Colloids and Surfaces B: Biointerfaces 101 (2013) 392–397

Contents lists available at SciVerse ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

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

Luis V. Rodríguez-Durán[a], Darío Spelzini[b], Valeria Boeris[b,*], Cristóbal N. Aguilar[a], Guillermo A. Picó[b]

[a] *Food Research Department, School of Chemistry, Universidad Autónoma de Coahuila, Blvd. Venustiano Carranza and J. Cárdenas s/n, ZIP 25280 Saltillo, Coahuila, Mexico*
[b] *Laboratorio de Fisicoquímica aplicada a Bioseparación – Rosario, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, FonCyT, CONICET, Suipacha 570, S2002RLK Rosario, Argentina*

ARTICLE INFO

Article history:
Received 19 January 2012
Received in revised form 4 June 2012
Accepted 18 July 2012
Available online 25 July 2012

Keywords:
Aqueous two-phase system
Tannase
Partition
Purification
Interaction
Downstream processing

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.

* Corresponding author at: Laboratorio de Fisicoquímica aplicada a Bioseparación, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 570, S2002RLK Rosario, Argentina. Tel.: +54 341 4804597x253; fax: +54 341 4804598.
E-mail address: valeriaboeris@conicet.gov.ar (V. Boeris).

0927-7765/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.colsurfb.2012.07.023

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