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Research Article

Refolding of laccase from *Trametes versicolor* using aqueous two phase systems: Effect of different additives

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ABSTRACT

Protein refolding is a strategy used to obtain active forms of proteins from inclusion bodies. On its part, laccase is an enzyme with potential for different biotechnological applications but there are few reports regarding its refolding which in many cases is considered inefficient due to the poor obtained refolding yields. Aqueous Two-Phase Systems (ATPS) have been used for the refolding of proteins getting acceptable recovery percentages since PEG presents capacity to avoid protein aggregation. In this work, 48 PEG-phosphate ATPS were analyzed to study the impact of different parameters (i.e. tie line length (TLL), volume ratio (V_R) and PEG molecular weight) upon the recovery and refolding of laccase. Additionally, since laccase is a metalloprotein, the use of additives (individually and in mixture) was studied with the aim of favoring refolding. Results showed that laccase presents a high affinity for the PEG-rich phase obtaining recovery values of up to 90%. Such affinity increases with increasing TLL and decreases when PEG molecular weight and V_R increase. In denatured state, this PEG-rich phase affinity decreases drastically. However, the use of additives such as L-cysteine, glutathione oxidized, cysteamine and Cu^{+2} was critical in improving refolding yield values up to 100%. The best conditions for the refolding of laccase were obtained using the PEG 400 g mol^{-1}, TLL 45% w/w, V_R 3 ATPS and a mixture of 2.5 mM cysteamine with 1 mM Cu^{+2}. To our knowledge, this is the first time that the use of additives and the behavior of the mixture of such additives to enhance refolding performance in ATPS is reported.

1. Introduction

The expansion of the genomic sequence databases has originated an increment in the large-scale production of recombinant proteins to meet the ever-growing demand of these molecules. However, many of these proteins are produced in non-native, insoluble, and biologically inactive conformations that are found in deposits inside the cell, called inclusion bodies [1–3]. The processes by which molecules regain their native conformations is a subject of fundamental and practical importance since to be used, proteins have to be refolded into correct secondary and tertiary structures, which are further stabilized by the formation of intramolecular disulfide bonds and hydrogen bridges [4].

Protein refolding has become the limiting step during the recovery of active proteins from inclusion bodies [5]. In traditional refolding processes, inclusion bodies are first solubilized in denaturing concentrations of urea or guanidine hydrochloride [4,5]. Then the selected denaturing agent is removed to start the procedure in what is considered the key step of the operation. In this context, several approaches have been reported to refold an inactive protein into an active one, such as size-exclusion chromatography, reversed micelle systems, zeolite absorbing systems, among others. However, in most cases there is a significant amount of protein precipitation, resulting in low recovery yields [6]. Consequently, several additives (amino acids, glycerol, sugars, among others) have been used to improve such yields [4].

In this context, the addition and/or the use of polymers in protein refolding operations is gaining scientific interest. For instance, Cleland and Wang (1990) showed that polyethylene glycol (PEG)

Abbreviations: ATPS, aqueous two-phase systems; PEG, polyethylene glycol; TLL, tie line length; V_R, volume ratio; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; K_P, partition coefficient; MW, molecular weight.

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binds to proteins as an intermediate preventing aggregation during refolding and promoting the correct conformation suggesting it has an analogous function to chaperones in *in vivo* protein folding [5,7]. On their part, Rahimpour et al., recovered recombinant xylanase in active state using PEG-phosphate aqueous two-phase systems (ATPS) showing the potential of this technique in the recovery of active proteins [5].

ATPS are formed by combining aqueous solutions of two incompatible polymers or by mixing a polymer and salt above a critical concentration [8,9]. In this technique, PEG is frequently used as one of the phase forming chemicals because it is available at low costs, forms a two-phase system with other neutral polymers as well as salts and, as it has been mentioned earlier, can enhance protein refolding. However, both protein partition and refolding depend on system design parameters such as polymer molecular weight, tie-line length (TLL), volume ratio (V_R) and pH. In this sense, our research group has previously reported the effect of these design parameters upon the recovery and refolding of denatured invertase [10]. Other studies have also reported variations in phase forming chemical selection like the use of stimuli-responsive and modified polymers with dextran and PEG to achieve better refolding and recovery yields in ATPS [11,12].

On the other hand, some natural compounds and synthetic polymers of low molecular weight are commonly added to refolding buffer to promote the correct refold of proteins avoid the formation of aggregates and an incomplete folding processes [13]. Such compounds are called additives. Additives like cysteine-cystine, oxidized and reduced glutathione, cysteamine and cystamine, arginine, sucrose and also some divalents ions like copper and zinc have been tested to refold proteins by dilution and chromatographic methods [14–17]. However, it is important to understand how the additives promote better protein refolding. This is specifically important with structurally complicated molecules whose refolding in ATPS could be improved.

Due to its environmental, industrial and economic importance, laccase from *Trametes versicolor* was selected as a model protein for this study. Laccases (E.C. 1.10.3.2) are multi-copper oxidases that catalyze the one electron oxidation of several aromatic substrates with the simultaneous reduction of dioxygen to two molecules of water [18]. Laccases are promising enzymes for industrial applications due to their broad spectrum of phenolic and non-phenolic substrates and the wide range of reactions that can be catalyzed [14]. There have been few successful efforts for laccase refolding. Furthermore, the cost and time of the whole process must be considered if the goal is to obtain a large-scale manufactured product [19]. Therefore, it is important to develop and improve methodologies for the recovery of this enzyme in active state using refolding techniques. In this work the recovery and refolding of laccase using PEG-phosphate ATPS and the influence of different parameters such as TLL, V_R and PEG molecular weight were studied. The effect of four additives: L-cysteine, glutathione oxidized, cysteamine and Cu^{+2} (individually or mixed) upon laccase refolding in ATPS was analyzed. It is believed that with this, new insights in improving protein refolding with the use of ATPS will be gained and could be extrapolated to other difficult-to-refold proteins.

2. Materials and methods

2.1. Materials

Laccase from *Trametes versicolor*, polyethylene glycol (PEG) of nominal molecular mass of 400, 1000, 3350 and 8000 g mol^{-1}; L-cysteine, cysteamine, copper, glutathione oxidized and guanidine hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium chloride, dibasic potassium phosphate (K_2HPO_4) and monobasic potassium phosphate (KH_2PO_4) were purchased from J.T. Baker (Center Valley, PA, USA). All other reagents used were of analytical grade.

2.2. Laccase enzymatic activity assay

The enzymatic activity of laccase was estimated following the change in optical density at 436 nm using 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) as substrate and the extinction coefficient (ε_{436} = 29,300 M^{-1} cm^{-1}) [20], using a microplate spectrophotometer (Biotek, VT, USA). Briefly, the assay mixture consisted of 222 μL of acetate buffer (0.1 M, pH 4.0), 25 μL of ABTS (1 mM; dissolved in ethanol) and 3 μL of sample. One unit of enzymatic activity was defined as the amount of enzyme required to oxide 1.0 μmol of substrate per minute.

2.3. Laccase denaturation

A curve for laccase denaturation was prepared using guanidine hydrochloride as chaotropic agent. 1.0 mg of laccase was diluted in 1.0 mL of guanidine hydrochloride solutions with concentrations ranging from 0.0 to 6.0 M (using increments of 0.25 M). Solutions were gently mixed for 1 h and enzymatic activity was measured [10]. All measurements were made in triplicate.

2.4. Effect of ATPS system design parameters upon partition and refolding of native and denatured laccase

In order to evaluate the effect of system parameters (molecular weight of PEG, TLL and V_R) upon enzyme partition behavior, 48 PEG-potassium phosphate ATPS were analyzed. The composition of each system has been reported previously [10]. Determined stock solutions of PEG of nominal molecular weights of 400 (liquid at room temperature and therefore used at 100% w/w), 1000 (50% w/w), 3350 (50% w/w) and 8000 (50% w/w) g mol^{-1}, potassium phosphate buffer (Na_2HPO_4–NaH_2PO_4, ratio 18:7, 40% w/w, pH 7.0), bi-distilled water and 0.2 g of either enzyme (native or denatured) in solution at a concentration of 10 mg/mL were mixed to have a total weight of 2.0 g. Tie line length (TLL) is defined by the concentration of the chemicals forming the phases (i.e. PEG and potassium phosphate) and values of 15, 25, 35 and 45% w/w were tested for each PEG molecular weight. Volume ratios (V_R; volume of the top phase/volume of the bottom phase) of 0.33, 1.0 and 3.0 were studied for each TLL value and pH was kept constant at 7.0. Systems were mixed lightly for 15 min for the native protein and 1 h for the denatured protein. Complete phase separation was accomplished by batch centrifugation at 10,000 rpm at 25 °C for 10 min, using an Allegra 64R Centrifuge (VWR, PA, USA). Graduated tubes were used for clear visualization of the phase volumes and each phase was carefully separated for further analysis. Total protein concentration in each phase was estimated by absorbance at 280 nm in a microplate reader (Synergy HT, Biotek, VT, USA); using a calibration curve prepared with laccase solutions ranging from 0.0 to 2.0 mg/mL. Enzyme phase recovery and corresponding K_P values (defined as the ratio of protein concentrations in the top and bottom phases of each ATPS) were calculated relative to the total amount of enzyme in the phase and that loaded initially into the system. To estimate protein refolding after ATPS incubation, enzymatic activity was measured in each phase. Assuming a two-state denaturation model [21], refolding percentages were calculated as:

$$\text{Percentageofrefolding}(\%) = (1-\alpha)\times 100 \quad (1)$$

$$\alpha = \frac{A_N - A_i}{A_N - A_U} \quad (2)$$