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Functional expression of *Coprinus cinereus* peroxidase in *Pichia pastoris*

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ABSTRACT

A *Coprinus cinereus* peroxidase (CiP) was successfully expressed by the methylotrophic yeast *Pichia pastoris*. The 1095-bp gene encoding peroxidase from *C. cinereus* was cloned with a highly inducible alcohol oxidase (*AOX1*) promoter and integrated into the genome of *P. pastoris*. The recombinant CiP (rCiP) fused with the α-mating factor pre-pro leader sequence derived from *Saccharomyces cerevisiae* accumulated neither inside the cell nor within the wall, and were efficiently secreted into the culture medium. SDS-PAGE and immunoblot analysis revealed that the rCiP was not hyper-glycosylated and its α-factor signal sequence was correctly processed. It was also found that the kinetic properties of rCiP were similar to those of native CiP. In order to produce large amounts of rCiP, the high cell density cultivation of recombinant *P. pastoris* was carried out in a fermentor with fed-batch mode. The peroxidase activity obtained in a 5 l fermentor cultivation became about 6 times (1200 U/ml) higher than that in shake-flask cultures (200 U/ml).

1. Introduction

Peroxidases (EC 1.11.1.7) are enzymes capable of oxidizing a broad range of compounds including phenols, aromatic amines, and reduced inorganic compounds with peroxide as the oxidant. The active sites of most peroxidases contain protoporphyrin IX (heme) with ferric iron. They occur in a wide variety of organisms, and their specificity and biological functions vary depending on the sources. They have been classified in two superfamilies, the mammalian superfamily and the plant superfamily. Based on sequence alignment, the members of the plant superfamily have been divided into three classes: Class I members are intracellular peroxidases such as yeast cytochrome *c* peroxidase; classes II and III are extracellular fungal peroxidases and extracellular plant peroxidases, respectively. Class II peroxidases are monomeric glycoproteins, containing two calcium ions and four conserved disulfide bridges, although the placement of the disulfides is different from class III enzymes [1,2].

Peroxidases have attracted industrial attention due to their various applications including clinical examination and waste-water treatment [3–5]. We have been applying peroxidases from various sources to the production of phenolic polymers [6–10]. Peroxidase from horseradish root (HRP) belonging to class III may be best known due to its high catalytic activity and its broad specificity for electron donors. However, since it was revealed that *Coprinus cinereus* peroxidase (CiP), a class II fungal peroxidase found in the culture filtrate of an ink cap basidiomycete, was similar to HRP, CiP has been drawing much attention [11–13]. CiP (essentially identical to *Arthromyces ramosus* peroxidase, ARP) exhibits a specific activity and maintains the broad substrate specificity of HRP. In addition, CiP comprises a single species of enzyme, whereas HRP consists of at least twelve isozymes with different catalytic properties, with an isozymic ratio that depends on the conditions under which the plant is cultivated over the period of several months required for its growth. For industrial applications of this fungal peroxidase, an efficient and economical production system is required. Thus far, *Saccharomyces cerevisiae* [14], *Aspergillus oryzae* [15], and *Aspergillus awamori* [16] have been attempted as a heterologous expression system for CiP/ARP production.

Pichia pastoris has been developed as an expression system of choice for foreign protein production on an industrial scale. This is based on the many advantages of the methylotrophic yeast: ease of genetic manipulation, high levels of protein expression, the ability to perform higher eukaryotic protein modifications, easy scale-up of fermentation, and simple purification of secreted recombinant proteins [17,18]. The *P. pastoris* expression system is being used successfully to produce various recombinant proteins, the most

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Abbreviations: YPDS, yeast extract peptone dextrose sorbitol; BMMY, buffered minimal methanol complex; BMGY, buffered minimal glycerol yeast extract medium; SEC, size exclusion chromatography; BCA, bicinchoninic acid; BSA, bovine serum albumin; PBS, phosphate buffer solution; NBT/BCIP, nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate; PVDF, polyvinylidene fluoride.

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recent example being the functional expression of horseradish peroxidase and manganese peroxidase [19–21].

In this work, we have performed the heterologous expression and secretion of CiP using *P. pastoris* as a cellular host. Characteristics of the produced recombinant CiP were compared with those of native CiP. To the best of our knowledge, there is not yet any article concentrating on a heterologous expression of rCiP in *P. pastoris*, even though Lokman et al. mentioned the production of ARP in *P. pastoris* [16].

2. Materials and methods

2.1. Materials

All chemicals were of reagent-grade purity. Classical peroxidase substrate ABTS (2,2′-azinobis(ethylbenzthiazoline-6-sulfonate)), and H_2O_2 were obtained from Sigma (USA). The restriction enzymes and ligase were purchased from New England BioLabs (USA), EXTaq from Takara Bio Inc. (Japan), and *Escherichia coli* HIT Competent Cells™-DH5α from RBC (Taiwan). *P. pastoris* strains X-33 (wild-type) and plasmid pPICZαA were obtained from Invitrogen (USA). The yeast transformation kit (MicroPulser™) and the plasmid miniprep kit were purchased from Bio-Rad (USA) and Takara Bio Inc. (Japan), respectively. Plasmid propagation was done in *E. coli* DH5α cells using low-salt LB medium (Zeocin 25 μg/ml).

2.2. Vector construction for CiP expression in P. pastoris

Total RNA was isolated from *C. cinereus* IFO 8371. The strain was stored on a potato dextrose agar slant at 4 °C, and the spores were routinely transferred to the fresh slant and incubated at 30 °C for 7 d every month. After incubation at 30 °C for 7 d, the strain was used in the experiment. The medium used for the peroxidase production contained 20 g/l glucose, 4 g/l peptone (Difco Lab., USA) and 2 g/l yeast extract (Difco Lab., USA). The culture was started by introducing the spore suspension, which was prepared by adding 5 ml of the medium to the slant culture and vibrating for 30 s, into 100 ml of the culture medium in a 500 ml Erlenmeyer flask. It was incubated on a rotary shaker at 150 rpm and 30 °C. The mycelium was harvested after 5 d and RNA was prepared by using an RNeasy Plant Mini Kit (Qiagen, USA). The cloning of the peroxidase cDNA from *C. cinereus* by RT-PCR was performed using the oligo dT and sequential PCR with appropriate primer sets. Reverse transcription was carried out using the "1st strand cDNA synthesis kit for RT-PCR (AMV)+" (Roche, Germany). The following primer set used: rCiP-N-*Eco*RI, 5′-CGGAATTCCAGGGTCCTGGAGGAGGCGGGTCAG-3′ and rCiP-C-*Not*I, 5′-ACGCGTCGACTCAAGGAGCAGGAGCGAGGGAGG-3′ to amplify the structural region of CiP. The PCR products were ligated to pPICZαA after the treatment with *Eco*RI and *Not*I as shown in Fig. 1.

2.3. Transformation into P. pastoris

The plasmids were isolated from *E. coli* and transformed into *P. pastoris* by electroporation according to the supplier's instructions (Invitrogen, USA). Before their transformation, the plasmids were linearized with SacI. Following their transformation, the linearized vectors were integrated into the *P. pastoris* genome via homologous recombination. The transformed cells were plated on YPDS medium (1% yeast extract, 2% peptone, 2% glucose, and 1 M sorbitol) supplemented with 100 μg/ml Zeocin. For the CiP construct, typically 4–6 transformants were picked and purified on new YPDS plates (supplemented with 100 μg/ml Zeocin) to isolate single colonies, which were then screened to identify the clones with the highest levels of CiP activity.

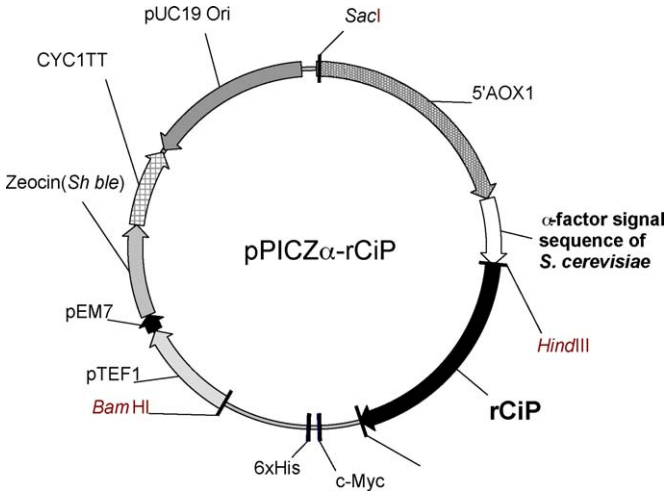

Fig. 1. Construction of the CiP expression vector in *Pichia pastoris*. The CiP gene was cloned into the vector pPICZαA with the α-mating factor pre-pro leader sequence derived from *Saccharomyces cerevisiae*.

2.4. CiP expression in P. pastoris X-33

Pichia cells were grown at 30 °C in shake flasks. pPICZα-rCiP harboring cells were first grown overnight in BMMY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, 400 μg/l biotin, 0.5% methanol). Sterile methanol (0.5%) was added every 24 h to maintain induction conditions.

2.5. Fermentation conditions

One milliliter of stock seed was inoculated into 200 ml of BMGY (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 400 μg/l biotin, 1% glycerol, 100 mM potassium phosphate, pH 6.0) in an 1 l shake flask. The cultivation lasted for 16–24 h at 30 °C and 250 rpm to reach an optical density (at 600 nm) of 2–6. As the inoculum, 200 ml of the propagated culture was used for a 5 l fermentor (New Brunswick Scientific, Edison, USA) containing 2 l basal salts medium (BSM) and 8.7 ml PTM1 trace salts. One liter of BSM consists of 26.7 ml 85% H_3PO_4, 0.93 g $CaSO_4$, 18.2 g K_2SO_4, 14.9 g $MgSO_4{\cdot}7H_2O$, 4.13 g KOH, and 40.0 g glycerol. One liter of PTM1 (filter-sterilized) consists of 6.0 g $CuSO_4{\cdot}5H_2O$, 0.08 g NaI, 3.0 g $MnSO_4{\cdot}H_2O$, 0.2 g $Na_2MoO_4{\cdot}2H_2O$, 0.02 g H_3BO_3, 0.5 g $CoCl_2$, 20.0 g $ZnCl_2$, 65.0 g $FeSO_4{\cdot}7H_2O$, 0.2 g biotin and 5.0 ml H_2SO_4. The fermentation was run in fed-batch mode at 30 °C, and pH was maintained at 5.0 using undiluted (28%) ammonium hydroxide. Dissolved oxygen (DO) was maintained above 20% saturation by adjusting agitation rate. When the initial glycerol (40 g/l) in batch phase was depleted, as indicated by an abrupt increase in DO reading, a 50% (w/v) glycerol solution containing 1.2% (v/v) PTM1 was fed at a feed rate of 18.15 ml/h/l broth for 4 h. Following this step, 100% methanol containing 1.2% (v/v) PTM1 and 0.05% (w/v) antifoam (KFO 673, KABO Chemicals, Cheyenne, USA) was fed at 15 ml/h/l broth.

2.6. Purification of recombinant CiP

Yeast cultures were harvested at the time point corresponding to the highest activity for CiP and centrifuged at 3880 × *g* for 20 min. The supernatant was concentrated with ultrafiltration (10 kDa MWCO, Amicon) desalted with 0.1 M phosphate buffer (pH 5.0). The desalted CiP was finally purified with a SEC column (BioSil-125, Bio-Rad) using 0.1 M phosphate buffer (pH 5.0) as the eluent. The fractions with the highest ABTS activity were combined and concentrated again with ultrafiltration (10 kDa MWCO, Amicon). The total protein concentration was determined using a BCA method with BSA standard.

2.7. CiP activity assay

The CiP activity was measured using ABTS and hydrogen peroxide. The cell suspension/purified protein samples were mixed with 2 ml of ABTS-H_2O_2 (0.5 mM ABTS and 2.9 mM H_2O_2, pH 5.0) in a quartz cuvette. An increase in absorbance at 420 nm (molar extinction coefficient of oxidized ABTS is $3.6 \times 10^4\ M^{-1}\ cm^{-1}$ [22]) was recorded using a UV–vis spectrometer (Shimadzu, Japan) at 25 °C.

2.8. Separation of yeast proteins

Membrane-associated proteins were separated from cytosolic proteins as described in the literature [23]. Briefly, cells (50 mg) were lysed in PBS (pH 7.4) containing complete protease inhibitor cocktail-EDTA free (Roche, Germany) by vortexing with glass beads. Subsequent centrifugation (4 °C, 30 min, 15,000 × *g*) separated the soluble fraction containing the cytosolic proteins from the pellets containing the membrane-associated protein fractions. The pellets were added to PBS with protease inhibitor cocktail, 2% SDS and centrifuged at 2300 × *g* for 5 min (4 °C). The membrane-associated proteins became present in the supernatant. The yields of the protein preparations were determined with the Total Protein Kit (Sigma, USA) according to the supplier's instruction.

2.9. SDS-PAGE and immunoblotting

The recombinant CiP were analyzed by 10% acrylamide gel (0.1%, w/v, SDS, 10%, w/v, acrylamide) and detected by silver staining or Coomassie blue staining. Immunoblot analysis was performed according to manufacturer's instructions using the 1-Step™ NBT/BCIP Western blotting system (Pierce Biotech, Rockford, USA). The rCiP was separated by electrophoresis in the denatured condition (0.1%, w/v, SDS, 10%, w/v, acrylamide), transferred to PVDF membrane (Amersham, UK). Anti-cCiP antibody (Peptron Co., Korea) raised in mouse by using commercially available peroxidase (Novozymes, Denmark) was used as the primary antibody followed by an anti-mouse goat IgG-alkaline phosphatase. Western blot of the supernatant samples was applied to the