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Expression and biochemical analysis of codon-optimized polyphenol oxidase from *Camellia sinensis* (L.) O. Kuntze in *E. coli*

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ABSTRACT

Polyphenol oxidases (PPOs) are copper-containing industrially important enzymes that catalyze the synthesis of many commercially important products by using polyphenols as substrate. *Camellia sinensis* polyphenol oxidase (CsPPO) is interesting because it oxidizes epicatechins to yield theaflavins and thearubigins. The present study aimed to optimize the expression of *CsPPO* in *Escherichia coli*. Because *CsPPO* had a large number of *E. coli* rare codons, it yielded a poor quantity of protein in *E. coli* Rosetta™ 2 cells, which have additional tRNAs for *E. coli* rare codons. Thus, synthetically constructed codon-optimized *CsPPO* was cloned into pET-47b(+) vector and expressed in a bacterial host. Ectopic expression led to the formation of inclusion bodies. However, extensive standardization of buffers and methods of refolding such as dialysis, on-column refolding, and rapid dilution yielded active PPO from solubilized inclusion bodies with copper content of 0.880 ± 0.095 atom/molecule of protein.

Experimental data produced maximum PPO activity in a rapid dilution buffer containing 0.5 M L-arginine. Refolded CsPPO had an optimum pH of 5.0 and K_m values of 3.10, 0.479, and 0.314 mM, and a V_{max} of 163.9, 82.64, and 142.8 U/mg of protein for catechol, catechin, and epicatechin, respectively.

1. Introduction

Polyphenol oxidases (PPOs) are nuclear-encoded copper-containing metalloproteins involved in either the hydroxylation of monophenols to o-diphenols (EC 1.14.18.1; monophenol monoxinase, tyrosinase, and cresolase) or dehydrogenation of o-diphenols to o-quinones (EC1.10.3.1, diphenol oxygen oxidoreductase and catecholase) [1]. The reaction products of PPO-catalyzed reactions have varied effects on cellular processes and have commercial uses. Labile quinones formed from PPO-catalyzed reactions result in the formation of several co-products. Labile quinones react with themselves to yield "melanoid" pigments and react with amino acids/proteins to form brown-colored complexes called tannins/melanins [2]. Products from PPO-catalyzed reactions could further react with proteins to form covalent condensations [3–7], resulting in changes in the biochemical properties of food proteins [8]. PPO causes browning of freshly cut fruits and vegetables, juices, and tissue culture explants [1]. Moreover, labile quinones react with DNA, proteins, amino acids, or lipids and damage them [9,10]. PPO production is induced by wounds and pathogens; moreover, the reaction products of PPOs play a role in plant defense against insects and plant pathogens [11]. Although the role of PPO in plant metabolism is not very clear, the quinones produced by PPOs may act as antibiotics and cytotoxins to pathogens [12]. Active quinones covalently modify plant proteins, thus affecting their nutritive value, which further increase the plant's resistance toward herbivorous insects [13]. Recently, the role of PPO in tyrosine metabolism, betalain biosynthesis, lignan biosynthesis, and aurone biosynthesis was described [14]. Although PPOs have been purified and characterized from a large number of plant and fungal species, mushroom tyrosinase is widely used at a commercial scale [15] for the production of L-dopa, a potential drug used for the treatment of Parkinson's disease [16]. More importantly, PPO has various industrial applications that extend from the food to the chemical industry, including the treatment of industrial effluents [17,18].

PPOs isolated from various sources have different affinities for different substrates [2,19,20]. Substrate affinity depends on the abundance and type of substrate present in a given source and the monophenolase or diphenolase activity of PPO. Substrate affinity of tea PPO toward catechins and gallocatechins plays a very important role in the synthesis of commercially important black tea pigments named theaflavins (TF) [21]. TFs are reported to have many medicinal properties [22].

The *PPO* gene generally belongs to a multigene family and has been reported in a number of plant species [1,23,24]. Various members of

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the *PPO* gene family are differentially expressed in plant parts and respond differentially to abiotic and biotic elicitors [25–29]. The complete coding sequence of *CsPPO* was first reported by our group (accession number AY659975.1.) and was later independently expressed in *Escherichia coli* by Wu et al. [30]. Their group reported that recombinant CsPPO appears as an inclusion body upon expression in *E. coli* despite the removal of chloroplast-targeting transit peptide. Although Wu et al. attempted to solubilize inclusion bodies in a suitable buffer, the specific activity of PPO was very low (19.01 U/mg protein) [30]. Because CsPPO is commercially important owing to its potential to synthesize TFs, the present investigation was conducted to develop a synthetic PPO ($CsPPO_{syn}$) of tea to improve its expression and activity in *E. coli*.

2. Materials and methods

2.1. Cloning and expression of CsPPO in E. coli

Previously, clonal variant of *CsPPO* gene was cloned in our laboratory, and the sequence of same has been submitted to NCBI with accession number FJ656220.1. *CsPPO* was cloned, after removing chloroplast transit peptide present at position 1–288 bp, into the expression vector pET-47b(+) (Novagen, Germany) according to the manufacturer's instructions. The expression for CsPPO was first tried in *E. coli* BL21(DE3), followed by *E. coli* Rosetta™2 after induction with 1.0 mM isopropyl β-D-1-thiogalactopyranoside (IPTG).

2.2. Construction of $CsPPO_{syn}$

Using the *CsPPO*, we constructed $CsPPO_{syn}$ after removing the chloroplast transit peptide at position 1–288 bp of *CsPPO*. In addition, codons were optimized for the expression of $CsPPO_{syn}$ in *E. coli* using OptGene™ (Ocimum Biosolutions, USA) as mentioned in supplementary data (Supplementary Fig. 1). $CsPPO_{syn}$ was synthesized from BIO BASIC INC, Canada, and cloned in to the expression vector pET-47b(+) (Novagen, Germany). $CsPPO_{syn}$ used the initiation codon of the vector as the gene's own initiation codon was eliminated because of the removal of the initial 288 nucleotides from the 5′-end of *CsPPO*. In addition, two nucleotides "CT" were introduced at the 5′-end immediately downstream to the restriction site to ensure inframe expression of the protein (Supplementary Fig. 1). $CsPPO_{syn}$ was cloned in vector pET-47b(+) in between *Sac*II and *Not*I restriction sites and expressed in *E. coli* BL21(DE3) (Novagen, Germany).

2.3. Expression of recombinant $CsPPO_{syn}$ in E. coli

E. coli BL21(DE3) cells containing the $CsPPO_{syn}$ construct were grown in LB broth until A_{600} reached 0.6. Subsequently, protein expression was induced with the addition of 1.0 mM IPTG. Because PPO is a copper-requiring protein, copper sulphate ($CuSO_4$) was added to a final concentration of 20 μM. The cultures were harvested after 5 h of induction at 37 °C. All the subsequent steps were performed at 4 °C. Cells were resuspended in resuspension buffer (50 mM sodium phosphate, 300 mM NaCl, pH 8.0). Lysozyme (1 mg ml^{-1} of buffer) was added to the cells, and the mixture was kept on ice for 30 min. The cells were disrupted by sonication for 10 min with a pulse on for 9.9 s followed by pulse off for 7.0 s. The sonicated suspension was centrifuged at 12,000 × *g*, and the soluble extract and pellet were analyzed by 12% SDS-PAGE [31] to detect the presence of CsPPO.

2.4. Solublization of CsPPO from inclusion bodies

Because the total protein of CsPPO was found in the pellet and we could barely obtain any protein in the soluble fraction, the pellet was washed with resuspension buffer containing 2% Triton X-100 and 5.0 mM DTT at room temperature with intermittent shaking for 1 h, followed by centrifugation at 12,000 × *g* for 10 min. The supernatant was discarded, and the pellet was washed with washing buffer (100 mM sodium phosphate, 10 mM Tris-Cl, and 2 M urea; pH 8.0), followed by centrifugation at 12,000 × *g* for 10 min. The resulting pellet was resuspended in 2 ml denaturing buffer(100 mM sodium phosphate, 10 mM Tris-Cl, and 8 M urea; pH 8.0), followed by centrifugation at 12,000 × *g* for 10 min. The pellet was discarded, and the supernatant was stored at −20 °C until further use for refolding. In addition, we used various strategies and refolding buffers to obtain functionally active CsPPO; the steps are discussed in the following subheads.

(i) Refolding by dialysis

Inclusion bodies dissolved in denaturing buffer were dialyzed overnight against 0.12 M NaCl alone [29] or 50 mM Tris-Cl and 0.12 M NaCl. Dialysis was also performed by supplementing the dialysis buffer with different concentrations of $(NH_4)_2SO_4$ and Triton X-100 in 50 mM potassium phosphate (K-PO_4) buffer (pH 7.5) and 0.12 M NaCl [32].

(ii) On-column refolding

On-column refolding was attempted on a Ni-NTA column. The column was equilibrated with 50 mM Tris-Cl containing 8 M urea. CsPPO inclusion bodies dissolved in 50 mM Tris-Cl (pH 8.0), and 8 M urea were loaded on to the column and eluted with 50 mM Tris-Cl buffer (pH 8.0), 250 mM imidazole, and 300 mM NaCl. Elution was also attempted with different concentrations of $(NH_4)_2SO_4$ and Triton X-100 and using 50 mM K-PO_4 buffer (pH 7.5) on Ni-NTA.

(iii) Refolding by rapid dilution

The protein was diluted 100 times with different refolding buffers (RB, detailed in Table 1) to a final concentration of 50 μg ml^{-1} at 4 °C and 15 °C and incubated for protein refolding for 24 h. Then the protein was concentrated using Amicon Ultra-15 filter (Millipore, USA) with a 10-kDa cutoff and used for the analysis of PPO activity.

2.5. PPO activity assay

The activity assay of PPO was performed as described by Halder *et al.* [33]. Briefly, the reaction mixture contained 50 mM sodium citrate buffer, pH 5.0, 50 mM catechol, and PPO aliquot at room temperature. Enzyme activity was assayed spectrophotometrically by monitoring the change in absorbance at 410 nm with time. The rate of reaction was estimated from the linear part of the curve. The change in absorbance of 0.001 per minute was defined as one unit (U) activity. Protein concentration was estimated by Bradford's method [34].

2.6. Copper content analysis

The enzyme samples were refolded in triplicates in RB8 (Table 1), followed by extensive dialysis in 5 mM potassium phosphate buffer, pH 7.5, containing 0.1 mM EDTA, and subsequently in buffer without EDTA. A control experiment was also performed, in which a denaturing buffer was added to RB8 instead of protein, and the solution was similarly dialyzed as mentioned above. The control and protein samples were subjected to diacid digestion. Nitric acid was added to samples and heated in a digestion unit at 145 °C for 1 h. Perchloric acid was added, and samples were heated at 240 °C for 20 min. Finally, the volume was made to 25 ml using dH_2O. The blank was also acid digested along with the sample, wherein water was added instead of protein or buffer control. The metal content of the enzyme was determined using inductively coupled plasma optical emission spectrometry (ICP-OES).

2.7. Determination of pH optima

PPO activity was assayed in triplicates in sodium citrate buffers of pH 4.0, 4.5, 5.0, and 5.5 and potassium phosphate buffers of pH 6.0 and 6.5. Separate controls reactions were also set up, in which enzyme was replaced with buffer while keeping other components same.