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Increasing the catalytic activity of Bilirubin oxidase from *Bacillus pumilus*: Importance of host strain and chaperones proteins

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ABSTRACT

Aggregation of recombinant proteins into inclusion bodies (IBs) is the main problem of the expression of multicopper oxidase in *Escherichia coli*. It is usually attributed to inefficient folding of proteins due to the lack of copper and/or unavailability of chaperone proteins. The general strategies reported to overcome this issue have been focused on increasing the intracellular copper concentration. Here we report a complementary method to optimize the expression in *E. coli* of a promising Bilirubin oxidase (BOD) isolated from *Bacillus pumilus*. First, as this BOD has a disulfide bridge, we switched *E.coli* strain from BL21 (DE3) to Origami B (DE3), known to promote the formation of disulfide bridges in the bacterial cytoplasm. In a second step, we investigate the effect of co-expression of chaperone proteins on the protein production and specific activity. Our strategy allowed increasing the final amount of enzyme by 858% and its catalytic rate constant by 83%.

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1. Introduction

Multicopper oxidases (MCOs), containing four coppers, have been widely studied because of their usefulness in biotechnological applications (Solomon et al., 1996). They catalyze the four electron reduction of O_2 to water without releasing H_2O_2. This feature makes them the perfect candidate to elaborate O_2 reducing cathode in glucose/O_2 biofuel cells (Mano, 2012; Mano and Edembe, 2013; Rasmussen et al., 2016) which may power in the near future implanted medical devices (Falk et al., 2013; Leech et al., 2012). Up to now, this reaction was mostly catalyzed by laccases but Bilirubin oxidases (BODs) are getting more attention because they have the advantage to be more active and stable in physiological conditions (Beyl et al., 2011; Kavanagh et al., 2008).

MCOs have been overexpressed in numerous organisms such as yeast (Durand et al., 2012a), fungi (Kunamneni et al., 2008) and bacteria (Piscitelli et al., 2010). The heterologous expression of MCOs in *E. coli* is challenging because of the formation of inclusion bodies (IBs) and because most of the produced enzymes are depleted incopper. Most of the work on bacterial MCOs has always been directed to solve the problem of copper depletion during production and numerous strategies have been developed to overcome this issue (Bento et al., 2005; Galli et al., 2004; Koschorreck et al., 2008; Martinez-Alonso et al., 2010). Among others, Durao et al. (2008) described a production method in *E. coli* using a microaerobic step during the expression of the enzyme. The transition to microaerobic condition led to a higher intracellular copper concentration which promoted the appropriate folding of fully copper loaded enzymes. This strategy resulted in an increase in the amount and the specific activity of purified CotA from *Bacillus subtilis*. More recently Gunne et al. (2013) proposed another strategy to increase the copper content in CotA based on the co-expression with copper binding chaperone protein (CopZ). This method increased the copper content and specific activity of CotA laccase from *B. licheniformis*. However, a large part of expressed proteins still aggregate into inclusion bodies which is a barrier for large-scale bacterial production of MCOs. In addition, there is a lack of publications in the literature devoted to the optimization of bacterial production of MCOs. Other strategies such as host optimization, co-expression of chaperone proteins and optimization of expression vector (Gopal and Kumar, 2013) may be applied to solve this issue.

The proper folding of newly expressed enzyme is assisted by chaperones proteins. When misfolding occurs, protein is degraded by proteases. When degradation fails and misfolded polypeptides accumulate in the bacterial cytoplasm, IBs are formed to prevent the formation of aggregates with proper folded proteins and to

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protect the bacterial cytoplasm (Villaverde and Carrio, 2003). This failure is probably due to the high amount of expressed recombinant proteins. DnaK/DnaJ/GrpE and GroEL/GroES are the main molecular chaperone systems in the cytoplasm of *E. coli*, responsible for the correct folding of the newly expressed enzyme. The DnaK/DnaJ/GrpE system is reported to prevent the misfolding of proteins by protecting their hydrophobic part during the synthesis (Schroder et al., 1993). DnaK/DnaJ/GrpE system works cooperatively with GroEL/GroES system and can transferred to it the partially folded polypeptide for subsequent folding (Walter, 2002) (Fig. S1). The first preliminary results of increasing the yield of recombinant proteins by increasing the amount of chaperone proteins in the bacterial cytoplasm were obtained using the heat-shock natural response (Lorimer, 1996; Thomas and Baneyx, 1996), or other stress like osmotic stress (Blackwell and Horgan, 1991). The overexpression of chaperone proteins has been successfully applied to antibodies (Choi et al., 2004) and enzymes such as esterases (Böttcher et al., 2006) or lipases (Pfeffer et al., 2007). However, results were protein-dependent and overexpression of chaperone proteins either promoted the proper folding or the degradation of recombinant proteins (Martinez-Alonso et al., 2010), indicating that chaperone proteins have different ways of regulation to protect the cytoplasm of bacteria from misolded proteins. In the case of overexpression of MCOs in *E. coli*, the intracellular copper concentration and copper chaperone proteins seem to play a role in the correct folding and in the copper content of the enzyme. However, the roles of folding protein chaperones have not been examined.

In this study, we investigated the expression of BOD from *Bacillus pumilus*, an attractive enzyme in various biotechnological applications including the development of enzymatic biofuel cells (Durand et al., 2012b). As this BOD has a disulfide bridge that could be crucial for the correct folding and the stability of the enzyme, *E. coli* strain was replaced by Origami B (DE3) in order to enhance the formation of disulfide bridges in the bacterial cytoplasm. Then, the role of chaperone proteins induced by natural response to osmotic stress or specific expression of the two major folding chaperone systems of *E. coli* were examined. This study highlights the crucial role of host strain and folding chaperone proteins during the expression of MCOs in *E. coli*.

2. Materials and methods

2.1. Chemicals and materials

2,2′-azino-bis-[3-ethylbenzthiazoline-6-sulfonicacid] (ABTS), 2,6-dimethoxyphenol (2,6-DMP), isopropyl—thiogalactoside (IPTG) and all other chemicals were of analytical grade or higher and purchased from Sigma (Sigma-Aldrich, Saint Louis, MO, USA) or Euromedex (Souffelweyersheim, France). The cross linker poly ethylen glycol diglycidylether (400) (PEGDGE) was purchased from Polysciences Inc. (Warrington, PA). All solutions were made with deionized water passed through an AQ 10 Milli-Q purification system from Millipore (Molsheim, France). AKTA purifier 10UV-900 and prepacked Hisprep 16/10 column were from GE Healthcare BioSciences AB (Uppsala, Sweden). Amicon Ultra filtration columns were from Millipore (Molsheim, France). Electrochemical experiments were performed using a bipotentiostat (CH Instruments, model CHI 842 B, Austin, TX, USA) and 5 mm diameter rotating glassy carbon electrodes. Circular dichroism spectra were recorded on a MOS 450 (Biologic, France).

2.2. Bacterial strains and plasmids

Plasmids pGKJE8, pKJE7 and pGRO7 for the expression of chaperone proteins were purchased from Takara (Dalian, China). Origami B (DE3) competent cells were purchased from Millipore (Molsheim, France). The ORF coding BOD from *B. pumilus* (accession no. A8FAG9) was inserted into pET21a vector as previously described (Durand et al., 2012b) and electroporated into Origami B (DE3) using an Eppendorf Eporator system (Eppendorf, France). Later this strain was transformed by electroporation with plasmids encoding for chaperone proteins (Fig. S1), pKJE7 for the expression of DnaK/DnaJ/GrpE system, pGro7 for the expression of GroEL/GroES system, and pG-KJE8 for both (Fig. S1).

2.3. BOD expression and purification

Lysogeny broth (LB) was used as the standard growth medium for all protein expression experiments. Starter cultures were grown overnight in LB containing 100 μg/mL Ampicillin at 37 °C and 190 rpm. Afterwards, 5L shake flasks containing 2L LB medium with pH adjusted to 6 and supplemented with 100 μg/mL Ampicillin were inoculated with 20 mL of the prepared culture and incubated at 37 °C and 190 rpm. For expression in osmotic stress conditions the media was supplemented with 600 mM of sorbitol and 20 mM of betaine. Before the induction of protein expression, culture was incubated at 37 °C with shaking to reach an optical density at 600 nm (OD_{600}) comprised between 0.4 and 0.6. Expression was induced at 22 °C by the addition of 500 μM of IPTG and 500 μM $CuSO_4$ and incubated for 24 h at 190 rpm. After 24 h of induction, the culture was transferred in a 2L bottle to achieve microaerobic conditions by switching off the agitation (Durao et al., 2008).

The same protocol was used for protein co-expressed with chaperone proteins except that the selection was performed with 100 μg/mL of Ampicillin and 30 μg/mL of chloramphenicol. When the culture reached an OD_{600} between 0.4 and 0.6, the chaperone proteins were induced for 1 h at 22 °C with 0.5 mg/mL of arabinose and 5 ng/mL of tetracycline (as recommended by the supplier) (Nishihara et al., 1998; Nishihara et al., 2000) with agitation before adding the IPTG and $CuSO_4$. The growth rate for each condition was determined at 600 nm (OD_{600}) every hour by withdrawing 500 μL of the solution. At the end of each production, samples were harvested and adjusted at 2 OD_{600} in 4 mL, centrifuged at 21 000*g* for 6 min and bacterial pellets were kept at −20 °C to study the soluble/insoluble fraction.

The cells were harvested by centrifugation at 6000*g* for 20 min at 4 °C, resuspended in 100 mL buffer 50 mM Na_2HPO_4, 500 mM NaCl, 20 mM imidazole pH 7.6 (Buffer A) and disrupted twice with a cell Disruptor (Constant System LTD, CellD) at 2200 bar. The extract was then centrifuged 45 min at 20 000*g* at 4 °C and sequentially filtered with 1.5 μm and 0.22 μm diameter syringe filters. The 100 mL supernatant was loaded on a HisprepFF 16/10 (GE Healthcare Life sciences, Uppsala Sweden) equilibrated with 5 column volume of the same buffer. The BOD was eluted with a linear gradient (1 mL/min) of 20 mM to 500 mM Imidazole in 50 mM Na_2HPO_4, 500 mM NaCl. The purification was continuously monitored at 280 nm and 600 nm to discriminate holo BOD from other proteins and were used to pool the enzymes which were then concentrated to 2 mL. Imidazole and NaCl were removed sequentially at 4 °C by dialysis (10 kDa) in two baths of 1L 50 mM Na_2HPO_4 pH 7.6 for 6 h and overnight.

2.4. SDS-PAGE

2.4.1. Purified enzymes

Enzymes were diluted at 2.5 μg in 15 μL H_2O and 5 μL sample buffer 4X (3.5 mL H_2O, 1 M Tris 0.5 M pH 6.8, 0.8 mL glycerol, 1.6 mL SDS 10%, 0.4 mL Β-mercaptoethanol, 1 mg bromophenol blue) and heated 30 min at 99 °C. The sample were centrifuged 5 s at 21

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