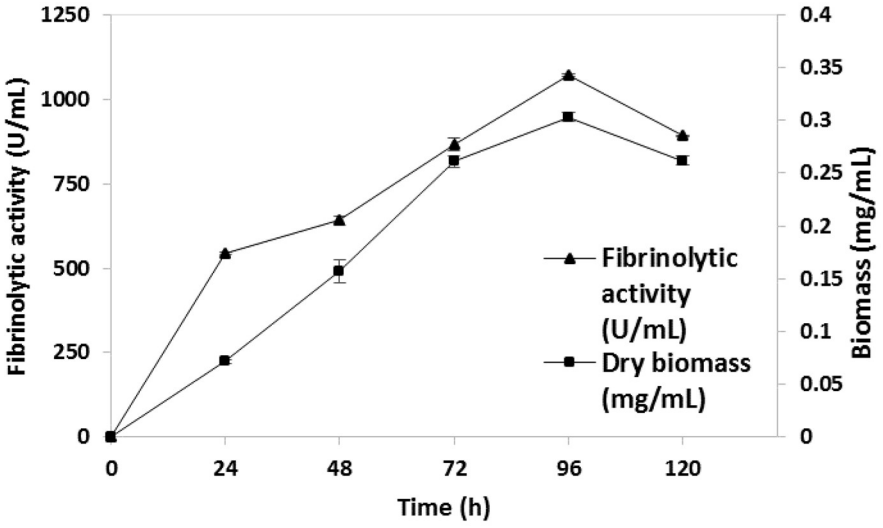

Figure 2. Biomass from *M. subtilissimus* UCP 1262 and fibrinolytic activity until 120 h of fermentation (Error bars based on standard deviation).

The optimum pH of the obtained fibrinolytic protease was 5.0 in the presence of 0.050 M sodium acetate buffer. The enzyme retained 60 % of its activity at pH 7.0 (0.050 M Tris-HCl) and 30 % activity at pH 8.5 (0.050 M Tris-HCl). A considerable loss of activity occurs at pH 3.0. Which is expected as the solubility of a protein will be minimal near this pI (Gaylord & Gibbs 1962). The enzyme was characterized as an acidic-neutral protease since it maintained activity when tested in the acidic-neutral range (pH 5.0-7.0). Similar results were obtained in the characterization of the fibrinolytic protease produced by *Schizophyllum commune* (Park et al. 2010) and *Aspergillus versicolor* (Zhao et al. 2022).

Influence of metal ions, protease inhibitors, and surfactants

Table IV shows the influence of metal ions over protease activity, which was stimulated in the presence of the following salts: $ZnSO_4$ (0.005, 0.010 and 0.020 M), $FeSO_4$ (0.010 and 0.020 M), and $CoCl_2$ (0.010 and 0.020 M). On the other hand, $CuSO_4$ (0.010 and 0.020 M) and $MgSO_4$ inhibited enzymatic activity. Similar to our results, $ZnSO_4$ and $CoCl_2$ were previously shown to stimulate the activity of a protease produced by *Schizophyllum commune* by 66 and 54 %, respectively (Park et al. 2010).

The enzyme present in the crude extract was subjected to the action of protease inhibitors such as PMSF, an inhibitor of serine proteases that induced a significant reduction of the enzymatic activity down to 43%. Table V shows the protease residual activity in the presence of inhibitors. Enzymatic activity was also inhibited by iodoacetic acid (64 %) but not in the presence of Pepstatin A, β- mercaptoethanol, or EDTA. These results allowed us to characterize the fibrinolytic protease from *Mucor subtilissimus* UCP 1262 as a serine protease. Similarly, Duan et al. (2022) produced a fibrinolytic

Table IV. Influence of the metal ions on fibrinolytic activity from *Mucor subtillissimus* UCP 1262.

METAL IONS	[ ]mM	Residual activity (%)
$ZnSO_4$	5	105
	10	193
	20	209
$MgSO_4$	5	108
	10	78
	20	76
$CuSO_4$	5	107
	10	74
	20	71
$FeSO_4$	5	60
	10	113
	20	339
$CoCl_2$	5	77
	10	106
	20	237

serine protease from *Neurospora crassa,* which PMSF highly inhibited. Shirasaka et al (2012) obtained similar results, where the protease isolated from fungi belonging to the genera *Aspergillus* was considerably inhibited by serine protease inhibitors PMSF and Pefabloc SC but not by the chelator agent EDTA.

The influence of surfactants on protease activity was also studied, as seen in Table VI. The enzyme showed a less activity loss in the presence of the non-ionic surfactant Triton X-100. The anionic surfactant SDS significantly increased fibrinolytic activity in the crude extract (up to 299 %). However, in the presence of other non-ionic surfactants with a longer chain, such as Tween 80, the activity was reduced to 37%. A significant decrease of activity in the presence of Tween 80 was also

Table V. Inhibitors influence on fibrinolytic activity from *Mucor subtillissimus* UCP 1262.

Inhibitors	Residual activity (%)
Iodoacetic acid	64
EDTA	156
PMSF	43
Pepstatin A	111
β-Mercaptoethanol	117

Table VI. Influence of the surfactants on fibrinolytic activity from *Mucor subtillissimus* UCP 1262.

Surfactants	[ ] %	Residual activity(%)
SDS	0.5	299
	1.0	209
	1.5	421
	2.0	136
Tween 80	0.5	74
	1.0	90
	1.5	37
	2.0	183
Tween 20	0.5	179
	1.0	117
	1.5	115
	2.0	102
Triton X-100	0.5	109
	1.0	97
	1.5	72
	2.0	77

reported for an alkaline serine protease from the thermophilic fungus *Myceliophthora sp* (Zanphorlin et al. 2011).

Substrate specificity

The amidolytic activity of the purified enzyme was measured using several chromogenic substrates, as described in item 2.6, and reached its highest level of fibrinolytic activity with N-Succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide suggesting that it is a chymotrypsin-like protease (Deng et al. 2018). This type of fibrinolytic enzyme has also been reported from *Neurospora sitophila* (Deng et al. 2018), *Armillaria mellea* (Lee et al. 2005), *Perenniporia fraxinea* mycelia (Kim et al. 2008), and *Fomitella fraxinea* (Lee et al. 2006). *Mucor subtilissimus* UCP 1262 also produced the same type of protease when grown under solid-state fermentation (Nascimento et al. 2017). The purified sample with fibrinolytic activity was subjected to protease inhibitors. For the serine protease inhibitor, PMSF, the fibrinolytic enzyme showed a residual activity of 36.5%. It has also been inhibited by iodoacetic acid (54.5%) but not in the presence of pepstatin A, β-mercaptoethanol, or EDTA, with residual activity of 93.9, 98.6, and 132% respectively. These results allowed for the characterization of fibrinolytic protease as a serine protease, similar to the one described by Deng et al. (2018).

Circular dichroism (CD) spectroscopic analysis of the fibrinolytic protease

The far-UV CD analysis was used to investigate the structural characteristics and conformational stability of the purified fibrinolytic protease through pH and thermal unfolding (Sales et al. 2015). Figures 3 and 4 show the spectra of the present fibrinolytic protease, expressed as its ellipticity and as a function of pH and temperature. As shown in the Figures, the bands in the spectra are negative at 220 nm of wavelength, which reveals that the fibrinolytic protein contains a significant amount of α-helical structure (Greenfield, 2006). The far-UV CD spectra of the fibrinolytic protease as a function of pH and temperature are shown in Figures 3 and 4.

The pH-induced denaturation of fibrinolytic protease

Far-UV CD spectra were measured at various pH values ranging from 2.0 to 10.0. The far-UV CD spectra did not change in the pH range of 3-7. At the extremely acidic pH (2.0), the secondary structure of this protein was noticeably disturbed (Figure 3a), and the protein reached a somewhat disordered conformation at the alkaline pH (8-10) (Figure 3b). The decrease in α-helical content and increase in β-sheet and unordered structure content suggested different modes of unfolding by temperature and pH (acid-induced denaturation).

The far-UV spectra of the fibrolase displayed essentially no change in the band shape or position over the pH range from 5 to 9. The spectral changes at pH 2 and 3 were consistent with the loss of α-helical structure. These differences in α-helix content are shown in Table VII, which brings the content of each secondary structure present in the fibrinolytic protease. This destabilization induced by changing pH could result from unfavorable changes in the electrostatic environment of the protein and, probably, can be caused by the loss of electrostatic interactions necessary to maintain the structure (Haq & Khan 2005).

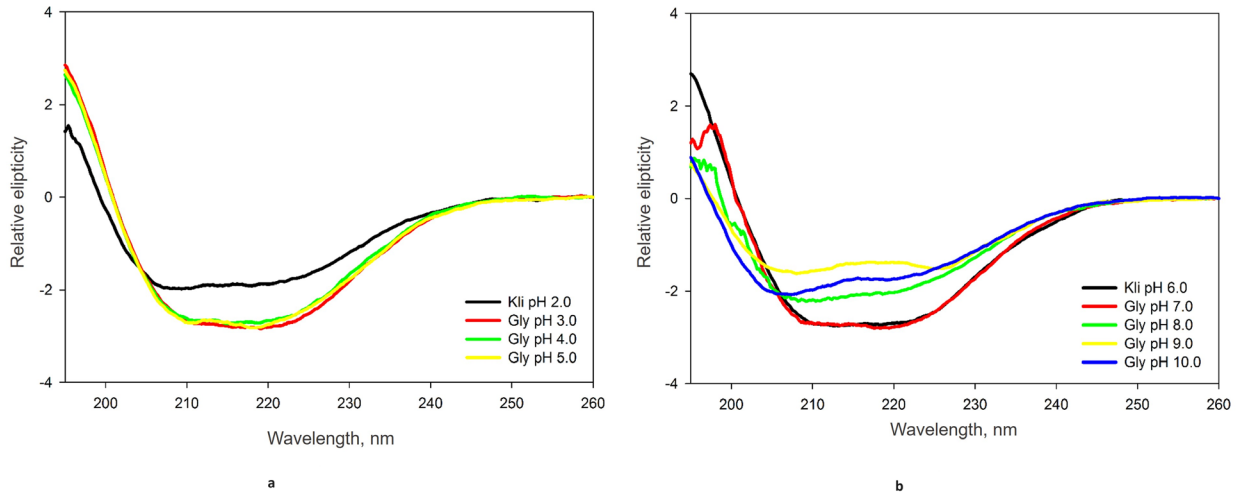

Figure 3. (a) Effect of different pH on the secondary structure of 0.2 mg/mL fibrinolytic protease monitored by far UV circular dichroism in 10 mM buffers pH 2.0 – 5.0 at 25 °C. (b) 10 mM buffers pH 6.0 – 10.0 at 25 °C.

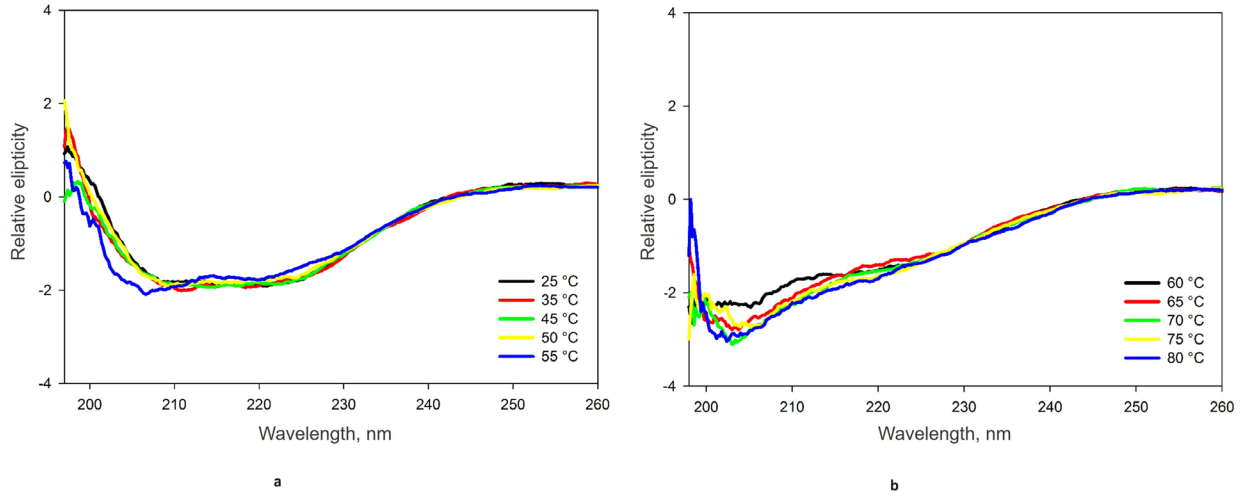

Figure 4. Thermal denaturation pattern of 0.2 mg/mL fibrinolytic protease in 10mM Tris buffer pH 7.5. (a) The circular dichroism signal of the protein sample was monitored at a temperature gradient of 1 °C/min from 25 to 55 °C (a) and from 60 to 80 °C (b).

Table VII. Secondary structures of the fibrinolytic protease from *Mucor subtilissimus* UCP 1262 under different pHs.

Secondary structure	pH 2	pH 3	pH 4	pH 5	pH 6	pH 7	pH 8	pH 9	pH 10
α-Helix(%)	26.1	24.5	28.2	32.6	28.2	28.5	19.8	15.3	17.2
Antiparallel (%)	16.9	6.4	9.8	9.2	10.2	11.9	12.3	13.4	11.5
Parallel (%)	0.5	13.9	8.9	7.3	12.2	6.2	6.0	7.6	8.9
Turn (%)	14.9	10.6	11.4	11.2	11.3	10.7	15.6	15.8	14.1
Others (%)	41.6	44.5	41.7	39.8	38.1	42.6	46.3	47.9	48.3

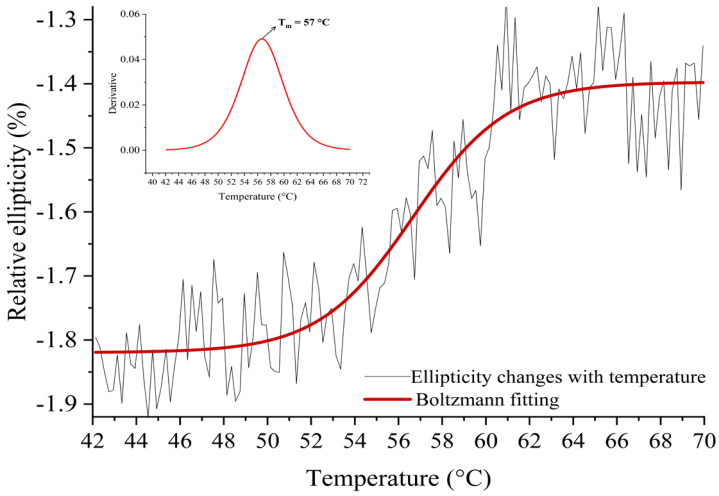

Figure 5. Circular dichroism signals at 222 nm of the protein sample from 42 °C to 70°C and its Boltzmann fitting along with the derivative of the fitting and the melting temperature.

Thermal denaturation of fibrinolytic protease

The fibrinolytic protease lost a significant amount of ordered secondary structure at temperatures above 60 °C, indicating the unfolding of the protein (Figure 4a). Monitoring changes in the secondary structure during thermal denaturation by far-UV CD spectroscopy showed a noticeable reduction of the ellipticity at 222 nm when the protein was heated to 60 °C (Figure 4b). The complete loss of the secondary structure was observed above 65 °C. The thermal denaturation curve (Figure 5) with a change in ellipticity at 222 nm indicated that the T_m (melting temperature) of the protein was 58.1 °C (according to the spectropolarimeter). The curve had a sigmoidal profile, and a Boltzmann fitting was also used to calculate T_m. From the fitting, a derivative (Figure 5) allowed to calculate T_m, which was found to be 57 °C, differing by only 1.1°C from the experimental determination. The denaturation enthalpy (ΔH) was 85.1 kcal/mol, and the denaturation entropy (ΔS) was 0.26 kcal/mol/K. These thermodynamical parameters were obtained directly from the spectropolarimeter and were defined at T_m as reference temperature (Blanco et al. 2007). The effect of temperature and pH on the activity and conformation of the thrombolytic protein fibrolase has been previously examined and results similar to ours were obtained (Pretzer et al. 1991), where fibrolase unfolded irreversibly, and its T_m was 50 °C, at pH 8.0 and 43 °C, at pH 5.0.

CONCLUSIONS

A novel potential therapeutical fibrinolytic enzyme for thrombosis treatment was produced, purified, and characterized and is reported in the present work. The fungal strain *Mucor subtilissimus* UCP 1262 was the highest producer of a fibrinolytic enzyme among 36 strains analyzed in this study and it was able to produce up to 1075 U/mL of fibrinolytic activity production, using wheat bran as substrate, 1% of nitrogen source and 1% of $CaCl_2$. Characterization of the enzyme allowed us to define the best conditions for the application, with the crude extract exhibiting maximum activity at pH 5.0 and 28 °C. The enzyme was found to be A chymotrypsin-like serine-protease with fibrinolytic activity from *Mucor subtilissimus* UCP 1262. The structure of the fibrinolytic protease was shown to contain a significant amount of α-helix and denatured with a *Tm* of 58.14 °C, denaturation enthalpy of 85.1 Kcal/mol, and denaturation entropy of 0.26 Kcal/mol·K.

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