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Short communication

Interaction of tannase from *Aspergillus niger* with polycations applied to its primary recovery

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ABSTRACT

The interaction of tannase (TAH) with chitosan, polyethyleneimine and Eudragit®E100 was studied. It was found that TAH selectively binds to these polycations (PC), probably due to the acid nature of the target protein. TAH could interact with these PC depending on the medium conditions. The effect of the interaction on the secondary and tertiary structure of TAH was assayed through circular dichroism and fluorescence spectroscopy. TAH was recovered from *Aspergillus niger* culture broth by means of precipitation and adsorption using chitosan.

1. Introduction

The production of proteins is a complex process which requires upstream and downstream steps in order to obtain the final product in the desired purified form. Bioseparation may include operations which are slow, multi-step, and thus very costly. This creates a bottleneck in some process development that brings about an urgent need for efficient and high-yield protein separation technologies.

The ability of synthetic and natural polyelectrolytes (PE) to interact with globular proteins is well known [1–4]. Proteins can interact with either PE soluble or PE insoluble. When one out of many of the proteins in a crude extract preferentially interacts with the PE, and a phase separation of the pair protein–PE is possible, the process could be used as a convenient strategy for the isolation and purification of the target protein [5–8]. Precipitation and adsorption are two of the separation operations which take advantage of the polyelectrolyte–protein interaction.

The mixture of a negatively charged protein and a water-soluble polycation (PC) may lead to the interaction between the macromolecules, the formation of a protein–PC complex and its further precipitation. The precipitate can be separated from the supernatant and then redissolved to recover the protein and the PC [9–11]. On the other hand, a negatively charged soluble protein can also interact with a non-soluble form of a PC. Thus, the adsorption of the protein onto the non-soluble PC surface and its further elution allows the recovery of the protein from a sample [2,10,12,13]. These operations play an important role in the downstream processing in biotechnology. A variety of charged polymers can be used to precipitate or adsorb proteins with a high industrial value [7,8,12–14]. The importance of the application of PE to alternative processes for the recovery of proteins lies not only in economic issues but also in the short operation time, the high yield and the low environmental impact [15,16].

However, it has been shown that, at least in some systems, the PPI may have an impact on the biological performance of the protein [4,17]. Thermal stability, enzymatic activity and structure of proteins can be affected by PPI [3]. This is important to consider before designing a purification protocol for a protein based on PPI. It is necessary to evaluate the protein stability and functionality while interacting with PE.

Tannin acylhydrolase, also known as tannase (EC 3.1.1.20), is a hydrolytic enzyme that catalyses the hydrolysis of gallotannins,

Abbreviations: TAH, Tannin acylhydrolase (tannase); PPI, protein–polyelectrolyte interaction; PE, polyelectrolyte; PC, polycation; CS, chitosan; EuE, Eudragit® E100; PEI, polyethyleneimine.

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releasing glucose and gallic acid. Tannins are natural compounds which have a number of phenolic hydroxyl groups and can precipitate proteins, which makes these compounds undesirable in any protein-containing medium. Tannase (TAH) is extensively used in 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. This enzyme was also proposed to be used in environmental biotechnology in the treatment of tannery effluents. Several microorganisms are potential sources of TAH, such as *Aspergillus niger* and *Rhizopus oryzae*. The TAH from *A. niger* has an isoelectric point of 3.8 [18,19]. TAH has traditionally been isolated using ammonium sulphate or solvent precipitation, gel permeation and ionic exchange chromatography [20–22], which allows the recovery of small amounts of enzyme. The development of scaling-up methodologies is necessary to obtain significant amounts of TAH to be used in biotechnological industrial processes. Recently, two alternative methods to recover TAH have been proposed: (1) the use of reverse micellar extraction [23] and (2) partition in aqueous two-phase systems [24].

The aim of this work was to evaluate the interaction between TAH and three PC: chitosan (CS), polyethyleneimine (PEI) and Eudragit®E100 (EuE) on TAH stability, enzymatic activity and structure and then, to recover TAH from a crude cell broth by means of precipitation and adsorption using these PC.

2. Materials and methods

2.1. Reagents

Eudragit® E100 was kindly donated by Etilfarma (Buenos Aires – Argentina). Chitosan from crab shells – practical grade (medium degree of deacetylation 85%), polyethyleneimine (50% aqueous solution, mean molecular mass 60,000) and pure lyophilized 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. Circular dichroism spectra

Circular dichroism (CD) scans of TAH in 20 mM acetate buffer pH 4.0 in the absence and presence of EuE, CS and PEI up to 25 ppm were carried out using a Jasco spectropolarimeter, model J-8150. The ellipticity values [θ] were obtained directly from the instrument. A 1 cm pathlength cell was used for the spectral range 200–250 nm. The scanning rate was 10 nm/min and the slitwidth was 1 nm. Eight scans for each sample were made. The CD signal was informed as the total signal (protein plus polyelectrolyte) minus the polyelectrolyte signal.

2.3. Native fluorescence emission spectra

Fluorescence emission spectra of TAH in 20 mM acetate buffer medium, pH 4.0 were obtained when exciting at 280 nm at increasing PC (EuE, CS and PEI) concentrations. The scanning rate was 10 nm/min and the data acquisition was performed every 0.1 nm with a 0.1 nm slit. The fluorescence spectra were obtained in an Amico Browman spectrofluorometer Series 2000 using a thermostatized quartz cell of 1 cm path length, and they were corrected using the software provided by the instrument manufacturer.

2.4. Quenching of the native fluorescence

The fluorescence quenching of the protein tryptophan (Trp) residues, measured at 340 nm (exciting at 280 nm), was carried out by titration of TAH with acrylamide in different media: 20 mM acetate buffer pH 4.0 in the absence and presence of EuE, CS and PEI 25 ppm. The data were analyzed applying the mathematical model of Stern Volmer, using the following equation:

$$\frac{F_0}{F_i} = 1 + K_D.[Q] \quad (1)$$

where F_0 and F_i are the protein fluorescence intensities in the absence and presence of the quencher respectively, K_D is the Stern Volmer constant (related to the lifetime of the fluorophore and the bimolecular quenching constant) and [Q] is the quencher – acrylamide – concentration.

2.5. Microorganism and culture medium

A. niger GH1 was used due to its ability to produce tannase. Spores were stored at −20 °C in a cryo-protector medium composed of glycerol and skim milk. The culture medium for TAH production contained: 1 g/L K_2HPO_4, 3 g/L $NaNO_3$, 0.5 g/L KCl, $MgSO_4{\cdot}7H_2O$; $FeSO_4{\cdot}7H_2O$. Salt solution was autoclaved 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. The culture media was filter-sterilized through 0.45 μm nylon membrane.

2.5.1. Cultures

Inocula were prepared by transferring the spores to potato dextrose agar and incubating them at 30 °C for 4 days. 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/mL.

2.6. Tannase production in solid-state fermentation

Polyurethane foam was used as a solid support to absorb the inoculated liquid medium. The foam was ground and washed with hot water (60 °C) twice 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.

2.7. Production and concentration of enzyme extract

An extract (about 1 L) containing extracellular TAH 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 r.p.m., 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).

2.8. Enzymatic activity

The enzymatic activity determination of TAH was carried out using tannic acid as substrate. The enzyme solution (100 μL) was incubated with 3 mL of 0.004% (w/v) tannic acid, in 20 mM acetate buffer (pH 5.0) at 25 °C. The absorbance at 310 nm (due to the remaining tannic acid) vs. time was measured.

2.9. Determination of the interaction conditions

Turbidity (Abs 420 nm) measurements were used to determine the pH of initial insoluble complex formation. The dependence of solution turbidity on pH was obtained by the addition of 0.1 M