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Partition in aqueous two-phase system: Its application in downstream processing of tannase from *Aspergillus niger*

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ABSTRACT

Tannase from *Aspergillus niger* was partitioned in aqueous two-phase systems composed by polyethyleneglycol of molar mass 400, 600 and 1000 and potassium phosphate. Tannase was found to be partitioned toward the salt-rich phase in all systems, with partition coefficients lower than 0.5. Partition coefficients values and low entropic and enthalpic changes associated with tannase partition suggest that the entropic effect may be the driving force of the concentration of the enzyme in the bottom phase due to the high molar mass of the enzyme. The process was significantly influenced by the top phase/bottom phase volume ratio. When the fungal culture broth was partitioned in these systems, a good performance was found, since the enzyme recovery in the bottom phase of the system composed by polyethyleneglycol 1000 was around 96% with a 7.0-fold increase in purity.

1. Introduction

Tannin acylhydrolase, also known as tannase (EC 3.1.1.20), is a hydrolytic enzyme that catalyses the hydrolysis of gallotannins, releasing glucose and gallic acid. Tannins, which are natural compounds with a number of phenolic hydroxyl groups, can precipitate proteins, making these compounds undesirable in any protein-containing media. Tannase (TAH) is extensively used in the food and medical industries. In the food industry, the enzyme is used in the manufacture of instant tea, as a clarifying agent of wine, fruit juices, and in the reduction of antinutritional effects of tannins in animal feed [1–3]. This enzyme has also been used in environmental biotechnology, as in the treatment of the tannery effluents. Several microorganisms are potential sources of tannase [4–7]. The enzyme has an isoelectric point of 3.8 [8]. TAH has traditionally been isolated using gel permeation and ionic exchange chromatography, which allows the recovery of small amounts of enzyme [9,10]. The development of scale-up techniques is necessary to obtain significant amounts of TAH to be used in biotechnological industrial processes.

A downstream process usually accounts for 50–80% of the total production costs of enzymes. Conventional methods used for protein purification are usually expensive because they involve several unit operations, high cost of the reactants and they are difficult to scale up; therefore, it is necessary to develop new methods in scaling up to obtain enzymes with industrial applications [11–13]. Hence, in recent years, there has been an ongoing interest in biotechnology for the development of innovative and integrative separation and purification methods that are both economically viable and gentle enough to preserve the biological activity of proteins [14–18].

Aqueous two-phase systems (ATPS) have been used for the industrial isolation of enzymes from their natural source. They are formed by mixing two flexible chain polymers in water or one polymer and a salt at certain concentrations [19–22]. Proteins are partitioned between the two phases with a partition coefficient that can be modified by changing the experimental conditions of the medium such as pH, salts, and ionic strength, among others [23–28]. ATPS have been used as a first purification step since such systems allow the removal of large amounts and different types of contaminants by a simple and economical unit operation. Partition in ATPS presents many advantages over conventional methods for the isolation and purification of proteins: partition equilibrium is reached very fast, it can be applied in scale up, it has the possibility

Abbreviations: TAH, tannin acylhydrolase (tannase); CD, circular dichroism; Trp, tryptophan; ATPS, aqueous two-phase systems; Pi, potassium phosphate; PEG400 PEG600 and PEG1000, polyethylene glycol of average molar mass 400, 600, and 1000, respectively; R, volume ratio; MM, molar mass.

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Table 1
Phase percentage composition (w/w), for PEG–potassium phosphate systems at pH 7.00. The ATPS compositions correspond, in all cases, to tie line 4.

System	Total system			Bottom phase			Top phase		
	Pi	PEG	H_2O	Pi	PEG	H_2O	Pi	PEG	H_2O
PEG 400	17.90	25.10	57.00	39.73	2.12	58.15	3.95	39.73	56.32
PEG 600	17.40	18.30	64.30	30.59	2.00	67.41	4.78	34.48	60.74
PEG 1000	17.00	19.00	64.00	30.81	0.56	68.63	3.70	36.37	59.93

of continuous state operation, it has low costs and the materials that make up this system are non-expensive and can be recycled.

The aim of this work is to apply liquid–liquid extraction in ATPS to a culture broth of *Aspergillus niger* to obtain partially purified TAH. To fulfill this goal, we first performed a study about the interaction between TAH and PEG and then we assayed the TAH partition in ATPS to optimize the conditions before the partition of the crude cell broth.

2. Materials and methods

2.1. Chemicals

Polyethylene glycol of average molar mass 400, 600 and 1000 (PEG400, PEG600 and PEG1000), tannase (TAH) from *Aspergillus ficcum* were purchased from Sigma Chem. Co. (USA). *A. niger* GH1 was provided by the UAdeC-DIA culture collection (Mexico). All the other reagents were of analytical quality.

2.2. Preparation of the aqueous biphasic system

To prepare the ATPS, stock solutions of the phase components: PEG 40% (w/w) and potassium phosphate (Pi) 27% (w/w) at pH 7.0 were mixed according to the binodal diagram previously reported [29], the phase composition is shown in Table 1. Low-speed centrifugation to speed up phase separation was used after thorough gentle mixing of the system components. The desired volumes of each phase were mixed to reconstitute several ATPS in which the partition was assayed.

2.3. Protein and enzyme partition in ATPS

The partition coefficients (K) of the TAH and of the total proteins were calculated as the ratio of the respective concentration in the top phase to that in the bottom phase. The partition coefficient of protein (K_p) is calculated from the following equation:

$$K_p = \frac{P_{\mathrm{TOP}}}{P_{\mathrm{BOTTOM}}} \tag{1}$$

where P_{TOP} and P_{BOTTOM} is the protein concentration in the top and the bottom phases respectively, the partition coefficient of enzyme (K_e) is defined by,

$$K_e = \frac{Act_{\mathrm{TOP}}}{Act_{\mathrm{BOTTOM}}} \tag{2}$$

where Act_{TOP} and Act_{BOTTOM} is the TAH activity in the top and the bottom phases respectively.

In order to evaluate the purification process, the purification factor (PF) and the recovery yield ($Y\%$) in the bottom phase were also calculated from the following equations:

$$PF = \frac{SAct_{\mathrm{BOTTOM}}}{SAct_{\mathrm{TOTAL}}} \tag{3}$$

$$Y\% = \frac{100}{1 + RK_e} \tag{4}$$

being $SAct_{\mathrm{BOTTOM}}$ and $SAct_{\mathrm{TOTAL}}$ the specific activity in the bottom phase and in the initial solution respectively; and R the top phase/bottom phase volume ratio.

2.4. Determination of the TAH activity

The enzymatic activity of TAH was determined using tannic acid as substrate. The enzyme solution (100 μL) was incubated with 3 mL of 0.004% (w/v) tannic acid, in 0.02 M acetate buffer (pH 5.0) at 25 °C. The decrease in absorbance at 310 nm (due to the remaining tannic acid) with time was measured [6,30].

2.5. Effect of PEG molar mass in the bottom and top phases on the native fluorescence emission of TAH

The fluorescence emission spectra of TAH in the presence of both phases of the assayed ATPS were obtained. A reference spectrum was obtained in 50 mM Pi buffer pH 7.00. The scanning rate was 1 nm/min and the data acquisition was each 0.1 nm with a slit of 0.1 nm. The fluorescence spectra were obtained in an Amico Browman spectrofluorometer Serie 2000 using a thermostatized cuvette of 1 cm pathlength and were corrected using the software provided by the instrument manufacturer.

2.6. Effect of PEG molar mass in the bottom and top phases on acrylamide quenching of the native TAH fluorescence

Fluorescence quenching of tryptophan (Trp) residues in TAH was carried out by titration with acrylamide in the presence of both phases of the ATPS assayed and also in 50 mM Pi buffer pH 7.00 (in the absence of the phases). The data were analyzed using the mathematical model for the sphere of action according to Lakowicz [31], applying the following equation:

$$\frac{F_0}{F} = 1 + K_D[Q]\exp\left(\frac{\nu[Q]N}{1000}\right) \tag{5}$$

where F_0 and F are the protein fluorescence excited at 280 nm in the absence and presence of quencher, respectively, being K_D the Stern Volmer constant related to the lifetime of the fluorophore and the bimolecular quenching constant, [Q] the quencher concentration, N is the Avogadro's number and ν is the volume of the "sphere of action", i.e., the sphere within which the probability of immediate quenching is unity, and whose radius is only slightly larger than the sum of the radius of the fluorophore and quencher.

2.7. Effect of PEG molar mass in the bottom and top phases on TAH secondary structure

Circular dichroism (CD) scans of TAH in the presence of the phases of the ATPS assayed and in 50 mM Pi buffer pH 7.00 were carried out using a Jasco spectropolarimeter, model J-815. Ellipticity values [θ] were obtained in millidegree (mdeg) directly from the instrument. The cell pathlength of 1.0 cm was used for the spectral range 200–250 nm. The scanning rate was 25 nm/min. Bandwidth was 1 nm. In all cases, five scans were carried out and the non-protein spectrum was subtracted. To estimate the content of secondary structure in TAH, the CD data were analyzed by the CONTIN algorithm [32].

2.8. Microorganism and culture medium

The *A. niger* strain used in the present study was selected for its ability to produce tannase. Spores were stored at −20 °C in a cryoprotector medium composed of glycerol and skim milk. The culture medium for tannase production contained (g/L): K_2HPO_4, 1; $NaNO_3$, 3; KCl, 0.5; $MgSO_4{\cdot}7H_2O$; $FeSO_4{\cdot}7H_2O$. Salt solution was autoclaved

Table 2
Biological TAH activity in different media. The values correspond to the average of three independent determinations and each error bar represents the standard deviation of the data.

Phase		Relative Biological activity (%)
Buffer Pi 50 mM pH 7.00		100
Top	PEG400/Pi	100 ± 2
	PEG600/Pi	110 ± 4
	PEG1000/Pi	78 ± 4
Bottom	PEG400/Pi	120 ± 5
	PEG600/Pi	160 ± 6
	PEG1000/Pi	131 ± 5

at 121 °C for 15 min and then cooled at room temperature. Tannic acid was added to a final concentration of 50 g/L, the pH was then adjusted at 4.0 with 1 M NaOH or 1 M H_3PO_4, and the culture media was filter-sterilized through 0.45 μm nylon membrane.

2.8.1. Cultures

Inocula were prepared by transferring the spores to potato dextrose agar and incubating them for 4 days at 30 °C. Spores were then scraped into a sterile 0.02% Tween-80 solution and counted in a Neubauer chamber. Liquid medium to be added to each solid-state fermentation support was inoculated with the spore suspension at 1×10^7 spores per mL.

2.9. Tannase production in solid-state fermentation

Polyurethane foam was used as solid support to absorb the inoculated liquid medium. The foam was ground and washed twice with hot water (60 °C) followed by a wash with cold water. The foam was then dried before autoclaving at 121 °C for 15 min. Erlenmeyer flasks containing 12 g of foam were impregnated with 28 mL of inoculated medium, prepared as described above. Flasks were incubated at 30 °C for 72 h before harvesting [8].

2.10. Production and concentration of enzyme extract

An extract (about 1 L) containing extracellular tannase was obtained by compressing polyurethane foam in a Buchner funnel. The polyurethane foam was washed with 50 mM acetate buffer pH 5.0 and compressed again. The extract was then centrifuged (15 min, 7000 rpm, Sigma 3–18 K H at 4 °C), filtered through 0.45 μm nylon membranes, and concentrated to 100 mL by ultrafiltration on an Amicon membrane with a 10 kDa molar mass cut-off (Millipore).

3. Results and discussion

3.1. Stability analysis of TAH

As a previous step to assay the partition of an enzyme in ATPS, it is necessary to test whether the medium composition (top and bottom phases) induces any conformational modification on the TAH structure. To do this, a commercial TAH was utilized.

3.1.1. Effect of PEG molar mass in the bottom and top phases on TAH biological activity

The effect of ATPS's phases on TAH enzymatic activity was assayed as shown in Table 2. TAH activity increased in the salt-rich phase in all the ATPS assayed and there was no significant variation on TAH activity in the top phases, except for PEG 1000, where a diminution of the relative activity was observed. These results suggest that the application of these ATPS is suitable for TAH purification since the presence of the phases does not have a negative effect for TAH activity.

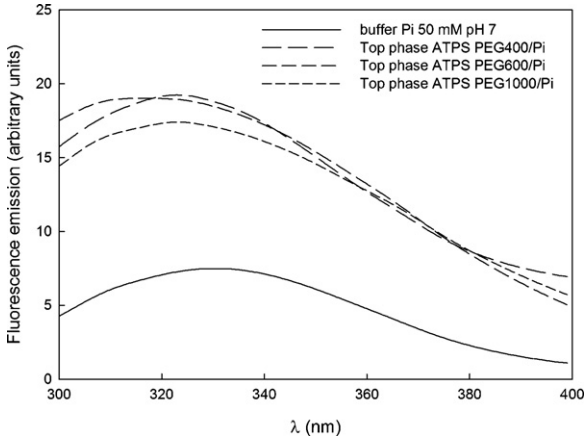

Fig. 1. TAH native emission fluorescence in 50 mM Pi buffer pH 7.00 and in the presence of top phases. Temperature: 25 °C. TAH concentration: 1 mg/mL. Excitation wavelength: 280 nm.

3.1.2. Effect of PEG molar mass in the bottom and top phases on the native TAH fluorescence emission

The tryptophan fluorescence emission of a protein is sensitive to changes in the polarizability that occurs in its environment; therefore, the fluorescence signal can be used as an optical probe to analyze the effect of a cosolute on the tertiary structure of the protein domain that contains the tryptophan [31,33,34]. The effect of the phases of the ATPS on the TAH fluorescence emission band was assayed. Fig. 1 shows the emission fluorescence spectra of TAH in the presence of the top phase for the different ATPS and also in 50 mM Pi buffer pH 7.00. PEG presence can be seen to induce a fluorescence shift to shorter wavelengths. This effect can be attributed to an increase in the hydrophobicity of the tryptophan environment that could be due to an interaction between Trp and the hydrophobic portion of the PEG molecule. Another change observed in the emission spectra is the increase in the fluorescence intensity in the presence of PEG-rich phases. This means a decrease of no-radiant processes with respect to TAH in the buffer medium. Considering that ionic species are generally responsible for decreasing the radiant process, this change in the spectra may be due either to salt exclusion from the Trp environment or to the increase in the viscosity of the media, thus preventing the interaction between the Trp and ionic species in the solution. The bottom phases did not vary TAH fluorescence spectra (data not shown), which suggested that the Trp environment was not modified in those media.

3.1.3. Quenching of the native TAH fluorescence by acrylamide

The fluorescence quenching of a fluorophore by a quencher is a technique that provides information about the quencher accessibility to the fluorophore. Moreover, this technique gives information about any modification in the tryptophan microenvironment induced by the cosolute presence [31,35]. The quenching of native TAH fluorescence using increasing concentrations of acrylamide was measured at 340 nm while exciting at 280 nm. Different media were assayed: 50 mM Pi buffer pH 7.00 and both top and bottom phases of the assayed ATPS. From the fitting of the data in a Stern Volmer plot (Fig. 2), the K_D and υ parameters values were calculated. The parameters obtained for the quenching of TAH in all the media were similar, except in the case of the top phase for the PEG400-phosphate system. In this case, the K_D value was 3.5 times higher than that for TAH in buffer. K_D value is related to the quencher capacity to access the tryptophan environment, so, the increase in K_D value by PEG400 suggests that the acrylamide intensively attacks the fluorophore, which may be due to a

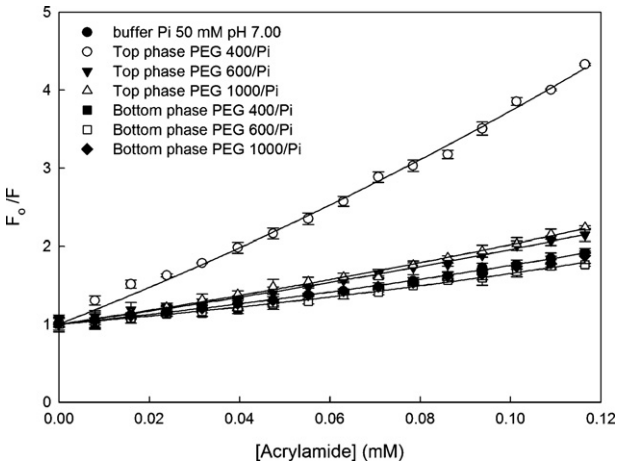

Fig. 2. Stern Volmer plot for the quenching of the native fluorescence of TAH (1 mg/mL) with increasing amount of acrylamide. Excitation wavelength: 280 nm. Emission wavelength: 340 nm. Temperature: 25 °C. The values correspond to the average of three independent determinations and each error bar represents the standard deviation of the data.

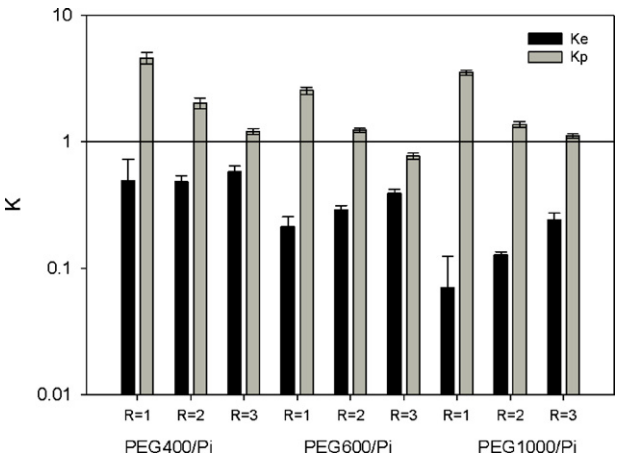

Fig. 4. Dependence of partition coefficient for TAH vs. PEG molar mass and top-bottom volume ratio. pH 7.00. Temperature 25 °C. The values correspond to the average of three independent determinations and each error bar represents the standard deviation of the data.

greater interaction between TAH and PEG400. υ values were about 4.3×10^6 nm^3, without correlation among the media assayed.

3.1.4. TAH secondary structure

Circular dichroism is a common tool for the analysis of the protein secondary structure; the CD spectrum in the far UV region (190–260 nm) is related to the protein alpha helix and the beta sheet content [32,36]. Fig. 3 shows the CD spectra of TAH in 50 mM Pi buffer pH 7.00 and in the presence of the top phases of the ATPS assayed. From these results, the content of the alpha helix, beta sheet and random coil were calculated applying the method previously reported. The secondary structure estimated for TAH was: 45% α-helix, 24% β-sheet, 14% turns and 16% random. It can be seen from the spectra, that only a slight modification in CD signal was induced by the top phases while the bottom phases did not show any change (data not shown). However, TAH retained the native secondary conformational state in the presence of the PEG-rich phases, since no variation in the fraction of the structure was observed. In the conditions assayed, the spectra in near UV region (260–320 nm) were very poor and did not provide valuable information about the tertiary structure of the protein.

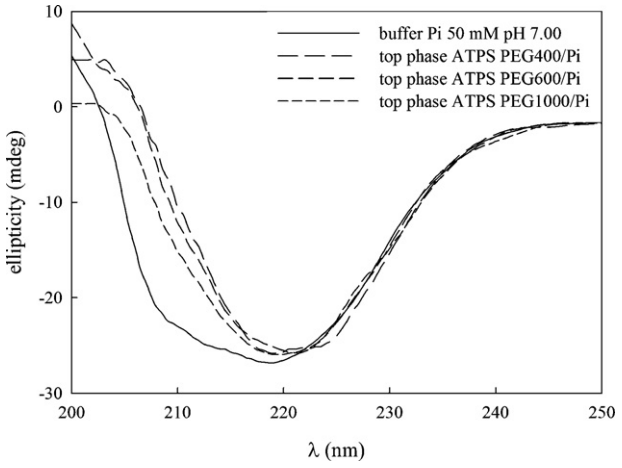

Fig. 3. CD spectra of TAH (1 mg/mL) in 50 mM Pi buffer pH 7.00 and in the presence of top phases. Temperature: 25 °C.

3.2. Profile of the TAH partition in ATPS composed by PEG and Pi

3.2.1. PEG molar mass effect on the TAH partition

Culture broth partition was assayed in systems containing PEG 400, 600, 1000 and 1450. In ATPS composed by PEG1450, no TAH activity was detected in the top phase, therefore, only partition coefficient values with PEGs of lower MM are reported. Ke and Kp were calculated with Eqs. (1) and (2); K values obtained for different ATPS are shown in Fig. 4. In most of the partitions assayed, K_p values were higher than 1, and K_e values were lower than 1, indicating that TAH was partially purified in the bottom phase. The contaminant proteins were preferentially partitioned to the PEG-rich phase.

The solute partition in PEG-salt two-phase system can be due to entropic contribution, electrostatic or hydrophobic interactions. Jonhanson et al. demonstrated that in PEG-salt systems, the phase-forming salt determines $\Delta\psi$ (Galvani-type interfacial electrostatic potential difference). In PEG–phosphate systems, this parameter is positive so that it favors the partitioning of net negatively charged proteins to the PEG-rich phase.

TAH is negatively charged (pI: 3.8) in the systems assayed (pH 7); however, the enzyme is favorably partitioned toward the bottom phase ($K_e < 1$ values were obtained). Therefore, the electrostatic interactions are not responsible for the partitioning of TAH toward the salt-rich phase [37].

Flory–Huggins theory yields a simple analytical expression for the partition coefficient. In the absence of enthalpic effects, Jonhanson et al. arrived at a simple relation to describe the entropic contribution to partition for the transference process of a solute from the polymer-rich phase to the salt-rich phase. They demonstrated that uneven partitioning will result if there is a difference between the top and the bottom phases in the number of molecules of solute per unit volume (i.e., the number density).

Under such conditions, the solute will partition to the phase with the highest number density. Entropic effects on solute partition are larger for PEG-salt systems, when the polymer is effectively localized in the top phase, causing the number density of the top phase to be lower than the salt-rich phase. This entropic driving force toward polymer free phase increases with the molar mass of the protein [38].

Another factor that takes part in the partitioning of a protein in ATPS is the hydrophobicity of the PEG molecule. Hachen et al. found a correlation between log K_e in PEG-salt ATPS and the hydrophobicity values derived from the solubility of protein in an ammonium sulphate solution. They showed that the more hydrophobic the

Table 3
Thermodynamic variables values associated to the TAH partition in ATPS, $R = 1$ for the process of transfer of TAH from salt-rich phase to polymer rich phase. Temperature: 25 °C. The values correspond to the average of three independent determinations and each error bar represents the standard deviation of the data.

ATPS	ΔH° (kcal/mol)	ΔS° (cal/mol K)
PEG400/Pi	−1.39 ± 0.06	−6.1 ± 0.5
PEG600/Pi	−7.04 ± 0.07	−26.7 ± 0.8
PEG1000/Pi	−0.74 ± 0.03	−7.7 ± 0.3

proteins are, the higher the Ke values. There is not solubility in ammonium sulphate data of *A. niger* TAH reported, but the K_e values measured in this work suggest that TAH has few hydrophobic patches in the surface [39].

The results show that when the MM of PEG increased, the partition coefficient of the enzyme significantly decreased. TAH showed an anomalous low partition coefficient in PEG–Pi systems considering that the PEG molecular weights assayed were very low. Proteins of high molar mass (around 70–80 kD, such as bovine albumin) show very low partition coefficients when they are partitioned in a system containing PEG 6000 or higher [40]. TAH from different sources show a variation in the MM; however, a MM of 225 kDa for *A. niger* TAH has been reported [41]. This high MM is the reason why the partition coefficients observed are low, although the systems are composed of PEGs of low MM.

Another characteristic of these systems is that the TAH and protein partition coefficients are highly dependent on the volume phase ratio; this effect is observed for all the PEGs assayed, as shown in Fig. 4. The dependence of the partition coefficient on the top phase/bottom phase volume ratio has been reported for enzymes such as chymosin and pepsin [27]. Taking into account the definition of partition constant, it should only depend on the temperature. However, the partition coefficients K_e and K_p are not true thermodynamic equilibrium constants, since they are calculated from the ratio between the enzymatic activity and protein concentration respectively in both phases without considering the (unknown) activity coefficients. The activity coefficients may be close to unity in diluted systems (i.e. K_e and K_p could be proximate to equilibrium constants); however, this is not the case, as we are working with a concentrated sample. The influence of top-bottom volume ratio on K values may be due to strong interactions between the different hydrophobic portion molecules which make up the cell broth. This interaction is highly modified by dilution of the homogenate. Similar results have been reported by Marcos et al. [40].

3.2.2. Temperature effect on TAH partition

The partition was assayed at two temperatures: 8 and 25 °C. The increase in temperature induced greater TAH transfer to the bottom phase; this effect was observed for all the assayed ATPS. This decrease in Ke indicates that the process of TAH partition is exothermic. By applying van't Hoff equation, the enthalpic and entropic changes for the partition process were calculated and shown in Table 3.

Low enthalpic and entropic changes are observed for TAH partition in comparison to those found for other macromolecules [42,43], suggesting that the interaction between TAH and the phase components is weak. This finding is in agreement with the idea that the partition mechanism is controlled by the exclusion volume effect.

3.2.3. TAH purification from culture broth by partition in ATPS

The TAH purification factor and recovery yield were calculated in all the assayed ATPS. The best conditions for the purification were obtained with the culture broth partition in ATPS composed by PEG1000-Pi and volume ratio 1. The values obtained at both temperatures were not significantly different, so the partition was carried out at 25 °C since it was easier and economical to be applied in scaling up. The purification performance is shown in Table 4.

Table 4
Purification table of the TAH from the culture broth by ATPS PEG1000/Pi. Temperature: 25 °C. The values correspond to the average of three independent determinations and each error bar represents the standard deviation of the data.

	Culture broth	Bottom phase
Volume (mL)	0.2	0.8
Act TAH (U/mL)	0.92 ± 0.03	0.22 ± 0.01
Act TAH (U)	0.184 ± 0.006	0.18 ± 0.01
[Total protein] (mg/mL)	7.01 ± 0.02	0.24 ± 0.08
Total protein amount (mg)	1.40 ± 0.05	0.192 ± 0.006
Specific activity (U/mg)	0.131 ± 0.004	0.92 ± 0.07
Purification factor	1	7.0 ± 0.5
Recovery yield (%)	100	96 ± 5

TAH is recovered in the salt-rich phase where the PEG concentration is lower, no additional steps being necessary for PEG separation. Moreover, it is noteworthy that the tannic acid content in the bottom phase was undetectable, since it was partitioned to the upper PEG-rich phase. This is particularly important since TAH is hard to separate from tannic acid.

Mahendran et al. purified tannase from *Paecilomyces variotii* 19.4 folds with traditional methods from cell-free culture filtrate using ammonium sulfate precipitation followed by ion exchange and gel filtration chromatography with a 56.1% yield [10]. In a previous work, we purified tannase from *A. niger* GH1 to apparent homogeneity by ultrafiltration, anion-exchange chromatography, and gel filtration that led to a purified enzyme with a final yield of 0.3% and a purification fold of 46 [41]. The traditional chromatographic methods for purification are more difficult to scale up and provide lower yields; TAH extraction in ATPS composed by PEG and Pi can be scalable to industrial purposes and provide an adequate TAH purification factor and high yield.

4. Conclusions

As a previous step to assay the partition of a protein in ATPS formed by PEG and a salt, the conformational state of the target enzyme in both phases must be analyzed. Both phases did not induce any significant changes in the secondary TAH structure and in most cases its biological activity did not decrease. It was found that PEG modified the TAH tryptophan environment inducing an increase in its hydrophobicity grade, which can be considered as an increase in the TAH stability.

A previous paper [38] postulated that protein partition in ATPS composed by PEG and Pi could be driven depending on two protein features: size and hydrophobicity. If the protein has low MM and its surface contains hydrophobic residues, an interaction between the protein and PEG driven by van der Waals forces takes place and the protein is preferentially partitioned to the PEG-rich phase. If the MM of the protein is high, the partition in ATPS is usually driven by the entropic effect and the protein is partitioned toward the salt-rich phase. This effect is more evident when the PEG MM increases. TAH from *A. niger* has a MM of about 225 kD, this high value confirms that the enzyme tends to be partitioned in favor of the salt-rich phase, and a partitioning coefficient lower than unity is observed. However, the partition coefficient of total proteins (impurities) present in the fungal homogenate was in favor of the PEG-rich phase and was not affected by PEG molar mass, showing a practically constant value. TAH recovery was strongly influenced by the top-bottom volume phase ratios. Previous reports [18,44] have demonstrated a similar behavior for different enzymes. The method is suitable for TAH isolation because a recovery of 96% on

the initial tannase activity was obtained in only one extraction with a purification factor of 7.0. Besides, tannic acid was eliminated.

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