



In vitro refolding of new recombinant bacterial laccase from *Bacillus sp.* HR03

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Abstract

A recombinant form of laccase (benzenediol oxygen oxidoreductases, EC 1.10.3.2) from *Bacillus sp.* HR03 was overexpressed in *Escherichia coli* BL-21(DE3). Inclusion body formation happens quite often during recombinant protein production. Refolding conditions to yield active enzymes— using dilution method —were optimized taking as a starting point for studying bacterial laccase. Solubilization of inclusion bodies was carried out in phosphate buffer pH 7, containing 8 M urea and 4 mM β -mercaptoethanol. The effect of different additives was investigated on the refolding procedure of denatured laccase. Also Kinetic parameters of soluble and refolded laccase were analyzed.

Keywords: Laccase; Refolding; Inclusion body; Intrinsic fluorescence;

Introduction

Laccases(monophenol, dihydroxyphenylalanine: oxygen oxidoreductase, EC 1.14.18.1) are a member of an important group, the multicopper oxidases, that catalyze the oxidation of a variety of aromatic substances to less hazardous compounds [1-3]. Although high-level expression of laccase has been achieved in

Escherichia coli, accumulation of recombinant proteins in the form of cytoplasmic inclusion bodies has been proved to be particularly problematic [4]. Therefore, one of the major challenges in bioprocess engineering has been carried out to convert this inactive and insoluble protein into soluble and correctly folded product in an efficient way [5, 6]. Attempts were made to alleviate this problem by refolding the

laccase from *Bacillus* sp. HR03. Based on our knowledge, this is the first report on optimization of refolding procedure of bacterial laccase from inclusion bodies.

Methods

The recombinant plasmid pET21a (+) containing laccase gene was transformed into *E. coli* BL21 (DE3). Bacterial pellets were resuspended in the 100 mM potassium phosphate buffer pH 7 (buffer A). The soluble and insoluble protein fractions were separated. The pellet was subsequently washed with buffer B (buffer A containing 2 M urea). The final washed pellet was solubilized and denatured in 5 ml of buffer C (100 mM potassium phosphate pH 7, containing 8 mM urea and 4 mM β -mercaptoethanol). The supernatant in this stage was used for refolding. In this article, refolding is attempted by removing the denaturant, via dilution and dialysis. The optimization of refolding medium was performed by varying type of buffer, pH, additives and divalent ions. Laccase activity was determined spectrophotometrically at 525 nm by following the oxidation of 0.05 mM syringaldazine in 100 mM potassium phosphate buffer pH 7.0 ($\epsilon = 65,000 \text{ M}^{-1} \text{ cm}^{-1}$) [7].

Results and discussion

A recombinant thermostable and thermoactive laccase from *Bacillus* HR03 in *E. coli* cells. Besides optimization of the cultivation conditions, the huge amount of this enzyme was expressed is an insoluble inactive form within inclusion bodies. As a starting and crucial step for each refolding reaction, the solubilization strategy has to be considered. The results here showed that the

highest levels of solubilized IBs were obtained using 8 M urea under neutral conditions (100 mM potassium phosphate buffer pH 7) and in the presence of a reducing agent (4 mM β -mercaptoethanol) (Fig. 1).

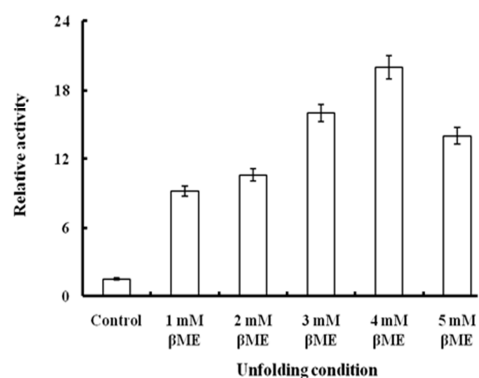


Fig. 1. Effect of various concentrations of β -mercaptoethanol (β ME) as a reducing agent on IB solubilization and yield of protein refolding. Denaturation was performed in 100 mM potassium phosphate (pH 7) containing 8 M urea and reducing agent.

The refolding parameters of laccase from *Bacillus* sp. HR03 were optimized in small-scale experiments using dilution method at 4 °C. The maximum recovery of refolding was obtained at Mix buffer (containing sodium citrate and phosphate buffer) at the pH

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