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Production of alpha-amylase from *Aspergillus oryzae* for several industrial applications in a single step

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ABSTRACT

A one-step method as a strategy of alpha-amylase concentration and purification was developed in this work. This methodology requires the use of a very low concentration of biodegradable polyelectrolyte (Eudragit® E-PO) and represents a low cost, fast, easy to scale up and non-polluting technology. Besides, this methodology allows recycling the polymer after precipitation.

The formation of reversible soluble/insoluble complexes between alpha-amylase and the polymer Eudragit® E-PO was studied, and their precipitation in selected conditions was applied with bioseparation purposes. Turbidimetric assays allowed to determine the pH range where the complexes are insoluble (4.50-7.00); pH 5.50 yielded the highest turbidity of the system. The presence of NaCl (0.05 M) in the medium totally dissociates the protein-polymer complexes.

When the adequate concentration of polymer was added under these conditions to a liquid culture of *Aspergillus oryzae*, purification factors of alpha-amylase up to 7.43 and recoveries of 88% were obtained in a simple step without previous clarification. These results demonstrate that this methodology is suitable for the concentration and production of alpha-amylase from this source and could be applied at the beginning of downstream processing.

1. Introduction

In their natural sources, proteins are normally found in very low concentrations or as part of a complex mixture of components. Besides, they can be unstable, thermolabile or sensitive to changes in variables of the medium such as pH, ionic strength, presence of cosolutes, etc.

The increasing demand of enzymes in different industrial activities (chemistry, food, textile, pharmaceutical, etc.) has promoted the development of purification technologies mainly if they are simple, low cost, fast, and clean. Alpha-amylase (α-Amy) is one of the enzymes with high demand for industrial applications. Starch depolymerisation by amylases is the basis for several processes such as glucose syrups production, bread making and brewing. It is also used as an additive in soaps, detergents and animal feeds, and in the treatment of paper, textiles, etc. [1,2]. Amylases are a class of hydrolases widely distributed in bacteria, fungi, plants and animals. They are members of family 13 in the classification of glycoside hydrolases according to Henrissat [3] which can specifically cleave the *O*-glycosidic bonds in starch.

Aspergillus oryzae is a filamentous fungus extensively used in fermentation industry because of its ability to secrete a variety of high-value industrial enzymes such as α-Amy, pectinase β-galactosydase, etc. [4–6]. The α-Amy synthesized from *A. oryzae* is an extracellular endoacting hydrolase that gives large oligosaccharides as products of starch degradation due to scission of internal α-1,4-linkages.

The complex formation between proteins and polyelectrolytes has been extensively studied [7–9]. due to its applications in the purification of proteins, control of protein release, enzyme immobilization and/or stabilization, etc. [10–18]. Precipitation of insoluble protein-polyelectrolyte complexes offers the basis for protein concentration and purification from a heterogeneous mixture. Furthermore, satisfying the requirements above mentioned, it has several advantages: it is easily scaled up, it requires simple equipment and a wide variety of alternative precipitants can be used [19].

A wide range of synthetic and natural [20–22] polyelectrolytes can interact with proteins to form stable protein-polyelectrolyte complexes that can be either soluble or insoluble depending on different experimental parameters such as concentration, number

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and distribution of charged sites on the components, protein-polyelectrolyte ratio, pH and ionic strength of the medium, etc. This kind of polymers is often called "intelligent polymers". The insoluble complexes can be easily separated by centrifugation or simple decantation [23–25].

Eudragits® are different types of enteric copolymers widely used in controlled drug delivery. Depending on the pH, these copolymers act as polyelectrolytes which make them suitable for different purposes from gastric or intestinal soluble drug formulations to insoluble but swellable delivery forms, regulated by the number of charged and non-ionized (ether) groups in the structure of these copolymers. Some of them, which are soluble in aqueous media with different pH values, can be considered as polycations (Eudragit® type E) and others as polyanions (Eudragit® types L and S). Eudragit® E-PO (EPO) is a cationic copolymer based on 2-dimethylaminoethyl methacrylate, methyl methacrylate and *n*-butyl methacrylate [26]. It is an enteric polymer which is commercially available and it is an interesting alternative to form reversible soluble/insoluble complexes with proteins, as a strategy of enzyme isolation.

The aim of this work was to develop a strategy of recovery of α-Amy from a culture supernatant of *A. oryzae* by one simple step by insoluble complex formation with the polymer EPO.

2. Materials and methods

2.1. Chemicals

α-Amy from *A. oryzae* was purchased from Sigma Chem. Co. (USA) and EPO molecular average mass 47 kDa was gently donated by Evonick, Argentina. Phosphate buffer solutions of different pH were prepared at concentration of 50 mM and were adjusted with NaOH or HCl.

2.2. Enzyme assay

The measurements of α-Amy activity were carried out through the commercial kit *Amylase 405, kinetic unitest*, which was purchased from Winner Lab., Rosario, Argentina. This kit makes use of a specific substrate of α-Amy: 2-chloro-*p*-nitrophenyl-α-D-maltotriose (CNP-G3). The enzyme hydrolyzes the substrate releasing 2-chloro-*p*-nitrophenol (CNP) which absorbs at 405 nm (λ_{405} = 12.9 mM^{-1} cm^{-1}) and the color development is directly proportional to enzymatic activity. Thus, the reaction was followed by measuring the absorbance at 405 nm for 5 min and activities were calculated from the initial linear portion of the Abs. vs. time curves [27] and expressed as "U". One unit of enzyme activity (U) was defined as the amount of enzyme required to hydrolyze 1 μmol of substrate per minute. The enzyme assays were performed at a constant temperature of 20 °C in medium phosphate buffer at the optimum pH of 6.00.

The enzymatic activity of α-Amy was evaluated in the absence and presence of EPO at different molar ratios chosen from the plateau of titration curves. In order to evaluate the stability of α-Amy in the presence of EPO, the enzyme was incubated at pH 5.50 with the polyelectrolyte and the activity was measured for 24 h.

2.3. Microorganism, maintenance of culture

A. oryzae NRRL 695, donated by the National Centre for Agricultural Utilization Research (ARS), USDA, USA, was used in this study. It was propagated on Potato-Glucose-Agar (PGA) medium at 30 °C. The plates were grown for five days [28]. Conidia suspensions in 10% glycerol were stored at −20 °C in 1 mL aliquots.

2.4. Inoculum preparation

Conidia of *A. oryzae* NRRL 695 from five-day-old cultures in PGA plates were harvested by the addition of 15 mL distilled water. Then it was appropriately diluted to the required density of conidia and used as the master suspension. The number of viable conidia in the inoculum was determined by the counting technique using the Thomas Cell [28].

2.5. Substrate and culture media

Starch was used as substrate and sole carbohydrate source. 100 mL of enzyme production medium was prepared in 250 mL Erlenmeyer flask, containing (g/L): starch: 17.00, peptone: 1.90, urea: 1.25, glycerol: 0.60, KH_2PO_4: 0.50, $MgSO_4$: 0.25, $(NH_4)_2SO_4$: 0.025 and distilled water. The initial pH was adjusted to a value of 5.00 after which the flasks were autoclaved at 121 °C for 20 min. After cooling, the medium was inoculated with the master conidia suspension to a final concentration of $\sim 1.00 \times 10^6$ conidia/mL. Then, the inoculated medium was kept on rotary shaker (150 rpm) at 30 °C for 96 h. At the end of the incubation, the suspension was filtrated and used as the crude enzyme for precipitation experiments [28]. The content of proteins in the culture supernatant was characterized by electrophoretic separation on 1013% SDS-polyacrylamide gel (SDS-PAGE), on a Bio-Rad minigel apparatus, stained with coomassie blue.

2.6. α-Amy turbidimetric titration curves with EPO at different pH and ionic strengths

The formation of the insoluble polymer-protein complex was followed by means of turbidimetric titration [7]. Buffer sodium phosphate solutions with a fixed protein concentration (40 μM) were titrated at 20 °C in a cubic 1 cm path length glass cell with the polymer stock solution, in the total volume of 2.5 mL. The concentration of EPO solution was 0.5% w/w. 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 (Abs.) at 400 nm vs. molar ratio EPO/α-Amy. We defined "stoichiometric polyelectrolyte/enzyme molar ratio" as the minimal EPO/α-Amy molar ratio in which the protein has been precipitated as an insoluble complex. It was calculated from the plot at the lowest polyelectrolyte concentration necessary to get a plateau. These values are important because they allow us to calculate the minimal polyelectrolyte amount necessary to fully precipitate the protein. The data have been expressed as the number of α-Amy moles bound per polyelectrolyte mol. The same was repeated at different ionic strengths by adding NaCl to phosphate buffer [24].

The time needed to form the complex was evaluated by measuring the time required to obtain the maximal absorbance at a fixed molar ratio [10].

2.7. Turbidimetric titration curves vs pH

Three different molar ratios from the plateau region of the titrations curves were selected. They were titrated with alkali and acid, at 20 °C. The protein concentration was 40 μM in the total volume of 8 mL. The Abs. at 400 nm was measured in a cubic 1 cm path length glass cell and plotted vs. pH. The same was repeated in the absence of α-Amy. These phase diagrams show the pH range where the polyelectrolyte-protein complex or the polyelectrolyte alone is soluble or insoluble.