High-level expression of Myrothecium verrucaria bilirubin oxidase in Pichia pastoris, and its facile purification and characterization

High-level expression of *Myrothecium verrucaria* bilirubin oxidase in *Pichia pastoris*, and its facile purification and characterization

Kunishige Kataoka*, Kazuhiro Tanaka, Yoko Sakai, Takeshi Sakurai

Division of Material Sciences, Graduate School of Natural Sciences and Technology, Kanazawa University, Kakuma, Kanazawa 920-1192, Japan

*Corresponding author

Tel.: +81-76-264-5683

Fax: +81-76-264-5742;

E-mail: kataoka@cacheibm.s.kanazawa-u.ac.jp

Abstract

Bilirubin oxidase (BO) from *Myrothecium verrucaria* (authentic BO) catalyzing the oxidation of bilirubin to biliverdine was overexpressed in the methylotrophic yeast, *Pichia pastoris*. The cDNA encoding BO was cloned into the *P. pastoris* expression vector pPIC9K under the control of the alcohol oxidase 1 (AOX1) promoter, and its protein product was secreted using the *Saccharomyces cerevisiae* α-mating factor signal sequence. The productivity of recombinant BO (rBO) in *P. pastoris* was approximately 5000 U per 1 liter of culture broth, being about 2.5- and 250-fold higher than rBO expressed in *Aspergillus oryzae* and *S. cerevisiae*, respectively. The calculated molecular mass of rBO consisting of 538 amino acids was 60493 Da, however, that of SDS-PAGE was 66 kDa because of non-native type *N*-linked sugar chains. The spectroscopic properties of rBO were typical of multicopper oxidase containing 4 Cu ions per protein molecule. The specific activity to oxidize bilirubin was 57 U/mg, having a value about twice that of authentic BO and rBO expressed in *A. oryzae*. Moreover, the thermostability of rBO expressed in *P. pastoris* was significantly high compared to the authentic BO previously reported. Accordingly, a heterologous expression system of rBO to meet clinical and industrial needs was constructed.

Key words: bilirubin oxidase; multicopper oxidase; *Pichia pastoris*; expression

Introduction

Bilirubin oxidase (BO)[1] [EC.1.3.3.5] catalyzes the oxidation of bilirubin to biliverdine *in vitro* concomitantly with the four-electron reduction of molecular oxygen to water (1). For this reason, BO has been used to determine the amount of bilirubin in serum for the clinical investigation of liver (2, 3). BO was purified and characterized from several basidiomycetes such as *Pleurotus ostreatus* (4) and *Trachyderma tsunodake* (5), and from filamentous fungi, *Myrothecium verrucaria* (1) and *Penicillium janthinellum* (6). The cDNA sequence from *P. ostreatus, T. tsunodake* and *M. verrucaria* indicates that basidiomycetous BO has considerable sequence similarity to fungal laccase (about 60% identity), however, *Myrothecium* BO has low homology with laccase (less than 30% identity) (1, 4). These BOs can oxidize phenolic compounds as fungal laccases; nevertheless, *P. ostreatus* and *M. verrucaria* have their own independent laccases (4, 7). Therefore, BO seems to be a member of the lignin-degrading enzyme system, but the physiological role of the enzyme remains unknown.

BO belongs to the multicopper oxidase superfamily, which includes laccase, ascorbate oxidase, ceruloplasmin, and other prokaryotic enzymes such as CueO and CotA (8-13). These oxidases contain four Cu ions per functional unit classified as types I, II and III Cus according to their spectroscopic and magnetic properties. Mononuclear type I Cu is separated by about 13 Å from

the trinuclear Cu center comprising of a type II Cu and a pair of type III Cus (8-13). Type I Cu functions as an electron mediator from the substrate to the trinuclear Cu center. The trinuclear Cu center is the site of binding and reducing a dioxygen molecule to two water molecules in the turnover process of the catalytic cycle.

Authentic BO is a monomeric enzyme with a molecular mass of 66 kDa. It contains *N*- and *O*-linked carbohydrate chains and 4 Cu ions essential for catalytic activity. cDNA encodes a precursor of authentic BO consisting of 572 amino acids, of which 38 amino acids are the signal (prepro) sequence and the following 534 amino acids are the mature enzyme (1). This cDNA has been previously expressed in a yeast, *Saccharomyces cerevisiae* under the control of the repressive acid phosphatase promotor, whereas the expression level of rBO was extremely low, less than 20 U/l culture (1). We therefore constructed another heterologous expression system of BO to use *Aspergillus oryzae* as the host (14). The expression level of BO and mutants using *A. oryzae* as the host differed; the yields ranged from 2 to 80 mg/l (maximum yield: 2000 U/l) of culture medium since the non-homologous recombination resulted in the accidental insertion of a BO gene cassette in the host genome and moreover, the copy number was unstable.

Recently, the methylotrophic yeast *Pichia pastoris* has become one of the dominant expression systems in molecular biology for its stable and high-level expression of heterologous proteins by using the tightly regulated

alcohol oxidase 1 (AOX1) gene promoter (15, 16). *P. pastoris* can be easily grown to a high cell density in defined minimal media, and is able to introduce eukaryotic post-translational modifications. With this yeast system, multicopper oxidases such as some laccases and ceruloplasmin have been expressed to perform structural and kinetic studies (17-21).

We here report the overexpression of *M. verrucaria* BO in *P. pastoris* at a high level applicable to the clinical investigation. Mature BO cDNA under the control of the AOX1 promoter and in-frame with α-mating factor secretion signal sequence was incorporated into the AOX1 chromosomal locus, and protein synthesis was induced with methanol. rBO secreted into culture medium was purified and characterized, comparing with authentic BO and rBO produced in *A. oryzae*.

Materials and methods

Materials

pNBC1, the plasmid containing cDNA coding for *Myrothecium verrucaria* BO was prepared as described previously (14, 22). The oligonucleotides were purchased from BEX (Japan). The Multi-Copy *Pichia* Expression Kit and medium used for *P. pastoris* cultivation were from Invitrogen (U.S.A.). Vectors pBluescript II SK^- (STRATAGENE, U.S.A.) and pPIC9K (Invitrogen) carrying the kanamycin resistance gene and α-mating factor secretion signal sequence, were used for cloning and recombinant protein

secretion, respectively. *Escherichia coli* JM109 (Takara Biochemicals, Japan) was used for gene manipulations and *P. pastoris* GS115 (Invitrogen) was used for protein expression. The antibiotic G418 disulfate was purchased from Nacalai Tesque (Japan). Endo H_f, the recombinant endoglycosidase H fused with the maltose-binding protein and amylose resin were from New England Biolabs (U.S.A.). All other chemicals were of analytical grade.

Plasmid construction and P. pastoris transformation

The gene fragment coding for mature BO was amplified using the two primers shown below and pNBC3 as a template.

BO-EcoN: 5'-TTCGAATTCGTTGCCCAGATCAGC-3'

Eco RI

BO-PstC: 5'-ATTAACTGCAGCTACTCGTCAGCT-3'

Pst I

The PCR products were digested with *Eco* RI and *Pst* I, and cloned into pBluescript II SK^- digested with the same enzymes. The resulting plasmid pBSBO was sequenced and digested with *Eco* RI and *Not* I. The 1.65-kbp gene fragment was ligated into pPIC9K to yield the expression plasmid pPICBO that contains the BO gene under the control of the AOX1 promoter and in-frame with α-factor secretion signal sequence.

The competent cells (*P. pastoris* GS115) were transformed with pPICBO linearized with the restriction endonucleases *Bpu* 1102I by pulsed electropolation. After the initial selection of His^+ transformants on the minimal dextrose medium (MD) agar plate (1.34% yeast nitrogen base, 4 x 10^{-5}% biotin, 2.0% D-glucose, and 1.5% agar), they were pooled and plated on the yeast extract peptone dextrose medium (YPD) agar plate (1% yeast extract, 2% peptone, 2% D-glucose, and 1.5% agar) containing an appropriate amount of G418. The G418-resistant colonies were re-transformed with pPICBO to generate G418 hyper-resistant colonies with multiple inserts of the BO gene. The copy number of the multiple integrant was estimated by dot blot hybridization of genomic DNA from *Pichia* recombinants with the probe for the BO gene, as described below.

Dot blot hybridization

The recombinant *Pichia* colonies were grown overnight at 30°C in 5 ml of YPD medium in a test tube. Aliquots of washed cells (1.0 X 10^6 – 6.3 X 10^4 cells) were then spotted onto a nitrocellulose filter (Optitran BA-S85, Schleicher & Schuell, U.S.A.) using a dot blot apparatus. Cell lysis and DNA fixation were performed according to the supplier's manual (Multi-Copy *Pichia* Expression Kit, Invitrogen).

The 1.6 kb BO gene fragment was used as a template to prepare a DIG-labeled probe by random priming using a *Bca*BEST DIG Labeling Kit

(Takara Biochemicals). The filter was incubated overnight with the probe at 65°C in a hybridization solution containing 5% blocking reagent (Roche, U.S.A.), 0.15 M NaCl, and 0.1 M Tris-HCl, pH 7.5. After hybridization, the blot was treated with anti-DIG Fab fragment AP conjugate and detected with nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate solution using DIG Nucleic Acid Detection Kit (Roche).

Expression and purification of rBO in P. pastoris

A single *P. pastoris* colony was inoculated into 4 ml YPD medium in a test tube and cultured overnight at 30°C with shaking. The pre-culture was transferred into 100 ml YPD medium supplemented 3 x 10^{-5}% $CuCl_2$ in a 500 ml shaking flask and grown for 24 hr at 30°C. The cells were harvested by centrifugation, and the cell pellet was resuspended to 800 ml of the buffered minimal methanol medium (BMM, 0.1 M potassium phosphate (pH 6.0), 1.34% yeast nitrogen base, 4 x 10^{-5}% biotin, and 0.5% methanol) supplemented 3 x 10^{-5}% $CuCl_2$ and 0.8% DL-alanine. Four hundred milliliters of each cell suspension were transferred into a sterile 2-liter baffled flask and grown for an additional 5 days at 25°C with shaking. In order to induce rBO, methanol was added every 24 hr to a final concentration of 0.5%. The culture supernatant (3.2 liters of the main culture) was collected by centrifugation.

The culture supernatants in which $(NH_4)_2SO_4$ was added to the final concentration of 1.7 M and clarified by centrifugation (10000 Xg for 10 min),

was applied onto a TOYOPEARL-Butyl 650M column (ϕ 5 x 20 cm) equilibrated with 50 mM potassium phosphate buffer (pH 6.0) containing 1.7 M $(NH_4)_2SO_4$. The protein was eluted with a linear gradient of 1.7 to 0.0 M $(NH_4)_2SO_4$. The active fractions were concentrated by ultrafiltration and loaded onto a HiLoad Superdex 200 pg 26/60 column (Amersham Bioscience, U.S.A.) equilibrated with 100 mM Tris-H_2SO_4 buffer (pH 8.5). The active fractions were pooled and analyzed by SDS-PAGE.

Enzyme and protein assays

The bilirubin oxidase activity of rBO was determined as described previously by measuring the decrease in absorbance at 440 nm (14). One unit was defined as the amount of enzyme that oxidizes 1 μmol of bilirubin per min. To study substrate specificity, 0.2 mg of various substrates was reacted with rBO in 1.0 ml reaction mixture containing 100 nmol of sodium phosphate (pH 6.5). Protein concentrations were determined with the BCA protein assay reagent (PIERCE, U.S.A.).

Spectroscopic measurements

Absorption spectra were measured on a JASCO Ubest 50 spectrometer and a Shimadzu MultiSpec-1500 spectrometer. CD spectra were measured on a JASCO J-500C spectropolarimeter. X-band EPR spectra were recorded on a JEOL JES-RE1X spectrometer at 77 K. The total amount of Cu contained in

rBO was determined by atomic absorption spectroscopy on a Varian SpectrAA-50 spectrometer.

Deglycosylation and N-terminal sequencing

Twenty micrograms of SDS-denatured rBO was treated with 5000 U of Endo H_f in 50 mM citrate buffer (pH 5.5) at 37°C. After reacting for 2hr, Endo H_f was removed using the amylose resin mini-column, and the deglycosylated rBO was subjected to SDS-PAGE. The N-terminal sequence of the purified enzyme was analyzed by automated Edoman degradation using an Applied Biosystems 476A gas-liquid phase protein sequencer.

Results and Discussion

Expression of rBO in P. pastoris

The precursor protein of authentic BO contains the prepro-sequence consisting of 38 amino acid residues attached upstream of the N-terminus of the mature protein. When the BO gene with its original signal sequence for secretion was expressed in *S. cerevisiae* under the control of the host's promoter, the expression level of BO was very low, 20 U/l of the culture broth. It has been demonstrated that the yeast α-factor prepro-sequence is useful for the secretion of many heterogeneous proteins in *S. cerevisiae* and *P. pastoris* (15, 23). We therefore constructed the expression plasmid pPICBO, in which the gene encoding the mature BO fused in frame with the gene encoding the α-mating

factor signal peptide was cloned into *P. pastoris* expression vector pPIC9K under the control of the *Pichia* AOX1 promoter. The transformation of *P. pastoris* was carried out by integrating the transformed DNA into the AOX1 locus by homologous recombination, and the stable multi-copied recombinant *P. pastoris* strains were selected for G418 resistance. Fig. 1 shows the results of the semi-quantitative dot blot assay for BO gene content in multiple integrants. By comparing the relative intensities of the spots, the G418 hyper-resistant strain (3.0 mg/ml resistant) obtained by repeated transformation showed about a four-fold stronger signal of the BO gene than the strain transformed once (the strain resistant to 0.5 mg/ml G418). Of course, the untransformed strain control had no signal for the BO gene. The culture supernatant of the high resistant strain showed, moreover, 10-fold high activity compared to the 0.5 mg/ml G418-resistant strain. From these results, the G418 hyper-resistant strain has 4 or more copies of the BO gene cassette, and seems suitable for BO expression.

During preliminary studies on the production of rBO in BMM medium, the pH of cultures decreased from 7.0 to 3.0 concomitantly with the decrease in total BO activity (data not shown). Since it has been reported that ammonia metabolized from alanine neutralized acidic end-products of methanol in *Pichia* culture broth (18), 0.8% of DL-alanine was added to the culture medium and the decrease of medium pH was avoided. Furthermore, the BO yield increased by decreasing the cultivation temperature from 30°C to 25°C. Taking advantage of the multi-copy insertion of the expression cassette and of the optimized growth

conditions, we succeeded in the highly efficient extracellular production of rBO, approximately 5000 U/l of the culture broth, with conventional flask culture. The enzyme productivity was about 2.5- and 250-fold higher than using *A. oryzae* and *S. cerevisiae*, respectively (1, 14).

Purification and chemical properties of rBO expressed in P. pastoris

rBO was purified to homogeneity from the culture broth supernatant by simple two-step chromatography, hydrophobic chromatography with TOYOPEARL-Butyl and size exclusion chromatography with Supedex 200, as summarized in Table 1. Anion exchange chromatography with DEAE-Sephacel and size exclusion chromatography with Sephadex G-100 have been applied to purify authentic BO and rBO expressed in *A. oryzae* (14). By modifying the purification methods, ca. 25 mg (1400 U) of rBO with a specific activity of 57 U/mg was obtained from 1-liter culture broth. The specific activity of rBO was significantly improved as was that realized by the previous heterologous expression of rBO in *A. oryzae* (24 U/mg). The specific activity of commercially available BO used in the clinical investigation was < 3 U/mg, although it increased to 30 U/mg when purified by disregarding the yield.

The SDS-PAGE analyses for each purification step indicated that oligomerization of the rBO molecule takes place, especially when concentrated at the size-exclusion chromatography step (data not shown). It has been reported that laccases from *Pyroporus coccineus*, *Coriolus versicolor* and

authentic BO catalyze a similar crosslinking reaction of proteins such as casein, gelatin, and bovine serum albumin at neutral pH (24). Highly concentrated rBO thus catalyzes self-oligomerization. Therefore, in order to minimize the loss of rBO in the purification steps, pH was maintained at 8.5 and chromatography was performed as promptly as possible. rBO purified was stored in Tris-H_2SO_4 buffer solution at pH 8.5.

The molecular mass of rBO expressed in *P. pastoris*, hereafter abbreviated as rBO-*Pichia* on SDS-PAGE was 66 kDa, a 2 kDa higher value than that of rBO expressed in *A. oryzae*, hereafter abbreviated to rBO-*Aspergillus* (Fig. 2). Both authentic BO and rBO-*Aspergillus* have two *N*-linked sugar chains, although the former has additional *O*-linked sugar chains (14). To examine the carbohydrate linkage, rBO-*Pichia* and rBO-*Aspergillus* were treated with an endoglycosidase H that cleaves the *N*-linked sugar chain. As shown in Fig. 2, both deglycosylated rBOs showed bands at 60 kDa (*vide infra*) on SDS-PAGE, indicating that rBO-*Pichia* contains only *N*-linked sugar chains.

The N-terminal sequence of rBO-*Pichia* was determined as Y-V-E-F-V-A-Q-I-S-P-Q. The α-factor signal sequence fused to the N-terminal of the mature BO was cleaved at the protease Ste13 recognition site nearest to the fused position, and the 4 amino acid residues, Y-V-E-F remained at the N-terminal of the mature BO. The calculated molecular mass was 60493 Da, correlating well with the molecular mass of deglycosylated rBO-*Pichia* by

SDS-PAGE (Fig. 2).

Spectroscopic properties of rBO

The total Cu content of rBO-*Pichia* was 3.8 per protein molecule as determined by atomic absorption spectroscopy (experimental error to determine the Cu content was ca. 10%), insuring all four Cus required to exert enzyme activity were incorporated into the active site. The electronic absorption spectrum of rBO-*Pichia* is shown in Fig. 3-A together with that of rBO-*Aspergillus*. rBO-*Pichia* was intense blue due to the charge transfer, S^{-}(Cys) → Cu^{2+}, originated in type I Cu at 600 nm (ε = 6000, pH 6.0) (14). The *d-d* transitions due to type I Cu were observed as a considerably strong shoulder band at about 740 nm (ε = 2500). Another shoulder at about 330 nm due to the charge transfer, OH^{-} → Cu^{2+}(type III) is typical of multicopper oxidase. The absorption and CD (not shown) spectra of both rBOs were very similar.

In the EPR spectrum of rBO-*Pichia,* type I and type II Cu signals are observed (Fig. 3-B). Both EPR signals showed an axial character with spin Hamiltonian parameters of g_{II} = 2.22, $g_{\perp}$ = 2.03, and A_{II} = 8.3 x 10^{-3} cm^{-1} for type I Cu and g_{II} = 2.35, $g_{\perp}$ = 2.07, and A_{II} = 8.4 x 10^{-3} cm^{-1} for type II Cu. The spin Hamiltonian parameters of type I Cu are the same as for authentic BO and rBO-*Aspergillus*. On the other hand, while the spin Hamiltonian parameters of type II Cu are identical to rBO-*Aspergillus*, they are different from those of authentic BO (g_{II} = 2.24, $g_{\perp}$ = 2.07, and A_{II} = 18.6 x 10^{-3} cm^{-1}). This difference

in the parameters for type II Cu between authentic BO and rBOs originated in the difference in their resting form as evidenced by the change of type II Cu EPR of rBOs to that of authentic BO after one reaction cycle (25). Double integration of EPR signals showed that two Cu ions, type I Cu and type II Cu, were EPR-detectable but two type III Cus were EPR-undetectable because of an antiferromagnetic interaction through a bridged OH^- between two Cu^{2+} ions to give a shoulder band at ca. 330 nm.

All the spectral features above indicate that the Cu-binding sites in rBO-*Pichia* were not affected by differences in carbohydrate content and the N-terminal extension of 4 amino acid residues.

Effect of temperature and pH on rBO activity and functional stability

The optimum pH of the oxidase activity of rBO-*Pichia* for bilirubin was determined as 7.5, identical to that of authentic BO. Furthermore, the incubation of rBO-*Pichia* over a pH range of 6 to 11 at 25°C for 10 min showed only slight decreases in the enzyme activity similar to authentic BO. Both authentic BO and rBO-*Pichia* showed maximum activity at 40°C (data not shown). Fig. 4 shows the inactivation curves of rBO-*Pichia* and authentic BO for incubation at a higher temperature of 60°C. The half-inactivation time of rBO-*Pichia*, $t_{1/2} = 90$ min, was 6-fold longer than that of authentic BO, $t_{1/2} = 15$ min. The non-native-type carbohydrates attached presumably identified this profound increase in the thermostability of rBO-*Pichia*.

Catalytic properties of rBO

The substrate specificity of rBO-*Pichia* was studied for various compounds, good substrates for fungal laccase, including diamines, aminophenols, phenols, and naphtols. Table 2 summarizes the substrate specificity of rBO-*Pichia* at pH 6.5. rBO-*Pichia* catalyzes the oxidation of bilirubin (α-bilirubin), ditaurobilirubin (γ-bilirubin), ABTS, *p*-phenylenediamine, *N*,*N*-dimethyl-*p*-phenlenediamine, and 1,3-dihydroxynaphthalene. In contrast to fungal laccases, aminophenols and methoxyphenols were poor substrates for rBO-*Pichia*.

The apparent K_m and k_{cat} values for bilirubin, ABTS, and *p*-phenylenediamine of authentic BO and rBO-*Pichia* are summarized in Table 3. There was no significant change in the K_m values of rBO-*Pichia*, compared with that of the authentic enzyme, while the k_{cat} values increased dramatically. The K_m values of rBO-*Pichia* were calculated as 0.10 mM for bilirubin and 0.14 mM for ditaurobilirubin from the double reciprocal plots of the initial reaction rate against substrate concentration. ABTS, a highly hydrophobic substrate as bilirubin, gave a small K_m value, whereas it was ca. 100-fold larger for *p*-pehnylendiamine, a relatively small-sized substrate. These results indicate that rBO-*Pichia* is suitable for hydrophobic substrates of considerable size.

Conclusion

In this study, we constructed a novel effective heterologous expression system of *M. verrucaria* BO with *P. pastoris* as the host. The methylotrophic yeast produced rBO-*Pichia* in the culture medium at a high level, 5000 U/l. rBO-*Pichia* was highly purified with simple two-step chromatography to exhibit specific activity two-fold higher than authentic BO and rBOs expressed in other hosts. Furthermore, rBO-*Pichia* showed significantly increased thermostability. The increased thermostability of rBO-*Pichia* is especially favorable for clinical investigation of the liver and its application to biofuel cells (26, 27).

Acknowledgments

This work was supported in part by Alfresa Pharma Corporation (AZWELL Inc.) for which we express our thanks. We also thank Dr. Shotoro Yamaguchi and Dr. Noriaki Tanaka, Amano Enzyme Inc. for their valuable discussions.

Footnotes

[1]*Abbreviations:* ABTS, 2,2'-azino-bis-(3-ethylbenziazoline-6-sulfonic acid); AOX1, alcohol oxidase 1; BO, bilirubin oxidase; CD, circular dichroism; EPR, electron paramagnetic resonance; rBO, recombinant bilirubin oxidase

References

1. S. Koikeda, K. Ando, H. Kaji, T. Inoue, S. Murao, K. Takeuchi, T. Samejima, Molecular cloning of the gene for bilirubin oxidase from *Myrothecium verrucaria* and its expression in yeast, *J. Biol. Chem.* **268** (1993) 18801-18809.

2. B.T. Doumas, F. Yein, B. Perry, B. Jendrzejczak, A. Kessner, Determination of the Sum of Bilirubin Sugar Conjugates in Plasma by Bilirubin Oxidase, *Clin. Chem.* **45** (1999) 1255-1260.

3. A. Kosaka, C. Yamamoto, M. Yoshitaka, K. Nakane, Enzymatic determination of bilirubin fractions in serum, *Clin. Biochem.* **20** (1987) 451-458.

4. I. Masuda-Nishimura, K. Ichikawa, O. Hatamoto, K. Abe, Y. Koyama, cDNA cloning of bilirubin oxidase from *Pleurotus ostreatus* strain Shinshu and its expression in *Aspergillus sojae*: an efficient screening of transformants, using the laccase activity of bilirubin oxidase, *J. Gen. Appl. Microbiol.* **45** (1999) 93-97.

5. K. Hiromi, Y. Yamaguchi, Y. Sugiura, H. Iwamoto, J. Hirose, Bilirubin oxidase from *Trachderma tsunodae* K-2593, a multi-copper enzyme, *Biosci. Biotech. Biochem.* **56** (1992) 1349-1350

6. Y. Seki, M. Takegichi, I. Okura, Purification and properties of bilirubin oxidase from *Penicillium janthinellum, J. Biotechnol.* **46** (1996) 145-151.

7. W.T. Sulistyaningdyah, J. Ogawa, H. Tanaka, C. Maeda, S. Shimizu, Characterization of alkaliphilic laccase activity in the culture supernatant of *Myrothecium verrucaria* 24G-4 in comparison with bilirubin oxidase, *FEMS Microbiol. Lett.* **230** (2004) 209-214.

8. A. Messerschmidt, Ascorbate oxidase, in: A. Messerschmidt, R. Huber, T. Poulos, K. Wieghardt (Eds.) Handbook of Metalloproteins, Vol. 2, John Wiley & Sons, Ltd., Chichester, 2001, pp. 1345-1358.

9. G.J. Davies, V. Ducros, Laccase, in: A. Messerschmidt, R. Huber, T. Poulos, K. Wieghardt (Eds.) Handbook of Metalloproteins, Vol. 2, John Wiley & Sons, Ltd., Chichester, 2001, pp. 1359-1368.

10. P.F. Lindley, Ceruloplasmin, in: A. Messerschmidt, R. Huber, T. Poulos, K. Wieghardt (Eds.) Handbook of Metalloproteins, Vol. 2, John Wiley & Sons, Ltd., Chichester, 2001, pp. 1369-1380.

11. H. Claus, Laccases and their occurrence in prokaryotes, *Arch. Microbiol.* **179** (2003) 145-150.

12. S.A. Roberts, A. Weichsel, G. Grass, K. Thakali, J.T. Hazzard, G. Tollin, C. Rensing,

W.R. Montfort, Crystal structure and electron transfer kinetics of CueO, a multicopper oxidase required for copper homeostasis in *Escherichia coli, Proc. Natl. Acad. Sci. U.S.A.* **99** (2002) 2766-2771.

13. F.J. Enguita, L.O. Martins, A.O. Henriques, M.A. Carrondo, Crystal structure of a bacterial endospore coat component. A laccase with enhanced thermostability properties, *J. Biol. Chem.* **278** (2003) 19416-19425.

14. A. Shimizu, J.H. Kwon, T. Sasaki, T. Satoh, N. Sakurai, T. Sakurai, S. Yamaguchi, T. Samejima, *Myrothecium verrucaria* bilirubin oxidase and its mutants for potential copper ligands, *Biochemistry* **38** (1999) 3034-3042.

15. J.M. Cregg, T.S. Vedvick, W.C. Raschke, Recent advances in the expression of foreign genes in *Pichia pastoris*, *Biotechnology (NY)* **11** (1993) 905–910.

16. M. Romanos, C. Scorer, K. Sreekrishna, J. Clare, The generation of multicopy recombinant strains, *Methods Mol. Biol.* **103** (1998) 55-72.

17. L.J. Jönsson, M. Saloheimo, M. Penttilä, Laccase from the white-rot fungus *Trametes versicolor*: cDNA cloning of *lcc1* and expression in *Pichia pastoris, Curr. Genet.* **32** (1997) 425-430.

18. J. O'Callaghan, M.M. O'Brien, K. McClean, A.D.W. Dobson, Optimisation of the expression of a *Trametes versicolor* laccase gene in *Pichia pastoris*, *J. Ind. Microbiol. Biotechnol.* **29** (2002) 55-59.

19 D.M. Soden, J. O'Callaghan, A.D.W. Dobson, Molecular cloning of a laccase isozyme gene from *Pleurotus sajor-caju* and expression in the heterologous *Pichia pastoris* host, *Microbiology* **148** (2002) 4003-4014.

20. W. Liu, Y. Chao, S. Liu, H. Bao, S. Qian, Molecular cloning and characterization of a laccase gene from the basidiomycete *Fome lignosus* and expression in *Pichia pastoris*, *Appl. Microbiol. Biotechnol.* **63** (2003) 174-181.

21. P. Bielli, G.C. Bellenchi, L. Calabrese, Site-directed mutagenesis of human ceruloplasmin: production of a proteolytically stable protein and structure-activity relationships of type 1 sites, *J. Biol. Chem.* **276** (2001) 2678-2685.

22. A. Shimizu, T. Sasaki, J.H. Kwon, A. Odaka, T. Satoh, N. Sakurai, T. Sakurai, S. Yamaguchi, T. Samejima, Site-directed mutagenesis of a possible type 1 copper ligand of bilirubin oxidase; a Met467Gln mutant shows stellacyanin-like properties, *J. Biochem. (Tokyo)* **125** (1999) 662-668.

23. L.L. Kiiskinen, M. Saloheimo, Molecular cloning and expression in *Saccharomyces cerevisiae* of a laccase gene from the ascomycete *Melanocarpus albomyces*, *Appl. Environ. Microbiol*. **70** (2004) 137-144.

24. S. Yamaguchi, Crosslinking of protein with enzyme, Japan Patent 11-276162 (Oct. 12. 1999)

25. T. Sakurai, L. Zhan, T. Fujita, K. Kataoka, A. Shimizu, T. Samejima, S. Yamaguchi, Authentic and recombinant bilirubin oxidases are in different resting forms, *Biosci. Biotechnol. Biochem*. **67** (2003) 1157-1159.

26. A.F. Mayer, R.C. Staples, Laccase: new functions for an old enzyme, *Phytochemistry* **60** (2002) 551-565.

27. S. Tsujimura, M. Fujita, H. Tatsumi, K. Kano, T. Ikeda, Bioelectrocatalysis-based dihydrogen/dioxygen fuel cell operating at physiological pH, *Phys. Chem. Chem. Phys*. 3 (2001) 1331-1335.

Figure legends

Fig. 1. Semi-quantitative dot blot analysis of the BO gene.

Lane 1, untransformed control (*P. pastoris* GS115); Lane 2, the strain resistant to 0.5 mg/ml G418; Lane 3, the strain resistant to 1.5 mg/ml G418; Lane 4, the strain resistant to 3.0 mg/ml G418.

Fig. 2. SDS-PAGE analysis of purified and deglycosylated BOs.

Lane 1, rBO-*Pichia*; Lane 2, rBO-*Aspergillus*; Lane 3, authentic BO; Lane 4, deglycosylated rBO-*Pichia*; Lane 5, deglycosylated rBO-*Aspergillus*; Lane M, standard proteins, 97.4 kDa (phosphorylase B), 66.2 kDa (bovine serum albumin), 45.0 kDa (ovalbumin), 31.0 kDa (carbonic anhydrase), 21.5 kDa (trypsin inhibitor).

Fig. 3. Electronic absorption (A) and X-band EPR spectra (B) of rBO-*Pichia* (solid line) and rBO-*Aspergillus* (dotted line).

Conditions: (A) 0.1 M potassium phosphate (pH 6.0) at room temperature; (B) 0.1 M potassium phosphate (pH 6.0) at 77K, power 2.0 mW; frequency, 9.1875 GHz; modulation, 100 kHz, 1.0 mT; amplitude, 320; filter 0.03 s; sweep time 4 min.

Fig. 4. Inactivation curves of rBO-*Pichia* (open circle) and authentic BO (filled circle) on incubation with 0.1 M potassium phosphate (pH 6.0) at 60°C.

Table 1 Purification of rBO

Purification step	Total Protein (mg)	Total activity (U)	Sp. activity (U/mg)	Yield (%)	Fold
Culture broth*	1680	15800	9.40	100	1.0
TOYOPEARL-Butyl	421	9650	22.9	61.1	2.4
Superdex 200	71.2	4070	57.2	25.8	6.1

*Three liters of culture broth were used for the purification of rBO-*Pichia*.

Table 2 Substrate specificity of rBO-*Pichia*

Substrate	Detection (nm)	Activity (U/mg)*
o-phenylenediamine	430	0.6
p-phenylenediamine	470	3.4
N,*N*-dimethyl-*p*-phenlenediamine	470	42.4
p-aminodiphenylamine	470	15.4
o-aminophenol	450	1.8
p-aminophenol	405	0.5
2-methoxyphenol	450	0.0
2,6-dimethoxyphenol	470	0.4
1,3-dihydroxynaphthalene	450	12.6
1,5-dihydroxynaphthalene	470	3.3

* One unit is the amount of enzyme that oxidizes the substrate to 1 absorbance unit per min at each wavelength.

Table 3 Kinetic constants

Substrate	pH	authentic BO [a]			rBO-*Pichia* [b]		
		K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}•s^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}•s^{-1}$)
bilirubin	8.0	0.12	117	980	0.10	200	2000
ditaurobilirubin [c]	5.5	0.37	94.3	250	0.14	233	1700
ABTS [c]	6.5	0.25	115	460	0.34	164	480
p-phenylenediamine [c]	6.5	9.8	60	6.1	9.6	90	9.4

[a] The specific activity of authentic BO used was 30 U/mg

[b] The specific activity of rBO-*Pichia* used was 57 U/mg

[c] Molecular extinction coefficients used to quantifying ditaurobilirubin (at 500 nm), ABTS (436 nm), and *p*-phenylenediamine (487 nm) were 11.1, 29.3, and 14.3 $mM^{-1}•cm^{-1}$, respectively.

Figure 1

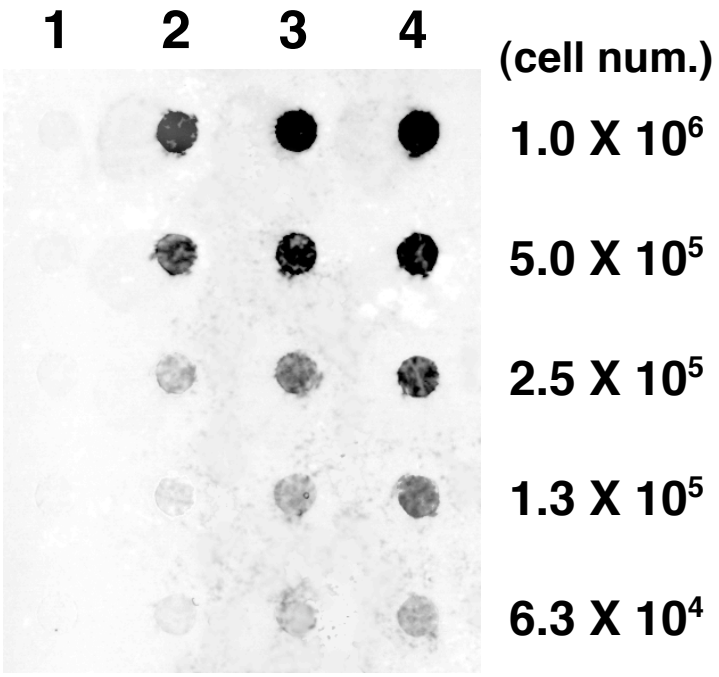


Figure 2

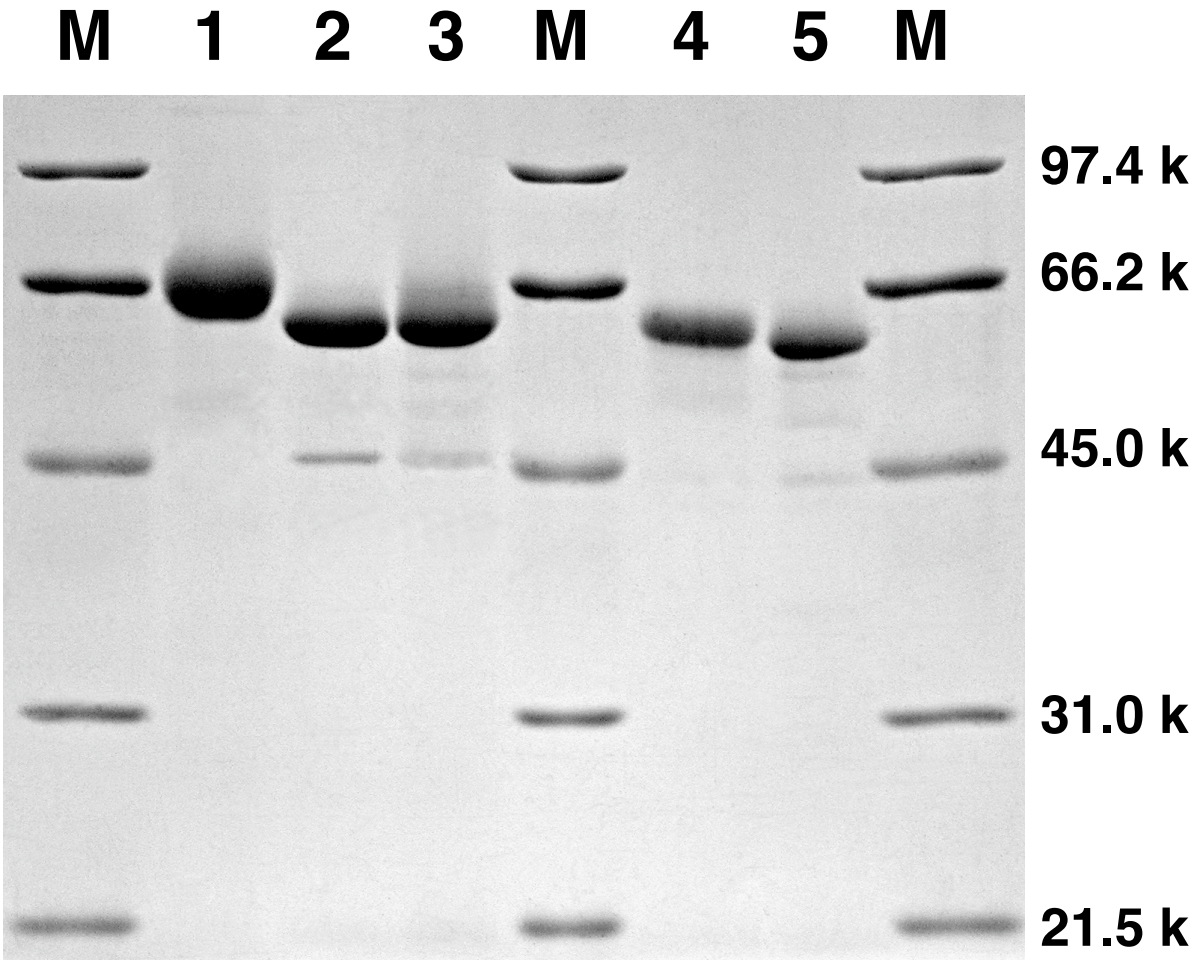


Figure 3

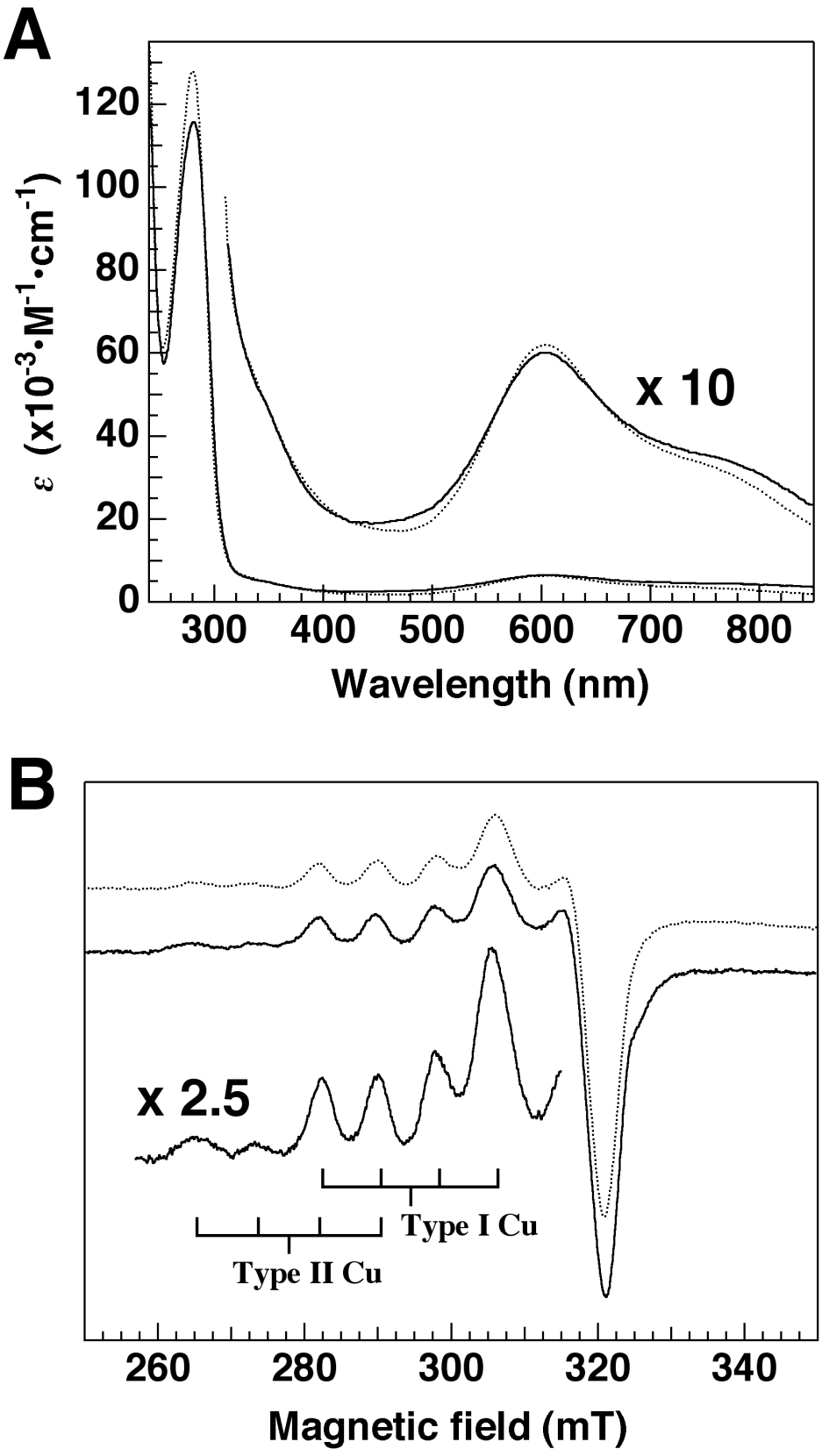
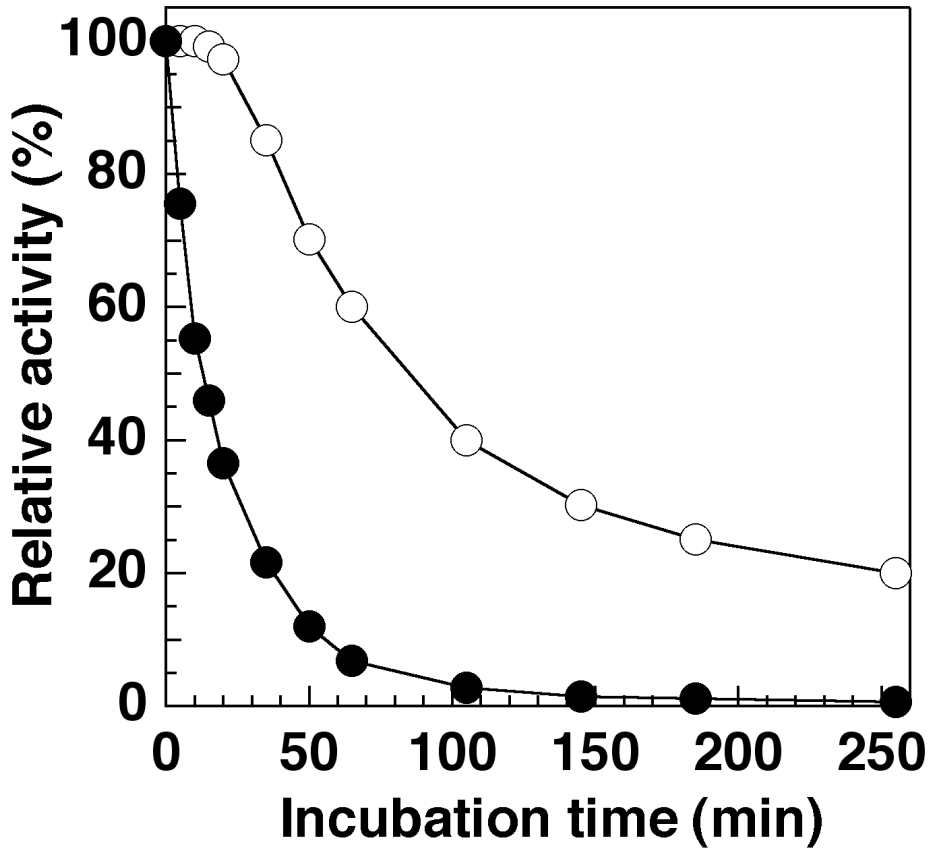

A
120
100
80
60
40
20
0
ε (x10-3•M-1•cm-1)
x 10
300
400
500
600
700
800
Wavelength (nm)
B
x 2.5
Type I Cu
Type II Cu
260
280
300
320
340
Magnetic field (mT)

Figure 4


Relative activity (%)
100
80
60
40
20
0
0
50
100
150
200
250
Incubation time (min)