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Effect of controlled redox potential and dissolved oxygen on the *in vitro* refolding of an *E. coli* alkaline phosphatase and chicken lysozyme

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ABSTRACT

The development of efficient purification strategies of recombinant active protein derived from inclusion bodies requires the knowledge of the effect of environmental variables, such as redox potential (RP) and dissolved oxygen tension (DOT), in order to control the protein folding process. However, that information is scarce and only few *in vitro* studies of the impact of such variables have been reported under constant controlled conditions. In this work, the effect of controlled RP and DOT on the refolding of *E. coli* alkaline phosphatase (AP) and chicken lysozyme (CL) enzymes were studied. Disulphide bonds of both enzymes were reduced in an instrumented vessel using 2-mercaptoethanol and nitrogen. In the latter case, guanidine hydrochloride was also used to denature the protein. Such conditions caused protein conformational changes, as determined by the intrinsic fluorescence spectra that correlated with a decrease on the activity in both cases. Reduced enzymes were then oxidized, under different constant and predetermined RP or DOT, by manipulating the gas composition in the vessel. Folding kinetics were followed as the recovery of enzyme activity. Results showed that the percentage of recovery and rate of increase of enzymatic activity directly depended on the RP and DOT. A higher folding efficiency was found under controlled DOT compared to controlled RP conditions. These results are useful for establishing protein folding strategies to improve the recovery of active protein from inclusion bodies.

1. Introduction

Development of new biopharmaceuticals requires the use of appropriate expression systems and efficient downstream strategies to obtain high product yields. Prokaryotic cells remain at present as a common alternative to produce recombinant proteins due to important advantages during up-stream processes, which include the ease of genetic manipulation and simplicity and low costs of fermentations [1]. However, recovery of recombinant proteins when expressed as inclusion bodies (IB) in bacterial cultures complicates downstream processing and limits productivities, as inefficient protein-folding stages are required to obtain an active protein [2]. Several promising approaches have been recently reported to improve the protein folding stage at laboratory scale, including high hydrostatic pressure technologies and chromatography refolding assisted by chaperones and oxidoreductases [3]. Likewise, novel monitoring strategies, such as the use of light scattering, have been useful to establish rational operation strategies for improving protein refolding processes [4]. Nonetheless, simple protein dilution followed by oxidation and recovery, still represents the most commonly used operations at the commercial scale for protein refolding [5,6]. However, such operations result in low yields of active protein and high production costs due to the long times spent during the refolding stage, and the use of considerable water volumes and corrosive reagents [6,7].

Traditional refolding of recombinant proteins at large scale generally involves three different steps: recovery of IB; solubilization and reduction of IB by caotropic and reducing agents; and oxidation and refolding of recombinant protein by dilution [8,9]. During the latter stage, an oxidative redox environment is needed to promote protein folding and formation of disulphide bonds. Accordingly, redox potential (RP) and/or dissolved oxygen concentration (DOT) are critical during traditional protein refolding process, however,

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both variables have received limited attention as a tool for improving folding yields.

The utility of controlling and monitoring redox potential, a measure of the total electron exchange between different compounds, has been demonstrated in a variety of biotechnological applications [10]. For instance, the importance of redox potential as a culture variable has been studied and its role during native protein folding within the cell is well known [11,12]. It has been shown that an adequate balance between reductive and oxidant species is necessary to optimize the oxidative folding of different proteins inside the cell [13–15]. In addition, the use of air or oxygen to promote protein folding or thiol oxidation during folding processes after dilution of IB is a common industrial practice. Menzella et al. [16] demonstrated that the use of a controlled environment by air oxidation and the use redox couples was useful to improve the prochymosin folding from inclusion bodies. Recently, it has been shown that DOT profiles, under a system with a controlled oxygen transfer rate, are indicative of product quality during the protein refolding stage [17].

The effect of constant RP or DOT on the *in vitro* refolding was systematically studied in this work by using an *E. coli* alkaline phosphatase (AP) and chicken lysozyme (CL) as model proteins. AP is a homodimer, with two disulphide bonds per monomer, that has been widely used as a convenient experimental model for following folding kinetics [15]. Likewise, CL is one of the most commonly used proteins to study refolding and bioprocess development [3,8]. This enzyme is a good model to evaluate the refolding process, as its activity is easily measured and depends on the formation of its four disulphide bonds. Pure AP and CL were used here in order to avoid the interference of cell debris and other compounds during folding when using crude IB. In the case of CL, guanidine hydrochloride (GnHCl), in addition to 2-mercapthoethanol (2-ME), was also employed to simulate typical conditions used during IB processing.

2. Materials and methods

E. coli AP (EC 3.1.3.1) (Sigma–Aldrich, P-5931), a metalloenzyme that catalyses the hydrolysis and transphosphorylation of a wide variety of phosphate monoesters [18], was used as a model protein. A stock solution of AP at 1 IU/μL was prepared using 30 mM Tris, 60 mM $MgCl_2$, and 50% (v/v) glycerol and maintained at −20 °C up to one month according to [15]. The AP working solution for the protein refolding reaction was kept at pH 10.4 and contained 100 mM glycine, 1 mM $ZnCl_2$, 1 mM $MgCl_2$, 5 μM $CuSO_4$ (used as catalyst for thiol oxidation), and AP at 30 mU/mL.

In the same way, the proteolytic enzyme lysozyme (Sigma–Aldrich, L4919) was used as a model [3]. Briefly, lysozyme at 10 mg/mL was dissolved using a denaturalizing buffer containing 50 mM Tris, 1 mM EDTA, pH 8.0, 10 mM of 2-ME and 4 M of GnHCl (Sigma–Aldrich, G4630). After 30 min at such conditions the enzyme was diluted 1:100 to obtain a concentration of 100 μg/ml in a modified refolding buffer (100 mM Tris, 1 mM EDTA, 100 mM NaCl) according to Antonio-Perez [3]. In this case, the redox couple O_2/H_2O, was considered as a substitute of glutathion.

To determine the effect of RP and DOT on folding kinetics, different sets of experiments were performed using pure AP or CL. In both cases, the enzymes were reduced and then oxidized while their enzymatic activities were monitored. Protein reduction and refolding were performed in an instrumented vessel (250 mL working volume) with RP (Ingold), DOT (Melter Toledo), and pH (Ingold) probes. All probes were calibrated according to standard procedures [19]. In the case of AP, temperature was controlled at 37 °C. and folding kinetics initiated at 100% DOT, pH 10.4, and 37 °C. For lysozyme, folding kinetics initiated at 100% DOT, pH 8.0, and 25 °C. In both cases, enzyme activities at this time point were taken as 100%. Such condition was referred as stage I (see Section 3). Once the monitored conditions were stable at 100% DOT, pure nitrogen was bubbled until a DOT of less than 1% was obtained. Such condition was referred as Stage II (see Section 3).

To create the most reducing environment, 2-ME reagent was added to a final concentration of 30 mM to reduce the AP. The reduction and denaturalizing conditions for 10 mg/mL of CL were 10 mM of 2-ME and 4 M GnHCl. After 30 min, CL was diluted 100× in the bioreactor to decrease the concentration of GnHCl and 2-ME and to reach an enzyme concentration of 100 μg/mL. Such conditions were considered because of their immediate effect on enzyme activity as well as on protein denaturation. For both enzymes, such stage was referred to as stage III (see Section 3).

After 20–60 min at reducing conditions (0% DOT), either RP or DOT were controlled at constant and predetermined values by manipulating the inlet oxygen and nitrogen composition of the gaseous stream that was sparged into the reaction vessel at a flow rate of 300 mL/min. Four constant values of RP (in the range of −370 to −330 mV) and four constant values of DOT (in the range of 20–150%, with respect to the air saturation) were tested in the case of AP. Three DOT conditions (20%, 50% and 150%) and two RP conditions (−155 and −120 mV) were tested in the case of CL. A set of three experiments was performed for each condition tested. This last was referred as stage IV (see Section 3).

Intrinsic fluorescence spectra of both enzymes were determined in a PerkinElmer LS 55 Luminiscence Spectrometer and used as a measure of the conformational protein changes during protein reduction (in the case of AP), and, protein reduction, denaturation and oxidation in the case of CL. The Fraction of Unfolded protein (Fu) was calculated using the following equation:

$$F_U = \frac{(f_N - f)}{(f_N - f_U)}$$

where: F_U, fraction of unfolded protein; f, observed fluorescence intensity at a given condition; f_U, fluorescence intensity when the protein is completely unfolded; f_N, fluorescence intensity of the native protein.

AP activity was determined by a standard colorimetric assay using *p*-nitrophenyl phosphate as substrate [20]. One enzymatic unit was defined as the quantity of enzyme needed to hydrolyze 1 mole of *p*-nitrophenyl phosphate in one minute at pH 10.4 and 37 °C. The percentage of recovered enzymatic activity during the oxidation stage was calculated as the maximum enzymatic activity at a predetermined condition with respect to the initial enzymatic activity immediately after AP reduction. The rate of increase of enzymatic activity during oxidation (RIEA) was determined from the slope of enzyme activity *versus* time. The concentration of 2-ME, used as a measure of environmental reduction during the folding kinetics, was determined by the Ellman reaction [21].

Lysozyme activity was measured at 25 °C by following the decrease in absorbance at 450 nm of 0.06 mg/mL *Micrococcus lysodeikticus* (ATCC 4698) in 0.1 M phosphate buffer at pH 7.0 [3]. In the assay, 50 μL of lysozyme sample were added to 950 μL of the substrate solution. Then, the decrease in absorbance was monitored every 1 s for 60 s using a Beckman DU650 spectrophotometer, and the specific enzyme activity was estimated by the initial rate per mg of protein. The refolding yield was determined by setting the specific activity of the native enzyme to 100%.

3. Results

Control runs without any protein were performed in both cases to determine the relationship between DOT and RP and the accuracy of the control system. It was observed that such a relationship depended on the buffer solution used in the folding system. In all the cases, a logarithmic relationship was found as shown in Table 1, as described by Nernst equation. Nevertheless, such a relationship is modified by the presence of reducing or caotropic compounds. These data were important to establish the redox controlled conditions. It has to be noted that each buffer solution had a different redox capacity, which is related to the salt concentration, as observed by the different equations obtained for different buffers. Therefore, a proper characterization is required to determine the behavior of redox potential with respect to DOT for the particular system used. Accordingly, experimental runs containing the enzyme were performed to establish the enzyme stability in both cases at different enzyme concentrations. In the case of CL, the resulting values were used as control during the first two stages of each folding reaction. No changes in either enzyme activity or Fu fraction were observed during such control runs.

Folding kinetics were followed at different constant RP values to study the effect of such a parameter on the AP protein

Table 1
Relationship between redox potential (RP) and dissolved oxygen tension (DOT) using different folding buffers. Equations show the logarithmic relationship between both parameters without the presence of any reductive agent. The equations shown are helpful to determine the buffer redox capacity of a solution.

Buffer solution	Equation
100 mM glycine, 1 mM $ZnCl_2$, 1 mM $MgCl_2$, 5 μM $CuSO_4$ pH 10.4	RP = 24.697 ln (DOT) − 444.75 r^2 = 0.998
Phosphate 0.1 M, pH 7.0	RP = 16.910 Ln (DOT) − 193.28 r^2 = 0.953
50 mM Tris, 1 mM EDTA, pH 8	RP = 31.039 Ln (DOT) − 268.66 r^2 = 0.981
100 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 8	RP = 9.021 Ln (DOT)+ 100.15 r^2 = 0.976