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HEALTH SCIENCES

Purification and characterization of fibrinolytic protease from *Streptomyces parvulus* by polyethylene glycol-phosphate aqueous two-phase system

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Abstract: Fibrinolytic proteases are a promising alternative in the pharmaceutical industry, they are used in the treatment of cardiovascular diseases, especially thrombosis. Microorganisms are the most interesting source of fibrinolytic proteases. The aim of this study was the production of fibrinolytic protease from *Streptomyces parvulus* DPUA 1573, the recovery of the protease by aqueous two-phase system and partial biochemical characterization of the enzyme. The aqueous two-phase system was performed according to a 2^4-full factorial design using polyethylene glycol molar mass, polyethylene glycol concentration, citrate concentration and pH as independent variables. It was analyzed the effect of different ions, surfactants, inhibitors, pH and temperature on enzyme activity. The best conditions for purifying the enzyme were 17.5% polyethylene glycol 8,000, 15% Phosphate and pH 8.0, it was obtained a partition coefficient of 7.33, a yield of 57.49% and a purification factor of 2.10-fold. There was an increase in enzyme activity in the presence of Fe^{2+} and a decrease in the presence of β-Mercaptoethanol, phenylmethylsulfonyl fluoride and Iodoacetic acid. The optimum pH was 7.0 and the optimum temperature was 40 ºC. The purified protease exhibited a molecular mass of 41 kDa. The fibrinolytic protease from *Streptomyces parvulus* proved to be a viable option for the development of a possible drug with fibrinolytic action.

Key words: Actinomycetes, protease, fibrinolysis, thrombolytic.

INTRODUCTION

Thrombosis is a cardiovascular disease with the highest morbidity and mortality worldwide. It is caused by unbalanced formation and accumulation of blood clots that block the normal flow blood (Preston et al. 2019). These clots are formed from conversion of fibrinogen to fibrin due to the proteolytic action of thrombin (Kattula et al. 2017). Current therapies against the formation of clots include antiplatelet drugs, anticoagulants and thrombolytic agents, however, several of these drugs promote haemorrhagic side-effects and exhibit high cost. Thus, a drug with the ability to quickly remove blood clots and restore blood flow is of paramount importance to the effective treatment

of thrombotic diseases. In this context, fibrinolytic enzymes are an alternative for the treatment of these diseases (Bray et al. 2020, Jiang et al. 2020).

Proteolytic enzymes with fibrinolytic activity are capable of degrading fibrin and have been studied as an alternative in therapy against thrombosis (Nascimento et al. 2016). Fibrinolytic enzymes with potential thrombolytic application have been extracted from various sources, such as fermented foods, microorganisms and animals (Katrolia et al. 2020, Chandramohan et al. 2019, Jiang et al. 2020).

Streptomyces is a genus of gram-positive aerobic bacteria belonging to the phylum of Actinobacteria, this phylum is characterized by the presence of diaminopimelic acid in the cell wall. *Streptomyces* spp. are scientifically known for their ability to produce different pharmaceutical products (e.g., antibiotics, anticancer drugs, immunosuppressive agents, anthelmintics and antiviral drugs) and several types of extracellular enzymes with industrial applications, among them, fibrinolytic proteases (Dhamodharan et al. 2019, Huguet-Tapia et al. 2016, Kumar et al. 2020, Osman et al. 2019, Tatar et al. 2020). However, microbial proteases to be used successfully, must pass through several purification steps. In some cases, technical and economic restrictions make these several steps an obstacle. It is necessary to reduce these process steps, especially for enzymes with pharmaceutical application. Alternatively, the aqueous two-phase system (ATPS) is a type of purification process with low cost, high efficiency and short term (Nadar et al. 2017).

ATPS is an ecofriendly technique that decreases the downstream steps and has in its composition a high level of water, which prevents the proteins denaturation, making the technique more interesting. The ATPS can be composed of one or more polymers, or a polymer and a salt for the formation of two immiscible phases. One of the most ordinary polymers used is Polyethylene Glycol (PEG) with great variation of molecular weight and low toxicity (Iqbal et al. 2016, Khan et al. 2019).

The Amazon holds an inestimable genetic resources bank, allowing the attainment of a varied products range, mainly biotechnological products from microorganisms (Bittencourt & Rios 2013). *Streptomyces parvulus* DPUA 1573 isolated from Amazonian lichens was studied by Batista et al. 2017, showing promise to produce fibrinolytic enzymes. However, studies are still needed on alternatives for the purification of the enzyme from the crude extract and its characterization. In the present study, fibrinolytic protease from *Streptomyces parvulus* DPUA 1573 was extracted by aqueous two-phase system and biochemical characterization of this fibrinolytic protease was performed.

MATERIALS AND METHODS

Bacterial strain and culture conditions

The strain of *Streptomyces parvulus* DPUA 1573 was isolated from lichens from Amazon and belongs to the culture collection of the Parasitology Department of Federal University of Amazonas (DPUA). The isolated microorganism was preserved in mineral oil and maintained at 25 °C in ISP-2 medium (Pridham et al. 1957).

Production of fibrinolytic protease

The enzyme was produced under submerged fermentation. Filamentous bacteria were grown in a 250 mL Erlenmeyer flask containing modified MS-2 medium (Porto et al. 1996). In the MS-2 medium modification, three types of flours (flours of passion fruit peel, orange flour and soy flour) were used

in different concentrations (w/w), 0.5, 1.5, 2.5 and 3.0%. The medium composition was one of the three types of flours, K_2HPO_4 (0.435 % w/v), and 1 mL of mineral solution containing $FeSO_4{\cdot}7H_2O$ (100 mg), $MnCl_2{\cdot}4H_2O$ (100 mg), and $ZnSO_44{\cdot}H_2O$ (100 mg) and distilled water q.s.p. 100 ml, NH_4Cl (0.1 % w/v), $MgSO_4{\cdot}7H_2O$ (0.06 % w/v), and glucose (1 % w/v). The microorganism was incubated with 10^8 UFC mL^{-1} in the medium. Cultivation was carried out for 48 h on a rotary shaker set at 28 ºC and 150 rpm.

Determination of total protein

Total protein concentration was determined by the Smith et al. 1985 method, using as standard bovine serum albumin (BSA).

Protease activity determination

Protease activity was measured as described by Ginther 1979. Assay mixtures of 1.0 mL, containing 0.2 M Tris hydrochloride, pH 7.2, 10^{-3} M $CaCl_2$, 1% azocasein and 150 µL of spent medium, were incubated at 28 ºC for 1 h. After stopping the reaction by the addition of 1.0 mL of 10% trichloroacetic acid, samples were centrifuged at 1,2000 x g for 15 min, and 0.8 mL of the supernatant was transferred into a second tube containing 0.2 mL of 1.8 N NaOH. Samples were finally blended in a vortex mixer, and the absorbance was measured at 420 nm. One unit of protease activity was defined as the amount of enzyme responsible for a 0.1 increase per hour in the absorbance.

Assay of fibrinolytic activity

Following the method of Astrup & Mullertz 1952, with minor modifications, a fibrin-agarose plate was made with 1% agarose, 0.1% human fibrinogen and 8 U mL^{-1} human thrombin, and 20 µL of crude extract was added to plate 1 h after its preparation. The plate was incubated at 37 °C for 18 h, the diameter of the lytic circle was measured after that time. Next, the area of the circle was calculated (in square millimeters). Following the method of Wang et al. 2011, 500 µL of fibrinogen dissolved in TRIS HCl-NaCl 0.15 M buffer pH 7.75 and 245 mM phosphate pH 7.0 was added to tubes containing 100 µL of 20 U ml^{-1} thrombin [T9326-150UN - Thrombin human, BioUltra, recombinant, expressed in HEK 293 cells, aqueous solution, $\geq$ 95% (SDS-PAGE) - Sigma-Aldrich], at 37 ºC. After 10 min, 100 µL of the crude extract was added to the tube. After 1 h at 37 ºC, the reaction was stopped by adding 700 µL of 0.2 M trichloroacetic acid. The reaction mixture was centrifuged at 15,000 ×g for 10 min, and the absorbency of the sample was read at 275 nm against a sample blank. A unit of fibrin degradation (U) was defined as the amount of enzyme capable of causing an increase of 0.01 per minute in absorbance.

Preparation of the aqueous two-phase systems

The aqueous two-phase systems (ATPS) were prepared at 20 ºC in 15 mL-graduated tubes with phosphate salts and polyethylene glycol (PEG) solutions. A statistical design 24 composed of 16 experiments added with 4 repetitions of the centre point, to estimate experimental errors (Bruns et al. 2006) (Table I) was made where the variables will be checked: pH, the molar mass and the PEG concentration, and the concentration of the phosphate, in the response variables: yield (Y), partition coefficient (K) and purification factor (PF), with four repetitions in the central points. Water was added and 2.0 g of crude extract up to a final weight of 10 g at 20 ºC. After addition of all ATPS components

and vortex shaking for 1min, the top and bottom phases were separated by settling for 60 min, and their respective volumes (Vt and Vb, respectively) measured. Both phases were finally assayed for determinations protein, protease activity and fibrinolytic activity.

Table I. Factor levels of the 2^4-experimental design used for the extraction of protease from *Streptomyces parvulus* DPUA 1573 by PEG/phosphate ATPS.

Variable	Level		
	Low (-1)	Central (0)	High (+1)
[a]MPEG $_{(g/mol)}$	1,500	4,000	8,000
[b]CPEG $_{(\% w/w)}$	12.5	15.0	17.5
[c]CPHO $_{(\% w/w)}$	10.0	12.5	15.0
pH	6.0	7.0	8.0

[a] PEG molar mass.
[b] PEG concentration.
[c] Sodium phosphate concentration.

Gel filtration chromatography

Gel filtration analysis of phase salt was performed using an Akta Avant 25 System (Pharmacia LKB Biotechnology, Uppsala, Sweden) on Superdex – G200 10/300 GL column, fractions were collected at a flow rate of 0.5 mL/min. The run was monitored at 215, 254 and 280 nm, and fractions were collected by automatic collector and the peaks were then submitted to fibrinolytic activity detection. The molecular weight estimation was achieved using the calibration curve of the column and weight markers (1.0 mg mL^{-1}): bovine serum albumin, carbonic anhydrase, albumin from chicken egg and a trypsin inhibitor. The standard curve was plotted using the UNICORN-6.0 software following the equation y= -0.2053x + 4.7654 (R2= 0.9946).

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

According to the Laemmli (1970) methodology, electrophoresis was performed with 12% polyacrylamide gel. The molecular mass marker used was Precision Plus Protein Dual Color Standards, 500 µL #1610374, containing the following molar masses (250, 150, 100, 75, 50, 37, 25, 20, 15, 10 kDa). Silver staining was used to detect protein bands.

Determination of ATPS parameters

The partition coefficient of protease was defined as the ratio of protease activity, expressed in U/mL, in the top phase (FAt) to that in the bottom phase (FAb):

$$K = \frac{FAt}{FAb} \quad (1)$$

The activity yield was determined as the ratio of total activity in the top or bottom phase to that in the crude extract (FAi) and expressed as percentage. For this purpose, FAt or FAb was multiplied by Vt or Vb, respectively, and FAi by the total volume of the crude extract:

$$Yt,b = \frac{FAt,b.Vt,b}{FAi.Vi} x100 \quad (2)$$

The specific activity (SA) was defined as the ratio of the enzyme activity (U/mL) to the protein concentration (mg/mL). The purification factor in the top or bottom phase was calculated as the ratio of the respective specific activity (SAt or SAb) to the specific activity of the crude extract (SAi), all expressed in U/mg:

$$PFt,b = \frac{SAt,b}{SAi} \quad (3)$$

Effect of metal ions, inhibitors, and surfactants

The effects of metal ions, inhibitors and surfactants were investigated by pre-incubating the purified enzyme in the presence of 5 mM of ions Ca^{2+}, Zn^{2+}, Fe^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Na^{+}, K^{+} 5 mM of inhibitors PMSF (fluoridemethylphenylsulfonyl), EDTA (ethylenediaminetetraacetic-acid), β-mercaptoethanol and Iodoacetic acid and 1% of surfactants Tween 20, Tween 80, Triton X-100, and SDS (Sodium dodecyl sulfate) during 1 h at 37 ºC. After incubation, the residual protease activity was measured, and the results were expressed as a percentage of the control activity. All experiments were carried out in triplicates.

Effects of pH and temperature

To measure the effects of pH on the activity, the purified enzyme was maintained for 60 min at 37 °C on the following different buffer solutions (0.1 M): sodium acetate buffer (pH 3.0 to 5.0), citrate phosphate buffer (pH 5.0 to 7.0), Tris–HCl buffer (pH 7.0 to 9.0), and glycine-NaOH buffer (pH 9.0 to 11.0). To measure pH stability, the enzyme was incubated in the buffers separately from pH 3.0 to 11.0 at 37 °C for 0, 30, 60, 120 and 180 min. After, the residual protease activity was determined. The effects of temperature on the activity were determined by incubating the enzyme at different temperatures (10, 20, 30, 37, 40, 50, 60 and 70 ºC) for 60 min and then measuring the residual activity. The thermal stability was investigated by assaying the residual protease activity in pH 7.5 Tris-HCl buffer at 10, 20, 30, 37, 40, 50, 60 and 70 ºC for 0, 30, 60, 120 and 180 min. All experiments were carried out in triplicates.

Experimental design and statistical analysis

A 2^4 full factorial design was utilized to evaluate the influence of four independent variables, namely PEG molar mass (x1), PEG concentration (x2), pH (x3), and phosphate salt concentration (x4) on the parameters of partition coefficient, activity yield and purification factor of the fibrinolytic enzyme. The experimental design included 16 runs and 4 repetitions at the central point, which were necessary to calculate the pure error. A linear regression model was employed to predict the response, according to eq. (4):

$$R = b0 + \sum bixi + \sum bjxj + \sum bijxix \quad (4)$$

where bo is the interception coefficient, bi and bj are the linear coefficients, bij are the interaction coefficients and xi and xj are the independent variables. The goodness of fit of the model was evaluated by the coefficient of determination (R2) and the analysis of variance (ANOVA); the first-order model equation was determined by Fischer's test. The experimental and predicted values were compared and the developed model validated with Statistica 8.0 software (StatSoft Inc., Tulsa, OK, USA).

RESULTS

The production of fibrinolytic protease by *Streptomyces parvulus* DPUA 1573 was carried out using three different substrates (Table II). The medium composed of passion fruit flour produced the greatest amount of fibrinolytic protease.

Table II. Production of fibrinolytic enzyme by *Streptomyces parvulus* DPUA 1573 using different substrates in liquid fermentation.

Substrate	SC (%)	PA[a] (U/mL)	FA[b] (U/mL)	Measures (mm)	FA[c] (mm2)
Orange flour	0.5	11.97 ± 0.52	10.75 ± 0.09	18.0 ± 0.01	254.47
Orange flour	2.5	20.83 ± 8.25	11.46 ± 0.08	20.01 ± 0.02	317.31
Passion fruit flour	0.5	33.70 ± 2.59	11.49 ± 0.53	20.0 ± 0.02	314.16
Passion fruit flour	2.5	33.93 ± 0.19	15.46 ± 0.15	20.01 ± 0.01	317.16
Soy flour	1.5	3.72 ± 0.19	3.48 ± 0.15	0	0
Soy flour	3.0	6.23 ± 0.19	8.71 ± 0.32	0	0

a Protease activity; b Fibrinolytic activity described by Wang et al. (2011); c Fibrinolytic activity described by Astrup & Mullertz (1952). All experiments were carried out in triplicates.

The results of protease extraction by PEG/Phosphate ATPS (Table III) reveal the preference of fibrinolytic protease for PEG-rich phase (top phase) for all assays (K > 1). The highest purification factor in the top phase value was found at C_{PEG} 17.5%, M_{PEG} 8,000 g mol^{-1} and pH 8.0 (assays 8 and 16). However, the best condition was found in assay 16, according to the combination of the highest specific activity index, partition coefficient, purification factor, yield and superior mass balance index.

The statistical analysis displayed the influence of the independent variables, namely pH, PEG molar mass (M_{PEG}), PEG concentration (C_{PEG}) and phosphate salt concentration (C_{PHO}) in the partition coefficient (K) and purification factor (FP) (Figure 1).

The chromatography profile of the fibrinolytic protease was verified by gel filtration chromatography (Figure 2). The enzyme was monitored under different conditions of absorbance 215, 254 and 280 nm. As can be seen, the absorbance peak appears in the range of 16 to 22 mL. The molecular weight of the enzyme was estimated to be approximately 42.65 kDa.

The activity of protease was partially inhibited by Zn^{2+} and was enhanced by Fe^{2+}. The activity was significantly suppressed by β-Mercaptoethanol, PMSF, and Iodoacetic acid (Table IV).

Table III. Production of fibrinolytic enzyme by *Streptomyces parvulus* DPUA 1573 using different substrates in liquid fermentation.

RUNS	M_{PEG} ($g.mol^{-1}$)	C_{PEG} (w/w)	C_{PHO} (w/w)	pH	PA_t	PA_b	K	Y_t	Y_b	FP_t	FP_b	$MB_{protein}$ (%)
1	1500	12.5	10	6	6.03	3.57	1.69	37.34	26.28	1.11	3.79	81.23
2	8000	12.5	10	6	7.40	3.67	2.02	41.44	31.34	1.31	4.08	78.65
3	1500	17.5	10	6	7.83	1.70	4.61	54.25	10.52	1.50	2.06	82.57
4	8000	17.5	10	6	6.67	1.80	3.70	44.21	13.26	1.66	3.25	61.36
5	1500	12.5	10	8	-	-	-	-	-	-	-	-
6	8000	12.5	10	8	8.83	2.37	3.73	52.07	17.44	1.64	1.89	81.9
7	1500	17.5	10	8	-	-	-	-	-	-	-	-
8	8000	17.5	10	8	7.20	1.70	4.24	46.68	12.02	2.33	1.49	56.21
9	1500	12.5	15	6	9.30	1.07	8.72	49.34	7.54	1.33	9.28	75.55
10	8000	12.5	15	6	8.33	1.80	4.63	36.84	15.92	1.49	10.14	52.56
11	1500	17.5	15	6	7.53	1.00	7.53	51.07	5.89	1.70	0.0	59.78
12	8000	17.5	15	6	7.23	1.20	6.03	34.11	10.26	1.72	0.0	36.49
13	1500	12.5	15	8	11.17	1.20	9.31	42.78	9.37	1.72	1.31	64.07
14	8000	12.5	15	8	9.57	1.53	6.24	42.29	14.46	1.86	1.93	60.51
15	1500	17.5	15	8	-	-	-	-	-	-	-	-
16	8000	17.5	15	8	10.27	1.40	7.33	57.49	10.73	2.10	1.58	68.48
17*	4000	15	12.5	7	9.03	1.70	5.31	50.58	13.03	1.68	3.32	67.98
18*	4000	15	12.5	7	9.03	1.70	5.31	50.58	13.03	1.65	4.40	67.11
19*	4000	15	12.5	7	8.53	1.73	4.92	47.78	13.54	1.59	3.39	68.13
20*	4000	15	12.5	7	8.53	1.73	4.92	47.78	13.54	1.56	4.48	67.23

M_{PEG} = PEG molar mass; C_{PEG} = PEG concentration; C_{PHO} = Sodium sulfate concentration; PA_t = Protease activity in the top phase; PA_b = Protease activity in the bottom phase; K = Partition coefficient; Y_t = Activity yield in the top phase; Y_b = Activity yield in the bottom phase; PF_t = Purification factor in the top phase; PF_b = Purification factor in the bottom phase; $MB_{protein}$ = Protein mass balance; * Central point system.

Results on the effect of pH on enzyme stability can be observed in Figure 3a. The optimum pH of protease was 7.0, and it was stable for 120 minutes at this pH (Figure 3b). The protease was stable in the pH 7.0 to 8.5 range.

The optimum temperature for enzymatic activity was 40 ºC (Figure 4a) and the protease was stable from 30 ºC to 60 ºC. The enzyme was stable for 30 minutes (Figure 4b) at 40 ºC, with residual activity of 85%.

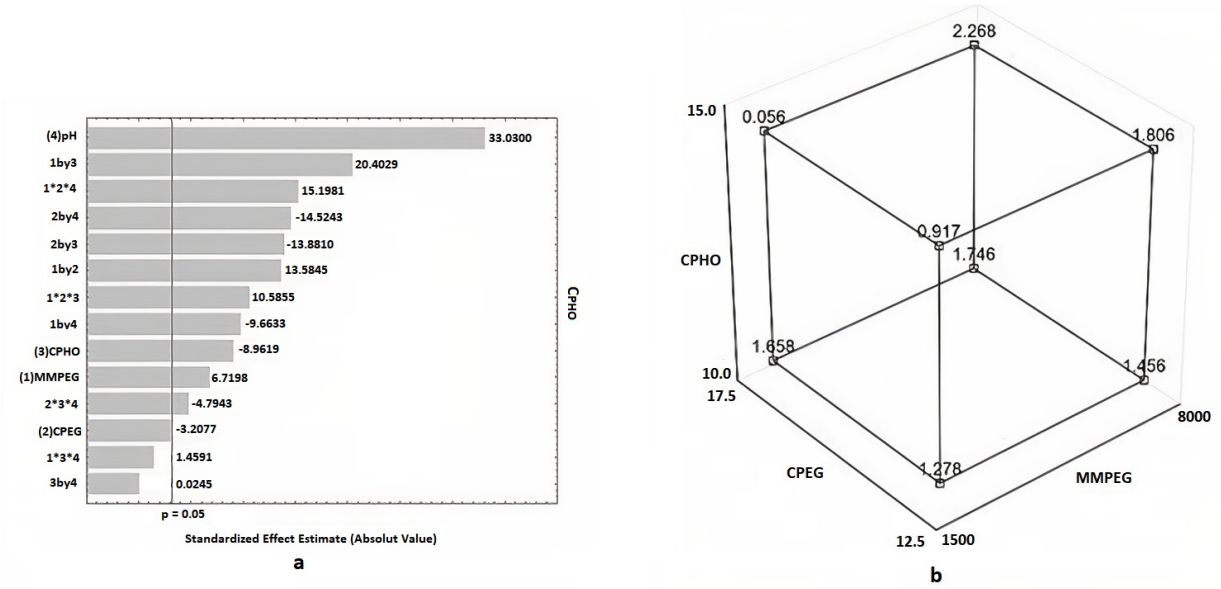

Figure 1. a - Effects of the independent variables (M_{PEG}, C_{PEG}, C_{PHO} and pH) on the partition coefficient (K) of fibrinolytic protease extracted by PEG/ phosphate ATPS according to the preliminary 2^4 full factorial design. b - Cubic plot of the effects of M_{PEG} and C_{PEG} and C_{PHO} on the purification factor (FP) in the PEG phase ATPS. M_{PEG} (1) = PEG molar mass; C_{PEG} (2) = PEG concentration; C_{PHO} (3) = phosphate concentration; pH (4) = potential of hydrogen.

DISCUSSION

It is known that the enzyme production by microorganisms is influenced by fermentation medium components, especially carbon and nitrogen sources and also by physical factors such as temperature, pH, incubation time and inoculum density. Furthermore, it is important to produce enzymes in inexpensive mediums that can be optimized on a large scale to make the process commercially viable. In this regard, different agro-industrial wastes, such as wheat bran, rice bran, green grass, ragi and corn flour can be used for enzyme production by microorganisms (Ravikumar et al. 2012).

It was evaluated three different types of substrates: orange peel flour, passion fruit flour and soy flour. As seen in Table II, the highest protease and fibrinolytic activity were obtained with the passion fruit flour 0.5 and 2.5%, the activity values were very close and without statistical significance. The crude extract obtained with the passion fruit flour 0.5% was selected because a less amount of substrate it was possible to obtain a crude extract with fibrinolytic activity very similar to using a larger amount of substrate, which makes the process more economical.

Other researchers have also tested different types of substrates for fibrinolytic enzymes production, such as Nascimento et al. 2015, who used different substrates, such as passion fruit peel, corncob, cassava peel, soybeans, Malpighia emarginata seeds, wheat bran and citrus pulp, it was obtained the higher fibrinolytic enzymes production by *Mucor subtillissimus* UCP 1262 using soy and wheat bran. Vijayaraghavan & Vincent 2015 used banana peel, cow manure, rice bran, wheat bran and green grass as substrates for fibrinolytic enzymes production by *Shewanella* sp, it was obtained the best results with cow dung and green grass. Ravikumar et al. 2012, using *Pleurotus sajor-caju* with different substrates (wheat bran, rice bran, green grass, corn flour and ragi) for protease production, obtained better results with corn flour.

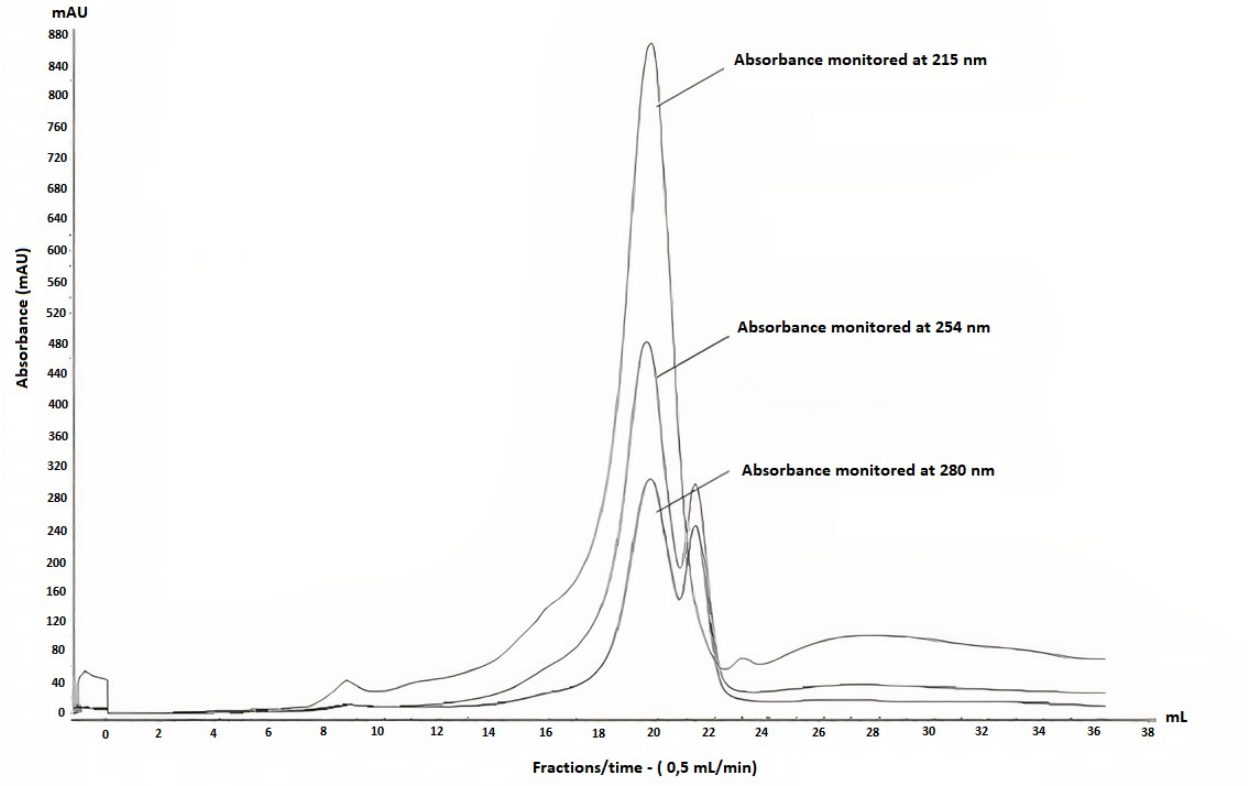

Figure 2. Chromatogram of fibrinolytic protease produced by *Streptomyces parvulus* DPUA 1573 and extracted by aqueous two-phase system.

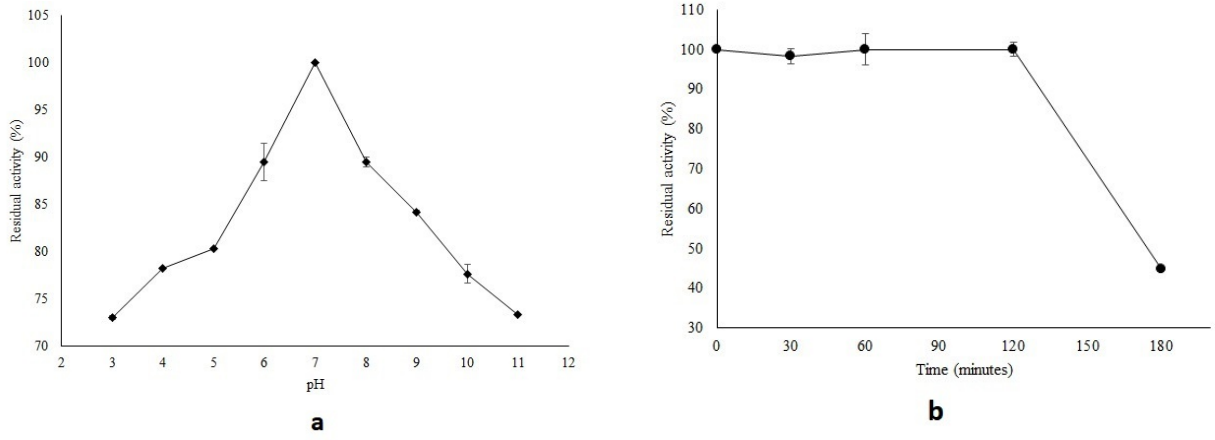

Figure 3. a - Effect of pH on enzyme activity. b - Stability of enzyme activity at pH 7,0.

Fibrinolytic activity produced from *Streptomyces parvulus* DPUA 1573 (317.31 mm^2) was higher than fibrinolytic activity found by Silva et al. 2016 (304 mm^2) from *Streptomyces* sp. DPUA 1576 and very close

Table IV. Effect of metal ions, protease inhibitors and surfactants on the enzyme activity.

Metal ion, inhibitors and surfactants	Residual activity (%)
Control	100.00
Ca2+	96.91 ± 4.36
Zn2+	76.23 ± 18.76
Fe2+	155.86 ± 6.54
Cu2+	96.91 ± 5.23
Mg2+	118.18 ± 0.01
Na+	106.82 ± 16.07
K+	104.55 ± 0.01
Tween 20	76.23 ± 11.78
Tween 80	108.02 ± 0.87
Triton X100	75.62 ± 12.65
SDSa	79.63 ± 1.74
EDTAb	104.55 ± 7.42
PMSFc	25.00 ± 16.07
β-Mercaptoethanol	9.09 ± 6.42
Iodoacetic acid	29.55 ± 3.21

SDS – Sodium Dodecyl Sulphate; EDTA – Ethylenediamietetraacetic acid; PMSF – Phenylmethylsulfonyl fluoride. All experiments were carried out in triplicates.

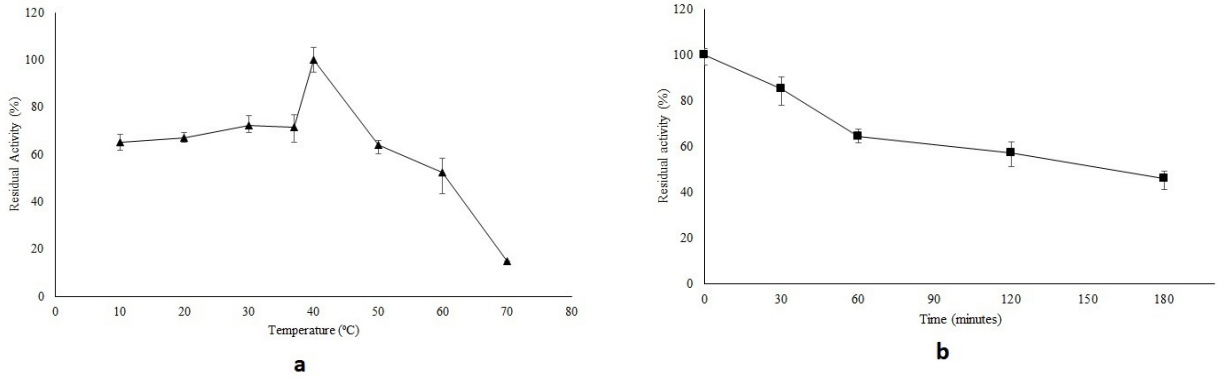

Figure 4. a - Effect of temperature on enzyme activity. b - Stability of enzyme activity at 40ºC.

to it was obtained by Nascimento et al. 2017 (125 mm^2) from *Mucor subtilissimus* UCP 1262 and less than obtained by de Souza et al. 2016 (660.52 mm^2) from *Bacillus amyloliquefaciens*.

Table III displays the results of extracting by ATPS, the assays 05, 07 and 15 did not show the separation of two phases. This behavior was explained by a shift in the binodal curve after the addition of crude extract, which caused critical points in upper and lower phases that were not sufficient for

phase formation (Silva et al. 2018). In all the remaining assays, the enzyme predominated in the top phase, demonstrated by the partition coefficient (K) greater than zero.

The partitioning of proteins by ATPS is influenced by several factors, such as the size and conformation of biomolecules, molecular charge, ionic properties, phase composition, electrical potential between phases, concentration of polymers and molecular size of the polymer (Ali et al. 2014, da Silva et al. 2018).

The variable with the greatest effect on partition coefficient was pH (Fig. 1), the pH induces the partitioning of proteins by altering biomolecules charge or proportion of charged (da Silva et al. 2017), it is greater the influence on the enzyme partition to the PEG phase the higher the pH value.

The interaction between variables PEG molar mass and salt concentration was also the most significant interaction, this means these variables exerted a synergistic effect, so K was improved when these variables had their levels increased to highest values. In the highest concentration of PEG (8,000), the enzyme was preferably partitioned in the top phase, a PEG-rich phase, probably because of enzyme hydrophobicity, since the PEG-rich phase is more hydrophobic than the salt-rich phase, combined with an effect "salting out" (salt-rich phase ionic strength) that caused decrease on protein solubility in the bottom phase. According to da Silva et al. 2018, the hydrophobic characteristics of the protein are considered the dominant influencing factor on partitioning efficiency.

This result, the enzyme being predominant in the top phase, agrees with that obtained in other studies where ATPS were used to purify fibrinolytic proteases, such as Silva et al. 2013 and Ali et al. 2014, who extracted fibrinolytic enzymes from *Streptomyces* sp., and *Auricularia polythicha*, respectively. Da Silva et al. 2017 extracted proteases from *Aspergillus tamarii* URM4634 and the protease showed high activity in PEG-rich phase, also with the variables PEG molar mass and salt concentration having positive effects on the partition coefficient. However, Sales et al. 2013 and Nascimento et al. 2016 extracting fibrinolytic proteases from *Bacillus* sp. UFPEDA 485 and *Mucor subtilissimus* UCP 1262, respectively, found high activity in the salt-rich phase.

The purification factor obtained by ATPS in best condition (2.10) was very close to that obtained by da Silva et al. 2018 (2.14) using ATPS PEG/Citrate, it was also higher than that found by Silva et al. 2013 (1.51) using ATPS PEG/Phosphate and lower than that found by Nascimento et al. 2016 (4.5) using ATPS PEG/Sulfate and da Silva et al. 2017 (3.95) using ATPS PEG/Citrate.

In the chromatography profile (Figure 2) it was possible to estimate the molecular weight of the fibrinolytic enzyme produced by *Streptomyces parvulus* DPUA 1573 in approximately 42.65 kDa, therefore, SDS-PAGE (Figure 5) shows it just one band with molar mass 40 kDa, thus, proving the effectiveness of purification methods and so which was close to fibrinolytic enzymes from marine algae *Codium* sp. (30–50 kDa) (Matsubara et al. 2000, Banerjee et al. 2013) and *Streptomyces* sp. CS684 (35kDa) (Simkhada et al. 2010).

As shown in Table IV, the enzyme activity was enhanced by the addition of Fe^{2+}, with 55.86% increase on enzyme activity. Moreover, the enzyme activity decreased 23.77% in the presence of Zn^{2+}. On the other hand, the activity of fibrinolytic proteases from *Xylaria curta* (Meshram et al. 2017) and from *Bacillus amyloliquefaciens* Jxnuwx-1 (Yang et al. 2019) were completely inhibited by Fe^{2+}. The enzyme was significantly inhibited by β-Mercaptoethanol, with a decrease of 90.91%, by PMSF, with a decrease of 75% and by Iodoacetic acid, with a decrease of 70.45%. These results suggest that the fibrinolytic enzyme under consideration is a serine protease. Similar outcome was described by Wang

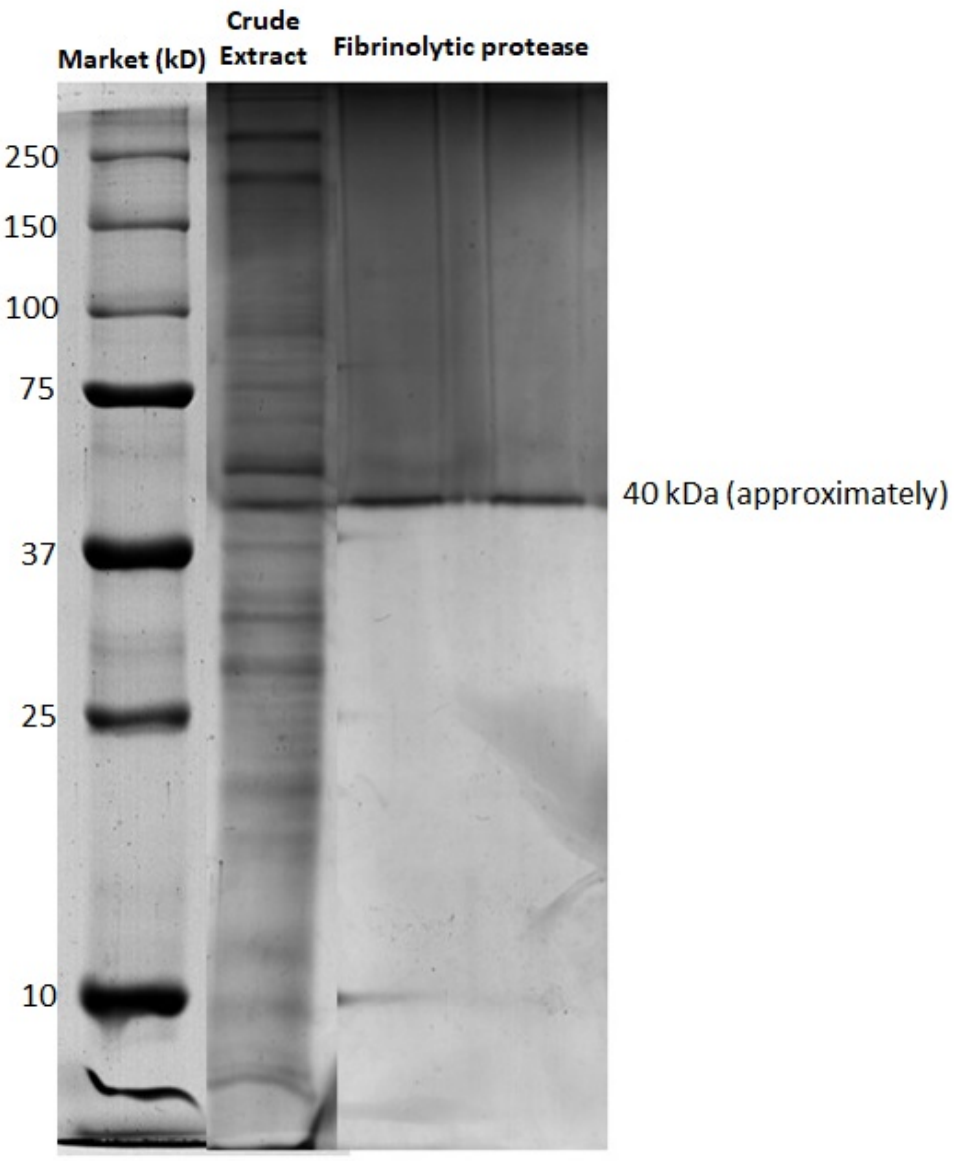

Figure 5. Molecular mass determination of the fibrinolytic protease by SDS-PAGE (12%) with protein molecular mass marker (Precision Plus Protein Dual Color Standards). Quantity of the fibrinolytic protease (µg): Crude Extract and fibrinolytic protease (5 µg lyophilized fibrinolytic enzyme).

et al. 2011, Ju et al. 2012, Moon et al. 2014, Nascimento et al. 2017 and Elhoul et al. 2015 from *Bacillus subtilis* TKU007, *Streptomyces* sp. XZNUM 00004, *Lyophyllum shimeji*, *Mucor subtilissimus* UCP 1262 and *Streptomyces koyangensis* TN650, respectively. The literature indicates that most of fibrinolytic enzymes of microbial origin are serine proteases (Sun et al. 2016).

Enzymes are usually inactivated by adding surfactants to the reaction solution by the action of the surfactants in the enzyme, causing a conformation change, thus decreasing the accessibility of the substrate (Wang et al. 2011). The protease activity from *Streptomyces parvulus* DPUA 1573 was moderately inhibited by Triton X100, Tween 20% and SDS, with a decrease of 24.38%, 23.77% and 20.37%, respectively.

The activity profile of purified protease was analyzed with various pH (Figure 3). The purified protease displayed an optimal pH of 7.0, which is very close to human physiological pH (almost pH 7.4) (Sun et al. 2016). It was stable in the pH range of 7.0–8.5, retaining 90% of its activity. The optimum pH was compatible with the findings of Deng et al. 2018 with proteases obtained from *Neurospora sitophila* and Yang et al. 2019 from *Bacillus amyloliquefaciens* and Hu et al. 2019 from *Bacillus subtilis* DC27. In optimum pH (7.0), the enzyme remained stable for 120 minutes (Figure 3b).

As shown in Figure 4a, the optimum temperature for enzymatic activity was 40 ºC. It exhibited thermostability in the temperature range of 30 – 60 °C, retaining > 80% of relative activity after a 30 min incubation at 40 ºC (Figure 4b). The optimal temperature agrees with Sun et al. 2016, Xin et al. 2018, Yang et al. 2019, Hu et al. 2019 and Silva et al. 2016 of fibrinolytic enzymes from *Streptomyces* sp. CC5, *Bacillus tequilensis*, *Bacillus amyloliquefaciens*, *Bacillus subtilis* DC27 and *Streptomyces* sp. CS684, respectively.

In 2017, Batista et al. carried out the biochemical characterization of fibrinolytic proteases from *Streptomyces parvulus* DPUA 1573, obtaining some different results from that found in the present study. However, in this work it was used passion fruit flour 0.5% as substrate. Batista et al. 2017 used soy flour 1.5% as a substrate, which could eventually induce the microorganism to produce enzymes with different biochemical characteristics, since the percentage of nitrogen and carbon are different in each type of substrate. Furthermore, in the studies carried out by Batista et al. 2017, the biochemical characterization tests were performed with the enzyme contained in the crude extract, so other enzymatic complexes also present could interfere with the results. In our work, the biochemical characterization was conducted with the enzyme obtained after the purification step using ATPS, which selected and extracted a specific fibrinolytic enzyme from the pool of enzymes contained in the crude extract.

CONCLUSIONS

To conclude, the fibrinolytic protease from *Streptomyces parvulus* DPUA 1573 isolated from Amazonian lichens was produced, purified by ATPS and characterized. Passion fruit flour (0.5% w/w) was used as an inexpensive substrate to produce fibrinolytic enzyme. The ATPS PEG/Phosphate in composition of 17.5% PEG 8,000 g mol^{-1}, 15% Phosphate (w/w) and pH 8.0 recovers serine protease from *Streptomyces parvulus* DPUA 1573 with purification factor of 2.10-fold and yield of 57.49%. The ATPS showed good performance as a first step for the purification of fibrinolytic protease from *Streptomyces parvulus* DPUA 1573. Furthermore, the optimal pH and temperature range was very close to human physiology. Therefore, the enzyme proved to be a viable option for the development of a possible drug with fibrinolytic action.

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