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Integration of ultrafiltration into an aqueous two-phase system in the keratinase purification

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ABSTRACT

Keratinases are proteases that have several important applications in the daily life. Although they have attracted the attention of researchers in the field of biotechnology, their use in industrial processes requires efficient purification techniques allowing scaling-up. Among these techniques, the aqueous two-phase system and ultrafiltration stand out. This study reports the purification of keratinase from *Bacillus* sp. P45 by aqueous two-phase system two-stage process integrated to ultrafiltration. In the aqueous two-phase system, the addition of sodium chloride in polyethylene glycol-potassium phosphate system favored the partitioning of keratinase. The keratinase purified by an aqueous two-phase system two-stage process integrated to ultrafiltration in diafiltration mode presented purification factor of 6.1-fold and enzymatic recovery of 56.3%, and the enzyme was separated from polyethylene glycol. The purified keratinase was capable of hydrolyzing both soluble and insoluble protein substrates more efficiently than the crude enzyme, evidencing the beneficial effect of enzyme purification on its catalytic activity.

1. Introduction

Keratins are insoluble and stable proteins found as major component of the skin and its appendages (hair, feathers, nails, hooves, horns and scales). These recalcitrant proteins are produced in large quantities by the agro-industrial processing and generally are not degraded by commonly known proteases such as pepsin, trypsin and papain. The microbial proteases that can hydrolyze keratin are defined as keratinases [1].

In recent years, keratinases have attracted the attention of biotech research, due to their numerous applications of industrial interest [2]. Keratinase can convert keratin present in the keratinous waste, producing protein hydrolysates that can be used as supplement in animal feed [3] and production of nitrogen fertilizers [4]. These enzymes can be used in detergents [5,6], pharmaceutical industry [7], leather industry [8], degradation of prions [9–11] and in the production of keratin peptides with antioxidant activity [12]. Since keratinases are proteases, they are well suited to take over the hitherto protease-dominant sectors (detergent, leather, and textiles). Overall, keratinases have broadened the horizons of protease market particularly in sectors where its application was limited [13].

The traditional process for keratinase purification generally involves chromatographic techniques such as ion exchange and gel filtration [2,14,15]. These techniques are costly, laborious and time consuming, usually being suitable for producing small quantities, and useful to study or characterize the catalytic properties of the bioproduct. However, industrial applications require more efficient and less expensive purification protocols, capable of scaling-up. The non-chromatographic methods may be an alternative, especially when high-purity bioproducts are not required. Among the non-chromatographic techniques, aqueous two-phase system (ATPS) and ultrafiltration (UF) are efficient methods for enzyme purification.

ATPS is a selective method to purify biomolecules formed by mixing two incompatible aqueous solutions, such as polyethylene glycol (PEG) and dextran or PEG and salt with water [16]. ATPS has been proposed as a useful technique for separation of a large number of bioproducts, including clavulanic acid [17], enzymes [18,19], DNA [20] and dye [21]. The ATPS promotes a mild environment for biomolecules and low interfacial tensions that do not denature

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these compounds [22]. When a solution containing biomolecules is mixed to ATPS, it usually tends to partition unequally between phases. The enzyme of interest migrates to one phase, while part of the contaminants migrates to the opposite phase, thereby facilitating purification. The addition of salts, which are not part of the initial composition of the ATPS based on polymer-salt systems, is one of the factors that strongly influence proteins partition [23]. Among these salts, sodium chloride (NaCl) is the most commonly added salt in ATPS at concentrations up to 8.8% (w/w) [23,24].

The high top phase concentration of PEG compromises the value of the product and presents practical problems in handling such a viscous phase [25]. Therefore, is interesting to separate the enzyme from the salt and/or the polymer using a non-denaturing method, such as ultrafiltration (UF). The UF can be operated in diafiltration mode, which contributes to a better separation and is often used to improve the purity of the solute, since the feed is diluted with solvent for reducing the concentration of permeable components and removing them from permeation through the membrane [26].

The keratinase purification by ATPS is quite recent, only Bach et al. [18] have reported the purification of this enzyme by ATPS. Regarding ATPS integrated into ultrafiltration, there are few reports, which are related to enzymes such as amyloglucosidase [27], lactoperoxidase [19], peroxidase [28] and natural dye C-phycocyanin [29]. Based on the current lacuna on keratinase purification using techniques to provide a bioproduct with high yield targeting industrial applications, the aim of this study was to establish an efficient purification protocol for keratinase integrating the UF into ATPS. For this purpose, the keratinase partitioning by ATPS was optimized, and the factors affecting the partitioning process was analyzed using a sequence of experimental designs (2^3 central composite design and 2^2 central composite rotational design). Aiming at a higher purification factor, the keratinase was submitted to a second step of aqueous two-phase system. To separate PEG/salts from the purified keratinase, ultrafiltration (diafiltration mode) was applied. Furthermore, the catalytic activity of purified keratinase in soluble and insoluble protein substrates was investigated.

2. Material and methods

2.1. Microorganism, inoculum and cultivation

Bacillus sp. P45 (GenBank accession number AY962474), maintained on brain-heart agar (BHA) at 4 °C, was used to produce the keratinase. For inoculum preparation, this strain was grown on BHA at 30 °C for 24 h. The cultures were scraped from the agar surface, added to a sterile 0.85% (w/v) NaCl solution, and mixed until a homogeneous suspension with $O.D._{600}$ of 0.5 was obtained. The enzyme was produced by submerged cultivation using feather meal as substrate as described by Daroit et al. [30]. After cultivation, the culture was then clarified by centrifugation (5000 × *g* for 20 min) and the supernatant containing the enzyme was used in the purification steps.

2.2. Purification steps

2.2.1. First step of aqueous two-phase system

The first step of ATPS was composed by appropriate amounts (% w/w) of polyethylene glycol (PEG) 1500 Da, stock potassium phosphate solution (40% (w/w), pH 7.0), sodium chloride (NaCl), deionized water and enzyme extract representing 20% (w/w) of total system. The components were mixed according to the appropriate concentration for each system, being the final mass equal to 40 g for 2^3 central composite design and 13 g for 2^2 central composite rotational design. After enzyme addition, the system was maintained at 10 °C to avoid denaturation. Thereafter, the system was centrifuged (5000 × *g* for 30 min) and allowed to stand until equilibrium between the phases was reached. The top and bottom volume phases were measured and quantified for enzymatic activity and protein concentration. For all experiments, control systems were performed as previously described, replacing the enzyme extract by deionized water.

The optimization of keratinase partition was performed through a sequence of experimental designs. A 2^3 central composite design (CCD) with three independent factors (X_1, PEG concentration; X_2, potassium phosphate concentration; X_3, NaCl concentration) at three variation levels was carried out (Table 2). Further, a 2^2 central composite rotational design (CCRD) was used to evaluate the significant independent variables derived from the CCD at five variation levels (Table 3). The concentration of potassium phosphate in the second design was fixed at 23% (w/w).

The obtained experimental data were analyzed by response surface regression procedure, using the second-order model (Eq. (1)):

$$y = \beta_0 + \sum_j \beta_j X_j + \sum_{i<j} \beta_{ij} X_i X_j + \sum_j \beta_{jj} X_j^2 + e \quad (1)$$

where y is the predicted response, β_0, β_j, β_{ij} and β_{jj} are regression coefficients for the intercept, linear, interaction and quadratic effects, respectively, and X_i and X_j are the coded independent variables. An analysis of variance (ANOVA) for the models was performed and the model significance was tested by Fisher's test (F-test). Parameters with less than 95% confidence ($p > 0.05$) were excluded and added to error term.

2.2.2. Second step of aqueous two-phase system

The second step of ATPS was composed by adding diluent and ammonium sulfate to the top phase from the first ATPS. The effect of diluent (100 mM tris–HCl buffer, pH 7.0, and distilled water) on keratinase purification was evaluated with ammonium sulfate concentration fixed in 36% (w/w). Thereafter, the effect of ammonium sulfate concentration (20, 24, 28, 32, 36 and 40% (w/w)) was assessed. To avoid denaturation, the systems were maintained at 10 °C. All the experiments were performed in triplicate and statistically analyzed by analysis of variance (ANOVA) followed by Tukey's or *t* tests.

2.2.3. Integration of ultrafiltration into an aqueous two-phase system

The integration of ultrafiltration (diafiltration mode) into an aqueous two-phase system was evaluated by two processes: (1) first step of ATPS followed by diafiltration; (2) first step of ATPS followed by a second step of ATPS and diafiltration. At the end of each step, both the purification factor and enzymatic recovery were evaluated.

The first ATPS was composed (w/w) by 3% PEG 1500 Da, 23% potassium phosphate pH 7.0, 8% NaCl, and 20% enzyme extract. The second ATPS was composed (w/w) by 36% top phase (1st ATPS), 36% tris–HCl buffer (100 mM, pH 7.0), and 28% $(NH_4)_2SO_4$.

The ultrafiltration process was operated in diafiltration mode, using stirred cell operated in the dead-end mode. The cell unit was pressurized by compressed nitrogen at a pressure of 1.5 kgf/cm^2 and the temperature was kept at 15 °C to avoid denaturation. The molecular mass cut-off of ultrafiltration membrane was 10 kDa (Millipore, regenerated cellulose). Ten milliliters of top phase containing the enzyme and 20 mL (two diavolumes) of tris–HCl buffer (pH 7.0, 100 mM) were added at the membrane module. Approximately 20 mL were permeated at each cycle; then, the process was stopped and a volume buffer was added at the same rate as permeate was generated, until 5 cycles have been completed. The enzyme