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Reversed micellar extraction of an extracellular protease from *Nocardiopsis* sp. fermentation broth

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Abstract

Extraction of an extracellular alkaline protease from *Nocardiopsis* sp. fermentation broth using reversed micelles of sodium di(2-ethylhexyl) sulfosuccinate (AOT) in isooctane was performed with equal phase volume ratio. This work describes the effects of pH, ionic strength and surfactant concentration on the enzyme transfer process from the aqueous to the organic phase by direct contact (5 min) between the two phases. The best conditions for extraction (38.4% of protein content with about 83.5% of activity) were obtained using 200 mM AOT, at pH 4.0 with 50 mM KCl. For back extraction, sodium carbonate buffer, at pH 5.0 with 100 mM of KCl allowed for the best conditions (47.2% of protein content with about 14.0% of activity). The low protease activity yield of 11.69% obtained for the total process, extraction and back-extraction, suggests enzyme denaturation or its allocation near the AOT hydrophobic tail.

Keywords: Protease; AOT; Protein recovery; Reversed micelles; Liquid–liquid extraction

1. Introduction

The liquid–liquid extraction using reversed micelles has been demonstrated as a potential method for biological products separation, since proteins were shown to be solubilized in organic solvents with surfactants, while maintaining their functional properties, and to be transferred between an aqueous solution and a reversed micellar organic phase. The major factors determining protein solubilization in reversed micellar systems are electrostatic interactions between the solutes and the charged surfactant heads and the aggregation properties of the surfactant. The protein extraction from the aqueous phase process by the reversed micelles and its back-extraction to an aqueous solution is critically dependent on several interrelated parameters. Selective solubilization of proteins can be achieved by manipulating the parameters of the system both in the micellar and aqueous phase, namely, pH and ionic strength of aqueous phase, nature of organic solvent, type and concentration of surfactant and temperature [1]. In the recent years, the extraction and purification of proteins using reversed micellar systems has been object of extensive studies with the aim of using it as a pre-purification step [2–3].

Proteases are hydrolytic enzymes showing a wide variety of industrial applications in the detergent, food, pharmaceutical, diagnostics, and fine chemical industries [4]. Among them, microbial proteases are responsible for approximately 65% of the total worldwide enzyme industry [5–6]. Recently, an alkaline protease from *Nocardiopsis* sp., isolated from a soil sample collected from the Northeast Brazil, has been reported to be stable at alkaline pH's between 8.0 and 10.5 and

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at 50 °C, suggesting its use in the detergent industry [7]. This enzyme was 26-fold purified by Sephadex G-75 chromatography with a 34% yield.

The aim of the present work was the extraction of this extracellular alkaline protease of *Nocardiopsis* sp. from its fermentation broth using sodium di(2-ethylhexyl) sulfosuccinate (AOT) reversed micelles in isooctane.

2. Materials and methods

2.1. Microorganism, cell growth and enzyme production

The *Nocardiopsis* sp., isolated from a soil sample collected in Pernambuco-Brazil, was maintained at 28 °C on ISP-2 slant medium [8] for about 96 h for sporulation. The spores thus obtained were suspended aseptically in a 0.9 % (w/v) saline solution with vigorous shaking. An Erlenmeyer flask (100 ml) containing 25 ml of MS-2 medium [9] was inoculated by transferring a suspension of spores (10^5 cells/ml) and incubated at 27 °C for 48 h in an orbital shaker (200 rpm). After the incubation, 12.5 ml of this culture was transferred to an Erlenmeyer flask (500 ml) containing 112.5 ml of MS-2 medium, and incubated again at 27°C for 48 h in an orbital shaker (200 rpm). Cells were harvested in the early stationary phase (48 h of cell growth), centrifuged and the supernatant containing the alkaline protease [7] was collected and termed as fermentation broth in this study.

2.2. Protease extraction and back-extraction with reversed micelles

The reversed micellar system was constituted by the anionic surfactant, sodium di(2-ethylhexyl)sulfosuccinate (AOT) in isooctane. Extraction and back-extraction procedures [10] were performed as follows: (1) to the fermentation broth (5 ml; KCl was added to a final concentration ranging from 50 to 200 mM) an equal volume of micellar phase (5 ml; AOT in isooctane at a concentration range of 50–250 mM) was added and both phases were mixed for 5 min at 700 rpm at 28 °C for protein extraction; the mixture was then centrifuged for 10 min at 2500 × *g*, for phase separation; (2) after extraction, the separated micellar phase (3 ml), containing the solubilized protein, was added to an equivalent volume of 200 mM buffered aqueous solution containing 100 mM KCl, at different pHs (sodium citrate pH 5.0, sodium phosphate pH 7.0 and sodium carbonate pH 10.0). The mixture was stirred for 5 min at 700 rpm, centrifuged for 10 min at 2500 × *g* for phase separation being the enzyme recovered in the aqueous phase.

2.3. Protease activity

Protease activity in the aqueous phase was spectrophotometrically measured according to Leighton et al. [11]. Azocasein (1.0%, w/v, in 200 mM Tris–HCl buffer, pH 7.6) was used as substrate. One unit of activity is defined as the amount of enzyme that produces an increase in optical density of 1.0 in 1 h at 440 nm. The enzyme activity in the organic phase was estimated by the difference between the activities from initial and residual aqueous phases.

2.4. Protein assays

The protein content in the aqueous and organic phases was spectrophotometrically determined using the bicinchoninic acid according to Smith et al. [12], with bovine serum albumin as the standard.

3. Results and discussion

3.1. Effect of AOT concentration and ionic strength on the extraction of the protease

One of the most striking features of micellar enzymology is the possibility of varying the dimensions of reversed micelles by changing the concentrations of either surfactant or water and the ionic strength of the reverse micellar water pool [13]. The study of the effect of AOT concentration on the protease extraction from fermentation broth, under 50 mM of KCl and pH 8.0 (original pH) condition, was carried out over a AOT concentration range of 50–250 mM (Fig. 1). It was found that the protein transferred to the micellar phase increased up to a 200 mM AOT concentration, remaining constant for higher values of surfactant concentration, whereas the enzymatic activity in the residual aqueous phase decreased in the whole range of tested AOT concentrations. These results suggest that with 200 mM AOT ca. 25% of the enzymatic activity was transferred to the organic phase being the recovered protein 31.63 ± 1.21% of total initial protein. Similar observations were reported for the ribonuclease A when the AOT concentration increased from 12.5 to 50 mM [14], for the amino acid phenylalanine when AOT increased from 50 to 200 mM [15] and for

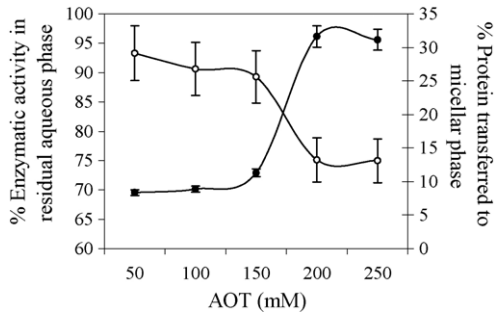

Fig. 1. Effect of AOT concentration on the protease during forward transfer. (◯) % Enzymatic activity in residual aqueous phase; (●) % protein transferred to micellar phase. Values are averages of three independent experiments (±95% confidence interval).