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Protease from Mucor *subtilissimus* UCP 1262: Evaluation of several specific protease activities and purification of a fibrinolytic enzyme

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Abstract: The industrial demand for proteolytic enzymes is stimulating the search for new enzyme sources. Fungal enzymes are preferred over bacterial enzymes, and more effective and easier to extract. The aim of this work was to evaluate the potential of protease production by solid state fermentation (SSF) of *Mucor subtilissimus* UCP 1262, evaluate different specific activities, purify and partially characterize the enzyme in terms of biochemical as to the optimal pH and temperature. Initially, the enzyme crude extract was screened for 3 different proteolytic activities, collagenolytic (161.4 U/mL), keratinolytic (39.6 U/mL) and fibrinolytic (26.1 U/mL) in addition to conventional proteinase activity. After ammonium sulfate precipitation, the active fractions with fibrinolytic activity were dialyzed in 15 mM Tris-HCl buffer, pH 8, loaded onto DEAE-Sephadex A50 ion-exchange column and gel filtrated through Superdex 75 HR10/300. The enzyme showed a fibrinolytic maximum activity at 40 C and pH 9,0. The purified enzyme showed activity against a chromogenic chymotrypsin substrate, SDS-PAGE showing a molecular mass of approximately 70 kDa and, the specific activity of 25.93 U/mg. These characteristics suggest that the enzyme could be and efficiently produced in a simple and low-cost way using *Mucor subtilissimus* UCP 1262 in SSF.

Key words: protease, *Mucor*, fibrinolytic activity, keratinase, collagenase, solid state fermentation.

INTRODUCTION

Proteases are one of the most important groups of enzymes found in all living organisms from bacteria to mammals (Shamsi et al. 2018). This class of enzymes belongs to the peptidyl-peptide hydrolases, which occupy a essential position with respect to their application, wide applicability in the medical field and industrial, representing approximately 60% of the enzymes sold worldwide (Suryia-Prabha et al. 2015, Al-Dhabi et al. 2020, Adetunji & Olaniran 2020). Because of their high efficiency, versatility in biotechnological applications, specificity and stability toward pH, salt, temperature, organic solvents, metal ions and surfactants, alkaline proteases are in high demand (Raval et al. 2014, Sarkar & Suthindhiran 2020, Fatima et al. 2008).

Peptidases are enzymes that have been produced by microorganisms in different types of culture media based on agro-industrial waste, such as wheat bran and soybean flour (Xiao et al. 2005, Meena et al. 2013, Semenova et al. 2020). The use of proteases for therapeutic applications has been one of the goals of the pharmaceutical industry in recent years, since the catalytic activity of these enzymes permits

the use of lower doses for treatments, with a target potential and greater efficiency, and reduce side effects of existing drugs, while maintaining the desired therapeutic benefits and reducing costs becoming interesting for industrial pharmaceutical industry, it is estimated that about 5 to 10% of all pharmaceutical targets for drug development are proteases (Al-Dhabi et al. 2020, Chimbekujwo et al. 2020). Over the last decade, the search for other proteases from various sources has been under way, being microorganisms are excellent sources of these enzymes because they have wide biochemical diversity, can be easily cultivated and maintained at low cost, and can be genetically manipulated to improve or modify the final product (Zheng et al. 2020, Barzkar 2020).

Solid state fermentation (SSF), which is defined as the fermentation on solid substrate, is carried out in the absence or near absence of free water, even though the substrate must possess enough moisture to support microbial growth and metabolism. The solid matrix could be either the source of nutrients or simply a support impregnated with proper nutrients that allow the development of microorganisms (Masutti et al. 2012, Olukomaiya et al. 2020). SSF is particularly advantageous for industrial enzyme production by filamentous fungi because it enables the use of agro-industrial residues as solid substrate, acting as carbon and energy source (Pirota et al. 2014, Garro et al. 2021). The aim of this work was the purification of proteases produced in SSF by *Mucor subtilissimus* UCP 1262 isolated from soil of the Brazilian Caatinga biome and its biochemical characterization.

MATERIALS AND METHODS

Fungal strain

Mucor subtilissimus UCP 1262 (SISGEN AA30B0B) was isolated from the Caatinga soil, Serra Talhada, PE-Brazil and deposited in the culture collection of the Catholic University of Pernambuco, Recife-PE, Brazil. This microorganism was maintained in Czapek medium. The microorganism was selected based on our previous data (Nascimento et al. 2015).

Inoculum preparation

Spores were collected using a sterile nutrient solution composed of 0.5% yeast extract, 1% glucose and 0.01% Tween 80 and diluted in 245 mM sodium phosphate buffer, pH 7.0. They were then counted in Neubauer chamber to a final concentration of 10^7 spores/mL.

Production of proteolytic enzymes by SSF

The substrate, after complete dehydration by drying at 65°C, was stored in plastic containers for subsequent use. The fungus *Mucor subtilissimus* UCP 1262 was inoculated to a final concentration of 10^7 spores/mL in 125 mL Erlenmeyer flasks, containing 5 g of wheat bran with a granulometry from 0.6 to 2.0 mm (moisture of 50%), and incubated at 25 °C for 72 h according Nascimento et al. (2015).

Enzyme extraction

The enzyme was extracted after 72 h of fermentation. After addition of 7.5 mL of 245 mM sodium phosphate buffer, pH 7, per g of substrate, flasks were placed in an orbital shaker (Model 430 –RD, Ethiktechnology, São Paulo, Brazil) at 150 rpm for 90 min at room temperature. After this period, the suspension was centrifuged (Frontier 5000 Multi Pro, Rio de Janeiro, Brazil) at 3,500 rpm for 10 min, and the supernatant used for determination of different enzyme activities.

Enzyme activities

Protease activity

Protease activity was determined by the method of Ginther (1979). An aliquot of the crude extract

(150 µL) was mixed with the substrate (250 µL) in incubated at 28 ºC in the dark for 1 hour, followed by the addition of 1 mL of 10% Tricloroacetic acid (TCA). Then, the reaction mixture was centrifuged (3.000 xg, 15 min), and the supernatant (800 µL) was homogenized with 200 µL of 1.8M NaOH. One unit of protease activity was defined as the amount of enzyme that produces an increase in the absorbance of 0.1 per hour at 420 nm. Experiments were performed in triplicate.

Collagenase activity

The assay for azo dye-impregnated collagen (Azocoll) was carried out according to a modified version of the method developed by Chavira et al. (1984). The Azocoll® (Sigma, St Louis MO, USA) was suspended in a Tris-HCl buffer solution (0.1 M pH 7.2), to reach a final concentration of 5 mg/mL. Subsequently, 150µL of crude extract and 150µL of buffer Tris-HCl (0.1M, 1 mM pH 7.2), were mixed with 270µl of the Azocoll® solution and kept in a water bath for 18h at 37 ºC. After that period, the samples were centrifuged at 10,000 xg for 15 min at 4ºC. One unit of collagenase activity was defined as the amount of enzyme per mL of crude extract that leads, after 1 h of incubation, to an increase in the absorbance of 0.01 at 520 nm, as a result of the formation of azo dye-linked soluble peptides.

Keratinase activity

Keratinase activity was determined according to Cheng-Gang et al. (2008). 1.0 mL of crude enzyme properly diluted in Tris-HCl buffer (0.05 mol/L, pH 8.0) was incubated with 1 mL keratin solution at 50 °C in a water bath for 10 min, and the reaction was stopped by adding 2.0 ml 0.4 mol/L trichloroacetic acid (TCA). After centrifugation at 1450×g for 30 min, the absorbance of the supernatant was determined at 280 nm (UV-2102, UNICO Shanghai Corp., China) against a control. The control was prepared by incubating the enzyme solution with 2.0 ml TCA without the addition of keratin solution. One unit of keratinase activity was defined as the amount of enzyme responsible for an increase in the absorbance of 0.01 at 595 nm after the reaction with keratin azure for 1 h at pH 8.0 and 50°C.

Fibrinolytic activity

Fibrinolytic activity was determined by the spectrophotometric (Uv Vis Spectro 580UVP, Marte Cientifica, São Paulo, Brazil) method described by Wang et al. (2011). In this assay, 0.4 mL of 0.72% fibrinogen was placed in a test tube with 0.1 mL of 245 mM phosphate buffer (pH 7.0) and incubated at 37°C for 5 min. Afterwards, 0.1 mL of a 20 U/mL thrombin solution [T9326-150UN - Thrombin human, BioUltra, recombinant, expressed in HEK 293 cells, aqueous solution, ≥95% (SDS-PAGE) - Sigma-Aldrich] was added. The solution was incubated at 37°C for 10 min, 0.1 mL of enzyme extracted by ATPS was added, and incubation continued at 37°C. This solution was again mixed after 20 and 40 min. After 60 min, 0.7 mL of 0.2 M trichloroacetic acid (TCA) was added and mixed. The reaction mixture was centrifuged at 15,000 × g for 10 min. Then, 1 mL of the supernatant was collected, and the absorbance was measured at 275 nm. 1 fibrin degradation unit (U) of enzyme activity was defined as the amount of enzyme able to cause a 0.01 increase per minute in the absorbance. Each experiment was performed in triplicate, and the results, after correction against blank samples, were expressed as mean values.

Amidolytic activity

Amidolytic activity was measured qualitatively by the method described by Kim et al. (1996) using the synthetic substrates: N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide

- Chymotrypsin substrate (S7388 Sigma) and Gly-Arg-p-nitroanilidedihydrochloride - Urokinase and plasmin substrate (G8148 Sigma). The mixture (0.8 mL) containing 30 µL of enzyme solution, 30 µL of chromogenic substrate and 140 µL of 20 mM Tris-HCl, pH 7.4 was incubated for 15 min at 37° C, in absorbance measurements at 405 nm.

Protein Determination

Protein content was determined by the method described by Bradford (1976) using bovine serum albumin (BSA) as a standard. Each experiment was performed in triplicate, and the results were expressed as mean values.

Purification of protease with fibrinolytic activity

After precipitation of the crude extract with ammonium sulphate up to 100% of saturation, the active fractions with fibrinolytic activity were loaded into a DEAE-Sephadex A50 ion-exchange column (25 x 12 x 2.0 cm) equilibrated with 150 mM Tris-HCl buffer, pH 8. The sample was then eluted with the same buffer containing 0.5 M potassium chloride. The protein-containing fraction was pooled, and the enzyme solution concentrated for further analysis. All the process was monitored at 280 nm absorbance. The main fractions with fibrinolytic activity were dialyzed in 15 mM Tris-HCl buffer, pH 8. The dialysate was concentrated by lyophilization and subsequently gel filtrated through Superdex 75 HR10/300, Äkta Avant 25 System (GE Healthcare, Uppsala, Sweden) that had previously been equilibrated with 100 mM Tris–HCl buffer, pH 8.0, at a flow rate of 0.5 mL/min. The fractions possessing protease activity were pooled and concentrated.

Effect of pH and optimal temperature on fibrinolytic activity

The effect of temperature on the optimal activity of the enzyme was evaluated by incubating the purified enzyme by gel chromatography filtration Superdex 75 HR10/300 at various temperatures ranging from 10 to 90 °C for 1 hour through fibrinolytic activity. For the pH assay, the same enzymatic preparation and activity dosage were performed, with the purified enzyme 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 9) and glycine-NaOH (pH 9.0 to 11.0) and incubated at 37 °C for 60 min.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a 12% polyacrylamide running gel according to the method of Laemmli (1970). The molecular mass was calibrated using a molecular mass marker (Low-Range Rainbow Molecular Weight Markers - GE Healthcare) as a standard. Protein bands were detected by staining with silver nitrate. The Molecular weight on the AKTA system was determined using the commercially available standards: albumin (65 kDa), ovalbumin (43 kDa), trypsin inhibitor (21.7 kDa) and lysozyme (14.3 kDa), all at a concentration of 0.6 mg/mL. The molecular weight was measured by calculating the retention time versus molecular weight of the standard (RT x MW).

Fibrin zymography

Fibrinolytic activity was assessed using a fibrin zymography gel according to Kim et al. (1998). Fibrinogen and thrombin were mixed with 12% polyacrylamide gel solution, and the mixture was loaded into fibrin gel for electrophoresis. After electrophoresis, the gel was washed with 2.5 % Triton X-100 for 1 h, rinsed three times

with distilled water, and incubated in reaction buffer (0.1 M glycine, pH 8.4) at 37 °C for 18 h. The staining and distaining procedures were the same described on SDS-PAGE section.

Statistical analysis

The statistical comparison between the control and sample were made using the non-parametric Mann-Whitney U Test ($p<0.05$) and R software was used for the analysis.

RESULTS AND DISCUSSIONS

Evaluation of enzyme activities

In previous study by Nascimento et al. (2015) we found that a wheat bran amount of 3g, a moisture content of 50%, a temperature of 30°C and a fermentation time of 72 hours were the best conditions for protease production by *M. subtilissimus* UCP 1262 therefore, they were used in these experiments for enzyme production.

The enzymatic activities measured in the crude extract can be seen in Table I. A similar study conducted by Sharkova et al. (2015) with fungal species with fibrinolytic and collagenase activities revealed that, although eighteen micromycetes exhibited proteolytic activity, species belonging to the *Mucor* genus showed specific collagenase activity but no appreciable proteolytic activity towards fibrin. Another study by Shirasaka et al. (2012) about protease production by *Aspergillus oryzae* KSK-3 isolated from commercial rice-koji for miso brewing, showed a fibrinolytic activity of 21.8 U/mL, less activity than that found in our work (26.1 U/mL). Kim (2003) observed that fourteen species of fungi associated with feather belonging to ten genera, including *Mucor*, showed keratinase activity in submerged fermentation, but the species that provided the best results were belonging to the genus *Aspergillus* with activity in the range 10-15 U/mL, below that of the crude extract produced in this work by *Mucor subtilissimus* (39.6 U/mL).

The enzyme produced by *Xylaria curta*, was studied by Meshram et al. (2016), performed fibrinolytic activities for different substrates, rice chaff 5.85 ± 0.67 U/mL, wheat bran 3.86 ± 0.67 U/mL, and egg shell 2.75 ± 0.38 U/mL, but none of the parameters demonstrated high activity when compared to that demonstrated by *Mucor subtilissimus*. Furthermore, Liu et al. (2016) presented an enzyme produced by *Neurospora sitophila* with fibrinogenolytic activity of 45 U/mL, which stands higher than the activities reported here. In addition, the literature also revealed an enzyme produced by *Penicillium* sp UCP 1286 (797 to 812 U/mL) (Wanderley et al. 2017), with stronger collagenase activity then reported by our data (161.4 U/mL).

The amidolytic activity of the purified enzyme was then assessed towards two chromogenic substrates. Since the highest degree of specificity was observed for

Table I. Enzymatic activities measured in the crude extract obtained by *Mucor subtilissimus* UCP 1262.

Enzymatic activity	Activity (U/mL)
Collagenase	161.4
Protease	102.9
Fibrinolytic	26.1
Keratinase	39.6
Amidolytic (N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide)	10.28

N-Succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide (10.48 U/mg), the fibrinolytic protease purified from *Mucor subtilissimus* UCP 1262 was characterized as chymotrypsin-like. Similar fibrinolytic activity was reported from *Armillaria mellea* (Lee et al. 2005), *Fomitella fraxinea* (Lee et al. 2006) and *Perenniporia fraxinea mycelia* (Kim et al. 2008), *Neurospora sitophila* (Deng et al. 2018), *Lyophyllum shimeji* (Moon et al. 2014). For further studies, specific fibrinolytic activity was used, due to the promising results.

Regarding the keratinolytic activity, the crude extract of *Mucor subtilissimus* showed an activity of 39.6 U/mL, much higher than that found by Anbu et al. (2005) that had a 6.2 U/mL keratinase activity with the saprobic anamorphic fungus *Scopulariopsis*. and after 35 days of fermentation, in our studies we achieved superior activities with only 72 hours of fermentation. Friedrich et al. (1999) using *Aspergillus flavus* obtained 0.781 U/mL of keratinolytic activity, that is, also below that found in our studies.

Enzyme purification

Among the enzymes with proteolytic activities detected in this work, the fibrinolytic enzyme was purified due to the ever-increasing interest in proteins used for a vast range of pharmaceutical applications. The ever-increasing interest in proteins used for a vast range of pharmaceutical applications becomes obvious considering the number of reports related to the production and purification of these types of macromolecules (Asenjo & Andrews 2012). Consequently, the fibrinolytic enzyme was purified by a combination of 3 chromatographic steps as summarized in Table II. The enzyme fraction obtained using 40-60% saturation with ammonium sulphate showed an increase in the fibrinolytic activity (10.08 U/mg) compared with the crude extract, being the fraction used for subsequent steps, while that reported by Shirasaka et al. (2012) for a fibrinolytic protease from *Aspergillus oryzae* KSK-3 using 0–60% saturation is similar to that obtained in the present work.

After anion exchange chromatography with DEAE Sephadex, the specific fibrinolytic activity was 19.96 U/mg. The main fractions with fibrinolytic activity collected after ion exchange chromatography were subjected to gel-filtration chromatography with Superdex 75 (HR10/300), resulting in three major fractions (Figure 1), only the first fraction (peak A), displayed fibrinolytic

Table II. Purification protocol adopted for purification of fibrinolytic protease from Mucor subtilissimus UCP 1262 and related results.

Purification step	Total Protein (mg)	Total Activity (U/mL)	Specific activity (U/mg)	Purification fold	Recovery (%)
Crude extract	107.20	498.25	4.64	1.00	100.00
Ammonium sulfate precipitation	42.73	430.75	10.08	2.17	86.45
DEAE Sephadex A 50 (Anion exchange)	5.41	108.00	19.96	4.30	21.67
Superdex 75 HR10/300 (Gel filtration)	0.93	40.12	43.13	9.29	8.05

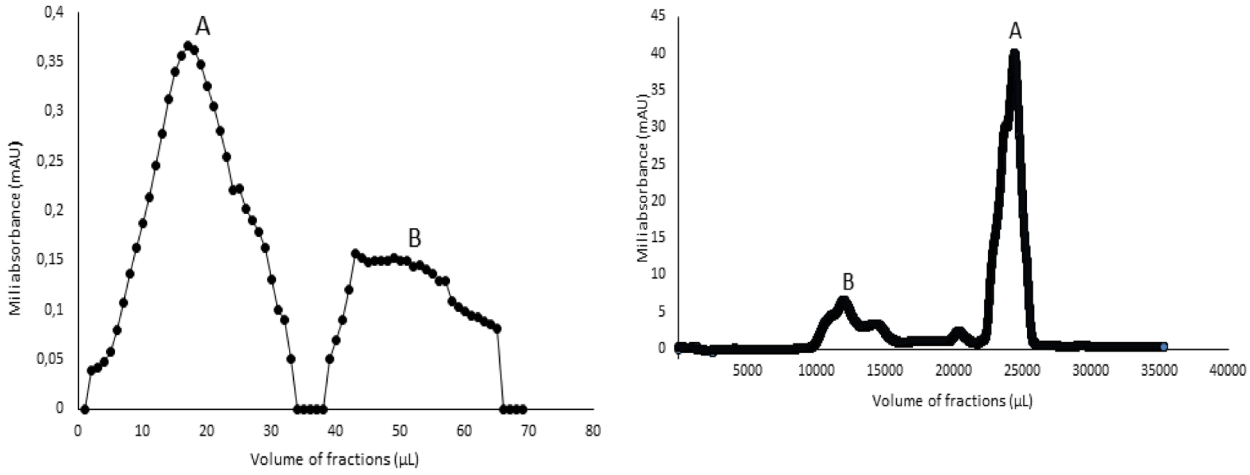

Figure 1. Gel-filtration chromatography with Superdex 75 HR 10/300. The main fractions with fibrinolytic activity collected after ion exchange chromatography were subjected to gel-filtration chromatography equilibrated with Tris-HCl/KCl 0.5 M buffer as the eluent. The fractions were collected for absorbance at 280nm. a- Peak containing the purified enzyme with fibrinolytic activity; b - Peak containing protein without fibrinolytic activity; c - Peak containing pigment extract without fibrinolytic activity.

activity (specific activity of 25.93 U/mg), with a percent recovery of 4.84%. Therefore, this work is promising, since with simple four purification steps, it was possible to yield and partially purify the fibrinolytic protease, when compared to other fibrinolytic proteases for example Shirasaka et al. (2012) used six steps of recovery for a fibrinolytic enzyme from *Aspergillus oryzae* KSK-3 however, only a percentage of recovery of 0.005% was possible. Liu et al. (2016), also, using the same gel-filtration system, purified a fibrinolytic enzyme from *Cordyceps militaris*, with 5.8% of recovery.

Effect of pH and temperature on the optimal activity of the fibrinolytic enzyme

The Figure 3 shows that the purified enzyme by Superdex 75 HR10/300 displayed its maximum activity at 40°C, confirming the results reported by Yang et al. (2019) for a fibrinolytic enzyme from *Bacillus amyloliquefaciens* Jxnuwx-1 which also showed an optimal enzyme activity of 41°C as Yao et al. (2018) obtained an optimal activity of 40 °C of a fibrinolytic protease by *Bacillus subtilis* JS2. The enzyme became less active

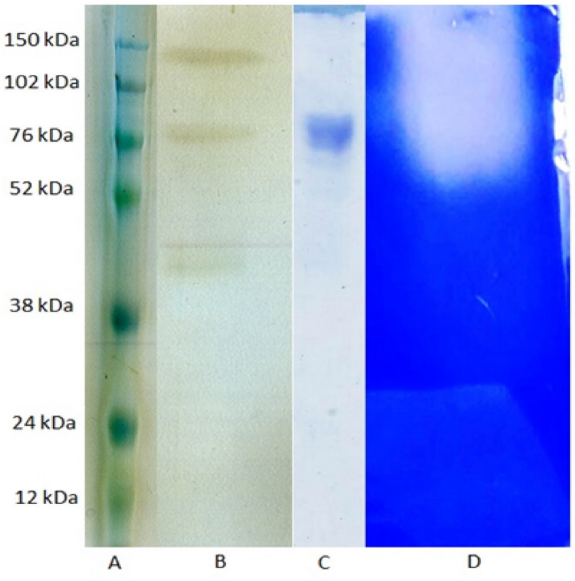

Figure 2. Molecular weight determination of fibrinolytic protease purified from *Mucor subtilissimus* UCP 1262 using by SDS-PAGE (a); (b) and fibrin zymography (c). (M) protein standard marker (Full Range RPN800E - GE Healthcare); (a) SDS-PAGE was carried out on a 12 % polyacrylamide gel and was stained with silver nitrate showed the purified fibrinolytic protease by DEAE Sephadex; (a) Purified fibrinolytic protease by Superdex 75 HR10/300; (c) Fibrin zymography was carried out on a 12 % polyacrylamide gel contain fibrin and was stained with Coomassie Brilliant Blue R-250.

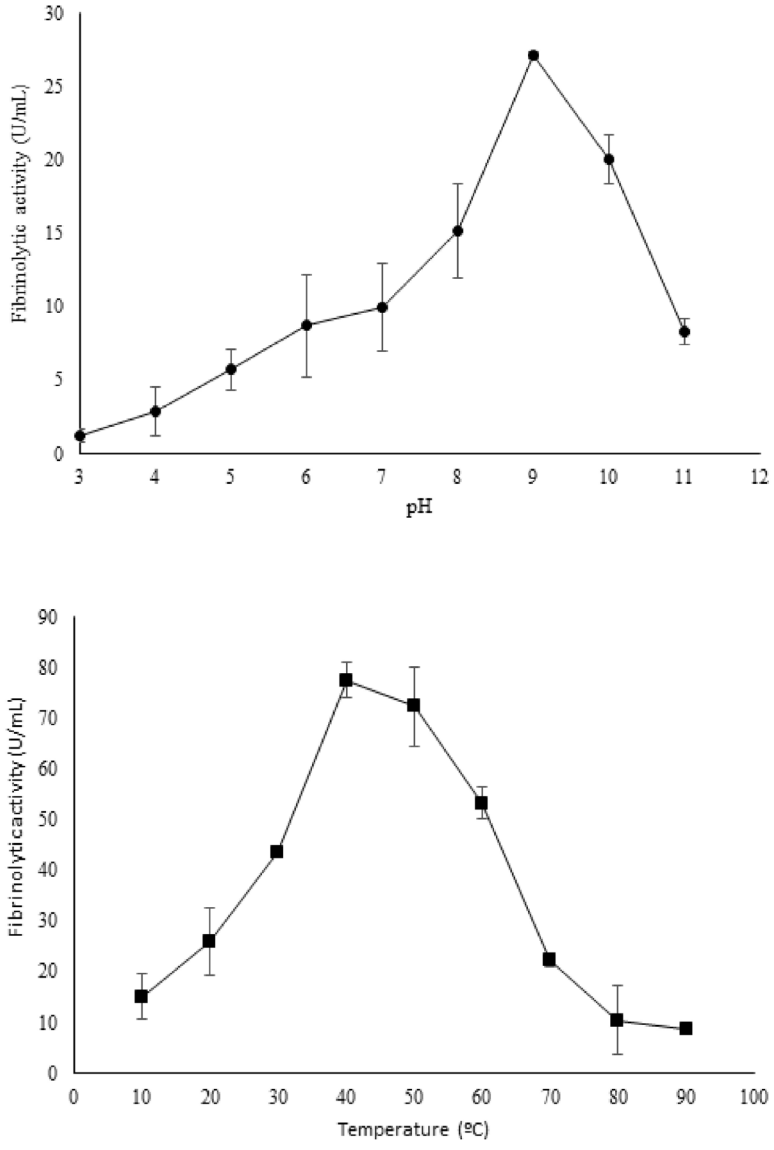

Figure 3. Optimum temperature (a) and optimum pH (b) on fibrinolytic activity of *Mucor subtilissimus* UCP 1262 of after gel filtrated through Superdex 75 HR10/300.

when temperature was raised to 70 °C, and was completely denatured at temperatures >80 °C.

The optimum pH was 9.0 (Figure 3) having a fibrinolytic activity of 26.30 U/mL. Protease activity was conserved in the range between pH 9.0 to 10 and thus is considered an alkaline fibrinolytic protease. At very acidic pH (3.0 - 5.0) the fibrinolytic activity was less than 5 U/mL and in the neutral (6.0 - 8.0) an average fibrinolytic activity of 12 U/mL. Fibrinolytic enzymes produced by *Bacillus subtilis* (Chang et al. 2012) and *Bacillus subtilis* ICTF-1 (Mahajan et al. 2012). also showed an optimum pH of 9 as the ideal for maximum fibrinolytic activity.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fibrin zymography

The SDS-PAGE analysis of the only fraction exhibiting fibrinolytic activity shows (Figure 2) a single homogenous band corresponding to a molecular weight of approximately 70 kDa. The fibrinolytic activity was confirmed by fibrin zymography (Figure 2) showing a clear sharp band with molecular weight close to

that revealed by SDS-PAGE and estimated by AKTA gel filtration. Such a molecular weight of the purified enzyme (70 kDa) is larger than the majority of known fibrinolytic enzymes described in the literature for *Armillaria mellea* (21 kDa) (Wu et al. 2009), *Fusarium* sp. BLB (27 kDa) (Ueda et al. 2007); *Fusarium* sp. CPCC (28 kDa), *Xylaria curta* (~33 kDa) (Meshram et al. 2016), *Ganoderma lucidum* (33.2 kDa) (Kumaran et al. 2011), *Neurospora sitophila* (34 kDa) (Liu et al. 2016), *Streptomyces* sp. (Streptomycetaceae) isolated from Amazonian lichens (39 kDa) (Silva et al. 2016) and *Cerrena albocinnamomea* (40 kDa) (Hamada et al. 2017) but close to the one reported for *Bionectria* sp. (80 kDa) (Rovati et al. 2010).

CONCLUSIONS

Among all the proteinase activities analyzed, the fibrinolytic was the most promising. A fibrinolytic enzyme purified from *Mucor subtilissimus* UCP 1262 exhibited similarity with a chymotrypsin like enzyme and exhibit a high degree of specificity toward fibrin in addition, the enzyme had its optimal temperature and pH defined. Therefore, the fungi *Mucor subtilissimus* UCP 1262 may be a source for fibrinolytic proteases to treat thrombosis soon.

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