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Enzymological Characterization of EpoA, a Laccase-Like Phenol Oxidase Produced by *Streptomyces griseus*

Kohki Endo, Yutaka Hayashi, Takahiro Hibi, Kuniaki Hosono, Teruhiko Beppu and Kenji Ueda*

Life Science Research Center, College of Bioresource Sciences, Nihon University, 1866 Kameino, Fujisawa 252-8510

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Laccase is an enzyme that catalyzes the oxidation of phenolic compounds by coupling the reduction of oxygen to water. While many laccases have been identified in plant and fungal species, enzymes of prokaryotic origin are poorly known. Here we report the enzymological characterization of EpoA, a laccase-like extracytoplasmic phenol oxidase produced by *Streptomyces griseus*. EpoA was expressed and purified with an *Escherichia coli* host-vector system as a recombinant protein fused with a C-terminal histidine-tag (rEpoA). Physicochemical analyses showed that rEpoA comprises a stable homotrimer containing all three types of copper (types 1–3). Various known laccase substrates were oxidized by rEpoA, while neither syringaldazine nor guaiacol served as substrates. Among the substrates examined, rEpoA most effectively oxidized *N*,*N*-dimethyl-*p*-phenylenediamine sulphate with a Km value of 0.42 mM. Several metal chelators caused marked inhibition of rEpoA activity, implying the presence of a metal center essential for the oxidase activity. The pH and temperature optima of rEpoA were 6.5 and 40°C, respectively. The enzyme retained 40% activity after preincubation at 70°C for 60 min. EpoA-like activities were detected in cell extracts of 8/40 environmental actinomycetes strains, which suggests that similar oxidases are widely distributed among this group of bacteria.

Key words: EpoA, laccase, phenol oxidase, *Streptomyces griseus*.

Streptomycetes are Gram-positive soil-habitating bacteria known for their capability of producing large numbers of useful substances. The organism performs secondary metabolism to synthesize many kinds of antibiotics and biologically active substances, which have important applications in medicinal, agricultural and chemical industries (*1*). The organism also produces a wide variety of useful enzymes such as proteases, nucleases and enzymes that hydrolyze polysaccharides, which benefit the saprophytic life cycle of the organism by catalyzing the degradation of many kinds of organic molecules (*2*). Another characteristic feature of this group of bacteria is the ability to perform complex morphological development resembling that of filamentous fungi (*3–5*). Early in the life cycle on solid medium, the organism undergoes vegetative growth as a branching multinucleoide substrate mycelium. In response to environmental and physiological signals, the substrate mycelium then produces aerial hyphae, which finally differentiate into spore chains by septum formation at regular intervals. The complex process of cellular development involves a coordinated gene expression programme that promotes a variety of physiological and structural changes.

We previously reported that an exogenous supply of copper causes a marked stimulation of both morphological and physiological differentiation in *Streptomyces* spp. (*6*, *7*). Our recent study on the effect of copper in *Streptomyces griseus* revealed the presence of a novel extracytoplasmic phenol oxidase, EpoA, and its involvement in the stimulation of morphogenesis (*8*). The marked EpoA activity exhibited by the wild-type strain was not detected or extremely reduced in several mutants defective in regulatory genes for morphogenesis, indicating that the expression of the enzyme is under the genetic control linking it to cellular development. The results strongly suggested that a byproduct generated during the melanin biosynthesis by EpoA upon oxidizing dihydroxyphenylalanine (DOPA) has an activity to stimulate morphogenesis in *Streptomyces* spp. (*8*).

Our preliminary observations of both sequence similarity and substrate selectivity, oxidizing DOPA and not tyrosine, imply that EpoA is a laccase (*8*). Laccase (benzenediol:oxygen oxidoreductases; EC 1.10.3.2) is a multicopper enzyme that catalyzes the oxidation of a number of aromatic substances by coupling the oxidation to the reduction of oxygen to water. The enzyme is widespread among fungal and plant species, and its characteristics have been studied extensively (*9–11*). Laccase is believed to be involved in various cellular and microbial activities such as conidial pigmentation and development, lignin formation and degradation, and fungal and plant pathogenicity (*12*, *13*). Furthermore, the broad substrate specificity of the enzyme lends high potential for wide industrial applications, such as in pulp delignification, dye bleaching, chemical synthesis and effluent detoxification (*14–18*).

While many eucaryotic laccases have been identified and studied, we know only a few examples of laccases of procaryotic origin. The two bacterial laccases identified and characterized up to now are PpoA from a marine bac-

*To whom correspondence should be addressed. Tel: +81-466-84-3936, Fax: +81-466-84-3935, E-mail: ueda@brs.nihon-u.ac.jp

terium *Marinomonas medeterranea* (*19*, *20*), and CotA from *Bacillus subtilis* (*21*, *22*). These enzymes show marked sequence similarity to eucaryotic laccase, and their biochemical properties as laccases have been precisely studied. Here we report the enzymological characteristics of EpoA. The enzyme was produced and purified as a recombinant protein with an *E. coli* host-vector system, and its physicochemical and biochemical properties were examined. The results show that EpoA has several unique features in both its structure and substrate specificity.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions—*Streptomyces griseus* IFO 13350, the source of *epoA*, was obtained from the Institute of Fermentation, Osaka. *Escherichia coli* BL21(DE3) pLysS (Novagen, Madison, WI) was used as a host for the expression of the recombinant protein of EpoA (rEpoA). pET-26b(+) (carrying kanamycin resistance; Novagen) was used for the efficient expression of *epoA*. pET-EpoA carries an *epoA* cassette on pET-26b(+) (see below). pUC-A, which carries *epoA* on pUC19 (*8*), was used as a template for the amplification of the *epoA* cassette. The standard DNA manipulation techniques were described by Maniatis *et al*. (*23*). *E. coli* BL21(DE3) harboring pET-EpoA was grown in Luria-Bertani (LB) medium [containing (grams per liter): Tryptonepeptone (DIFCO, Detroit, Mich.), 10; Yeast extract (DIFCO) 5; NaCl (Kokusan, Tokyo) 5] supplemented with 50 μg/ml kanamycin (Wako Pure Chemicals, Tokyo). For the efficient production of rEpoA, LB medium supplemented with 10 μM copper sulfate (Kokusan) was used. The environmental actinomycetes strains were isolated from soil collected at Fujisawa, and cultured on Bennett's/maltose agar medium [containing (grams per liter): Yeast extract (DIFCO), 1; Meat extract (Kyokuto, Tokyo), 1; NZ amine (Wako), 2; maltose, 10 (Kokusan); agar, 15 (Kokusan) (pH7.2)].

Construction of Expression Plasmid—The DNA fragment encoding EpoA including the region for the signal sequence was amplified by the standard PCR technique with primers [5′-CC*CATATG*GACCGAAGGACC (corresponding to +1–+15 bp when the A residue of the translational initiation codon of EpoA (ATG) was numbered as +1; italic letters indicate the restriction site for *Nde*I)] and [CC*CTCGAG*GTGCTGGTGCTCCGCGGC (corresponding to +1027–+1044 bp when the A residue of the translational initiation codon of EpoA (ATG) was numbered as +1; italic letters indicate a restriction site for *Xho*I)]. The amplicon was recovered as an *Nde*I/*Xho*I-digested fragment and inserted between the *Nde*I–*Xho*I sites of pET-26b(+). The oligonucleotide design at the *Xho*I restriction site allowed the translational fusion of EpoA to a C-terminal histidine-tag. The plasmid thus formed, pET-EpoA, carries *epoA* in downstream from the T7 promoter, which directs the expression of rEpoA. rEpoA consisted of an N-terminal signal sequence, a mature part of EpoA and an C-terminal histidine-tag. Enzymes used for DNA manipulation were purchased from Takara Shuzo (Kyoto).

In-Gel Activity Stain—The chromogenic substrate *N,N*-dimethyl-*p*-phenylenediamine sulphate (DMP) was used for the detection and quantification of EpoA activity. Since rEpoA showed the same activity in an SDS–polyacrylamide gel as did the native enzyme of *S. griseus* (*8*), the enzyme fractions were electrophoresed in SDS–polyacrylamide gels (containing 10–12.5% polyacrylamide), and subjected to the activity stain. Gels after electrophoresis were washed with deionized water for 30 min and then immersed in buffer C [prepared by mixing 370 mM citric acid and 126 mM Na_2HPO_4 to adjust the pH to 6.5] dissolved with 0.125 mg/ml DMP and 0.125mg/ml 1-naphthol for approximately 30 min at 25°C. EpoA activity was detected by the formation of an indigo precipitate.

Production of rEpoA—*E. coli* BL21(DE3) cells harboring pET-EpoA were used as the source of rEpoA. SDS–polyacrylamide gel electrophoresis was used to detect enzyme activities, monitor protein purification and estimate molecular sizes. Both the high and low molecular weight markers were purchased from Pharmacia Biotech (Uppsala, Sweden). For estimation of protein purification, gels were stained with Coomassie Brilliant Blue (CBB) R-250. Protein concentrations were measured with a Bio-Rad (Hercules, CA) protein assay kit with bovine serum albumin as the standard. *E. coli* BL21(DE3) harboring pET-EpoA was cultured in 3 liter LB liquid medium added with 10 μM CuSO4 at 30°C on a rotary shaker (100 rpm). 1 mM IPTG was added to the culture when the optical density at 600 nm reached 0.3, and the cells were harvested by centrifugation when the optical density reached 1.3.

Purification of rEpoA—(i) Preparation of cell extract: The *E. coli* cells were washed with buffer A [containing; 10 mM Tris, 1 mM EDTA, and 10% glycerol (pH 7.0)] and disrupted by sonication. The disrupted cells were then centrifuged at 10,000 ×*g* for 30 min at 4°C, and the supernatant containing 170 mg protein was used as the cell extract.

(ii) DEAE-Toyopearl column chromatography: The cell extract was applied to a DEAE-Toyopearl column (50 mm × 160 mm; Tosoh, Tokyo) previously equilibrated with buffer A. After the column was washed with 0.25 liters of buffer A, proteins were eluted with a linear gradient of KCl (0–0.25 M) in a total volume of 0.25 liters of buffer A at a flow rate of 2.4 ml/min. Fractions containing 6.3 mg proteins were recovered through this step.

(iii) His-Tag column chromatography: The fractions after DEAE column chromatography were then applied to a HiTrap Chelating column (Pharmacia) equilibrated with buffer B [containing; 0.5 M NaCl and 0.1 mM imidazol in 200 mM sodium phosphate buffer (pH 7.4)] according to manufacturer's recommendations. Prior to application to the column, the samples were dialyzed against buffer B to prevent an ion exchange effect. Fractions containing 1.1 mg proteins were recovered through this step. The N-terminal amino acid sequence of the purified rEpoA was determined following the standard protocol with a pulsed liquid sequencer (model 494HT; Applied Biosystems, Foster City, CA)

Copper Characterization and Isoelectric Electrophoresis—rEpoA (1.0 mg/ml) dissolved in buffer A was used for the spectroscopic characterization of the Cu(II) centers. Spectrophotometric measurements were carried out on a Hitachi UV-3210 spectrophotometer (Hitachi, Tokyo). For the copper content of the rEpoA active complex, 60 μg of

Table 1. **Activities of EpoA against various substrates.**

Substrate	EpoA[a]
N,*N*-Dimethyl-*p*-phenylenediamine sulfate (DMP)	58.3
p-phenylenediamine	19.1
chlorogenic acid	15.5
dihydroxyphenylalanine (DOPA)	13.7
pyrogallol	12.7
4-aminoantipyrine	10.9
m-methoxyphenol	9.0

[a]All values are the averages of duplicate measurements that vary by less than 5%; activities are shown as μM O_2 consumed/min/mg protein.

Table 2. **Effect of laccase inhibitors[a].**

Inhibitor	Concentration (mM)	EpoA activity remaining (%)
ethylenediaminetetraacetic acid	1.0	67
bathocuproinedisulfonic acid	1.0	39
dimethyldithiocarbamate trihydrate	1.0	13
	0.1	37
2,2-bipyridyl	1.0	61
hydroxylamine	1.0	62
2,4-dithio-6-pyrimidine	1.0	31
sodium azide	1.0	68
	0.1	73
sodium cyanide	1.0	0
	0.1	38

[a]All values are the averages of duplicate measurements that vary by less than 5%.

the purified rEpoA was analyzed with a Polarized Zeeman Atomic Absorption Spectrophotometer (model Z-5010, Hitachi) following the protocol recommended by the manufacturer. The electron spin resonance (ESR) spectrum of rEpoA (0.1 mg/ml in buffer A) was recorded by an ESR spectrophotometer (model JES-FA100, JEOL, Tokyo) under the following condition: microwave frequency, 9.13 GHz; microwave power, 10 mM; modulation frequency, 100 kHz, sweep time, 2 min. Isoelectric point electrophoresis was performed on a Multiphor II Electrophoresis Unit (Pharmacia Biotech) with an Immobiline Dry Strip (pH 3–11) following the standard protocol recommended by the manufacturer.

Quantification of EpoA Activity and Effects of pH, Temperature and Inhibitors—The EpoA activity was quantified following the method previously described by Clutterback (*24*). To 0.1 ml of enzyme solution (1.0 mg/ml) was added 0.1 ml of filtrated aqueous DMP (20 mg/ml) and 2.5 ml of buffer C (for contents, see above). The initial increasing rate of absorbance at 550 nm was scanned during 100 s of incubation at 45°C to determine the K_m value and to examine the effect of inhibitors and temperatures. In order to determine the pH optimum, buffer C was prepared in the range of pH 3–9 with various mixing ratios of 370 mM citric acid and 126 mM Na_2HPO_4. The activities expressed as the increase in absorbance per min were multiplied by a factor of 0.2 to convert to standard units (μmol substrate oxidized per min) as described by Clutterbuck (*24*). All compounds used in the inhibition study were purchased from Wako Pure Chemicals except for bathocuproine disulfonic acid (Sigma-Aldrich Fine Chemicals, St. Louis, Missouri). Inhibitor concentrations were 1.0 or 0.1 mM (see Table 2) in the reaction mixture with DMP as a substrate. Leucoberbelin blue and catalase used to detect peroxidase activity were purchased from Sigma-Aldrich.

Oxidation of Various Substrates—Oxidizing activities of rEpoA against various substrates were examined by measuring the decrease in dissolved oxygen concentration with an oxygen electrode (Biott, Tokyo). The reaction mixture contained 3 mM of each substrate in 5.4 ml of buffer C and 0.1 ml of purified rEpoA (0.1 mg/ml). The initial decrease in dissolved oxygen was monitored during 100 s of incubation at 25°C. The activities were expressed as the concentration of oxygen (μM) consumed by 1 mg rEpoA per min. Catechol, 4-aminoantipyrine, hydroquinone and 2,3-dimethoxyphenol were purchased from Sigma-Aldrich. Chlorogenic acid was purchased from ICN Biomedicals, Inc (Irvine, CA). All other substrates examined (Table 1) were purchased from Wako Pure Chemicals.

Isolation of Environmental Actinomycetes Strains and Detection of Enzyme Activities—Soil collected in a vegetative field (Fujisawa, Kanagawa) was suspended in adequate volumes of sterile distilled water, and the suspensions were plated onto Bennett's/maltose solid medium after appropriate dilution. Plates were incubated at 28°C for 3–7 days and pure colonies showing the typical appearances of actinomycetes were isolated. Among the isolates thus obtained, 40 strains with different macroscopic appearances were randomly selected and examined for the presence of phenol oxidase activities. Each strain was inoculated into a confluent lawn on a plate of Bennett's/maltose solid medium whose surface was covered with a sterile cellophane sheet. Mycelia were collected after incubation at 28°C for 3–7 days, washed with 10 ml of buffer A, and re-suspended in 3 ml of buffer A. The suspension was then sonicated until the cells were fully disrupted and centrifuged at 10,000 ×*g* for 10 min. 30 μl of each supernatant was subsequently applied to an SDS–polyacrylamide gel and stained with DMP by the in-gel activity stain method.

RESULTS

Sequence Similarity of EpoA—Previously, we reported that EpoA shows significant similarity to laccase, and functional domain search in the database revealed the presence of a multicopper oxidase signature in its C-terminal portion (*8*). Subsequently, we found that EpoA carries all three domains involved in copper binding that are conserved among laccases by manually aligning related sequences. As shown in Fig. 1, all the predicted residues for copper binding are entirely conserved in EpoA. We assume that the standard homology search in the protein database failed to show the presence of the conserved domains, because of the slightly weak similarity and the small molecular size.

Expression and Purification of EpoA in E. coli—To characterize precisely the enzymological properties, we expressed and purified EpoA as a recombinant protein (rEpoA). *epoA* of *S. griseus*, including the region for the signal sequence, was cloned into pET-26b(+), an *E. coli* expression plasmid. *E. coli* BL21(DE3) cells harboring

Fig. 1. **The copper-binding domains of laccase.** The conserved amino acid residues are shaded in black. Arrows and accompanying numbers indicate the putative amino acid residues for copper-binding and the corresponding types of copper (type 1–3), respectively. NCR, *Neurospora crassa* laccase (accession no. A28523); PAN, *Podospora anserina* laccase (2224342A); GGR, *Gaeumannomyces graminis* laccase-3 (AJ417687); CVE, *Coriolus versicolor* laccase (D13372); Ppo, *M. medeterranea* PpoA (2616352A); Cot, *B. subtilis* CotA (2408231AG); Epo, *S. griseus* EpoA (AB056583). The total numbers of amino acids in each enzyme is shown in the parentheses.

the expression plasmid produced rEpoA as a soluble protein in the cytoplasmic fraction (Fig. 2). As previously observed for native EpoA from *S. griseus* (*8*), rEpoA migrated to a position above the upper size limit of the low molecular weight marker (94 kDa) in the SDS–polyacrylamide gel and showed DMP oxidizing activity. A notable feature was that active rEpoA was efficiently produced when 10 μM copper sulfate was added to the *E. coli* culture medium (Fig. 2, lane 2). In contrast, an inactive 38 kDa protein corresponding to the monomeric rEpoA (see below) accumulated in cells when they were cultured in medium without the addition of copper (lane 1). The active rEpoA was purified to homogeneity by DEAE and Ni-affinity column chromatography (Fig. 2, lanes 3 and 4). Both column chromatographies efficiently eliminated contaminating proteins.

Physicochemical Properties of EpoA—(i) Molecular weight: The stable migration of active rEpoA in SDS–polyacrylamide gels made it possible to estimate its molecular size (Fig. 3). The apparent molecular weight of the active rEpoA was approximately 114 kDa (lane 1). After boiling for 20 min, the protein migrated as a single band at approximately 38 kDa, which corresponds to the molecular weight of monomeric rEpoA deduced from the amino acid sequence. The N-terminal amino acid sequences of both the 114 and 38 kDa proteins were identical to that deduced for the nucleotide sequence of *epoA*. The result indicates that rEpoA comprises a homotrimer. The boiled fraction showed no oxidase activity.

(ii) Isoelectric point and copper content: Isoelectric focusing electrophoresis of the rEpoA active complex showed the presence of three isomers with p*I*s of 5.3, 5.4, and 5.6. Atomic absorption analysis of the purified rEpoA showed the presence of 0.3 μg of copper per 45 μg of protein, which corresponds to a content of 4 copper ions per subunit.

(iii) Spectral characteristics: The UV-visible spectrum of rEpoA shows peaks of absorption at around 600 nm, which suggests the presence of a type 1 'blue' Cu(II) (Fig. 4A). ESR spectroscopy of rEpoA exhibited a typical laccase profile, with $A_{//} = 6.12 \times 10^{-3}$ /cm, $g_{//} = 2.23$, $g_{\perp} = 2.07$ for type 1 Cu(II) and an $A_{//} = 9.62 \times 10^{-3}$/cm, $g_{//} = 2.35$, $g_{\perp} = 2.07$ for type 2 Cu(II) (Fig. 4B).

Biochemical Properties of rEpoA—(i) Substrate specificity: Table 1 shows the laccase substrates oxidized by the purified rEpoA. Among the substrates examined, DMP was most effectively oxidized. As previously observed for native EpoA (*8*), rEpoA generated a melanin pigment upon oxidizing DOPA, while it did not oxidize tyrosine. EpoA did not oxidize syringic acid, 2,6-dimethoxyphenol, 2,3-dimethoxyphenol, guaiacol, *p*-hydroquinone, catechol, *o*-phenylenediamine, cresol, gallic acid, or syringaldazine.

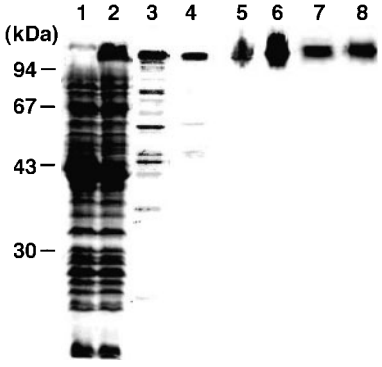

Fig. 2. **Monitoring the purification of rEpoA.** SDS–polyacrylamide gels with CBB staining (lanes 1–4) and in-gel activity stain (lanes 5–8) are shown. Lanes: 1 and 5, crude extract of *E. coli* BL21 (DE3) harboring pET-EpoA cultured in LB medium without additional copper; lanes 2 and 6, crude extract of *E. coli* BL21 (DE3) harboring pET-EpoA cultured in LB medium with 10 μM copper sulfate; lanes 3 and 7, active fraction after DEAE-column chromatography; lanes 4 and 8, active fraction after Ni-affinity column chromatography.

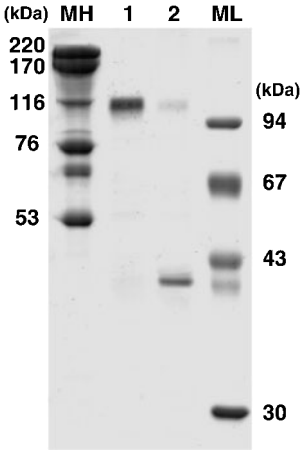

Fig. 3. **Determination of the molecular mass of rEpoA.** The purified rEpoA with (lane 2) or without (lane 1) boiling treatment was electrophoresed in an SDS–polyacrylamide gel followed by CBB staining. MH, high molecular weight markers; ML, low molecular weight marker.

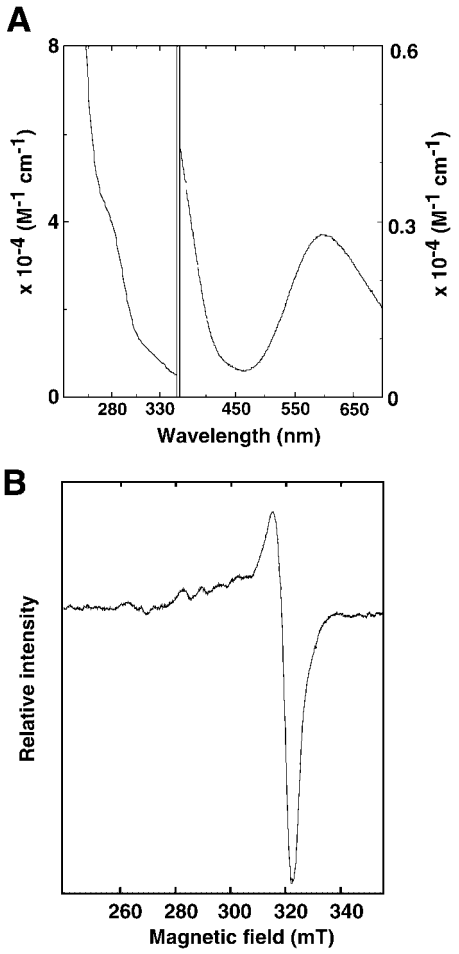

Fig. 4. **Optical absorption spectrum (A) and ESR spectrum (B) of purified rEpoA.**

(ii) Michaelis constant and inhibition studies: The kinetic data for rEpoA with DMP as a substrate (Fig. 5) are K_m = 0.42 mM and V_{max} = 0.85 nmol/min. The reaction was inhibited by several laccase inhibitors including copper chelating agents (Table 2). Marked inhibition was observed with dimethyldithiolcarbamate and sodium cyanide, suggesting the presence of a metal center essential for the oxidase activity. Dimethyldithiolcarbamate inhibited rEpoA activity in a concentration-dependent manner with IC_{50} = 42 μM, and showed a mixed-type of inhibition with K_i = 90 μM (Fig. 5). The oxidase activity of rEpoA was not affected by the presence of 1 μg/ml catalase or 0.1–10 mM hydrogen peroxide, which excludes the possibility that the enzyme is a peroxidase. Further, to assess whether EpoA is a manganese oxidase, 1 mM leucoberbelin blue, a specific indicator of Mn(III)-Mn(VII) (*25*), was added to the rEpoA/DMP reaction mixture in the presence of 1 mM $MnCl_2$. The assay did not detect manganese oxide in the reaction, which rules out the possibility that EpoA is a manganese oxidase.

(iii) Effects of pH and temperature on rEpoA activity and stability: The reactivity profile of EpoA against DMP was determined over the pH range 4.0–9.0 and temperature range 10–45°C (Fig. 6). The results showed that the enzyme has pH optimum of 6.5 and a temperature optimum of 40°C. The stability of rEpoA against pre-incubation at various temperatures was also assessed (Fig. 6). The results revealed the high stability of the enzyme, with 40% activity remaining after incubation at 70°C for 60 min.

Distribution of Similar Oxidase Activities in Environmental Strains—To assess the distribution of EpoA-like enzymes in actinomycetes, various environmental strains were isolated and examined for the presence of DMP-oxidizing activity. The cell-free extracts of 40 strains with different macroscopic appearances were subjected to SDS-polyacrylamide gel electrophoresis followed by in-gel staining with DMP as a substrate. As shown in Fig. 7, the extracts from 8 strains contained DMP-oxidizing activity. The enzymes of 5 strains showed apparent molecular masses equivalent to EpoA (>94 kDa) while the enzymes of the other 3 strains were of lower molecular sizes.

DISCUSSION

We succeeded in expressing and purifying a recombinant protein of EpoA with an *E. coli* host-vector system. Our recent study showed that the majority of native EpoA is secreted as a mature enzyme via the function of a signal peptide in *S. griseus* (*8*). We also expressed a recombinant protein of the mature form of EpoA with the same host-vector system and obtained preliminary data indicating that it shows identical features to the enzyme that carries the signal sequence examined in this study. Because of the higher yield of the resultant purified protein, we

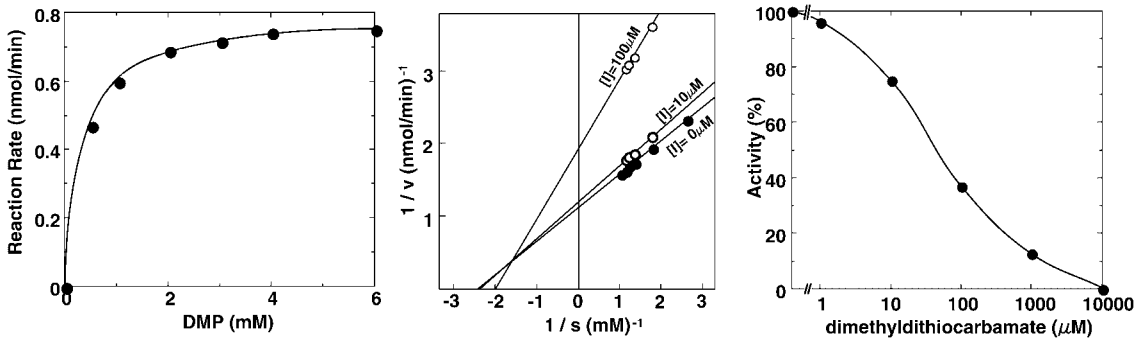

Fig. 5. **Kinetics of rEpoA inhibition by dimethyldithiocarbamate.** Lineweaver-Burk plot of the oxidation of DMP by rEpoA at different concentrations of dimethyldithiocarbamate (middle panel).

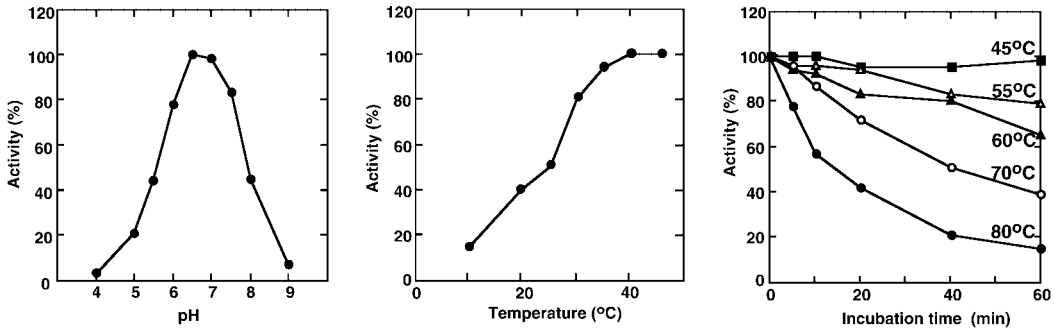

Fig. 6. **Effect of pH and temperature on the activity of purified rEpoA.** Enzyme activities at various pH values (left) and temperatures (middle), and after preincubation at various temperatures (right) are shown. 100% activity is the maximum activity at pH 6.5, 40°C.

used the latter form of the enzyme for precise characterization.

Although the amino acid sequence similarity of EpoA to laccase family members is low, the current study fully confirms its physicochemical and biochemical properties as a laccase or a related enzyme. Although the presence of procaryotic laccases is highly predicted because of their possible essential role in degrading various macromolecules in natural environments, few instances of such enzymes have been reported until now. This study clearly demonstrates that a form of laccase enzyme is produced by *S. griseus*, a soil bacterium. Regardless of the small molecular size in comparison with other laccases, the characteristics of the copper contained in rEpoA are similar to those of typical laccases. We assume that the conserved amino acid residues found in EpoA serve as sites for copper-binding as in eucaryotic laccases, but mutational studies are needed to finally determine the role of these residues.

While known laccases of eucaryotic origin are active as monomers or dimers (*12*), we found that rEpoA comprises a homotrimer. As far as we know, this is the first instance of a trimeric laccase. The homocomplex structure is probably essential for oxidase activity, since the putative monomeric molecule that accumulates in culture in the absence of additional copper (Fig. 2, lane 1) does not oxidize DMP. The finding that activity is retained after long-time incubation at high temperatures indicates that the conformation of EpoA complex is highly stable. The enhanced production of the trimeric molecule in *E. coli* cells cultured in the presence of additional copper implies that copper facilitates subunit assembly and/or stabilizes the trimeric structure. Currently we have no clear explanation for the isoelectric point heterogeneity of rEpoA, but a possible interpretation could involve variation in the redox state of the copper atoms that occurs during the course of isoelectric focusing. A similar situation was reported for cytochrome oxidase of *Pseudomonas* (*26*). Future precise studies of the conformation of EpoA essential for oxidase activity may reveal a novel molecular mechanism specific to the bacterial enzyme.

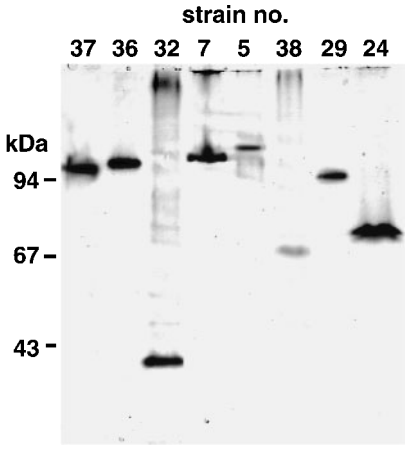

Fig. 7. **Detection of EpoA-like activities in environmental actinomycetes strains.** Cell extracts of each strain were subjected to SDS-PAGE followed by in-gel activity staining with DMP as a substrate.

Laccase is known to have broad substrate specificity, although the range differs from enzyme to enzyme (*12*). While rEpoA is capable of oxidizing several laccase substrates, the enzyme appears to have a relatively narrow range of substrate specificity, because it does not oxidize a number of compounds, including guaiacol and syringaldazine, which are known model laccase substrates. The fact that we could not detect a positive reaction of rEpoA with *p*-diphenolic compounds may indicate that the enzyme is a subspecies of the laccase family. Currently we have no additional information to determine whether the substrate specificity of EpoA is actually narrow or it has a different reaction spectrum from that of eucaryotic laccase.

Regardless of the poor understanding of prokaryotic laccases to date, the oxidase activities detected in environmental actinomyces strains suggest that similar enzymes are widespread among this group of bacteria. Our previous studies have shown that phenol oxidases are involved in morphogenesis in *Streptomyces* spp. (*8*, *27*). As suggested in fungi, these enzymes could also play some role in degrading phenolic compounds to benefit the saprophytic life cycle of soil bacteria in the natural environment. *Streptomyces* and related bacteria may prove to be abundant sources of laccases and similar useful enzymes. Studies on related enzymes should contribute not only to a precise understanding of the molecular function of this novel bacterial enzyme, but also to industrial applications for laccases.

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