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Catalytic Properties of an Organic Solvent–Resistant Tyrosinase from *Streptomyces* sp. REN-21 and Its High-Level Production in *E. coli*

Masaaki Ito[1,*] and Kuniyo Inouye[2]

[1]Central Laboratory, Rengo Co., Ltd., Fukushima-ku, Osaka 553-0007; and [2]Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502

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An organic solvent-resistant tyrosinase (OSRT) from *Streptomyces* sp. REN-21 is a unique enzyme showing high activity in the presence of organic solvents. The OSRT-catalyzed oxidation of monophenols such as tyrosine-containing peptides and proteins was examined. The catalytic properties of OSRT were compared with those of mushroom tyrosinase. OSRT was shown to oxidize Gly-L-Tyr most effectively among four peptide substrates tested. On the other hand, mushroom tyrosinase showed the highest activity toward L-Tyr-Gly under the condition of 1 mM substrate. OSRT oxidized several proteins, including casein and hemoglobin, with relatively higher activity compared with mushroom tyrosinase under the condition of 1% (w/v) substrate. Thus, it was clarified that the catalytic properties of OSRT toward tyrosine-containing peptides and proteins are different from those of mushroom tyrosinase under these conditions. The OSRT-encoding gene operon was cloned, and found to consist of two genes, designated ORF-OSRT and ORF-393. The former encodes apo-OSRT, and the latter encodes the putative activator protein of apo-OSRT. A binuclear copper-binding site (type-3 copper site) characteristic of tyrosinases is contained in the deduced amino acid sequence for apo-OSRT. A high-level production system for the OSRT was constructed using pET20b(+) and *Escherichia coli* BL21(DE3)pLysS. Approximately 54 mg of active OSRT was synthesized in a 1-liter broth culture by this system. The properties of the recombinant OSRT were similar to those of the wild-type enzyme. In conclusion, we succeeded in constructing a high-level production system for OSRT.

Key words: high-level production, organic solvent–resistance, oxidation, *Streptomyces*, tyrosinase.

Abbreviations: BCA, bicinchoninic acid; BSA, bovine serum albumin; DHI, 5,6-dihydroxyindole; DIG, digoxigenin; SDS-PAGE, SDS–polyacrylamide gel electrophoresis; L-DOPA, L-β-(3,4-dihydroxyphenyl)alanine; OSRT, organic solvent-resistant tyrosinase.

Tyrosinase (monophenol, *o*-diphenol:oxygen oxidoreductase, EC 1.14.18.1) is a copper enzyme distributed in a wide range of organisms including bacteria, fungi, plants, insects, and other animals. It is a key enzyme in the process of melanin biosynthesis from L-tyrosine (*1*, *2*). Tyrosinase has mainly two different catalytic activities. One is a diphenol oxidase activity acting on a variety of substituted *o*-diphenols to yield the corresponding *o*-quinones. Another is a monophenol oxidase activity that catalyzes the *o*-hydroxylation of monophenols to *o*-diphenols. Thus, tyrosinase catalyzes the straightforward formation of *o*-quinones either from monophenols or *o*-diphenols, which then are oxidized spontaneously and polymerized to form a melanin pigment. The enzyme also catalyzes the *o*-hydroxylation of aromatic amines and the oxidation of *o*-aminophenols to *o*-quinoneimines (*3*). Tyrosinases contain a single binuclear copper-binding site (type-3 copper site) as the active center, similar to hemocyanins and catechol oxidases. However, only tyrosinases are able to *o*-hydroxylate monophenols. Recent electron paramagnetic resonance studies of the active copper site of tyrosinase from *Streptomyces antibioticus* suggest the mechanism of this unique tyrosinase activity (*4*, *5*).

Enzymatic oxidative polymerizations of phenol derivatives using oxidoreductases as catalysts have been reported (*6–11*). This enzymatic process is expected to provide an alternative way for the preparation of phenolic polymers without the use of toxic formaldehyde, which is a monomer for the production of conventional phenolic resins. Polymerizations using enzymes are usually performed in the presence of an organic solvent to increase solubility of the products. An organic solvent-resistant tyrosinase from *Streptomyces* sp. REN-21 (OSRT) is a unique tyrosinase with high activity and stability in the presence of water-miscible solvents (*12*, *13*). In the presence of 50% ethanol or methanol, this enzyme shows approximately 50% of its control activity. The inactivation of OSRT induced by ethanol, acetone, or dimethylsulfoxide at up to 30% (v/v) organic solvent content is substantially reversible. This suggests that OSRT could be useful for the synthesis of phenolic polymers in the presence of water-miscible organic solvents

*To whom correspondence should be addressed at the present address: Life Science Laboratory, Shimadzu Corporation, Nakagyo-ku, Kyoto 604-8511. Tel: +81-75-823-1351, Fax: +81-75-823-1364, E-mail: ma-itou@shimadzu.co.jp

such as ethanol, methanol, 1-propanol, acetone, and dimethylsulfoxide.

Recently, we reported the catalytic properties of OSRT in water-in-oil microemulsions of a water/AOT/isooctane system (*14*). Although the properties of OSRT in the presence of water-miscible organic solvents and in water-in-oil microemulsions have been well studied for industrial applications, the general catalytic properties of OSRT toward monophenol substrates, such as tyrosine-containing peptides and proteins, in the absence of water-miscible organic solvents are not yet clarified. It is well known that mushroom tyrosinase oxidizes various tyrosine-containing peptides and proteins (*15–21*). In this study, the oxidation of peptides and proteins by OSRT is described, and the results are compared with those of mushroom tyrosinase.

Streptomyces sp. REN-21 produces only small amounts of OSRT in culture (approximately 0.8 mg/1-liter culture filtrate). Accordingly, it is difficult to obtain a large quantity of OSRT for the synthesis of phenolic polymers in the presence of organic solvents. To use OSRT for industrial applications, an effective production system for this enzyme is necessary. In this study, we also describe the cloning of the OSRT-encoding operon, nucleotide sequence analysis of the genes, and construction of a high-level production system in *Escherichia coli* for OSRT.

MATERIALS AND METHODS

Materials—L-Tyrosine, L-Tyr-Gly, L-Tyr-Gly-Gly, and Gly-L-Tyr were purchased from Sigma (St. Louis, MO, USA). The DIG (Digoxigenin) DNA labeling and detection kit was obtained from Roche Diagnostics (Darmstadt, Germany). The BCA (bicinchoninic acid) protein assay kit was purchased from Pierce Chemical (Rockford, IL, USA). Restriction enzymes and DNA-modifying enzymes were purchased from Nippon Gene (Toyama) and Toyobo (Osaka). The BigDye terminator cycle sequencing kit was from Applied Biosystems (Foster City, USA). OSRT was prepared as described previously (*12*). It gave a single protein band on SDS–polyacrylamide gel electrophoresis (SDS-PAGE). Mushroom tyrosinase was purchased from Sigma, and was used without further purification. All other chemicals were of reagent grade and purchased from Kishida Chemical (Osaka).

Assay for Tyrosinase Activity—Tyrosinase activity was assayed by the oxygen-uptake method (*22*). Oxygen uptake coupled to the oxidation of substrate was measured using a YSI Model 5300 biological oxygen monitor (YSI Inc., OH, Yellow Springs, USA). Substrate solution in 50 mM sodium phosphate buffer (pH 6.5) was allowed to equilibrate with the air by stirring (120 rpm) at 30°C for 10 min. The enzyme reaction was started by the addition of the enzyme solution (20 μl) to the substrate solution (4.98 ml). The final volume of the reaction mixture was 5.0 ml. The rate of oxygen uptake was recorded, and the enzyme activity was calculated from the initial slope (diphenol oxidase activity) or from the maximum slope after a lag period observed at the start of the reaction (monophenol oxidase activity). One unit of activity toward diphenolic substrates such as L-DOPA (diphenol oxidase activity) was defined as the amount of enzyme required for the uptake of 0.5 μmol of O_2 per min under these conditions. On the other hand, one unit of the activity toward monophenolic substrates such as L-tyrosine (monophenol oxidase activity) was defined as the amount of enzyme required for the uptake of 1 μmol of O_2 per min.

Kinetic Analyses—Kinetic analysis was done by the oxygen-uptake method by using various concentrations of substrate in 50 mM sodium phosphate buffer (pH 6.5). The reaction rate (v) of the monophenol oxidase activity was measured from the oxygen-uptake coupled to the oxidation of substrate. The Michaelis constant, K_m, and the catalytic constant, k_{cat}, were estimated from Lineweaver-Burk plots (1/v *vs.* 1/[s] plots), with at least six initial substrate concentrations. Protein concentration was determined by BCA protein assay with bovine serum albumin (BSA) as the standard.

Cloning of the OSRT-Encoding Operon—In order to obtain the partial gene fragment, the polymerase chain reaction (PCR) was done with KOD plus DNA polymerase (Toyobo) using primers designed from the amino acid sequence around the copper-binding sites of tyrosinases. The 400-bp DNA fragment amplified by PCR using F1 (5′-GCG/C CTG/T CCG/C TAC/T TGGGAC/T TGG-3′) as a sense primer and R2 (5′-CCAG/A AAG/C ACG/C GGA/G TCA/G TTC/G GG-3′) as an antisense primer (denaturation at 97°C for 15 s, annealing at 50°C for 30 s, extension at 68°C for 1.5 min, 30 cycles) was labeled with DIG using the DIG DNA labeling and detection kit, and used as a probe for hybridization. The genomic DNA of *Streptomyces* sp. REN-21 was digested with some restriction enzymes and analyzed by Southern blotting with the probe. Each DNA fragment around 1.5 kbp obtained by *Sma*I digestion and around 3.5 kbp obtained by *Sph*I digestion was self-ligated, and then inverse PCR was done using Inv-F3 (5′-CAA-CAACGTCCACGTGTGGAT-3′) as a sense primer and Inv-R4 (5′-GGTCCAGTCCCAGTACGGCAA-3′) as an antisense primer (denaturation at 97°C for 15 s, annealing at 55°C for 30 s, extension at 68°C for 4 min, 30 cycles). Genomic DNA was digested with *Bam*HI and then the DNA fragments around 7.5 kbp were recovered. To obtain a full-length OSRT-encoding gene operon, PCR was done using F5 (5′-TCCGCTACTGATCGTCCATTC-3′) as a sense primer, R6 (5′-CTGTGGCGTGCTGTGTTCTAC-3′) as an antisense primer, and 7.5-kbp DNA fragments as the templates. The amplified fragment was ligated into the *Sma*I site of pUC19, and then transformed into *E. coli* DH5α cells. The resulting right-oriented plasmid was designated pUC-OSRT. DNA sequencing was done using the BigDye terminator cycle sequencing kit with an ABI PRISM 310 genetic analyzer (Foster City, CA, USA).

Construction of a High-Level Production System for OSRT—The OSRT-encoding gene operon containing the open reading frames for the gene (ORF-OSRT) encoding apo-OSRT and the gene (ORF-393) encoding a putative activator protein of apo-OSRT was amplified using ex-F7Nde containing an *Nde*I site (5′-CCATATGTCGTC-CAACGTGAGCCGAC-3′) as a sense primer, ex-R8Eco containing an *Eco*RI site (5′-GGAATTCTCACGCG-TACGTGTAGTACGCGCTG-3′) as an antisense primer, and pUC-OSRT as the template (denaturation at 97°C for 15 s, annealing at 60°C for 30 s, extension at 68°C for 3 min, 30 cycles). The amplified DNA fragment was digested with *Nde*I and *Eco*RI, and then ligated into the *Nde*I–*Eco*RI site of pET20b (+) (Novagen, Darmstadt,

Germany). The resulting plasmid was used to transform *E. coli* BL21 (DE3) pLysS cells.

Production and Partial Purification of the Recombinant OSRT—E. coli BL21 (DE3) pLysS cells harboring the recombinant plasmid were inoculated into 100 ml of LB broth (1.0% polypeptone, 0.5% yeast extract, 0.5% NaCl, pH 7.0) containing 1 mM CuSO4 and 100 μg/ml ampicillin in a 500-ml flask, and maintained with shaking (100 strokes/min) at 25°C for 16 h. After cultivation, the cells were collected by centrifugation (8,000 × *g* for 5 min) and suspended in 50 mM sodium phosphate buffer, pH 7.0 (Buffer A). The suspension was sonicated and centrifuged (20,000 × *g* for 1 h), and the supernatant was applied to a column (50 mm inner diameter × 350 mm) of DEAE-cellulose (Wako Pure Chemicals, Osaka) equilibrated with Buffer A. The enzyme was eluted with the same buffer at a flow rate of 2 ml/min. Fractions containing tyrosinase activity were pooled and precipitated with ammonium sulfate (80% saturation). The precipitates were collected by centrifugation (30,000 × *g*, 20 min), dissolved in Buffer A, and dialyzed against 5 mM sodium phosphate buffer, pH 8.2 (Buffer B). The dialysate was applied to a column (26 mm inner diameter × 200 mm) of QAE-Sephadex A-50 (Amersham Biosciences, Uppsala, Sweden) equilibrated with Buffer B. The column was washed with the same buffer, and then the enzyme was eluted with a linear gradient of 5–500 mM sodium phosphate. The flow rate was 0.8 ml/min. Fractions containing tyrosinase activity were pooled as a recombinant OSRT solution and stored at –80°C until use.

Analysis of the N-Terminal Amino Acid Sequence—The recombinant OSRT was electrophoresed in an SDS–polyacrylamide gel, and then electrophoretically transferred to a polyvinylidene difluoride membrane. The protein on the membrane was stained with Coomassie Brilliant Blue R-250. The protein band was sequenced with an ABI Procise 491HT protein sequencer (Foster City, USA) by the method of Matsudaira (*23*).

RESULTS

Substrate Specificity toward Tyrosine-Containing Substrates—The substrate specificities of OSRT and mushroom tyrosinase toward 1 mM tyrosine-containing peptides are summarized in Table 1. The hydroxylation of monophenols such as L-tyrosine by OSRT showed a characteristic lag period (*12*), similar to the case with other tyrosinases (*24, 25*). OSRT had the highest activity toward Gly-L-Tyr under the condition of fixed 1 mM substrate. The oxidation rates of L-Tyr-Gly and L-Tyr-Gly-Gly were similar to that of L-tyrosine. On the other hand, mushroom tyrosinase had the highest activity toward L-Tyr-Gly, and relatively broad substrate specificity toward the four substrates tested in comparison with OSRT under the experimental conditions used.

Kinetic Analyses—The kinetic analysis of OSRT toward tyrosine-containing peptide substrates was done using L-Tyr-Gly, L-Tyr-Gly-Gly, or Gly-L-Tyr (Table 2). The kinetic parameters of L-tyrosine for OSRT were already determined as K_m = 1.25 mM, k_{cat} = 81.4 s^{-1}, k_{cat}/K_m = 65.1 mM^{-1} s^{-1} (*12*). The corresponding values listed in Table 2 are in good agreement with these values. As to k_{cat}, the smallest value was observed for L-tyrosine and the largest for Gly-L-Tyr. On the other hand, the K_m for L-tyrosine was the smallest, while the K_m values for the others did not vary much. These values are in good agreement with the results for substrate specificity of OSRT toward 1 mM tyrosine-containing peptides.

Table 1. **Substrate specificities of OSRT and mushroom tyrosinase toward tyrosine-containing peptide substrates.**

Substrate	Relative activity (%)	
	OSRT	Mushroom tyrosinase
L-Tyrosine	100	100
L-Tyr-Gly	110	157
L-Tyr-Gly-Gly	103	130
Gly-L-Tyr	338	132

Monophenol oxidase activity was measured by the oxygen up-take method described in "MATERIALS AND METHODS." The concentration of each peptide substrate was 1 mM. Monophenol oxidase activities are shown as the percent of the value of L-tyrosine (OSRT: 3.5 μM/min, mushroom tyrosinase: 21.2 μM/min).

Table 2. **Kinetic parameters for the oxidation of tyrosine-containing peptide substrates by OSRT.**

	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
L-Tyr	1.0 ± 0.1	57 ± 4	57 ± 4
L-Tyr-Gly	3.0 ± 0.2	116 ± 5	39 ± 2
L-Tyr-Gly-Gly	3.7 ± 0.2	163 ± 7	44 ± 3
Gly-L-Tyr	2.9 ± 0.1	340 ± 12	117 ± 7

Table 3. **Oxidation of various proteins by OSRT and mushroom tyrosinase.**

Protein	Relative activity (%)	
	OSRT	Mushroom tyrosinase
L-Tyrosine	100	100
BSA	2.3	0.3
Casein	40.0	0.8
Gelatin	105	4.9
Ovalbumin	2.9	1.2
Lysozyme	0.9	0.3
Hemoglobin	46.9	1.2

Monophenol oxidase activity was measured by the oxygen up-take method described in "MATERIALS AND METHODS." The concentration of L-tyrosine was 1 mM. The concentration of the proteins was 1% (w/v). Monophenol oxidase activities are shown as the percent of the value of L-tyrosine (OSRT: 3.5 μM/min, mushroom tyrosinase: 21.2 μM/min).

Oxidation of Various Proteins by Tyrosinases—The substrate specificities of OSRT and mushroom tyrosinase toward various proteins at 1% (w/v) are summarized in Table 3. All proteins used in this experiment (BSA, casein, gelatin, ovalbumin, lysozyme, and hemoglobin) were oxidized by OSRT or mushroom tyrosinase with different oxidation rates under the conditions of fixed 1% substrate concentration. Of these proteins, gelatin, hemoglobin, and casein were oxidized effectively by OSRT. Especially, the oxidation rate of 1% gelatin was similar to that of 1 mM L-tyrosine. Less activity was observed with BSA, ovalbumin, and lysozyme. On the other hand, mushroom tyrosinase had relatively low activity toward the proteins used in this experiment under the same conditions.

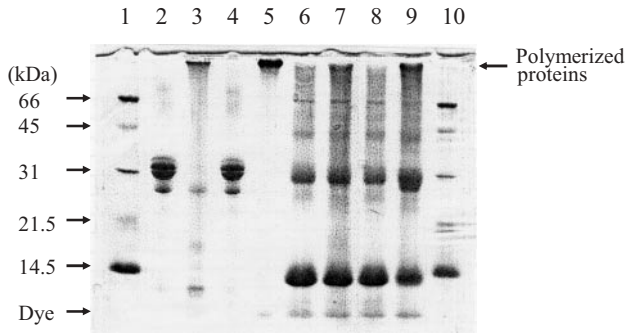

Fig. 1. **SDS-PAGE of the oxidation products of casein and hemoglobin by tyrosinases**. Casein and hemoglobin (1% w/v) in 50 mM sodium phosphate buffer (pH 6.5) were incubated at 30°C for 20 h in the absence or presence of OSRT or mushroom tyrosinase. Each enzyme was added to the reaction solution at a final concentration of 230 units/ml as estimated from the L-DOPA oxidase activity measured by the oxygen-uptake method. SDS-PAGE was performed in a 15% polyacrylamide slab gel using 2 μl of each reaction sample. After electrophoresis, the proteins were stained with Coomassie Brilliant Blue R-250. Lanes 1 and 10, standard proteins; lanes 2 and 4, casein in the absence of enzyme; lane 3, casein in the presence of OSRT; lane 5, casein in the presence of mushroom tyrosinase; lanes 6 and 8, hemoglobin in the absence enzyme; lane 7, hemoglobin in the presence of OSRT; lane 9, hemoglobin in the presence of mushroom tyrosinase. BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.5 kDa) were used as molecular standards.

No correlation was observed between the oxidation rate and the tyrosine contents of the proteins. After reaction at 30°C for 20 h, the proteins oxidized by OSRT or mushroom tyrosinase were analyzed by SDS-PAGE. Polymerization was observed for casein by the addition of OSRT or mushroom tyrosinase resulting in inseparable high molecular weights on SDS-PAGE (Fig. 1). In the case of hemoglobin, partial polymerization was observed in the presence of OSRT or mushroom tyrosinase. No molecular weight change was observed in the case of gelatin, BSA, ovalbumin, or lysozyme (data not shown). These characteristics were not related to the oxidization rates for the proteins by tyrosinases.

Cloning of the OSRT-Encoding Operon—Oligonucleotide primers were synthesized from the consensus binuclear copper- binding site (type-3 copper site) among tyrosinases. A 400-bp DNA fragment was amplified by PCR using the genomic DNA of *Strepotomyces* sp. REN-21 as a template (data not shown). The fragment was labeled with DIG and used as a probe for hybridization. Genomic DNA of *Streptomyces* sp. REN-21 was digested with some restriction enzymes and analyzed by Southern blotting with the probe. A 1.5-kbp fragment of *Sma*I-digested DNA hybridized with the probe (data not shown). *Sph*I-digested 3.5-kbp fragments and *Bam*HI-digested 7.5-kbp fragments were also hybridized with the probe (data not shown). The *Sma*I-digested 1.5-kbp fragments and *Sph*I-digested 3.5-kbp fragments were purified and self-ligated with Ligation High. Then, inverse PCR was done, and the sequence of the amplified DNA fragment was analyzed. Two open reading frames designated ORF-393 and ORF-OSRT were present in the sequence (Fig. 2). ORF-393 consisted of 393 nucleotides (starting with an ATG initiation codon at position 1 and terminating with a TAA translational stop codon at position 394) coding for a polypeptide chain of 131 amino acids with a calculated molecular weight of 13,743. ORF-OSRT is located downstream of ORF-393. The ORF-OSRT consisted of 819 nucleotides (starting with an ATG codon at position 457 and terminating with a TAA codon at position 1276) coding for a polypeptide chain of 273 amino acids with a calculated molecular weight of 30,860.

The N-terminal amino acid sequence of the wild-type enzyme started at Ala2 of the deduced amino acid sequence of OSRT. This result suggests that the N-terminal Met is eliminated from apo-OSRT by post-translational modification. The N-terminal amino acid sequence of wild-type OSRT has been reported as AVRKNQANLTASE- (*12*). Only one amino acid, Thr13, in the deduced amino acid sequence of ORF-OSRT is changed to Ser in the sequence of the wild-type enzyme. This difference may be caused by misreading the sequence analysis of the wild-type enzyme. The deduced amino acid sequence of ORF-OSRT shows high identity to the sequences of tyrosinases from *Streptomyces* species, *S. lavendulae* MA406 A-1 (68.5%) (*26*), *S. anitibioticus* (67.0%) (*27*), *S. castaneoglobisporus* (65.2%) (*28*), and *S. glaucescens* (63.4%) (*29*), *S. avermitilis* (51.6%) (*30*), *S. coelicolor* A3(2) (50.5%) (*31*) (data not shown). The amino acid sequence of OSRT contains the binuclear copper-binding site characteristic of tyrosinases. The sequence around the binding site of OSRT was compared to those of other *Streptomyces* tyrosinases (Fig. 3). The deduced amino acid sequence of ORF-393 shows high identity to those of putative tyrosinase activating proteins from *S. glaucescens* (51.9%) (*29*), *S. antibioticus* (49.6%) (*27*), *S. castaneoglobisporus* (40.5%) (*28*), *S. coelicolor* A3(2) (32.1%) (*31*), *S. avermitilis* (27.5%) (*30*), and *S. lavendulae* MA406 A-1 (26.7%) (*26*). S.D. sequences similar to the *E. coli* ribosome-binding site are present 11 nucleotides upstream from the initiation sites of ORF-393 (position 1) and ORF-OSRT (position 457) (*32*). The putative promoter sequence is located upstream from the potential S.D. sequence for ORF-393. A palindromic sequence, that is, a potential terminator sequence, is located downstream from the stop codon TAA at position 1276 of ORF-OSRT (Fig. 2).

Production of the Recombinant OSRT—A high-level production system for OSRT was constructed using the full-length OSRT-encoding gene operon, pET20b (+) vector, and *E. coli* BL21 (DE3) pLysS. Recombinant OSRT was successfully produced in *E. coli* with a productivity of about 54 mg/1-liter broth culture. The productivity of recombinant OSRT was calculated from the L-DOPA oxidase activity in the *E. coli* extract by a colorimetric method with specific activity of the purified wild-type enzyme (130 units/mg) (*12*). No activity was found in the culture filtrate. All activity was found in the bacterial sonicate. The optimum pH of the recombinant OSRT was around pH 7.0 using L-DOPA as a substrate. The recombinant OSRT had 45% activity of the control in the presence of 50% ethanol. The enzyme solution was kept at 30°C for 20 h with 30% ethanol. The activity of the recombinant OSRT was not affected under these conditions. The properties of the partially-purified recombinant OSRT (purity: >90% on SDS-PAGE) were similar to those of wild-type OSRT (*12*). The N-terminal amino acid sequence of the recombinant OSRT was NH_2-Ala-Val-Arg-Lys-Asn-. The sequence of the

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                                                        ACATCGGACGCCCCGCAG  -301
GAAGATCTTTCGGCAAACCCCCTCGCGGCCGCCAAGTTTCCTGACTCGGCGTCAACAACG  -241
TCACGAGTCGGAAATTCCTCTCTCCGCCGCTTGTTGACCGTTCATTTACCCAATGTCCCA  -181
AAACCGGTCAACCGTTTTCCTCCCCCTTCCCGATCGTGCGAGAGTCCGCCCCGCACGGAT  -121
TGATCACGAAATACAACAAGCAACCGTCATCAAAAGGGATTTACGGCTGCATTCGTGATG   -61
ACTTCCGCTACTGATCGTCCATTCCGCGCCTTGTCGGCGCGAACCCGAGGGGGACCGACC    -1
ATGTCGTCCAACGTGAGCCGACGCATTGTGCTCAGAGGAACCGCCGCTGCCGCCGCCGGC    60
M  S  S  N  V  S  R  R  I  V  L  R  G  T  A  A  A  A  A  G
GTGGCGCTGCTCGCCACCGGCGCCCGGGCGGCCGACCACACCGGCCACACCATGGACCAC   120
V  A  L  L  A  T  G  A  R  A  A  D  H  T  G  H  T  M  D  H
ACCGGGCACGAGGGCCACGGCACCGGGCTCGCGGCGTTCGACGAGGTCTACAAGGGCCGC   180
T  G  H  E  G  H  G  T  G  L  A  A  F  D  E  V  Y  K  G  R
CGCATCGTCGGCAAGCCGGTGACGAGCGGCGAGCACGCGGCCCACGGTGGCTTCGCCGTC   240
R  I  V  G  K  P  V  T  S  G  E  H  A  A  H  G  G  F  A  V
ACGATCGACGGCAAGGAGCTGCACGTCATGCGGAACGCCGACGGCACCTGGATCAGCGTC   300
T  I  D  G  K  E  L  H  V  M  R  N  A  D  G  T  W  I  S  V
ATCAACCACTACCAGGTCTTCCCCGAGCCGCTGTCGCTCACCCGCGCCGCCGTCGACAAG   360
I  N  H  Y  Q  V  F  P  E  P  L  S  L  T  R  A  A  V  D  K
CTCCGGGGCGCCTCGCTCGTCCCGATCCAGATGTAACCGGCGCACTGCCGCACGCACCCC   420
L  R  G  A  S  L  V  P  I  Q  M  *
GCGACCCACACTCTCACCCACGAGGACACGAACCCCATGGCAGTGCGCAAGAACCAGGCA   480
                                 M  A  V  R  K  N  Q  A
AACCTGACCGCCACCGAGAAGCGGACCTTCGTCAACGCCCTGCTGGAGCTCAAGCGCACC   540
N  L  T  A  T  E  K  R  T  F  V  N  A  L  L  E  L  K  R  T
GGCGTCTACGACGGATTCGTCCGCACGCACGACCAGTTCATCATGAGCGACCGCGACGAC   600
G  V  Y  D  G  F  V  R  T  H  D  Q  F  I  M  S  D  R  D  D
AGCGACCGCGTCGGCCACCGCTCGCCGTCGTTCGTCCCCTGGCACCGCCGCTTCCTGATC   660
S  D  R  V  G  H  R  S  P  S  F  V  P  W  H  R  R  F  L  I
CAGTTCGAGCAGGAGCTCCAGAAGGTCGACTCCTCGATCACGCTGCCCTACTGGGACTGG   720
Q  F  E  Q  E  L  Q  K  V  D  S  S  I  T  L  P  Y  W  D  W
ACCGTCGACCGCTCGGTCAACTCCTCGATCTTCGGCGCCGACTTCATGGGCGGCAACGGC   780
T  V  D  R  S  V  N  S  S  I  F  G  A  D  F  M  G  G  N  G
CGCAGCCGTGACGCCCAGGTCACCACCGGTCCCTTCGCCTACTCCACGGGCAAGTGGAAC   840
R  S  R  D  A  Q  V  T  T  G  P  F  A  Y  S  T  G  K  W  N
ATCAACGTCCAGGCCGAGAACAGCCCCTTCCTCCAGCGCGAGATGGGCGCCGCGGGCACG   900
I  N  V  Q  A  E  N  S  P  F  L  Q  R  E  M  G  A  A  G  T
CTGCCCACCCGCGCCCAGGTCGACGCCGTCCTCGCCATGCCGGTCTACGACACGGCCCCG   960
L  P  T  R  A  Q  V  D  A  V  L  A  M  P  V  Y  D  T  A  P
TGGAACAGCGCCTCCGAGGGCAGCTTCCGCAACTACCTGGAGGGCTGGCGCGGCGCCGGC  1020
W  N  S  A  S  E  G  S  F  R  N  Y  L  E  G  W  R  G  A  G
CGCGTCCACAACAACGTCCACGTGTGGATGGGCGGCCACATGGCCACCGGCGTCTCCCCC  1080
R  V  H  N  N  V  H  V  W  M  G  G  H  M  A  T  G  V  S  P
AACGACCCGGTCTTCTGGCTGCACCACGCCCACATCGACAAGCTGTGGTCCGACTGGCAG  1140
N  D  P  V  F  W  L  H  H  A  H  I  D  K  L  W  S  D  W  Q
CGCATGCACCCCTCCTCGGCCTACCTGCCGGCCGCCGGCACCGCGAACGTCGTCGACCTC  1200
R  M  H  P  S  S  A  Y  L  P  A  A  G  T  A  N  V  V  D  L
AACGACACCATGCGTCCGTGGAACAACGTCACCCCGGCCCAGATGCTCGACCACAGCGCG  1260
N  D  T  M  R  P  W  N  N  V  T  P  A  Q  M  L  D  H  S  A
TACTACACGTACGCGTAAGGCACGGCCTGCCCGTACGGCCTCGGCGTATCCGTACGGCAC  1320
Y  Y  T  Y  A  *
GGCGTGCACGTAAGACCACGGCGTACCCGTAGAACACAGCACGCCACAGGCGCACGCGCG  1380
CCCGGCCCGGTGCGGGGAACCGCACCGGGCCGGCGGTCGTGATGGGGGACGAGGGCCGCC  1440
ACGGGCCC
```

Fig. 2. **Nucleotide sequence and deduced amino acid sequence of the 1.8-kbp fragment containing the OSRT-encoding operon from *Streptomyces* sp. REN-21**. The nucleotide sequence is numbered from the initiation codon (ATG) of ORF-393. The putative promoter sequences are underlined. Boxes indicate potential S.D. sequences. Shaded boxes show putative transcription terminator regions. Asterisks indicate termination codons.

recombinant OSRT was identical to that of the wild-type enzyme.

DISCUSSION

OSRT from *Streptomyces* sp. REN-21 is a unique enzyme with high activity and stability in the presence of water-miscible organic solvents such as ethanol, methanol, acetone, and dimethylsulfoxide (*12, 13*).

We evaluated the catalytic properties of OSRT by analyzing the oxidation of various tyrosine-containing peptides and proteins by OSRT. Furthermore, the properties of OSRT were compared with those of mushroom tyrosinase. OSRT oxidized tyrosine-containing peptides and proteins with different oxidation rates. This result indicates that OSRT catalyzes not only the oxidation of free tyrosine, but also that of tyrosine residues in peptides and proteins, similar to mushroom tyrosinase (*15–21*). OSRT was shown to oxidize Gly-L-Tyr most effectively among four substrates tested. The k_{cat}/K_m value of OSRT for Gly-L-Tyr was 2–3-fold higher than those for other substrates. On the other hand, mushroom tyrosinase showed high activity toward L-Tyr-Gly under the condition of fixed 1 mM substrate. Thus, the specificity of OSRT toward tyrosine-containing peptides is different from that of mushroom tyrosinase. OSRT oxidized several proteins well, including casein and hemoglobin, in comparison with mushroom tyrosinase under the condition of fixed 1%

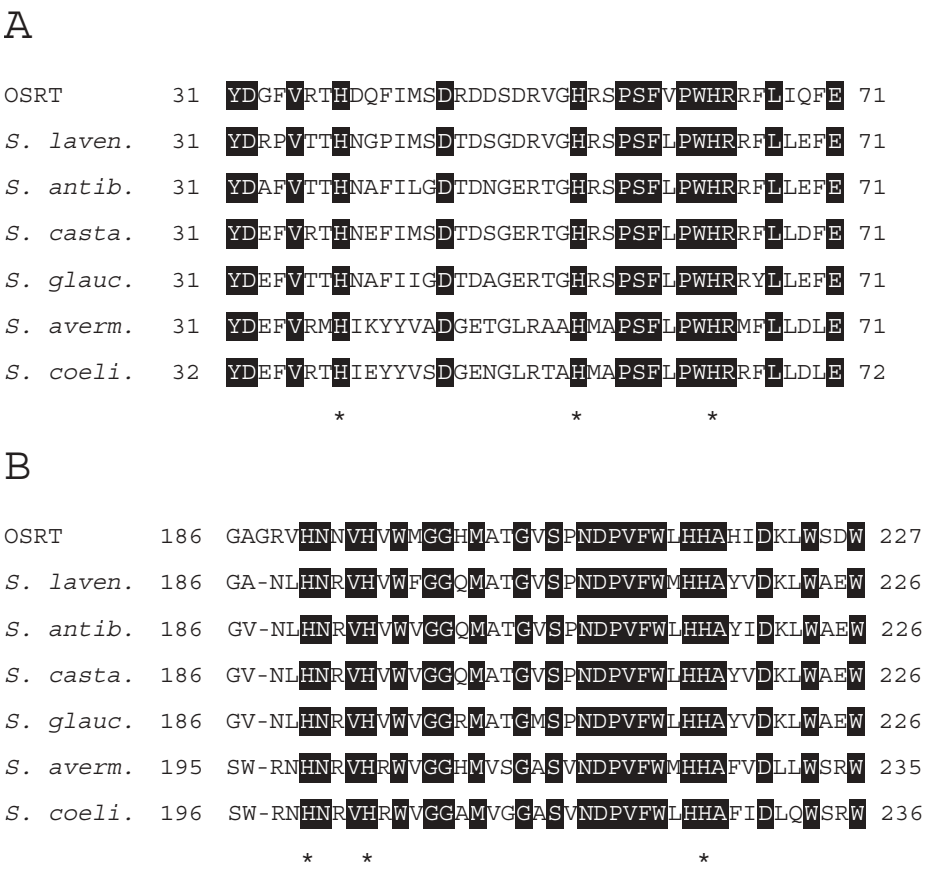

Fig. 3. **Alignment of the putative copper-binding motifs of OSRT and other tyrosinases**. Amino acid residues corresponding to potential binuclear copper-binding sites [CuA(A) and CuB(B)] are shown. Identical residues are boxed. Histidine residues potentially serving as copper ligands are marked with asterisks. Abbreviations: OSRT, *Streptomyces* sp. REN-21 OSRT (this paper); *S. laven*., *Streptomyces lavendulae* MA406 A-1 tyrosinase (*26*); *S. antib*., *Streptomyces antibioticus* tyrosinase (*27*); *S. casta, S. castaneoglobisporus* tyrosinase (*28*); *S. glauc, Streptomyces glaucescens* tyrosinase (*29*); *S. averm*., *Streptomyces avermitilis* MA4680 tyrosinase (*30*); *S. coeli., Streptomyces coelicolor* A3(2) tyrosinase (*31*).

substrate. Thus, it was clarified that the catalytic properties of OSRT toward tyrosine-containing peptides and proteins are different from those of mushroom tyrosinase.

OSRT and mushroom tyrosinase polymerized casein to inseparable high molecular weights on SDS-PAGE. On the other hand, gelatin, BSA, ovalbumin, and lysozyme were not polymerized by OSRT or mushroom tyrosinase. These characteristics were not related to the oxidization rates for proteins by tyrosinases. Tyrosinase catalyzes the oxidation of L-tyrosine to L-DOPA, which undergoes cyclization and decarboxylation to 5,6-dihydroxyindole (DHI). Further oxidation of DHI to indole-5,6-quinone leads to melanin synthesis. Tyrosinase-induced cross-linking of L-tyrosine takes place mainly through DHI. No direct oligomerization products of L-tyrosine are observed (*33*). Unless L-tyrosine is the N-terminal amino acid, the amino group of L-tyrosine is no longer available to participate in cyclization to form an indole ring. Thus, tyrosine cross-linking via DHI cannot take place in tyrosine-containing peptides as it does with Gly-L-Tyr. Melanogenesis from tyrosine-containing peptides could take place only through direct coupling of the benzene rings in the tyrosine side chain, as observed in the past in radical-induced cross-linking reactions (*34, 35*). Further analysis of the oxidation products of tyrosine-containing peptides and proteins is required.

Since tyrosinases catalyze the oxidation of a variety of monophenolic- and diphenolic-compounds, they have received great attention for biotechnological applications. OSRT is a tyrosinase with high activity and stability in the presence of water-miscible organic solvents. Thus, it might be usable for the synthesis of phenolic polymers in the presence of organic solvents. But the productivity of OSRT by *Streptomyces* sp. REN-21 is very low (approximately 0.8 mg/1 culture filtrate). Therefore, we attempted to construct a recombinant expression system for OSRT in *E. coli*. First, we cloned an OSRT-encoding operon by PCR with primers designed based on the amino acid sequence around the copper-binding sites of tyrosinases. The operon consisted of two genes, designated ORF-OSRT and ORF-393, which encoded apo-OSRT and the putative activator protein of apo-OSRT, respectively, similar to the melanin-synthesizing operons from *Streptomyces* species (*26–29*).

When the DNA for the OSRT-encoding operon composed of ORF-OSRT and ORF-393 was ligated to the *Nde*I–*Eco*RI sites of pET20b(+), the active form of OSRT (54 mg/1-liter broth) was effectively expressed in LB medium containing 1 mM $CuSO_4$. On the other hand, less activity (8%) was observed in the absence of $CuSO_4$, suggesting that OSRT is a copper-dependent enzyme. The recombinant OSRT showed the same N-terminal amino acid sequence as the wild-type OSRT. Furthermore, the activity and stability of the partially-purified recombinant OSRT in the presence of ethanol or methanol were similar to those of the wild-type OSRT. These results suggest that the recombinant OSRT is essentially the same as the wild-type enzyme. Recently, an efficient method for the overexpression of *Streptomyces castaneoglobisporus* tyrosinase in *E. coli* has been reported (*36*), although the expression level of this system is approximately 11 mg/1-liter broth as a heterodimer complex of tyrosinase and its activator protein. The expression level

of our system for OSRT is much higher than that of the system for *S. castaneoglobisporus* tyrosinase. Thus, we have succeeded in constructing a large-scale production system for OSRT. It has been shown that a large quantity of OSRT for polymerization experiments involving phenolic compounds can be produced using our system.

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