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An efficient in vitro refolding of recombinant bacterial laccase in *Escherichia coli*

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ABSTRACT

Laccases (benzenediol oxygen oxidoreductases, EC 1.10.3.2) are important multicopper enzymes that are used in many biotechnological processes. A recombinant form of laccase from *Bacillus* sp. HR03 was overexpressed in *Escherichia coli* BL-21(DE3). Inclusion body (IB) formation happens quite often during recombinant protein production. Hence, developing a protocol for efficient refolding of proteins from inclusion bodies to provide large amounts of active protein could be advantageous for structural and functional studies. Here, we have tried to find an efficient method of refolding for this bacterial enzyme. Solubilization of inclusion bodies was carried out in phosphate buffer pH 7, containing 8 M urea and 4 mM β-mercaptoethanol and refolding was performed using the dilution method. The effect of different additives was investigated on the refolding procedure of denaturated laccase. Mix buffer (phosphate buffer and citrate buffer, 100 mM) containing 4 mM $ZnSO_4$ and 100 mM sorbitol was selected as an optimized refolding buffer. Also Kinetic parameters of soluble and refolded laccase were analyzed.

1. Introduction

Laccases (monophenol, dihydroxyphenylalanine: oxygen oxidoreductase, EC 1.14.18.1) are members of the multicupper oxidase family of enzymes that catalyze the oxidation of a variety of aromatic substances to less hazardous compounds [1–3]. 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 by these enzymes [4,5]. Although high-level expression of laccases has been achieved in *Escherichia coli*, accumulation of recombinant proteins in the form of cytoplasmic inclusion bodies is particularly problematic [6]. Therefore, one of the major challenges in bioprocess engineering is efficient conversion of these inactive and insoluble protein aggregates into soluble and correctly folded enzymes [7,8].

As we have previously reported, a novel laccase gene was isolated from a local *Bacillus* sp. HR03 (accession number FJ663050). Sequence analysis of the isolated gene showed highest similarity to the thermostable laccase (CotA) from *Bacillus subtilis*. The sequences of *Bacillus* species were obtained from National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) and multiple sequence alignment was performed with ClustalW version 1.82. Studies on the native enzyme, revealed a number of unusual biochemical properties [9]. Compared to other bacterial laccases, this enzyme showed a different profile of thermoinactivation. This isolated laccase was active when incubated at high temperature, which makes the enzyme interesting for the biotechnological applications. Unfortunately, enzyme expression in *E. coli* yields a small fraction of active protein, which seems to be inadequate for many structural and biochemical studies, while a remarkable fraction aggregates in inclusion bodies. Although there have been relatively few successful efforts for refolding of fungal laccase from inclusion body (IB), but other studies have found that bacterial laccases (e.g. *Bacillus subtilis)* cannot be recovered from the IBs and only a soluble fraction representing about 10% of the total heterologous laccase can be purified [10]. Expression of laccase from *Bacillus* HR03 also resulted in a small fraction of soluble enzyme which seems to be inadequate for many structurel and biochemical studies. Here, we have tried to refold the laccase from *Bacillus* sp. HR03 to gain more active enzyme molecules after heterologous expression. Based on our knowledge, this is the first report on optimization of refolding procedure of a bacterial laccase from inclusion bodies. Intrinsic fluorescence intensity was also used to investigate the conformational changes during the process of refolding.

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2. Materials and methods

2.1. Materials

Isopropyl-β-D-thiogalactopyranoside (IPTG), dithiothreitol (DTT) and oxidized glutathione (GSSG) were purchased from Sigma Aldrich (St. Louis, MO, USA). *Escherichia coli* BL21 (DE3) cells, pET21a (+) vector were purchased from Novagen (USA). All other reagents were purchased from Merck (Darmstadt, Germany) and were of analytical grade.

2.2. Protein expression

The recombinant plasmid pET21a (+) containing laccase gene was transformed into *E. coli* BL21 (DE3). A selected lac$^+$ transformant strain was grown in Luria-Bertani (LB) medium supplemented with 100 μg/mL ampicillin at 37 °C. When the optical density at 600 nm (OD600) reached about 0.5, IPTG and $CuSO_4$ were added to a final concentration of 0.1 mM and 2 mM, respectively. The temperature was reduced to 18 °C. After 4 h shaker was turned off to provide a micro aeration condition. Cells were subsequently harvested by centrifugation (8000 × *g*, 15 min, 4 °C).

2.3. Separations of inclusion bodies

Bacterial pellets were resuspended in the 100 mM potassium phosphate buffer pH 7 (buffer A). The solution was sonicated for six pulses of 40 s. The soluble and insoluble protein fractions were separated by centrifugation at 8000 × *g* for 30 min. The pellet was subsequently washed with buffer B (buffer A containing 2 M urea) that allow efficient removal of nonspecific proteins from inclusion bodies. The final washed pellet was solubilized and denatured in 5 mL of buffer C (100 mM potassium phosphate pH 7, containing 8 M urea and 4 mM β-mercaptoethanol) and then centrifuged at 8000 × *g* for 30 min. The supernatant in this stage was used for refolding.

2.4. Purification

Unfolded protein was loaded on Ni-NTA agarose column (Amersham Biosciences) that had been equilibrated with buffer C. Bound proteins were eluted with buffer C containing 200 mM imidazole and the purity was ensured by SDS-PAGE according to the Laemmli method [11]. The gel was stained by Coomassie brilliant blue R-250. Protein concentration was determined by the Bradford method [12].

2.5. In vitro refolding of laccase using dilution procedure

The refolding procedure was carried out through gradual removing of the denaturant, via dilution and dialysis. The solubilized laccase (0.06 mg/mL) was added to the refolding medium and incubated at 4 °C for 6 h. To optimize the refolding process, small-scale assays were carried out in 1 mL reactions, where concentration of different components and reaction conditions were variable (following the order indicated below) glycerol (0–15%), Triton X100 (0–3%), PEG (0–3 mg/mL), sorbitol, sodium chloride, glucose, lactose, trehalose, immidazole, glycine and L-arginine (0–400 mM), time (0–24 h), protein (0.02–0.15 mg/mL), pH (5–9) and metals (0–6 mM) were used to find the optimal condition of refolding. Refolding yield is formally defined as the percentage of soluble protein following dilution refolding, as assessed by enzyme activity measurements and compared with activity of soluble form.

2.6. In vitro refolding of laccase using one-step dialysis strategies

Denatured-reduced protein (2 mL at 0.15 mg/mL) was loaded into a dialysis bag having a membrane molecular weight cutoff of 10 kDa, and then dialyzed against refolding buffer under stirring condition at 4 °C. The optimization of refolding medium was also performed using different types of buffer, pH, additives and divalent ions as mentioned in Section 2.4.

2.7. Spectroscopic studies

The purification of refolded enzyme was carried out. Purity of the protein was confirmed by using SDS-PAGE according to Laemmli method. Protein concentration was determined using Bradford method. Fluorescence of refolded laccase was measured on a Perkin Elmer luminescence spectrometer LS 55. The excitation wavelength was set at 280 nm and the emission spectra were recorded from 300 to 400 nm. The excitation and emission slit were both set to 10 nm.

2.8. Laccase activity

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 (ε = 65,000 M^{-1} cm^{-1}) Syringaldazine was dissolved in absolute methanol [13]. All assays were performed in triplicates. Furthermore, the purity of the refolded protein was analyzed using SDS–PAGE. Then coomassie brilliant blue staining was performed according to Laemmli method. The protein concentration was determined by the Bradford assay using BSA as the standard [12]. For comparison of catalytic parameters of the soluble and refolded laccase forms, the soluble laccase was obtained as previously reported [9].

3. Results and discussion

3.1. Expression of the recombinant laccase in E. coli BL21 (DE3)

Recombinant laccase from *Bacillus* HR03 was expressed in *E. coli* cells. A remarkable amount of this enzyme was expressed is an insoluble inactive form within inclusion bodies. Therefore, different refolding methods for large-scale in vitro activation were investigated [7]. *E. coli* cells were sediment from 500 mL medium culture and lysed by sonication. Since the IBs have higher densities, high-speed centrifugation results in separation of these aggregates from contaminating cellular fragments/proteins. The wash step is necessary to remove contaminants, especially proteins, which may have absorbed onto the hydrophobic surface of the inclusion bodies during the procedure.

3.2. Dependence of the IB solubilization efficiency to applied conditions

As a starting and crucial step for each refolding reaction, the solubilization strategy has to be considered. The washed and purified inclusion bodies were resuspended and incubated in a buffer containing a strong denaturant (Fig. 1A). The IB solubilization was

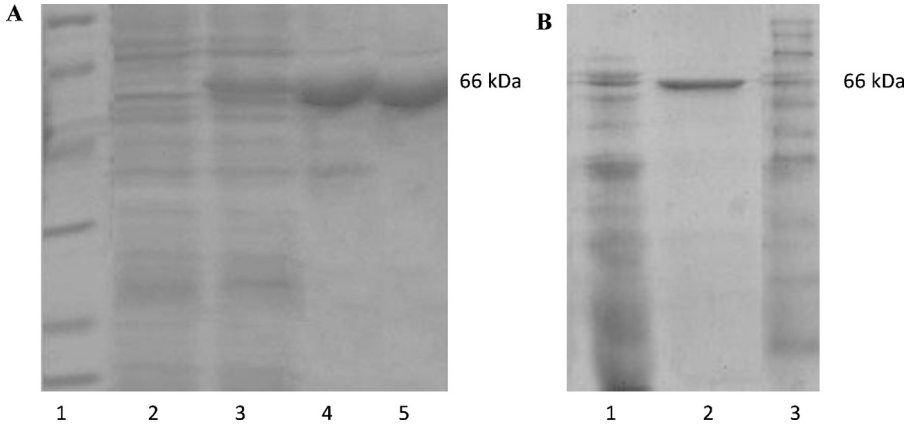

Fig. 1. SDS-PAGE analysis of laccase from *E. coli*. (A) Lane 1 marker, lanes 2 pellet after induction, lane 3 soluble inclusion bodies before and lane 4 after washing with 2 M urea and purification. (B) Lane 1 soluble enzyme after expression, lane 2 purified refolded enzyme and lane 4 marker.