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Adsorption onto alumina and stabilization of cysteine proteinases from crude extract of *Solanum granuloso-leprosum* fruits

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ABSTRACT

Proteolytic biocatalysts extracted from the ripe fruits of *Solanum granuloso-leprosum*, a native Uruguayan tree of the Solanaceae family, were stabilized by adsorption onto gamma-alumina supports. Conditions for preparing the biocatalyst–adsorbant system were evaluated for different crude extract (CE) protein concentrations and at different pHs and temperatures. All systems assayed reached equilibrium within 2–4 h. The best proteolytic activity (measured as direct enzymatic activity) was found to be at pH 6.0 and 5 °C. The biocatalyst–adsorbant system was more stable upon storage than the free enzyme in solution, which loses activity over time due to autolytic processes. Stability (resistance to desorption) was studied after ten cycles of incubation at different pHs and temperatures. There was a general tendency to retain higher activity (more than 60%) when the incubation conditions were the same as those used for preparing the biocatalyst–adsorbant system. The best conditions for using this system to treat industrial effluents were evaluated using milk whey from cheese-making and hemoglobin (the main constituent of wastewater from cattle slaughter-houses) as trial substrates.

1. Introduction

Enzymes are extremely important biocatalysts because they are highly substrate-specific (including stereospecific and regiospecific), highly active under ambient conditions and biodegradable [1]. Because proteolytic enzymes are labile molecules, studies of their stability and stabilization potential are a central issue in biotechnology today. Their stability is often restricted due to denaturation and/or autoproteolysis, which cause a loss of activity [2]. Several strategies exist to increase enzyme stability, including chemical modification of the enzyme's structure, derivatization, and immobilization. Of these, immobilization onto a solid carrier (adsorption, entrapment or covalent linkage to an insoluble support) is perhaps the most commonly used strategy to improve enzyme stability [3–7]. Immobilization by adsorption can be highly selective because it involves affinity phenomena between two surfaces [8]. Among the different transition aluminae known, gamma-alumina (γ-Al_2O_3) is perhaps the most important with direct application as a catalyst and catalyst support. The usefulness of this oxide can be attributed to its favorable combination of textural properties (such as surface area, pore volume, and pore size distribution) and its acid/base characteristics [9].

The number of applications of immobilized biocatalysts in industry is growing daily because of the advantages they confer over their soluble counterparts (increased resistance to temperature, pH, and organic solvents; recovery and re-use of the enzyme) [10].

Interest in proteolytic biocatalysts is rising due to the wide use of protein hydrolysates (for example, in food technology) for their nutritional and functional properties [11,12]. Protein hydrolysates are less harmful environmental pollutants than untransformed effluents containing proteins. In some cases, biotransformation of waste products converts them into useful raw materials for other industries [13]. Interest in plant proteases is growing because high-activity enzyme preparations can be obtained from natural, renewable sources. [14]. The source of the biocatalysts used in this study is the ripe fruit of a native tree known as "gravitinga" [15], wild tomato [16], or "fumo bravo" [17] (*Solanum granuloso-leprosum* Dunal [18]). This species is commonly found as secondary forestation, and it is geographically distributed in the northwest of Argentina, the whole of Uruguay and Paraguay, and the south of Brazil. At present, "gravitinga" is mainly used in the restoration of degraded ecosystems.

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We have previously characterized the major proteolytic enzymes present in the crude extract of mature fruits of *Solanum ganuloso-leprosum* Dunal [19,20].

These enzymes, which had different isoelectric points (pI) but similar molecular weights (MWs), showed marked instability (manifested as a gradual loss of proteolytic activity) when stored at temperatures above 4 °C. However, when the enzymes were adsorbed onto alumina, proteolytic activity was maintained, creating a stable enzyme–adsorbant system with potential applications in biotechnological processes.

In this study, we examined the possible causes of this loss of enzymatic activity in solution. The main goal was to improve the stability of these proteolytic enzymes by adsorption. The pH and temperature influence on the adsorption process and biocatalyst–adsorbant system were studied.

2. Materials and methods

2.1. Materials

Alumina beads (gamma configuration, BET surface: 300 m^2/g and particle size: 2–5 mm), azocasein, Coomassie Brilliant Blue R-250, cysteine, hemoglobin, Tris base, mono- and di-basic phosphate, citric acid, glycine, and trichloroacetic acid (TCA) were purchased from Sigma Chemical Company (St. Louis, MO, USA). Acrylamide, bisacrylamide, and low-range molecular weight standards were obtained from Bio-Rad (Hercules, CA, USA). Acid whey was purchased from local milk industries.

2.2. Preparation of the crude extract

Ripe fruits of *Solanum granuloso-leprosum* were gathered from trees grown as an experimental crop in Montevideo, Uruguay. The ripe fruits were ground together with abrasives (sand) and then centrifuged at 6654 × *g* for 30 min at 4 °C. The supernatant was filtered through gauze and called "crude extract." The crude extract (CE) with a protein content of 4.6 mg/mL, pH 5.2 ± 0.2 and 250 EU/mL was divided into aliquots and stored at −20 °C.

2.3. Protein determination

The protein concentration of the extracts was determined by the Bradford method [21] using bovine serum albumin (BSA) as the protein standard.

2.4. Enzymatic activity

Reaction mixtures containing 340 μL of an appropriate dilution of the CE (in 0.2 M phosphate buffer, pH 7.5) or 0.1 g of the biocatalyst–alumina system, 340 μL of azocasein solution (1%, w/v in distilled water), 340 μL of 0.2 M phosphate buffer, pH 7.5 and 15 mM β-mercaptoethanol were incubated for 20 min at 37 °C with manual agitation every 2 min. The reaction was stopped by adding 340 μL of TCA (10%, w/v in distilled water). After centrifuging for 20 min at 20,600 × *g*, absorbance was measured at 337 nm. One enzymatic unit (EU) was defined as the amount of enzyme producing an increase of one absorbance unit per minute at 337 nm in assay conditions modified from the method of Andrew and Asenjo [22].

2.4.1. Indirect method

The proteolytic activity of the CE was determined before and after adsorption, and the difference was taken as the theoretical amount of enzyme adsorbed.

2.4.2. Direct method

The enzyme activity of the biocatalyst–adsorbant was determined as real adsorbed enzyme units.

2.5. Adsorption

An appropriate dilution of crude extract with a specific activity of 54 EU/mg (azocasein assay) was incubated with adsorbant (previously hydrated with distilled water) with agitation (200 rpm) in a bioreactor for as long as necessary to attain equilibrium. The ratio of the enzyme solution volume (in mL) to the adsorbant mass (in g) was 6:5. This parameter was selected based on previous experience immobilizing other enzymes onto alumina [23].

2.6. Effect of protein concentration on adsorption

Crude extract (CE) dilutions containing protein concentrations of 0.35, 0.7, 1.4, and 2.8 mg/mL were adsorbed onto gamma-alumina beads. Adsorption was carried out at 5 °C in 0.2 M Tris buffer (pH 7.8).

The progress of the adsorption process was monitored by UV spectrophotometric measurements of the CE solution in contact with the support. The measurements were performed at 280 nm in a Shimadzu UV-1603 spectrophotometer. Aliquots of 0.1 mL of the supernatant solution were withdrawn at 30-min intervals throughout the adsorption period, appropriately diluted, centrifuged at 5867 × *g* for 10 min and filtered through Whatman filters (0.45 μm) prior to spectrophotometric measurement.

At the end of the adsorption period, the supernatant was discarded, and the biocatalyst alumina system was washed with distilled water. The recovered solid was then dried to constant weight at room temperature (20 °C).

2.7. Effects of pH and temperature on the adsorption process

Adsorption experiments were performed with CE diluted in 0.2 M citrate–phosphate buffer (pH 4.4), distilled water (pH 6.0 ± 0.2), or 0.2 M Tris buffer (pH 7.8), all with beta-mercaptoethanol to a final concentration of 20 mM. The final protein concentration in all cases was 0.7 mg/mL.

Adsorptions were carried out at 5 °C and 25 °C. The progress of the adsorption process was monitored by UV measurement at 280 nm as described in Section 2.6.

2.8. Effects of pH and temperature on proteolytic activity of the system

Biocatalyst–adsorbant systems were prepared as described in Section 2.5 at three defined pH values (pH 4.4, 6.0 and 7.8). Adsorption experiments were carried out at 5 °C, 25 °C and 39 °C. After 3 h, supernatants were removed and the solids were washed and dried to constant weight at room temperature. The amount of enzyme adsorbed was determined by direct measurement of activity as described in Section 2.6.

2.9. Effects of temperature and pH on the stability of the system

Three grams of each enzyme–adsorbant system prepared at pH 4.4, 6.0 or 7.8 at 5 °C or 25 °C (a total of six systems) was placed in contact with 20 mL of citrate–phosphate buffer (pH 4.4), distilled water (pH 6.0 + 0.2), or 0.2 M Tris buffer (pH 7.8). The mixtures were stirred at 200 revolutions per minute for 20 min. Each experiment was carried out at 5 °C, 25 °C and 39 °C.

This procedure was repeated ten consecutive times changing the buffer solution every new cycle.

The systems prepared at pH 7.8, 5 °C and 25 °C were monitored by measuring enzymatic activity directly after cycles 1, 2, 3, 4, 6, 7 and 10.

Adsorbed enzyme activity was measured directly for all systems at the end of the 10th cycle.

2.10. Stability

The stability of the CE was determined by measuring residual enzymatic activity after pre-incubating the extracts diluted 1/20 in distilled water (final pH 6.0 ± 0.2) at 5 °C and 20 °C for 0, 24, 48 and 168 h.

2.11. Electrophoresis

Samples of CE left at 20 °C for 0, 24, 48 and 168 h (the same conditions used for the stability studies) were analyzed by 12.5% SDS-PAGE. The voltage was kept constant at 30 V during stacking and then increased to 90 V and kept constant for the resolving gel.

The protein bands were visualized by staining with Coomassie Brilliant Blue R-250.

2.12. Stability upon storage of biocatalyst–alumina systems

Biocatalyst–alumina systems were stored at 5 °C or 20 °C. Residual enzymatic activity was determined at 0, 24, 48, and 168 h.

2.13. Hydrolysis of different substrates

Three grams of biocatalyst–adsorbant system or 3.0 mL of CE (equivalent to 750 EU) was placed in contact with 15 mL of 1% (w/v) azocasein, 1% (w/v) hemoglobin, or acid whey (with a protein content of 7%) at pH 7.1 and 37 °C for 45 min. TCA (1 mL of 12.5%) was added to 0.5 mL of the reaction mixture and then centrifuged at 5867 × *g* for 10 min. Free amino groups were measured according to the TNBS method [24].

2.14. Hydrolysis of whey proteins at different pH values

An amount of biocatalyst–adsorbant system equivalent to 250 EU (1 g) was placed in contact with 15 mL of acid whey solution (protein content 7%) at pH 4.3, 6.0, or 7.1.

The hydrolysis process was carried out for 45 min at 37 °C. TCA (1 mL of 12.5%) was added to 0.5 mL of the reaction mixture and then centrifuged at 5867 × *g* for 10 min. Free amino groups were measured according to the TNBS method [24].