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Purification of a fibrinolytic protease from *Mucor subtilissimus* UCP 1262 by aqueous two-phase systems (PEG/sulfate)

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ABSTRACT

A fibrinolytic protease from *M. subtilissimus* UCP 1262 was recovered and partially purified by polyethylene glycol (PEG)/sodium sulfate aqueous two-phase systems (ATPS). The simultaneous influence of PEG molar mass, PEG concentration and sulfate concentration on the enzyme recovery was first investigated using a 2^3 full factorial design, and the Response Surface Methodology used to identify the optimum conditions for enzyme extraction by ATPS. Once the best PEG molar mass for the process had been selected (6000 g/mol), a two-factor central composite rotary design was applied to better evaluate the effects of the other two independent variables. The fibrinolytic enzyme was shown to preferentially partition to the bottom phase with a partition coefficient (K) ranging from 0.2 to 0.7. The best results in terms of enzyme purification were obtained with the system formed by 30.0% (w/w) PEG 6000 g/mol and 13.2% (w/w) sodium sulfate, which ensured a purification factor of 10.0, K of 0.2 and activity yield of 102.0%. SDS–PAGE and fibrin zymography showed that the purified protease has a molecular mass of 97 kDa and an apparent isoelectric point of 5.4. When submitted to assays with different substrates and inhibitors, it showed selectivity for succinyl-L-ala-ala-pro-L-phenylalanine-*p*-nitroanilide and was almost completely inhibited by phenylmethylsulfonyl fluoride, behaving as a chymotrypsin-like protease. At the optimum temperature of 37° C, the enzyme residual activity was 94 and 68% of the initial one after 120 and 150 min of incubation, respectively. This study demonstrated that *M. subtilissimus* protease has potent fibrinolytic activity compared with similar enzymes produced by solid-state fermentation, therefore it may be used as an agent for the prevention and therapy of thrombosis. Furthermore, it appears to have the advantages of low cost production and simple purification.

1. Introduction

Mucor subtilissimus is a dimorphic fungus of the phylum of Zygomycota that can switch between yeast and filamentous growth structures depending on the environmental stimulus received [1]. This fungus belongs to the order of Mucorales also comprising the genera *Rhizopus*, *Mucor*, *Rhizomucor*, *Cunninghamella* and *Absidia*, which are known to have a potential for the production of milk-clotting enzymes, lipases, amylases and extracellular proteases [2].

Proteases are a large and complex group of enzymes that play an important nutritional and regulatory role in nature [3]. These enzymes are considered the most important group of industrial enzymes with a great variety of industrial and biotechnological applications accounting for about 60% of the total enzyme

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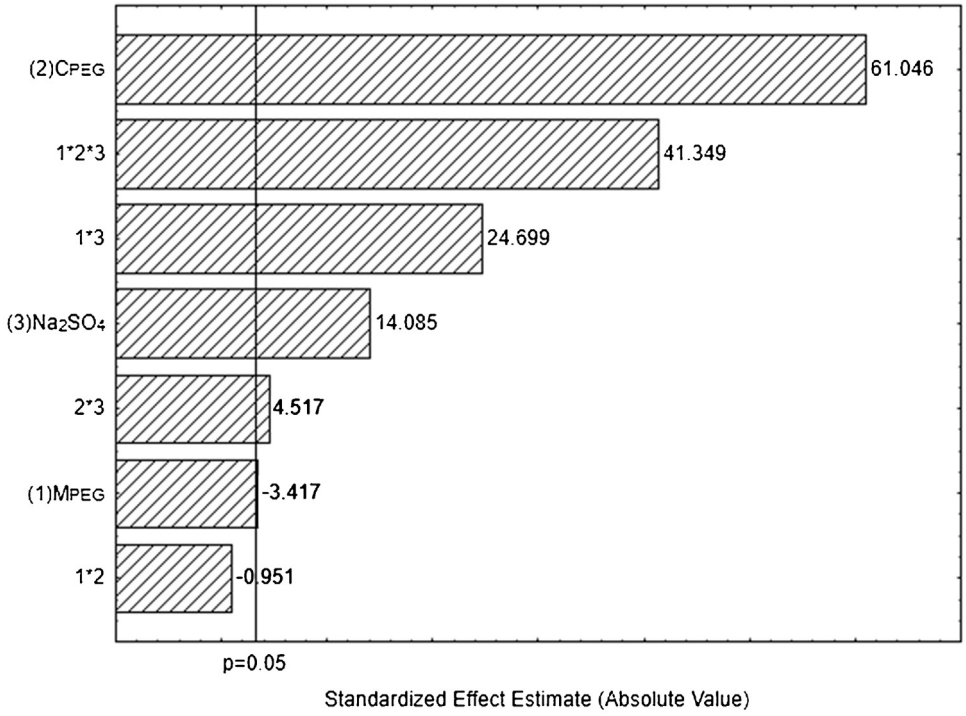

Fig. 1. Effects of the independent variables (M_{PEG}, C_{PEG} and C_{Na2SO4}) on the partition coefficient (K) of fibrinolytic protease extracted/recovered by PEG/sodium sulfate ATPS according to the preliminary 2^3 full factorial design.

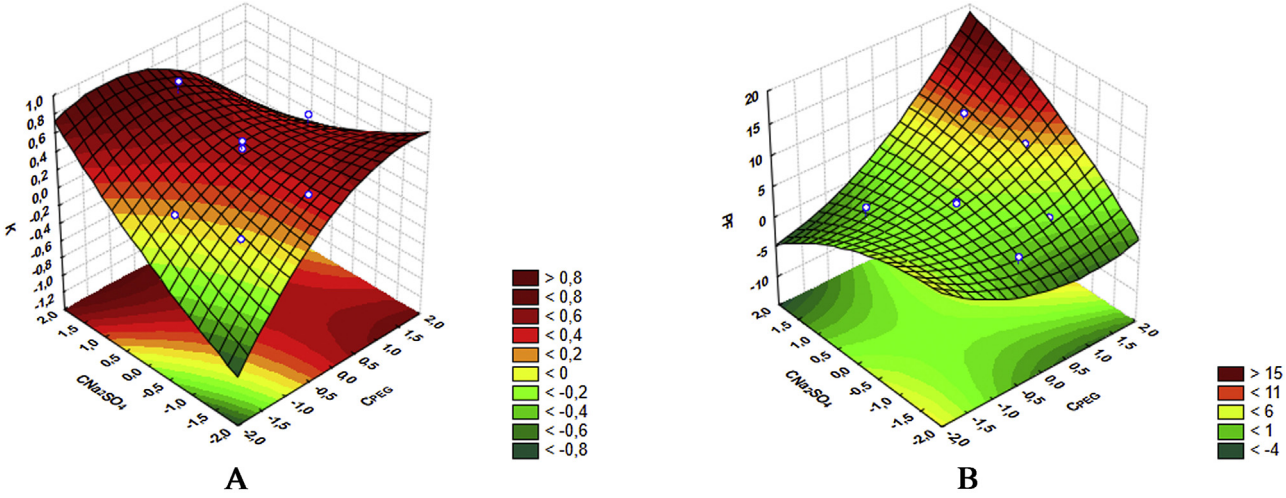

Fig. 2. Three-dimensional contour plots showing the interactive effects of the concentrations of PEG (C_{PEG}) and sodium sulfate (C_{Na2SO4}): (A) on the partition coefficient (K) and (B) purification factor (PF) of fibrinolytic protease.

market worldwide and 40% of the total enzyme sales [3–5]. Fibrinolytic proteases are proteases that degrade fibrin, the major protein component of blood clots, whose accumulation leads to thrombosis that is responsible for cardiovascular diseases including myocardial infarction [6].

In recent years, several studies have been made to search for new sources of thrombolytic agents such as bacteria, algae, plants, worms, snake venom, insects, and fungi [6–10]. Filamentous fungi have been shown to be a good choice for the production of fibrinolytic enzymes, since they produce them in large amounts mostly extracellularly, which makes their extraction/purification easier [11].

Partitioning in aqueous two-phase systems (ATPS) is an effective method for separating and purifying mixtures of biomolecules [12–15], which is suitable for large-scale production in an environmentally friendly way. Traditional techniques such as chromatography and ammonium sulfate precipitation are slowly being replaced, especially in the primary steps of proteases purification, by extraction/purification methods like ATPS, because of their lower costs of production and operation [6,12,16,17].

Aiming to optimize the conditions of purification of these enzymes, to reduce their costs, the amount of work and time, some theoretical tools have been used. Among these, the Response Surface Methodology (RSM) is a collection of mathematical and statistical techniques that are useful for modeling and analysis in applications where a response of interest (or output) is influenced by several factors. The objective of RSM is to optimize such a response [18–20] through experimental design, model fitting,

validation and optimization steps, using a minimum number of experiments for a large number of factors. It was already applied successfully to improve the production and/or purification of several enzymes [21,22].

Based on such a background, the objective of this study was to select the optimal operation conditions for the purification of fibrinolytic protease from *Mucor subtilissimus* UCP 1262 by ATPS using RSM.

2. Material and methods

2.1. Microorganism

The filamentous fungi *Mucor subtilissimus* UCP 1262 used in this study was isolated from the soil of Caatinga, PE-Brazil, deposited and gentle provided by the Catholic University of Pernambuco (UCP) Culture Collection. It was maintained on Czapek medium at 30 °C for 7 days and stored in mineral oil.

2.2. Preparation of inoculum

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

2.3. Production of fibrinolytic protease by solid-state fermentation

125 mL-Erlenmeyer flasks containing 3.0 g of wheat bran as a substrate (moisture content of 50%) were sterilized by autoclaving at 121 °C, 1 atm for 20 min, inoculated with the above suspension of *M. subtilissimus* UCP 1262 spores, and incubated at 25 °C for 72 h. Both the microorganism and the substrate were chosen based on previous work [23].

2.4. Enzyme extraction

Enzyme extraction was performed after 72 h of fermentation. For this purpose, 7.5 mL of 245 mM sodium phosphate buffer, pH 7, were added per g of substrate, and the flasks were placed in an orbital shaker at 150 rpm for 90 min at room temperature, as previously described [23]. Samples were then centrifuged at 3500 rpm for 10 min, and the supernatant was used for determination of protease and fibrinolytic activities.

2.5. Protease activity

Protease activity was measured as described by Ginther [24]. 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 °C for 1 h. After stopping the reaction by the addition of 1.0 mL of 10% trichloroacetic acid, samples were centrifuged at 3000*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.

2.6. Fibrinolytic activity

The fibrinolytic activity (*FA*) was determined using the spectrophotometric method described by Wang et al. [25]. Briefly, 0.4 mL of 0.15 M TRIS HCl-NaCl, pH 7.75, with 0.72% fibrinogen was placed in a test tube containing 0.1 mL of 245 mM phosphate buffer, pH 7, and incubated at 37 °C for 5 min. After addition of 0.1 mL of 20 U/mL thrombin solution and further incubation for 10 min, 0.1 mL of diluted enzyme solution was added. After addition of 0.7 mL of 0.2 M trichloroacetic acid, the resulting solution was incubated at 37 °C for 1 h and homogenized for 20, 40 and 60 min. The reaction mixture was then centrifuged at 15,000 × *g* for 10 min, and 1.0 mL of the supernatant collected to read the absorbance at 275 nm. One 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.

2.7. Protein determination

Total protein content of samples was determined according to Bradford [26] using Coomassie Brilliant Blue G-250 as a dye and Bovine Serum Albumin as a standard. Each experiment was performed in triplicate, and the results, after correction against blank samples, were expressed as mean values.

2.8. Preparation of the aqueous two-phase systems

Aqueous two-phase systems (ATPS) were prepared at 25 ± 1 °C in 15 mL-graduated tubes with equal masses of sodium sulfate and PEG solutions with different concentrations. Water was added to 2.0 g of crude extract up to a final weight of 10 g at 37 °C. After addition of all ATPS components and vortex shaking for 1.0 min, the top and bottom phases were separated by settling for 60 min, and their respective volumes (V_t and V_b, respectively) measured. Both phases were finally assayed for protein and fibrinolytic activity determinations.

2.9. Determination of ATPS parameters

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

$$K = \frac{FA_t}{FA_b} \quad (1)$$

The activity yield was determined as the ratio of total activity in the top or bottom phase to that in the crude extract (FA_i) and expressed as percentage. For this purpose, FA_t or FA_b was multiplied by V_t or V_b, respectively, and FA_i by the total volume of the crude extract (V_i):

$$Y_{t,b} = \left(\frac{FA_{t,b}.V_{t,b}}{FA_i.V_i}\right) \times 100 \quad (2)$$

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 (SA_t or SA_b) to the specific activity of the crude extract (SA_i), all expressed in U/mg:

$$PF_{t,b} = \frac{SA_{t,b}}{SA_i} \quad (3)$$

2.10. Factorial design for preliminary selection of purification parameters

The influence of the independent variables, namely PEG molar mass (M_{PEG}), PEG concentration (C_{PEG}), and sodium sulfate concentration (C_{Na2SO4}) on the responses, namely *K*, FA_b, Y_b and PF_b, was first investigated according to a 2^3 factorial design with four

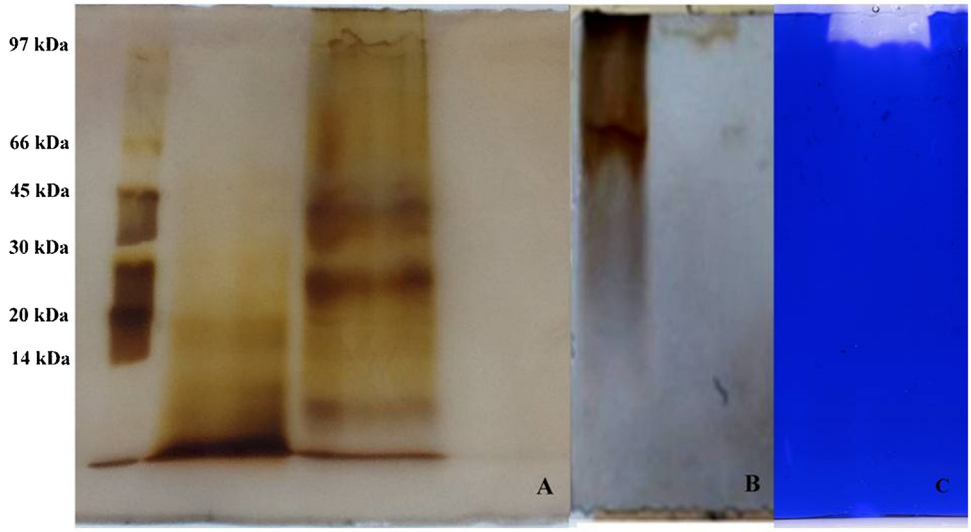

Fig. 3. Molecular mass determination of fibrinolytic protease by SDS-PAGE (12%) and fibrin zymography. (A) SDS-PAGE: Lane 1, protein molecular mass marker; Lane 2, precipitated crude extract; Lane 3, crude extract. (B) SDS-PAGE: Lane 1, protein molecular mass marker; Lane 2, PEG-rich top phase of fibrinolytic protease recovered by ATPS; Lane 3, salt-rich bottom phase of fibrinolytic protease recovered by ATPS. (C) Fibrin zymography (12%) of fibrinolytic protease partitioned to the bottom phase of ATPS.

replicates at the central point to allow for the estimation of pure experimental error [27].

2.11. Determination of the optimal conditions for proteases purification by two-factor central composite rotary design

To find the optimum conditions for purification of fibrinolytic protease by ATPS, we used a two-factor central composite rotary design (CCRD), where C_{PEG} and C_{Na2SO4} were selected as the independent variables, and the fibrinolytic activity, activity yield and purification factor in the bottom phase and the partition coefficient as the responses. Such a design was centered on those values of variables that had ensured the highest purification factor according to the previous factorial design. The coded values for the independent variables were −1.4142 (lowest level), −1, 0, +1, and +1.4142 (highest level). The complete design consisted of 12 experiments with four replicates at the central point carried out in random order. The Response Surface Methodology (RSM) was used to better visualize and compare results [18–21].

2.12. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a 12% polyacrylamide running gel according to the method of Laemmli [28]. The molecular mass was calibrated using as a standard a molecular mass marker (GE Healthcare 17044601, São Paulo, SP, Brazil) containing (a) phosphorylase b: 67 μg, relative molecular mass (Mr) of 97,000, rabbit muscle (source), (b) albumin: 83 μg, 66,000 (Mr), bovine serum (source), (c) ovalbumin: 147 μg, 45,000 (Mr), chicken egg white (source), and (d) carbonic anhydrase: 83 μg, 30,000 (Mr), bovine erythrocyte (source). Protein bands were detected by staining with Coomassie Brilliant Blue G-250.

2.13. 2D two-dimensional electrophoresis

The fibrinolytic enzyme purified by ATPS was dialyzed in 24 mM buffer sodium phosphate, pH 7.0, lyophilized and resolubilized in buffer-2 [7 M urea; 2 M thiourea; 85 mM DL-dithiothreitol (DTT); 2.5% (v/v) Triton X–100 ($C_{34}H_{62}O_{11}$); 0.5% (v/v) Pharmalyte™; 10% (v/v) isopropanol]. The resulting solution was used to rehydrate 18 cm-long strips (IPG ReadyStrip, Bio-Rad, Rio de Janeiro, Brazil) containing fixed, linear pH gradient (IPG), pH 3–10/pH 4–7, for 17 h. The rehydrated strips were subjected to isoelectric focusing in IPGPhor system (GE Healthcare, São Paulo, SP, Brazil) in manifold system. The focus was performed in four successive steps that depended on the pH range at which the focus was carried out, namely pH 3–10: step-and-hold 500 V/1H, gradient of 500V–1000 V/1 h, gradient–1000 V 8000 V/3h45, and step-and-hold 8000 V/45 min. Strips were then put into a solution containing DTT to disrupt disulfide bridges (S—S bonds) present in the protein, SDS to facilitate the access to the internal parts of the protein due to disruption of its tertiary structure, and iodoacetamide to avoid reoxidation of thiol groups (alkylation).

2.14. Fibrin zymography

Fibrinolytic activity was analyzed by means of a fibrin zymography gel as described by Kim et al. [29]. Fibrinogen and thrombin were mixed with 12% polyacrylamide gel solution, and electrophoresis of protease solution was carried out. A molecular mass marker was uses as a standard. After electrophoresis, the gel was washed with 2.5% Triton X-100 for 1 h, rinsed thrice with distilled water, and incubated in the reaction buffer (0.1 M glycine, pH 8.4) at 37 °C for 18 h. The gel was stained with Coomassie blue for 1 h and then destained. The digested bands were visualized as the non-stained regions of the fibrin gel.

2.15. Amidolytic activity

Amidolytic activity was measured as described by Kim et al. [30] using synthetic substrates, namely succinyl-L-ala-ala-pro-L-phenylalanine-*p*-nitroanilide (SAPNA, S7388 Sigma), gly-arg-*p*-nitroanilide dihydrochloride (G8148 Sigma) and Nα-benzoyl-DL-arginine 4-nitroanilide hydrochloride (B4875 Sigma).

2.16. Effect of metal ions on protease activity

Protease activity of the enzyme purified from the salt-rich bottom phase was evaluated in the presence of metal ions described as inhibitors or activators of protease activity, whose effect was evaluated at concentrations of 2.5, 5.0 and 10 mM. The crude extract was exposed to the following ions: zinc (Zn^{++}), magnesium (Mg^{++}), copper (Cu^{++}), ferrous (Fe^{++}), calcium (Ca^{++}), manganese (Mg^{++}),

sodium (Na^+), potassium (K^+) and cobalt (Co^{++}),·and incubated at 37 °C for 60 min. The ions were dissolved in TRIS-HCl, pH 7.75, containing 150 mM NaCl.

2.17. Effect of inhibitors on protease activity

To evaluate the effect of inhibitors on protease activity, the enzyme purified from the salt-rich bottom phase was exposed to the following inhibitors: phenylmethylsulfonyl fluoride (PMSF), 2-mercaptoethanol, ethylenediaminetetraacetic acid (EDTA-acetic-$C_{10}H_{16}N_2O_8$), pepstatin A and iodoacetic acid. Each inhibitor was dissolved according to the protocol provided by Sigma and incubated for 60 min at 37 °C with the enzyme solution at a fixed concentration (5.0 mM).

2.18. Effect of temperature on protease activity and stability

The effect of temperature was investigated by incubating the enzyme purified from the salt-rich bottom phase at the temperatures 10, 20, 30, 40, 50, 60, 70, 80 and 90 °C. To determine the stability to temperature, aliquots were withdrawn at the beginning and after 15, 30, 60, 90, 120, 150 and 180 min and then submitted to determination of protease activity.

2.19. Statistical analysis

Statistical analysis of results obtained through the factorial experimental design and central composite rotary design were performed using the software Statistical 8.0. In order to compare the means of the effects of inhibitors on protease activity, we used the Student *t*-test for independent samples. The results were considered statistically significant at $p \leq 0.05$.

3. Results and discussion

3.1. Preliminary selection of ATPS for fibrinolytic protease extraction

The experimental results of the partition coefficient (K) of the fibrinolytic protease listed in Table 1 show that in most of the PEG/Na_2SO_4 ATPS runs the enzyme partitioned preferentially to the bottom salt-rich phase ($K < 1$), consistently with the observations of Sales et al. [31] for fibrinolytic proteases from *Bacillus* sp. UFPEDA 485. On the other hand, values of $K > 1$ in runs 3, 4, 7 and 8 point out that, at the highest PEG concentration (C_{PEG} = 30.0% w/w), the enzyme preferentially partitioned to the top PEG-rich phase likely due its high hydrophobicity [12,31,32] or to a salting out effect [6,31,32]. As shown in Fig. 1, C_{PEG} influenced positively the enzyme partition although also sodium sulfate concentration (C_{Na2SO4}) and PEG molar mass (M_{PEG}) exerted weaker positive and negative effects, respectively. Similarly, Medeiros e Silva et al. [6] observed a positive effect of C_{PEG} and a negative one of M_{PEG} on the partition of fibrinolytic proteases in PEG/phosphate ATPS.

The highest values of the activity yield in the bottom phase (Y_b) (106.0–130.0%) were obtained in the central point runs carried out at M_{PEG} 6000 g/mol, C_{PEG} 24.0% (w/w) and C_{Na2SO4} 11.6% (w/w) (Table 1). Yield values higher than 100% as in the present case may be associated with removal of contaminants and inhibitors during the partition, which increases the enzyme activity [33,34]. Central point runs also ensured the highest values of the purification factor in the bottom phase (PF_b of 3.2 and 4.5), which are in agreement with those (up to 4.0) reported for a fibrinolytic protease extracted by PEG/potassium phosphate ATPS [35].

3.2. Optimization of fibrinolytic protease partition by two-factor central composite rotary design

As previously mentioned, the conditions of fibrinolytic protease partition were optimized through a set of 12 additional runs carried out according to a two-factor central composite rotary design (CCRD), where C_{PEG} and C_{Na2SO4} were selected as the independent variables and FA_b, K, Y_b and PF_b as the responses. One can see in Table 2, which summarizes the conditions and results of these runs, that all these responses varied in very wide ranges ($34.6 \leq FA_b \leq 94.2$ U/mL, $0.0 \leq K \leq 0.7$, $47.5 \leq Y_b \leq 177.0\%$ and $1.6 \leq PF_b \leq 10.0$), suggesting strong influence of the selected variables on them. The run 4, carried out using C_{PEG} = 30.0% (w/w) and C_{Na2SO4} = 13.2% (w/w), allowed for the best results in terms of purification factor (PF_b = 10.0) as well as a quite high activity yield (Y_b = 102.0%) in the bottom phase (Table 2). This PF_b value is higher than those obtained at the beginning of this work using the preliminary factorial design (PF_b = 3.2 up to 4.5) as well as those reported for the extraction of fibrinolytic protease from *Streptomyces* sp. DPUA1576 by PEG/phosphate ATPS (1.51) [6].

Table 3 lists the linear (L) and quadratic (Q) effects of the independent variables as well as their interactions on the selected responses. One can see that the only statistically significant effects on Y_b and FA_b were the linear one of C_{PEG} and the quadratic one of C_{Na2SO4}, respectively, being both negative. From Fig. 2, which illustrates, as an example, the three-dimensional graph obtained applying the Response Surface Methodology to the experimental results of K and PF_b, it is evident the significant combined effect of C_{PEG} and C_{Na2SO4} on these responses. As reported by Ashipala and He [21], the fibrinolytic activity of an enzyme produced from *Bacillus subtilis* DC-2, after extraction by PEG 4000/Na_2SO_4 ATPS performed according to a similar CCRD, was mainly dependent on C_{PEG} and C_{Na2SO4} besides pH.

Since PF_b, which is by far the most important response for the purposes of this work, has given the best results compared to literature, it was submitted to the analysis of variance. The *F*-value for the overall regression model was significant at 5% level, the coefficient of determination satisfactory (R^2 = 0.8584) and the lack of fit insignificant (Table 4), hence indicating that the first-order model with interaction was adequate to represent the response surface of this response for fibrinolytic protease obtained according to the selected CCRD.

3.3. 2D two-dimensional SDS-PAGE and fibrin zymography

SDS-PAGE and fibrin zymography were performed to check the purity degree of fibrinolytic protease from *Mucor subtilissimus* UCP 1262 that preferentially partitioned to the bottom phase after extraction by ATPS under the best conditions selected according to the CCRD, namely C_{PEG} of 30.0% (w/w) and C_{Na2SO4} of 13.2% (w/w). One can see in Fig. 3B that the enzyme had a molecular mass (97 kDa) consistent with those reported for novel fibrinolytic enzymes of *Schizophyllum commune* BL23 (66–97 kDa) [36] and *Bionectria* sp. isolated from Las Yungas rainforest (80–173 kDa) [37], but significantly higher than those reported for fibrinolytic enzymes from *Fusarium* sp. BLB (20–30 kDa) [38], *Rhizopus chinensis* 12 (17–18 kDa) [39], *Pleurotus eryngii* (14 kDa) [40] and *Schizophyllum commune* [41] (21 kDa). The above molecular mass value is practically coincident with that revealed by fibrin zymography of the enzyme partitioned to the bottom phase (Fig. 3C). In addition, SDS-PAGE (12%) revealed an apparent isoelectric point of 5.4 (result not shown), which is characteristic of an acidic protease.

Table 1
Results of fibrinolytic protease extraction/recovery by PEG/sodium sulfate ATPS according to the preliminary 2^3 full factorial design.

Run	M_{PEG} [a] (g/mol)	C_{PEG} [b] (%w/w)	C_{Na2SO4} [c] (%w/w)	FA_t [d] (U/mL)	FA_b [e] (U/mL)	K [f]	Y_t [g] (%)	Y_b [h] (%)	PF_t [i]	PF_b [j]
1	4000	18.0	10.0	11.7	44.2	0.2	19.0	73.3	1.0	0.8
2	8000	18.0	10.0	24.2	47.1	0.5	35.1	81.4	1.5	0.9
3	4000	30.0	10.0	19.2	9.5	2.0	40.4	9.61	1.0	0.8
4	8000	30.0	10.0	15.8	12.1	1.3	32.85	12.53	0.7	1.0
5	4000	18.0	13.2	20.8	28.7	0.7	28.8	49.7	1.7	0.6
6	8000	18.0	13.2	14.6	37.5	0.3	20.2	64.8	0.7	1.1
7	4000	30.0	13.2	17.5	15.0	2.3	169.0	35.0	3.5	2.0
8	8000	30.0	13.2	11.2	5.00	2.2	22.6	5.1	0.6	0.6
9[k]	6000	24.0	11.6	23.7	57.1	0.8	128.0	130.0	2.5	3.2
10[k]	6000	24.0	11.6	20.8	52.9	0.8	121.0	124.0	2.6	3.6
11[k]	6000	24.0	11.6	20.4	57.1	0.5	70.2	106.0	1.5	3.2
12[k]	6000	24.0	11.6	22.1	55.0	0.5	78.9	116.0	2.0	4.5

[a] M_{PEG} = PEG molar mass.
[b] C_{PEG} = PEG concentration.
[c] C_{Na2SO4} = Sodium sulfate concentration.
[d] FA_t = Fibrinolytic activity in the top phase.
[e] FA_b = Fibrinolytic activity in the bottom phase.
[f] K = Partition coefficient.
[g] Y_t = Activity yield in the top phase.
[h] Y_b = Activity yield in the bottom phase.
[i] PF_t = Purification factor in the top phase.
[j] PF_b = Purification factor in the bottom phase.
[k] Central point runs.

Table 2
Results of the two-factor central composite rotary design used to optimize the extraction/recovery of fibrinolytic protease by PEG 6000/sodium sulfate ATPS.

Run	C_{PEG} [a] (% w/w)	C_{Na2SO4} [b] (% w/w)	FA_b [c] (U/mL)	K [d]	Y_b [e] (%)	PF_b [f]
1	18.0	10.0	94.2	0.0	177.0	2.0
2	18.0	13.2	71.7	0.4	138.0	2.2
3	30.0	10.0	93.3	0.4	78.0	1.9
4	30.0	13.2	76.2	0.2	102.0	10.0
5	15.6	11.6	84.2	0.1	176.0	2.1
6	32.4	11.6	51.2	0.4	47.5	7.7
7	24.0	9.36	57.5	0.3	74.5	1.3
8	24.0	13.8	34.6	0.7	56.4	1.6
9[g]	24.0	11.6	90.0	0.4	143.0	3.8
10[g]	24.0	11.6	89.6	0.4	127.0	3.6
11[g]	24.0	11.6	87.9	0.5	99.3	2.0
12[g]	24.0	11.6	88.3	0.3	118.0	3.4

[a] C_{PEG} = PEG 6000 concentration.
[b] C_{Na2SO4} = Sodium sulfate concentration.
[c] FA_b = Fibrinolytic activity in the bottom phase.
[d] K = Partition coefficient.
[e] Y_b = Activity yield in the bottom phase.
[f] PF_b = Purification factor in the bottom phase.
[g] Central point runs.

Table 3
Estimated effects for the responses of fibrinolytic protease extraction/recovery by PEG 6000/sodium sulfate ATPS according to the two-factor central composite rotary design.

Factor	K[a]	Y_b[b](%)	PF_b[c]	FA_b[d](U/mL)
C_{PEG}[e] (L)[f]	3.24*	−6.13*	6.91*	−0.28
C_{PEG}[e] (Q)[g]	−4.08*	0.51	3.43*	1.04
C_{Na2SO4}[h] (L)[f]	3.90*	0.79	3.85*	0.18
C_{Na2SO4}[h] (Q)[g]	1.06	2.69	−2.11	−3.72*
C_{PEG}[e] (L)[f] x C_{Na2SO4}[h] (L)[f]	−4.06*	1.73	4.90*	−2.62

[a] K = Partition coefficient.
[b] Y_b = Activity yield in the bottom phase.
[c] PF_b = Purification factor in the bottom phase.
[d] FA_b = Fibrinolytic activity in the bottom phase.
[e] C_{PEG} = PEG 6000 concentration.
[f] L = Linear effects.
[g] Q = Quadratic effects.
[h] C_{Na2SO4} = Sodium sulfate concentration.
* Statistically significant effects (P-value < 0.05).

3.4. Amidolytic activity and effect of inhibitors on protease activity

As far as the amidolytic activity of the purified fibrinolytic protease is concerned, succinyl-L-ala-ala-pro-L-phenylalanine-*p*-nitroanilide (SAPNA), a typical substrate of chymotrypsin, was shown to be the most effective among all other substrates tested

Table 4
Analysis of variance of the effects on the purification factor in the salt-rich bottom phase obtained by PEG 6000/sodium sulfate ATPS according to the two-factor central composite rotary design.

Factor	SS[a]	DF[b]	MS[c]	*F*-value	*P*-value
C_{PEG}[d] (L)[e,*]	30.3801	1	30.3801	47.7621	0.0062
C_{PEG}[d] (Q)[f,*]	7.5192	1	7.5192	11.8214	0.0412
C_{Na2SO4}[g] (L)[e,*]	9.4769	1	9.4769	14.8991	0.0307
C_{Na2SO4}[g] (Q)[f]	2.8499	1	2.8499	4.4805	0.1245
C_{PEG}[d] (L)[e] x C_{Na2SO4}[g] (L)[e,*]	15.3275	1	15.3275	24.0972	0.0161
Lack of fit	9.2865	3	3.0955	4.8666	0.1131
Pure error	1.9082	3	0.6360	–	–
Total SS[a]	79.1095	11	–	–	–

[a] SS = Sum of squares.
[b] DF = Degree of freedom.
[c] MS = Mean square.
[d] C_{PEG} = PEG 6000 concentration.
[e] L = Linear effects.
[f] Q = Quadratic effects.
[g] C_{Na2SO4} = Sodium sulfate concentration.
[*] Statistically significant effects (*P*-value < 0.05).

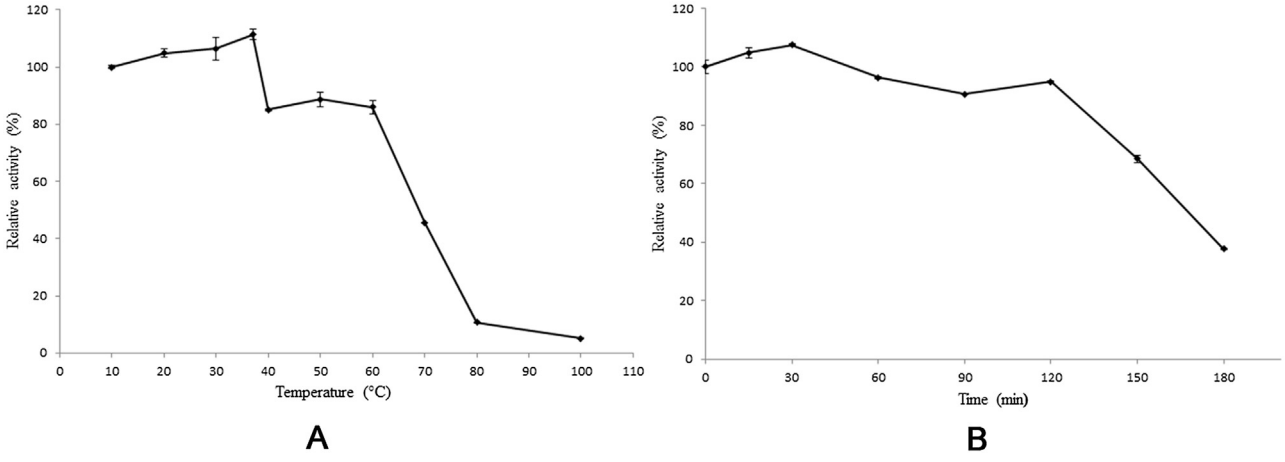

Fig. 4. (A) Effect of temperature on the activity of fibrinolytic protease purified by ATPS. (B) Stability of purified fibrinolytic protease at 37° C versus time. Relative activities do refer to the activity at 10 °C in (A) and the starting activity in (B).

Table 5
Amidolytic activity of fibrinolytic protease partitioned to the salt-rich bottom phase of PEG 6000/sodium sulfate ATPS.

Substrate	Enzyme	Amidolytic activity
S7388	Chymotrypsin	positive effect
G8148	Plasmin and urokinase	negative effect
B4875	Trypsin	negative effect

Table 6
Effect of inhibitors on protease activity of fibrinolytic protease partitioned to the salt-rich bottom phase of PEG 6000/sodium sulfate ATPS.

Inhibitor	Residual activity (%)
Control	100
EDTA	100
β-Mercaptoethanol	97.7
PMSF[a]	0.00[*]
Pepstatin A	91.3
Iodoacetic acid	100

[a] PMSF = phenylmethylsulfonyl fluoride.
[*] Statistically significant effects (*P*-value < 0.05).

(Table 5), which suggests that the enzyme may be a chymotrypsin-like protease, similar to other fibrinolytic enzymes [42].

On the other hand, the effect of various inhibitors on protease activity is summarized in Table 6. Our fibrinolytic protease was completely inhibited by phenylmethylsulfonyl fluoride (PMSF), a well-known inhibitor of serine protease. This result corroborates those of Peng et al. [43], who purified a fibrinolytic enzyme that behaved similarly to serine protease.

Table 7
Effect of metal ions on protease activity of fibrinolytic protease partitioned to the salt-rich bottom phase of PEG 6000/sodium sulfate ATPS.

	Residual activity (%)		
Metal ion	2.5 mM	5.0 mM	10 mM
Control	100.00	100.00	100.00
K^+	93.86	93.86	100.0
Ca^{++}	119.09[*]	107.50[*]	102.27
Mn^{++}	109.31[*]	107.50[*]	100.90
Zn^{++}	89.77[*]	76.36[*]	30.00[*]
Mg^{++}	102.50	92.73	110.91[*]
Co^{++}	65.68[*]	72.50[*]	61.59[*]
Cu^{++}	66.13[*]	57.95[*]	54.09[*]
Fe^{++}	75.68[*]	66.59[*]	52.95[*]
Na^+	91.59	91.59	98.40

[*] Statistically significant effects (*P*-value < 0.05).

3.5. *Effect of metal ions on protease activity*

The effect of different metal ions was also investigated on the residual activity of protease from *M. subtilissimus* extracted by ATPS using PEG 6000 (30% w/w) and Na_2SO_4 (13.2% w/w). As shown in Table 7, this activity was enhanced by Ca^{++} and Mn^{++} both at concentrations of 2.5 and 5.0 mM, and by Mg^{++} at concentration

of 10.0 mM, confirming the results reported by Kim et al. [44] for a similar enzyme, whereas it was strongly inhibited by Co^{++}, Cu^{++} and Fe^{++} at all the concentrations tested. This result is consistent with those of other researchers, who observed inhibiting effects of Cu^{++} [45], Fe^{++} [46], Cu^{++} and Co^{++} [47] on the activities of other purified fibrinolytic enzymes. On the contrary, the other metal ions (K^{+}, Na^{+} and Zn^{++}) did not exert any significant effect on residual enzyme activity.

3.6. Effect of temperature on protease activity and stability

Fig. 4 shows that the purified enzyme displayed its maximum activity at 37 °C, confirming the results reported by Lu et al. [45] for a novel fibrinolytic enzyme from *Paenibacillus polymyxa* EJS-3 (HIPC-I) purified by chromatography methods. This comparison underscores the relevance of the results of this work based on the use of a cheap purification method like ATPS in comparison with those obtained employing more expensive and time-consuming multi-steps methods. The enzyme became less active when temperature was raised to 60 °C, and was completely denatured at temperatures >80 °C (Fig. 4A).

Thermal stability studies showed that the purified fibrinolytic protease was quite stable at 37 °C (optimal temperature for fibrinolytic activity), and its residual activity was 94 and 68% of the initial one after 120 and 150 min respectively (Fig. 4B).

4. Conclusions

Aqueous two-phase systems (ATPS) using PEG 6000 and Na_2SO_4 were used to select the optimal conditions to recovery a fibrinolytic acidic protease from *Mucor subtlilissimus* UCP 1262. To this purpose, extraction tests were carried out according to a two-factor central composite rotary design, where the concentrations of PEG (C_{PEG}) and Na_2SO_4 (C_{Na2SO4}) were used as the independent variables, and the partition coefficient, the fibronolytic activity, activity yield and purification factor in the bottom phase as the responses. Optimal conditions were shown to be C_{PEG} of 30.0% (w/w) and C_{Na2SO4} of 13.2% (w/w). Results clearly showed a high purification degree of the enzyme in the salt-rich bottom phase. The enzyme, which was characterized as a chymotrypsin-like protease, exhibited an optimal temperature for fibrinolytic activity of 37 °C, at which it was quite stable, and had its activity increased in the presence of some metal ions (Ca^{++}, Mn^{++} and Mg^{++}) or decreased in the presence of others (Co^{++}, Cu^{++} and Fe^{++}). This work demonstrates the potential of ATPS as primary recovery step for the extraction/purification of fibrinolytic proteases.

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