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Extraction of lipase from *Aspergillus niger* by insoluble complex formation with anionic and cationic polyelectrolytes

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ABSTRACT

The insoluble complex formation between lipase from *Aspergillus niger* and the electrically charged polymers, polyacrylic acid (PAA), poly-vinil sulfonate (PVS) and chitosan (CHI), was studied by using turbidimetric and enzymatic methods on a commercial lyophilized (Ly) and a filtrate of solid culture medium (SCM). It could be shown that both electrostatic interactions as hydrophobic are involved in the formation of insoluble complexes. The kinetics of the complex formation were determined. Lipase enzymatic activity is maintained through time in the presence of polyelectrolytes.

On the Ly the three polymers produced insoluble complex, with a stoichiometric ratio (polymer mass per mass of Ly from *Aspergillus niger*) of PAA/Ly: 0.035, PVS/Ly: 0.099 and CHI/Ly: 0.071 mg/mg Ly. For the anionic polyelectrolytes, the PAA presents slightly better results than PVS to be used when the protein concentration is similar to the lyophilized.

The filtrate of the SCM has a total protein concentration much lower than commercial lyophilized. Working with CHI as cationic polymer a recovery of the activity in the re-dissolved precipitate higher than 80%, with purification factors greater than 3 were achieved, both at 8 and 20 °C. Therefore, this methodology could be used as a first step of purification.

1. Introduction

From Morawetz and Hugues work [1], it is now recognized that globular proteins can form tight complexes with polyelectrolytes. These associations may result in soluble species [2], complex coacervation [3], precipitation [4], or gelation [5]. The formation of complexes affects both the state of aggregation of the species and the environment immediately surrounding the bound moieties of the partners. Therefore, their detection reflects either the increase of the average size and the molecular weight of the species or a perturbation at a molecular level. Either an estimate of the light scattered by the samples using a spectrophotometer to measure the turbidity or a rapid measurement of the viscosity appears to be the simplest and most sensitive technique to be used for any kind of aggregation [6].

All the interactions (electrostatic, hydrophobic, hydrogen bonds, steric effects) can take place simultaneously among the polypeptide chains and parts of another polymer, directing both protein folding and complexation [6]. The practical approach of the protein–polyelectrolyte complex formation includes protein separation and protein recovery [7], immobilization or stabilization of enzymes [8], modification of protein–substrate affinity [9]. Today, technological development requires the production of biological catalysts for many industrial processes. This has led to the development of techniques to obtain large-scale enzyme. The need to generate enzymes for industrial use along with the synthesis of new polymers capable of interacting with them has allowed the development of rapid and simple methods of purification thus allowing the production of these macromolecules from natural sources or recombinant organisms where they are expressed.

Lipases (EC 3.1.1.3) are a group of enzymes which catalyze the hydrolysis and synthesis of triglycerides in vivo, producing or consuming fatty acid esters and even the synthesis of these ones in low water content environments. This feature has increased the biotechnological interest in these enzymes for a number of industrially significant biotransformations. Potential applications include modification of sugars, synthesis of flavor esters for the food industry, the resolution of racemic mixtures and biofuel production [10]. From an industrial standpoint, fungi are more interesting sources of these enzymes than animals or plants for their potential use in biotechnology due to their availability and high stability. Lipase

Abbreviations: PVS, poly (vinyl sulfonic acid, sodium salt); PAA, poly (acrylic acid) partial sodium salt; CHI, chitosan; Ly, lyophilized; pNPL, 4-nitrophenyl laurate; pNP, p-nitrophenol; SCM, solid culture medium.

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from *Asperghillus niger* is mainly used in industry [11]. Knowledge of the medium conditions that stabilize the enzyme is of great interest from the theoretical and practical point of view for the design and development of a biotechnological process where the macromolecule participates as a catalyst.

The aim of this study was to investigate the polyelectrolyte–enzyme complex formation on a commercial lyophilized to be applied to the isolation of lipase from a solid culture medium. We have used spectroscopic techniques and enzymatic activity measurements to obtain information about the interaction between lipase with two negatively charged polyelectrolytes (polyacrylate and poly-vinyl sulfonate) and a positively charged polymer (chitosan).

2. Materials and methods

2.1. Chemicals

Lipase from a lyophilized culture medium of *A. niger* (Ly) was purchased from Sigma Chem. Co. (USA) and used without further purification. Its solutions (50 mg/mL) were prepared by direct weighing into 50 mM potassium phosphate buffer at pH 7. Poly-acrylic acid (PAA) of 240,000 Da molecular weight, 1.09 g/cm^3 density and poly-vinyl sodium sulfonate (PVS) of 170,000 Da molecular weight, 1.267 g/cm^3 density were purchased from Aldrich (USA). Both polymers were used in solutions 1% (w/w) in 50 mM phosphate buffer at the appropriate pH for the assay.

Chitosan (CHI)—minimum 75% desacetylation grade, given by the manufacturer—was purchased from Sigma (MO, USA) and used without further purification. The concentration of the working solution was 0.05% (w/v) in 50 mM phosphate buffer at pH 6 from a CHI solution in 1% (v/v) acetic acid.

All other chemicals were of analytical grade. The specific substrate for the lipase determination, 4-nitrophenyl laurate (pNPL), was acquired from Sigma Chem. Co. (USA).

2.2. Lipase assay

Lipase activity was determined by the enzymatic capacity to hydrolyze the substrate 4-nitrophenyl laurate (pNPL), which liberates p-nitrophenol (pNP) that absorbs at 400 nm at pH 7. A solution 25 mM of pNPL in ethanol–water (1:1) was prepared. The reaction was carried out for 30 min at 37 °C, with 100 μL of an adequate dilution of the enzymatic sample in 800 μL of 50 mM phosphate buffer at pH 7. Then, 100 μL substrate was added to achieve the final saturation concentration (0.4 mM). After that, 250 μL 1 M Na_2CO_3 was added and kept for 15 min at 0 °C. The mixture was centrifuged at 16,000 g for 15 min and the supernatant was read at 400 nm. pNP molar absorptivity coefficients were determined on each of the media studied (data not shown). Blanks without the enzyme were assayed [12]. One unit of lipase activity is the amount of enzyme that liberates 1 μmol of pNP/min from pNPL per mg of protein under the above mentioned conditions.

A Jasco V-550 spectrophotometer was used to absorbance measurements in all determinations of this work. The pH values in all cases were tested with a Hanna Instrument HI 9017 microprocessor pH meter.

2.3. Lipase turbidimetric titration curves with polymers at different pH and ionic strengths

The formation of the insoluble polymer–protein complex was followed by means of turbidimetric titration [13,14]. Turbidity measurements were obtained by absorbance measurements at 420 nm which is the parameter indicated by the spectrophotometer. Buffer sodium phosphate solutions with a fixed protein concentration were titrated at 20 °C in a cubic 1 cm path-length glass cell with the polymer stock solution. Solutions were 1% (w/w) of PAA 240,000 and 1% (w/w) of PVS 170,000. In the case of the positively charged polymer (CHI), the stock solution was 0.05% (w/v). To avoid changes in pH during titration, both protein and polyelectrolyte solutions were adjusted to the same pH value. The complex formation was followed through a plot of absorbance at 420 nm vs. added amount of PAA, PVS or CHI. We defined "stoichiometric polymer/protein ratio" as the minimal ratio needed in which the protein has been precipitated as an insoluble complex. It was calculated from the plot at the lowest polymer concentration necessary to reach a plateau. These values are important because they allow us to calculate the minimal polymer amount necessary for maximum protein precipitation. The data have been expressed as mg of polyelectrolyte per mg of Ly.

The time needed to form the complex was evaluated by measuring the time required to obtain the maximal absorbance of the high mass ratio of polymer–protein [15]. A mass ratio from the plateau region of the titration curves was selected for each polymer.

Polymers were titrated with alkali and acid, the absorbance at 420 nm was plotted vs. pH. The same graph was plotted in the presence of different ionic strengths adding NaCl to phosphate buffer [16].

2.4. Lipase precipitation with PAA, PVS and CHI from commercial lyophilized

A solution of polymer (PAA and PVS) and lyophilized at an adequate ratio of mg of polymer per mg of Ly (0.035 mg PAA/mg Ly, 0.099 mg PVS/mg Ly) in 50 mM phosphate buffer of pH 3 was prepared. The precipitate formed was incubated for 1 h at 4 °C for PAA or 1 h at 20 °C for PVS and centrifuged at 815 × *g* for 10 min. The supernatant of the precipitate was separated and re-dissolved in 50 mM phosphate buffer pH 7. The activity in the supernatant and in the re-dissolved precipitate was measured.

An aliquot 0.05% (w/v) CHI prepared in 50 mM phosphate buffer of pH 6 was added to a solution of Ly in 50 mM phosphate buffer, at pH 6, until a relationship 0.084 mg CHI/mg Ly was obtained. The precipitate formed was incubated for 8 min at 8 °C and 20 °C and centrifuged at 815 g for 10 min. The supernatant of the precipitate was separated. Then, it was re-dissolved in 50 mM phosphate buffer with 0.5 M NaCl pH 6 until the solution reaches initial volume but re-dissolution of the precipitate was not completed. To achieve these goals, different strategies were tested: the decrease of pH, the decrease to 0.071 mg CHI/mg Ly of the ratio polymer/protein and an increase of ionic strength. Finally, the re-dissolution of the precipitate was achieve in 50 mM phosphate buffer with 1.0 M NaCl pH 3.8 and completed with 50 mM phosphate buffer pH 6. Since the conditions for re-dissolution are extreme, the enzymatic activity at low pH and high ionic strength were compared with the Ly activity in 50 mM phosphate buffer pH 6 and no difference was observed (data not shown). The activity in the supernatant and in the re-dissolved precipitate was measured.

2.5. Lipase production from A. niger

Strains of *A. niger* NRRL3 were provided by the culture collection of Agricultural Research Service, USDA. The production of lipase from *A. niger* NRRL3 strain was carried out in a solid culture medium (SCM) according to Adham and Ahmed [11] with some modifications. The organism was reactivated on a potato-dextrose agar medium (PDA) at 30 °C for 5 days. The spores were removed from the PDA with sterile distilled water, with the help of a sterile magnetic stirrer. The numbers of spores were counted using a Thoma counter chamber type. A spore suspension was prepared in sterile distilled water to get a concentration of 10^6 spores/mL in each specific medium. This solution was used as a source of inoculum. The culture medium (pH: 6) components were: 1% glucose, 1% olive oil, 3% casein peptone, 0.05% $MgSO_4 \cdot H_2O$, 0.05% KCl and 0.2% K_2HPO_4. This medium was autoclaved at 121 °C for 15 min and was inoculated when it reached room temperature. The SCM was carried out in a 250 mL Erlenmeyer with 6 g of milled polyurethane (particle size: 1.68–3.36 mm, approximately); it was sterilized and 14 mL of culture medium were added. Then, the SCM was homogenized and incubated for 5 days at 30 °C. The extract was obtained by the addition of 30 mL of 50 mM phosphate buffer at pH 7 to the SCM, compressed with a syringe, filtered and centrifuged at 6000 rpm for 20 min at 8 °C [16]. The protein content was estimated by the Warburg and Christian method [17].

3. Results and discussion

3.1. Lipase/polymer titration curves

The isoelectric point of lipase is near pH 5; under this pH value, its charge is positive. The interaction with negatively charged polyelectrolytes (PAA and PVS) was analyzed at pHs 3, 4 and 5 at different fixed protein concentrations of lipase and at various polymer concentrations. Fig. 1A shows only the lipase titration curves at pH 3 as absorbance at 420 nm vs. added amount of PAA or PVS on a fixed amount of Ly. With the addition of low polymer volumes, it was observed that the absorbance increased with an increase in the total polymer concentration; however, at high polymer/protein ratios, there was a plateau in the curve, which depends on polymer/protein molar ratio and the pH of the medium. Maximum turbidity was observed at pH 3 with both polymers, being almost negligible at the other pHs assayed (data not shown). The plateau was reached at 0.035 mg PAA/mg Ly and 0.099 mg PVS/mg Ly. PAA is more efficient as a precipitant agent than PVS. These values correspond to the stoichiometric polymer/protein ratio. For PAA, the curves follow a sigmoid function or hyperbolic function for PVS, which shows that in the complex formation there is a cooperative process or a saturation process respectively.

Chitosan was selected to interact with lipase above its isoelectric point, where the protein is charged negatively. Fig. 1B shows the lipase titration curves at pH 6 as absorbance at 420 nm vs. amount of CHI added on two fixed amount of Ly. The curves followed a

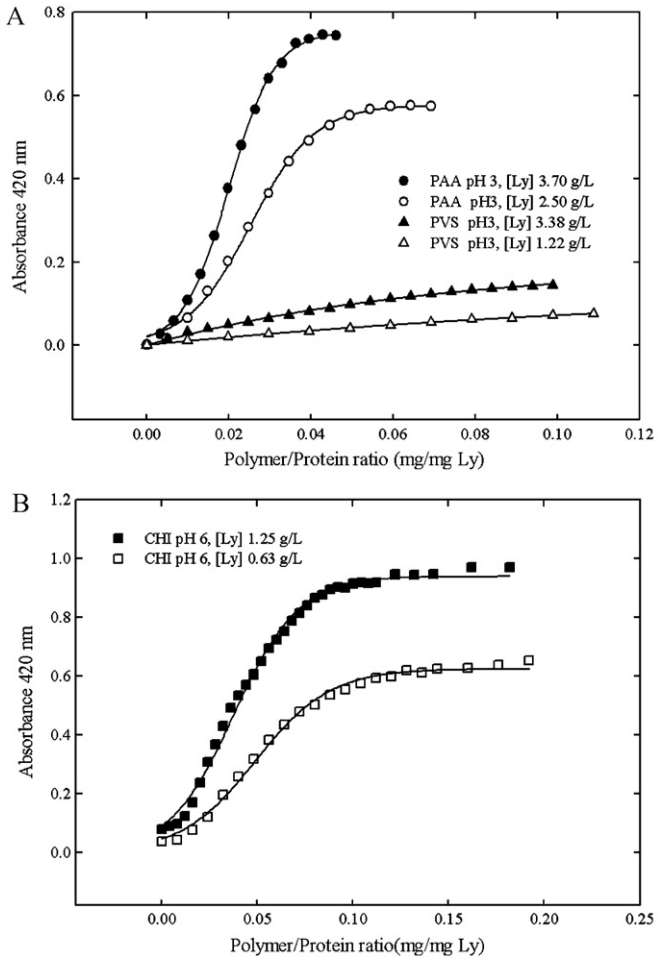

Fig. 1. Turbidimetric titration curves of a fixed concentration of lyophilized (Ly) from *Aspergillus niger* vs. (A) PAA and PVS in 50 mM phosphate buffer, pH 3 and (B) CHI in 50 mM acetate buffer, pH 6. $T = 20\,^{\circ}C$.

sigmoid function, increased with an increase in the total polymer concentration and the plateau was reached at 0.084 mg CHI/mg Ly.

A comparison between the two figures shows that the absorbance values obtained are much larger in the case of CHI–Ly complex than in the other two complexes. Therefore, the precipitation is more evident with CHI despite a greater ratio of polymer/lyophilized. The shapes of the curves show similarities between the type of precipitation of the CHI/Ly and PAA/Ly complexes, these settings correspond to sigmoid functions which show the presence of cooperative processes.

The precipitation curves obtained for the polymers allowed us to determine the mass ratio between the polymer and the enzyme, which is necessary to precipitate most of the present protein, to specific pH medium.

3.2. Precipitation curve in pH function of lipase with PAA, PVS and CHI

Taking into account previous results, three mass ratios were chosen for each polymer and phase diagrams were carried out. One of the chosen ratios was the stoichiometric polymer/protein ratio, the remaining two were bigger and smaller respectively. Phase diagrams were also made both for the protein and for the polymer. Fig. 2 shows a precipitation curve in pH function of Ly with PAA, PVS and CHI; and the pH variation effect on the insoluble complex formation obtained for one PAA/Ly, PVS/Ly or CHI/Ly fixed ratio. The other mass ratios assayed showed similar behavior

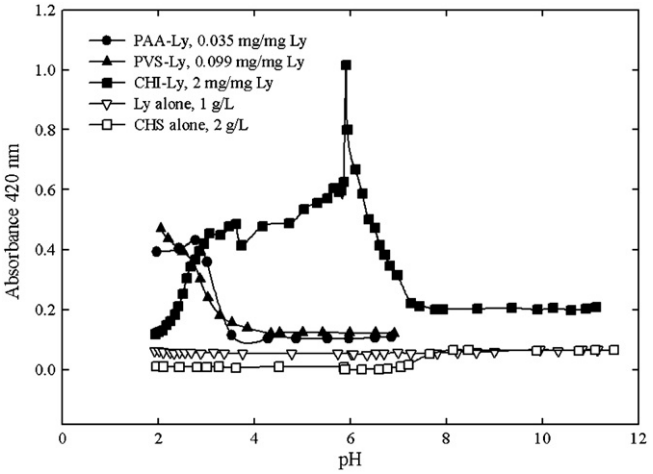

Fig. 2. Phase diagrams of the polymers alone and complexed.

(overlapping curves) for all polymers, which indicates that the insolubility range is not affected by the assayed mass ratios.

For PVS and PAA, the increase of pH above 3 induced a dramatic decrease in the maximum absorbance values, which suggested a minor amount of complex formation. This result was observed in the previous experiences.

The phase diagram of lipase with CHI indicates that complex formation is maximal around pH 6 (5–7 range).

Enzymatic stability at different times (up to 24 h) for different pHs (2–9 range) was previously measured. Results (data not shown) indicate that the enzymatic activity is constant over time at pHs 2, 3 and 9 and is slightly modified in time at pHs 4, 5, 6, 7 and 8.

3.3. Ionic strength effect on precipitation curve

Complex turbidity was measured as a function of the polymer volume added at different ionic strengths (NaCl from 0.25 M to 1 M). The pH ranges of insoluble complexes were not modified by the presence of NaCl in the three cases. Results are shown in Fig. 3A and B. They are different when the polymers are CHI and PAA or PVS.

The increase of ionic strength reduces the turbidity to values close to zero for NaCl concentration 1 M for CHI and PAA. This fact indicates that the formation of lipase–PAA and lipase–CHI complexes is directed primarily by electrostatic interactions and the precipitate could be re-dissolved at high ionic strength.

For PVS, high concentrations of NaCl fail to reduce complex turbidity even when they reach values of 1 M. This effect may suggest that hydrophobic interactions could be involved between hydrocarbonated framework of polyelectrolyte and hydrophobic groups of enzyme [18]. That is the reason why the complex was dissolved by a pH change. From these curves, the optimum pH interval in which the polymer–protein complex is insoluble was determined but no data could be provided as regards particles size.

3.4. Kinetics of the polymer–Ly complex formation

In systems where proteins interact with polymers is frequently found that complex formation is not instantaneous but there is an interaction time required to reach maximum turbidity [19]. The time needed to form the complex at two temperatures was evaluated measuring the time required to obtain the maximal absorbance. The results are shown in Fig. 4A and B. The PAA–Ly (0.035 mg PAA/mg Ly) system required 60 min to reach the maximum turbidity at $4\,^{\circ}C$ and 30 min at $20\,^{\circ}C$. Moreover, this maximum is greater at $4\,^{\circ}C$ than at $20\,^{\circ}C$, which suggests that

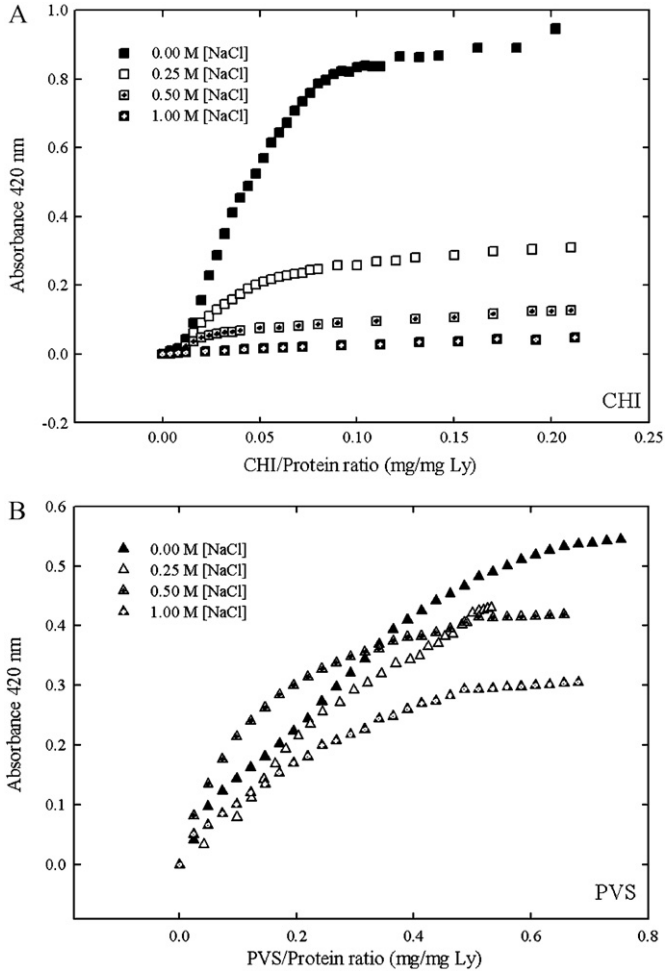

Fig. 3. Turbidimetric titration curves of Ly from *Aspergillus niger* with (A) CHI and (B) PVS at different ionic strengths. CHI/Ly mass ratios: 0.084 mg/mL, PVS/Ly mass ratios: 0.099 mg/mL. $T = 20\,^{\circ}C$.

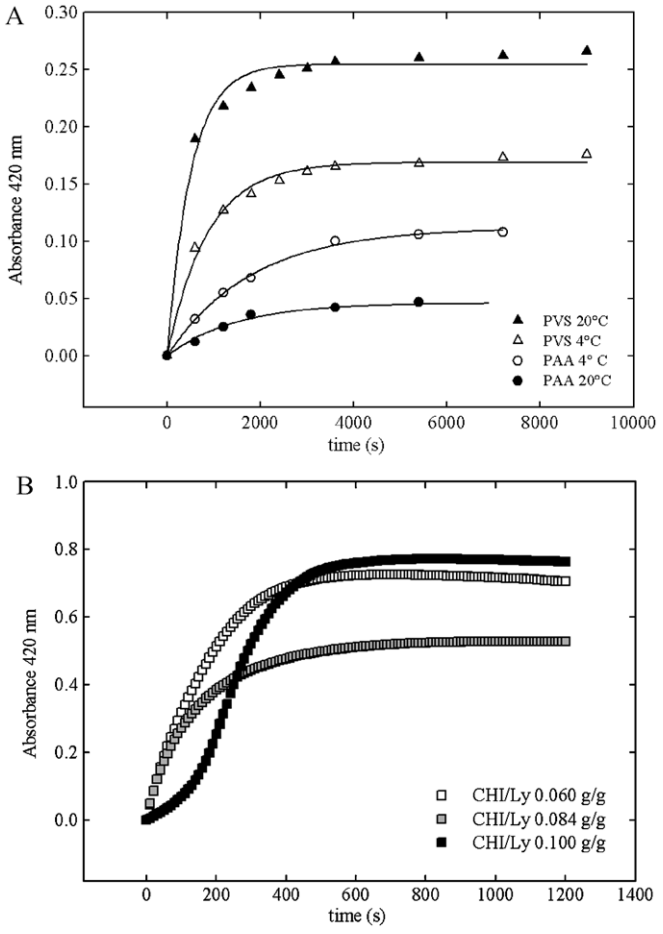

Fig. 4. Precipitation kinetics. PAA/Ly mass ratio: 0.035 mg/mg Ly and PVS/Ly mass ratio: 0.099 mg/mg Ly. $T = 20\,^{\circ}C$.

complex formation is exothermic. The PVS–Ly (0.099 mg PVS/mg Ly) system required 40 min to reach the maximum turbidity at two temperatures assayed. The maximum was greater at 20 °C than at 4 °C, suggesting the presence of an endothermic process. The CHI–Ly interaction was much faster than PVS/Ly and PAA/Ly but it changed according to an increase in the CHI/Ly ratio. Behavior with temperature is equal to PAA (data not shown). Therefore, the CHI/Ly complex formation is exothermic and when the polymer/protein rate increases, a mechanism of saturation changes into a cooperative process.

The slow kinetics should be considered in the design and purification method because the time variable should be considered a constraint.

3.5. Lipase biological activity through time, in the presence and absence of polymers

The enzymatic activity in the presence or absence of polymer is an indicator of the protein stability. There are polymers that increase or decrease the enzymatic stability. The lipase activities of the control, in the polymer/Ly precipitate and in the supernatant were measured. The precipitate was re-dissolved in the same original volume. The mass ratios used were the ones corresponding to the stoichiometric ratio of each complex. They were chosen to verify the effect of the polymer on the enzymatic activity through time. The presence of the polymer did not modify the enzymatic activity of lipase because the sum of activity in the precipitate and the supernatant were approximately equal to control at all the times assayed. For the CHI/Ly complex, the activity was constant until the first 5 h and there is an increase (43% and 25% in presence and absence of polymer, respectively) in the activity at 24 h. For the PAA and PVS/Ly complex, there is a slight reduction in the activity until the first 2 h, then it increases and decreases again at 24 h. (data not shown).

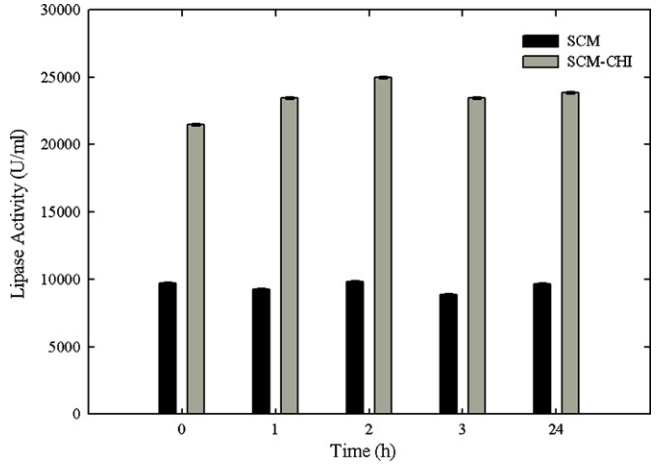

Fig. 5. Lipase activity vs. time for a fungus filtrates in presence and absence of CHI.

Table 1
Purification table of fungal filtrate with CHI.

Temperature	Fraction	Total protein (mg/L)	Recovery (%)	Purification factor
8 °C	Precipitate	18.55	80.72	3.11
	Supernatant	55.80	15.69	0.20
	Control	71.45		
20 °C	Precipitate	23.32	94.71	3.24
	Supernatant	62.37	3.66	0.05
	Control	79.77		

Fig. 5 shows the enzymatic activity measurement on SCM filtrated for 24 h, in the presence and absence of CHI. The filtrated activity remained constant, the polymer increases the lipase activity considerably and mildly at the 2 h. This increase of the activity by polymer presence was taken into account when the recovery and purification factors were calculated accordingly, which is shown in Table 1.

3.6. Lipase precipitation from lyophilized

Fig. 6 shows the recovery of biological activity and purification factors of lipase after precipitation from commercial lyophilized with PAA, PVS and CHI. Activities measured after re-dissolution were compared with a control group which was prepared at identical medium conditions. Activity percentages were calculated taking into account the control lipase solution. Lipase activity recovery was not important for any of the three polymers, being slightly higher for PAA (55.8%). The purification factors were all relatively low. However, PAA presents rather better results than PVS and CHI and can be used as a first step of purification for the concentration of the enzyme from a medium with high protein concentration.

3.7. Lipase precipitation from culture medium

The precipitation with the three polymers was assayed on the SCM according to the best conditions found in the proposed earlier experiences.

When the anionic polymers were added to the culture media, precipitate formation was not observed under any of the conditions tested. This could be due to the characteristics of the SCM, such as ionic strength or low total protein concentration. The same results were achieved even when testing conditions were different from those assayed with lyophilized. The order of addition of components, pH, volumes and concentrations of the polymers and the times of precipitation were modified but in no case, was complex formation observed.

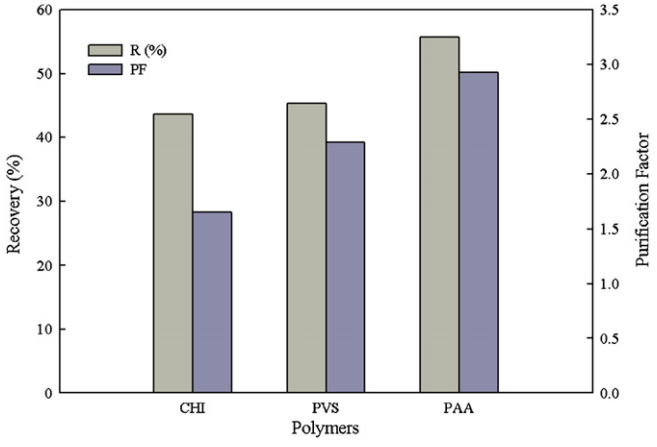

Fig. 6. Recovery and purification factor for the precipitation with the three polymers on commercial lyophilized.

SCM has a total protein concentration 15 times less than the lyophilized used in previous assays. Therefore, the volume of SCM used for lipase precipitation with CHI was increased 3 times compared to that used for Ly (Section 2.4). Precipitation reaction time was also modified because it was incomplete. Precipitation of lipase from SCM filtrate with CHI was carried out in a volume of 4 mL, on 250 μL of SCM filtrate. The CHI/total protein ratio in the reaction medium was 1.18 mg CHI/mg TP. The precipitate formed was incubated for 1 h at two different temperatures, 8 and 20 °C and centrifuged at 815 × g for 10 min. In Table 1 the purification factors and recovery can be observed. The recovery of activity in the re-dissolved precipitate was higher than 80% with purification factors above 3, both for 8 as to 20 °C. This is a good result taking account that also the SCM has major yield of enzyme that liquid culture media [16].

4. Conclusion

The protein concentration depending on the enzymatic source, natural or commercial, is a determining factor in the methodology of precipitation. Considering the simplicity in the preparation of solid culture media, that the CHI is a natural polymer and the results obtained, this methodology could be used as a first step for the isolation of extracellular lipase from *A. niger*. Therefore, the precipitation of protein–polymer complex represents an alternative method, with different comparative advantages over traditional methods of purification.

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