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Interference of salts used on aqueous two-phase systems on the quantification of total proteins

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ABSTRACT

In this study the interference of potassium phosphate, sodium citrate, sodium chloride and sodium nitrate salts on protein quantification by Bradford's method was assessed. Potassium phosphate and sodium citrate salts are commonly used in aqueous two-phase systems for enzyme purification. Results showed that the presence of potassium phosphate and sodium citrate salts increase the absorbance of the samples, when compared with the samples without any salt. The increase in absorptivity of the solution induces errors on protein quantification, which are propagated to the calculations of specific enzyme activity and consequently on purification factor. The presence of sodium chloride and sodium nitrate practically did not affect the absorbance of inulinase, probably the metals present in the enzyme extract did not interact with the added salts.

1. Introduction

Aqueous two-phase systems (ATPSs) are generally obtained by mixing two aqueous solutions of different constituents that become immiscible under certain critical conditions such as temperature and concentration. Both phases are composed mainly of water, and each one is enriched in a different component [1]. Due to the high percentage of water present in their composition, ATPSs can provide a gentle environment for the extraction and recovery of sensitive biological materials such as proteins [2].

To evaluate the protein purification methods it is necessary to quantify the total protein in the samples, which will be used to calculate the specific enzymatic activity and also the purification factor. Fast and sensitive quantification methods are preferred, like spectroscopic, chemical and colorimetric assays. The colorimetric method proposed by Bradford in 1976 [3] is the most accepted for protein quantification in enzyme purification by aqueous two-phase systems [3–6], due to its simplicity and sensitivity. This assay involves the binding of the Coomassie Brilliant Blue G-250 dye to proteins. In practice, an acidic solution of Coomassie is added to a protein solution, and the absorbance of the resulting mixture is measured at 595 nm and compared with the absorbance of the free dye solution (without protein) [2,3].

Recent studies have showed that the Bradford's method can suffer interference on the color intensity by some ionic compounds commonly used in aqueous two-phase systems [2] for increasing the ionic strength of the aqueous phase. These systems are usually applied to enzyme purification, and the salt interference on the protein quantification will yield misleading results of specific activity and purification factors [4].

In this context, the present study aimed to evaluate the influence of different salts commonly used in aqueous two-phase system on the quantification of proteins by Bradford's method. Crude enzymatic extracts were used as proteins source simulating real systems purification.

2. Material and methods

2.1. Production of inulinase

The enzyme was produced by solid-state fermentation of sugarcane bagasse with *Kluyveromyces marxianus* NRRL Y-7571. The solid

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substrate was supplemented with 15% (w/w) sugarcane molasses, 30% (w/w) corn steep liquor and 20% (w/w) soybean meal [7,8]. Initial moisture of the bagasse was adjusted to 65% as described on literature [7]. Sugarcane molasses was pre-treated with a solution 200 g L^{-1} in sulfuric acid pH 5.0. This solution was set to rest for 24 h. Thereafter, final pH was adjusted to 4.0 with sulfuric acid. The solution was centrifuged at 15,000 × *g* for 15 min at 4 °C [9]. The fermentations were carried out in a fixed bed bioreactor with 2 kg of dry sugarcane bagasse. The bioreactor was loaded with 3 kg of bagasse with moisture content at 65% (w/w) and sterilized at 121 °C for 20 min. The bagasse was inoculated with 765 mL of a cell suspension previously prepared. After 24 h the bagasse was removed from the reactor and the enzyme extracted with sodium acetate buffer pH 4.8 100 mM.

2.2. Production of keratinase

Bacillus sp. P45 was used to produce the keratinase enzyme, and the microorganism is registered in Gen Bank with the accession number AY962474. For inoculum preparation, this strain was inoculated on BHI (brain heart infusion) plates and incubated at 30 °C for 24 h. The cultures were scraped from the agar surface, added to a sterile solution of sodium chloride (NaCl) (0.85% (w/v)), and mixed until a homogeneous suspension with $O.D._{600}$ of 0.5 was obtained [10]. The enzyme was produced by submerged cultivation [10] using feather meal as substrate. The culture medium was composed of (g L^{-1}): feather meal (43) and ammonium chloride (NH_4Cl) (1.9) prepared in mineral medium (NaCl (0.5), dibasic potassium phosphate (K_2HPO_4) (0.3) and monobasic potassium phosphate (KH_2PO_4) (0.4). The initial pH of the medium was adjusted to 7.0 and the cultivation initiated with 1% (v/v) inoculum. The cultivation conditions were 30 °C, 125 rpm for 48 h. The enzymatic extract was obtained by cell centrifugation (5000 × *g* for 20 min).

2.3. Protein quantification

Protein determinations were carried out by adding 100 μL sample to 5 mL of Bradford's reagent. After mixing, the samples were left to react by 2 min at room temperature (25 °C) and the absorbance of the sample was measured at 595 nm.

2.4. Sample preparation

The samples were prepared by diluting the enzymatic extract with appropriate buffer to reach different protein concentrations. Potassium phosphate, sodium citrate, sodium chloride and sodium nitrate were added to the extract solutions to achieve different concentrations of the salts. Each test was run in triplicates.

2.5. Determination of calcium (Ca), magnesium (Mg), iron (Fe) and aluminum (Al) in the crude inulinase extract

Concentrations of Ca, Mg, Fe and Al were determined by atomic absorption spectroscopy. For this, 2 mL of crude extract was subjected to acid digestion step with 1 mL of concentrated nitric acid at 120 °C by 1 h. Subsequently, the extract was diluted into 50 mL of deionized water.

2.6. Determination of the specific activity and purification factor

Specific activity (SA) was calculated by dividing the enzyme activity by the protein content in the sample. Purification factor (PF) was calculated by the ratio between the specific activity of the purified sample and the specific activity of the crude sample [11].

3. Results and discussion

Figs. 1 and 2 present the absorbances as a function of salt concentration for different enzymatic extracts. The absorbance for all the enzyme samples with salts increased when compared with the samples without salts. The increase in the absorbance in the Bradford's reaction was linearly proportional to the salt concentration in the samples, within the studied concentrations (0–100 mg). It is thus possible to fit a model to predict the increment that the salt present in the solution could induce in the Bradford's test. Negative interference, that is a decrease in color intensity to the Bradford's method generated by the addition of salts were observed by other authors [2].

These interferences may be associated with the dye binding procedure of Bradford's reagent (Coomassie Brilliant Blue G-250) with the protein, which is regulated by the electrostatic interaction between the dye ionized sulfonic groups ($-SO_3^-$) and the positively charged functional groups of enzyme. The presence of

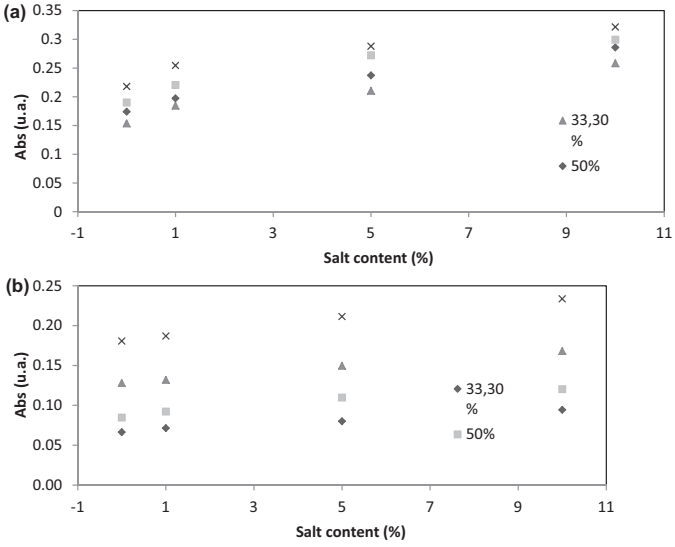

Fig. 1. Interference of potassium phosphate on protein quantification by Bradford's method for (a) inulinase and (b) keratinase samples.

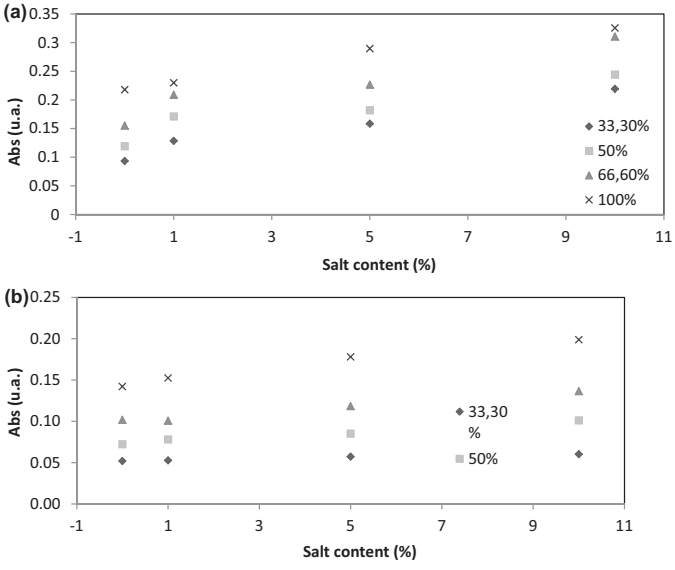

Fig. 2. Interference of sodium citrate on protein quantification by Bradford's method for (a) inulinase and (b) keratinase samples.