Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 322 (2004) 126–132
BBRC
www.elsevier.com/locate/ybbrc

Role of H164 in a unique dye-decolorizing heme peroxidase DyP

Yasushi Sugano, Yosuke Ishii, Makoto Shoda *

Chemical Resources Laboratory, Tokyo Institute of Technology, R1-29-4259 Nagatsuta, Midori-ku, Yokohama 226-8503, Japan

Received 24 April 2004
Available online 31 July 2004

Abstract

The expression system of a unique dye-decolorizing peroxidase DyP in *Escherichia coli* has been constructed. The molecular mass of the expressed DyP (eDyP) is 47 kDa, indicating no any modification with saccharides. The characteristics of eDyP were almost the same as those of native DyP from a fungus *Thanatephorus cucumeris* Dec 1 and recombinant DyP with *Aspergillus oryzae* except thermostability. As H164 was suggested to be the proximal histidine based on the preliminary X-ray crystallographic analysis of DyP, the site-directed mutations H164A and H166A (residue near H164) were introduced into the gene encoding DyP. The specific activity and RZ value of the purified H164A were 1.52 U/mg and 0.11, respectively, which were 99.8% and 95% lower than those of eDyP, respectively. On the contrary, those of H166A were not different from those of eDyP. Therefore, H164 was confirmed to be the proximal histidine.

Keywords: *Thanatephorus cucumeris*; Peroxidase DyP; Proximal histidine

DyP is a unique dye-decolorizing peroxidase from a fungus *Thanatephorus cucumeris* Dec 1 (formerly called *Geotrichum candidum* Dec 1) [1–5]. According to the classification of plant peroxidase superfamily proposed by Welinder [6], DyP is classified to class II. Representative peroxidases of class II are lignin peroxidase (LiP) and manganese peroxidase (MnP). LiP [7] and MnP [8] degrade non-phenolic compounds such as veratryl alcohol and phenolic compounds such as 2,6-dimethoxyphenol and guaiacol with Mn^{2+}, respectively. Interestingly, DyP degrades phenolic compounds, such as 2,6-dimethoxyphenol and guaiacol without Mn^{2+} different from MnP. In contrast, DyP does not degrade non-phenolic compounds different from LiP. Furthermore, amino acid sequence homology of DyP shows low similarity to any other proteins registered in the DNA Data Bank of Japan except two proteins. The highest identity to DyP was 55% for *Termitomyces albuminosus* peroxidase [9] and *Polyporaceae* sp. hypothetical peroxidase (unpublished work) by PSI-BLAST search [10]. However, DyP and those two proteins showed no homology to other fungal peroxidases such as LiP and MnP. Another unique characteristic of DyP is to decompose several synthetic dyes [2]. Especially, the effective decolorization for anthraquinone dyes is different from most of other decolorizing enzymes.

One of the most popular dye-decolorizing peroxidases, LiP, decolorizes azo dyes and its decolorizing mechanism has been proposed [11–15]. Versatile peroxidase [16–18] and manganese independent peroxidase [19–22] that have been reported as dye-decolorizing peroxidase also degrade azo dyes mainly.

According to intensive characterization of plant peroxidase superfamily, the peroxidases have one proximal histidine, one distal histidine, and one essential arginine [6]. These three residues are conserved without exception among plant peroxidase superfamily [6]. The most interesting point of the DyP structure is that no typical heme binding region conserved among plant peroxidase superfamily was observed. In our previous report, H164 (the 164th histidine from the N-terminus of mature DyP)

* Corresponding author. Fax: +81 45 924 5976.
E-mail address: mshoda@res.titech.ac.jp (M. Shoda).

0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.07.090

was proposed to be a proximal His, based on the preliminary X-ray crystallographic analysis of DyP [5].

Therefore, in this report, the relation between H164 and enzyme activity of DyP has been researched. The purpose of this study was to clarify the role of H164 apart from structural analysis. Site-directed mutagenesis using *Escherichia coli* transformation system is effective for taking the useful information for active sites. Therefore, the expression of DyP in *E. coli* is indispensable. We present here the recombinant DyP expression system in *E. coli* and clarify the role of H164.

Materials and methods

Chemicals. All chemical regents were of analytical grade and obtained from Kanto Chemical (Tokyo, Japan), Wako Junyaku (Tokyo, Japan) or Sigma-Aldrich (Tokyo, Japan) unless otherwise specified. Synthetic dyes were kindly provided from Bayer Japan (Tokyo, Japan) or Nippon Kayaku (Tokyo, Japan).

Microorganisms. *E. coli* JM109 (*recA*1 *supE*44 *endA*1 *hsdR*17 *gyrA*96 *relA*1 *thi*Δ(*lac-proAB*) F′[*traD*36 *proAB*$^{+}$ *lacI*^{q} *lacZ*ΔM15]) containing plasmid pYES92 was used as the source of *dyp*. *E. coli* DH5α (*supE44* Δ*lac*U169 (*ϕ*80*lacZ* ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1) and BL21(DE3) (*hsdS gal* (*λcts*857 *ind*1 *Sam*7 *nin*5 *lac*UV5-T7 gene*1*)) were used for amplification of *dyp* and expression of DyP, respectively. *E. coli* BMH71-18 (*Δ*(*lac-proAB*) *supE thi*-1 *mutS* 215:: Tn10(tet^{r})/F′[*traD* 36 *proAB*$^{+}$ *lacIq lacZ*ΔM15]) and MV1184 (*Δ*(*lac-proAB*) *ara rpsL thi*(*ϕ*80*lacZ*ΔM15) *Δ*(*srl-recA*) 306::Tn10(tet^{r})/F′[*traD*36 *proAB* + *lacIq lacZ*ΔM15]) were used for the preparation of the mutant DyP. *T. cucumeris* Dec 1 [1] and *A. oryzae* RD005 [4] were used to prepare native DyP (nDyP) and recombinant DyP (aDyP), respectively.

Media. Luria–Bertani (LB), LB-ampicillin (LB-amp), SOB, and SOC media were prepared according to the standard protocol [23] to culture *E. coli*. For Dec 1 culture, potato glucose broth (potato extract 100 g, glucose 20 g, in 1000 ml H_2O, pH 5.5) was used. RD005 was grown on the medium described in the previous report [5].

Plasmids. pUC18 was used for cloning and sequencing of *dyp*. pET25b(+) was obtained from Takara (Tokyo, Japan) and used for expression of *dyp*. pYES92 harboring cDNA of *dyp*, which was constructed using pYES2 (Invitrogen, Tokyo, Japan) by us (unpublished data), was used as the source of recombinant *dyp*. pKF-18k was used for selection of site-directed mutation.

Polymerase chain reactions. Polymerase chain reaction (PCR) was done using Thermal Cycler 480 (Takara, Tokyo, Japan). KOD polymerase (Toyobo, Tokyo, Japan) was used. Primers (dyp-n; GACC ATGGCCAACGATACAATTCTGC, dyp-m; ATCCGTGAACTGA TCCAAGAAG, dyp-e; AGTGCCATGGAACCGGAGC) were synthesized by Sigma Genosys (Tokyo, Japan). The dyp-n is identical to the mature N-terminal sequence of *dyp* except the sequence underlined. The underlined sequence of dyp-n is designed to correspond to *Nco*I site of pET25b(+). Therefore, one Met residue was added to the N-terminus of the mature DyP.

Construction of pET-DyP expression vector. Amplification of a 0.4 kb partial region (including one *Nco*I site) of *dyp* was carried out by PCR using KOD polymerase (Toyobo, Tokyo, Japan). Reaction conditions were as follows: 10 ng plasmid pYES92, each 20 pmol of oligonucleotides dyp-n and dyp-m, 200 μM of each dNTP, 5 μl of 10× KOD buffer, and 2.5 U of KOD polymerase in a final volume of 50 μl. Initial denaturation at 95 °C for 5 min was followed by 25 thermal cycles of denaturation (30 s at 94 °C), annealing (30 s at 50 °C), and extension (1 min at 72 °C). The reaction solution was applied to 0.7% agarose gel electrophoresis. The 0.4 kb fragment was cut out and extracted and purified from the gel by QIAEX II Gel Extraction kit (Qiagen, Tokyo, Japan). The fragment was ligated to *Sma*I site of pUC18 with DNA Ligation kit ver. 2 (Takara, Tokyo, Japan) and *E. coli* DH5α was transformed by the resultant plasmid. The nucleotide sequences of several transformants were confirmed by Sanger method [24] with the DNA sequencer model 4000 L (Li-COR, Lincoln, NE, USA) according to the supplier's protocol. The plasmid which was identical to 0.4 kb of the cDNA sequence of *dyp* was named pUCdf. The pUCdf was treated with *Nco*I and applied to 0.7% agarose gel electrophoresis. The fragment was ligated to *Nco*I site of pET25b(+) and the resultant plasmid was named pET-DyPF. Both pYES92 and pET-DyPF were double digested with *Kpn*I and *Not*I and applied on 0.7% agarose gel electrophoresis. The 0.9 kb fragment from pYES92 was ligated to pET-DyPF of which 0.9 kb *Kpn*I–*Not*I fragment was removed and then *E. coli* BL21(DE3) was transformed with the resultant plasmid (pET-DyP). To confirm the construction of the full length *dyp*, PCR has been done with primers dyp-n and dyp-e using KOD polymerase (Toyobo, Tokyo, Japan) according to the above PCR condition. The reaction solution was applied to 0.7% agarose gel electrophoresis. The 1.5 kb fragment was confirmed to be perfect match to the cDNA sequence of *dyp*.

Protein assay. Protein concentrations were determined by the Bradford method [25] with the Protein Assay Kit II (Bio-Rad, Tokyo, Japan) with bovine serum albumin as the standard protein.

Enzyme assay. Reactive blue 5 (RB5), a representative anthraquinone dye, was used as the authentic substrate. The substrate solution consists of 125 μM RB5 in 25 mM citrate buffer (pH 3.2). An adequate amount of the enzyme solution was mixed with the substrate solution, and then H_2O_2 was added to give a final concentration of 0.2 mM. The total volume of the enzyme reaction mixture was adjusted to 3 ml. Enzyme activity was calculated from the decrease in absorbance at 600 nm using molar extinction coefficient of RB5 (ε_{600} = 8000 $M^{-1}cm^{-1}$). One unit of enzyme activity was defined as the amount of enzyme that decolorized 1 μmol of RB5 at 30 °C for 1 min.

Preparation of nDyP and aDyP. nDyP and aDyP were purified from the method described in our previous report [2,4,5].

Expression and purification of recombinant DyP in E. coli. *E. coli* BL21(DE3) containing pET-DyP was precultured at 37 °C for 15 h at 120 spm using 20 ml LB-amp medium in a test tube. The 1.5 ml of the preculture was inoculated to 600 ml of fresh LB-amp including 16 μM hemin and cultured at 25 °C at 120 spm until the OD_{600} reached 0.6. Then, 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture and the incubation was further continued for 8 h. The bacteria were harvested by centrifuge (4100*g* for 20 min) at 4 °C. The cell pellets were resuspended and washed twice in 200 ml buffer A (2 mM EDTA and 2 mM DTT in 25 mM Tris–HCl, pH 8.0) by centrifuge (4100*g* for 20 min). The washed cell was resuspended in 60 ml buffer A and applied to a French Press apparatus (Ohtake Seisakusho, Tokyo, Japan). The supernatant was obtained from the solution by centrifuge at 4 °C, 10,000 rpm for 15 min, kept at 4 °C for 5 days, and then applied to the following purification steps. After the supernatant was adjusted to 10% saturation with solid ammonium sulfate, the precipitate was removed by centrifuge (5500*g*, 30 min). The obtained supernatant was adjusted to 50% saturation with solid ammonium sulfate and left to stand overnight. The precipitate collected by centrifuge (10,000*g*, 15 min) was dissolved in 20 ml of 25 mM piperazine buffer (pH 5.5). The solution was dialyzed against 2 L of the same buffer. For QAE-Toyopearl (Tosoh, Tokyo, Japan) chromatography, the column (1.6 × 10 cm) of QAE-Toyopearl was equilibrated with 25 mM piperazine buffer (pH 5.5). The dialyzed solution was applied to the column and washed with 60 ml of equilibrated buffer. The column was eluted with a continuous linear gradient of 0–0.5 M NaCl in the same buffer (total volume, 180 ml). The flow speed and the volume of one fraction were 3 ml/min and 6 ml, respectively. The five most active fractions (30 ml) detected by the enzyme assay were collected and pooled for subsequent Mono Q chromatography. A column (0.5 × 5 cm) of Mono Q (Amersham Bioscience, Tokyo, Japan) that

had been equilibrated with 25 mM piperazine buffer (pH 5.5) was used. The active fractions (30 ml) obtained by QAE-Toyopearl chromatography were concentrated to 3 ml by ultrafiltration with Centriprep 10 (Millipore, Tokyo, Japan) and then diluted to 30 ml with 25 mM piperazine buffer (pH 5.5). This concentration and dilution steps were repeated to reduce the NaCl concentration. They were then applied to the column and washed with 3 ml of the same buffer. The elution was carried out with a linear gradient of 0–0.25 M NaCl in 25 mM piperazine buffer (pH 5.5, total volume, 15 ml) and then eluted with a linear gradient of 0.25–0.5 M NaCl in 25 mM piperazine buffer (pH 5.5, total volume, 5 ml). The flow speed and the volume of one fraction was 1 ml/min and 1 ml, respectively. The most active fraction was supplied to the subsequent Poros PE chromatography. A column (0.46×10 cm) of Poros PE (Nippon Perseptive, Tokyo, Japan), which is a hydrophobic column, had been equilibrated with 25 mM piperazine buffer (pH 5.5) containing 1.5 M ammonium sulfate was used. Ammonium sulfate was added to the active Mono Q fraction and its concentration was adjusted to 1.5 M. This solution was then applied to the column and washed with 5 ml of the same buffer. The elution was carried out with a linear gradient of 1.5–0 M ammonium sulfate in 25 mM piperazine buffer (pH 5.5, total volume, 25 ml). The flow speed and the volume of one fraction was 1 ml/min and 1 ml, respectively. The two most active fractions were collected and applied to Superdex 75 column (1.6×60 cm, Amersham Bioscience, Tokyo, Japan) that had been equilibrated with 25 mM piperazine buffer (pH 5.5) containing 0.2 M NaCl. The flow rate and fraction size were 1 ml/min and 2 ml, respectively.

Molecular mass M_r determination. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed with a 10% gel in accordance with the Laemmli method [26]. Furthermore, the M_r of the native protein was determined by a gel filtration method with Hi-Load Superdex 75 HR16/60 (16×60 cm) at a flow rate of 1 ml/min with Fast Protein Liquid Chromatography (FPLC) system.

Western blotting. The purified DyP (0.1 μg) was run on SDS–PAGE and electrotransferred to a commercial membrane (Immobilon-P; Millipore, Tokyo, Japan) with a horizontal blotting apparatus (ATTO, Tokyo, Japan). The membrane was treated with anti-DyP antibody prepared from rabbit serum using Amplified Alkaline Phosphatase Goat Anti-Rabbit Immun-Blot Assay kit (Bio-Rad, Tokyo, Japan) according to the supplier's protocol.

N-terminal sequence analysis. SDS–PAGE of DyP was performed according to the above-described method and then the DyP on the gel was electrotransferred to Immobilon-P. A part of the membrane blotted DyP was cut out and then amino acid sequencing analysis was performed with an amino acid sequencing apparatus (PPSQ-21; Shimadzu, Kyoto, Japan) according to the standard method [27].

Characterization of DyP. Thermostability, pH profile, and substrate specificities were tested by the same method as described in the previous report [2,4].

Construction and expression of mutant DyP. To confirm the heme binding site of DyP, site-directed mutagenesis of DyP was done with site-directed mutagenesis system, Mutan-Express Km (Takara). Mutants H164A (the 164th histidine was changed to alanine) and H166A (the 166th histidine was changed to alanine) were constructed as follows. *E. coli* DH5α and BMH71-18 *mut*S were used for construction of the expression vector. Template DNA was constructed with pKF-18k harboring the *Kpn*I–*Bam*HI fragment of *dyp*. The resultant plasmid was named pKF-KB. For constructing H164A and H166A, Primer164 (5′-CGAAGTGTTCAGCACCAGC-3′) and Primer166 (5′-GAACC CGAAGGCTTCATGACCAG-3′) were designed. One microliter of pKF-KB, 1 μl of selection primer provided by the supplier, 1 μl of Primer164 or Primer166, 2 μl of annealing buffer, and 15 μl water were mixed, heated at 100 °C for 3 min, then left on ice for 5 min. Three microliters of extension buffer, 1 μl T4 DNA ligase, 1 μl T4 DNA polymerase, and 5 μl water were added and left for 2 h at 25 °C. *E. coli* BMH71-18 *mut*S was transformed by using 10 μl of the resultant DNA solution according to the standard method (20). The transformant was gently stroked at 37 °C for 1 h in 1 ml SOC and then 2 ml SOC and 100 μg/ml kanamycin (final concentration) were further added and incubated overnight. *E. coli* MV1184 was transformed by the extracted plasmid from the culture broth and several kanamycin resistance clones were obtained and resultant plasmids were named pKF-H164A and pKF-H166A. The two plasmids were treated with *Kpn*I and *Eco*RI and then 0.4 kb *Kpn*I–*Eco*RI fragments were isolated. Each fragment was ligated to the pET-DyP in which *Kpn*I–*Eco*RI fragment was removed beforehand. The resultant plasmids were named pET-H164A and pET-H166A.

Expression of mutants H164A and H166A. For expression of mutants H164A and H166A, *E. coli* BL21(DE3) containing pET-H164A and pET-H166A were used. Culture condition and purification steps were the same as those of BL21(DE3) containing pET-DyP.

Results and discussion

Expression of eDyP in E. coli

DyP expressed in *E. coli* was named eDyP. The pET vector expression system is controlled by bacteriophage T7 transcription and translation signals and has *pelB* leader peptide for secretion to periplasmic space of *E. coli*. In general, the *pelB* leader peptide is removed by signal peptidase after the secretion but often it remained in the N-terminus of the expressed protein. In case of eDyP, the ratio of eDyP having *pelB* leader peptide was high when the incubation temperature was over 30 °C (data not shown), presumably because secretion speed or transformation efficiency was too large to remove *pelB* leader peptide by signal peptidase. Therefore, we lowered the incubation temperature (25 °C) and concentration of IPTG (0.1 mM) and succeeded in taking the mature eDyP without *pelB* leader peptide predominantly.

Most of eDyP seemed to be expressed as inclusion body. However, we could not recover the enough active eDyP from the inclusion body because the efficiency of the refolding of the inclusion body was below 1%. On the other hand, eDyP was also expressed in the soluble fraction of the cell. Therefore, we purified eDyP from the soluble disrupted cell solution and 14.8 U eDyP was obtained. The purification steps are summarized in Table 1. Total activity increased gradually until QAE-Toyopearl column chromatography step. The solution was treated with ammonium sulfate and the precipitate obtained was dialyzed. The dialyzed sample was applied to QAE-Toyopearl column chromatography. The active fractions were eluted around 0.18 M NaCl and subjected to Mono Q column chromatography. The active fraction also eluted around 0.18 M NaCl was then subjected to Poros PE column chromatography. The active fractions eluted around 0.6 M ammonium sulfate were then subjected to Superdex 75 column chromatography. The active fractions were eluted at 57–63 ml from the elution start. Three fractions were confirmed to a single band on SDS–PAGE and Western

Table 1
Purification of eDyP from *E. coli*

Purification step	Protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)
Disrupted cell	257	14.8	0.058	1
$(NH_4)_2SO_4$ precipitate	116	55.3	0.48	8.3
Dialysis	34.2	127	3.7	63.8
QAE-Toyopearl	8.38	192	22.9	395
Mono Q	0.98	132	134	2310
Poros PE	0.19	94.8	499	8601
Superdex 75	0.07	57.6	834	14,385

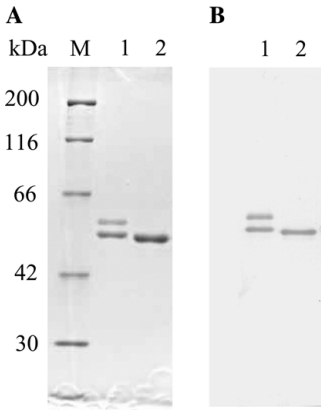

Fig. 1. (A) SDS–PAGE of purified eDyP and aDyP. The enzymes were subjected to electrophoresis on a 10% polyacrylamide gel at pH 8.0 in a Tris–glycine buffer, and the protein was stained with Coomassie brilliant blue R-250. Myosin (200 kDa), β-galactosidase (116 kDa), bovine serum albumin (66 kDa), aldolase (42 kDa), and carbonic anhydrase (30 kDa) were used as standard molecular markers. Top and bottom bands of aDyP are glycosylated and deglycosylated proteins, respectively. Lanes: M, molecular mass standards; 1, 1 μg aDyP; 2, 1.2 μg eDyP. (B) Western blot of purified eDyP and aDyP. Anti-DyP antibody was prepared from rabbit serum. Lanes: 1, 0.1 μg aDyP; 2, 0.12 μg eDyP.

blotting as shown in Fig. 1. The specific activity and Reinheitzahl (RZ) value (A_{407}/A_{280}) were 834 U/mg and 2.41, respectively. The specific activity and RZ value are higher than those of native DyP from *T. cucumeris* Dec 1 (nDyP) and are similar to those of recombinant DyP from *A. oryzae* (aDyP). This means that the purity of the final purification product of eDyP is enough compared with those of aDyP and nDyP.

M_r and N-terminal sequence of the enzyme

The values of M_r of the purified eDyP were 47 and 50 kDa from SDS–PAGE and gel filtration chromatography, respectively. Those sizes are smaller than that of nDyP (60 kDa) or aDyP (58 kDa). However, it is inconsistent with the molecular mass calculated from the deduced amino acid sequence of the cDNA (47,463 Da). It means that the polypeptide of eDyP is not modified. Generally, no protein was modified with glycosylation in *E. coli*. To the contrary, nDyP and aDyP are found to be glycosylated [2,4,5] because the origin is eukaryote. The first 20 amino acid residues of the N-terminal sequence of mature eDyP were determined to be MANDTILPLN. The Met residue of the N-terminus was designed beforehand to make an *Nco*I site and the cleavage site of signal peptidase. Therefore, this Met residue was remained in mature eDyP.

Generally, glycosylation was considered not to be influenced on enzyme activity but stability. Therefore, we investigated the characteristics of eDyP.

pH profile

The optimum pH for the enzyme activity of the recombinant DyP from *E. coli* was 3.2. This is the same as nDyP and aDyP. pH profile was also the same pattern as aDyP and nDyP [2,4].

Substrate specificities among eDyP, aDyP, and nDyP

The dyes which were decolorized by Dec 1 and standard substrates of veratryl alcohol for LiP and guaiacol, and 2,6-dimethoxyphenol for MnP were selected. The characteristics of substrate specificities of eDyP, aDyP, and nDyP are shown in Table 2. Substrate specificities were almost the same among three DyPs. eDyP did not lose its activity at 30 and 40 °C. The activity lowered slightly and retained 83% of full activity after heating 60 min at 50 °C. In contrast, the activity drastically lowered at 60 and 70 °C as shown in Fig. 2. The activity retained only 4% and 10% of full activity after heating 120 min at 60 °C and 15 min at 70 °C, respectively. As shown in Table 2 the activity of nDyP after 60 min heating at 60 °C was 92% of full activity whereas those of eDyP and aDyP were only 42%, and 44%, respectively. This difference is deduced to be due to the efficiency and types of glycosylation of each DyP. nDyP has both O-linked and N-linked glycans, but aDyP has only N-linked glycan and eDyP has neither N-linked nor O-linked glycan. Nie et al. [28] reported that O-linked glycan was important for the thermostability of a manganese peroxidase from *Phanerochaete chrysosporium*. If this is the case, it is reasonable that only nDyP has high thermostability at 60 °C. As eDyP has the same activity

Table 2
Comparison of characteristics of eDyP, aDyP, and nDyP

Test of characteristics	Value for:		
	eDyP	aDyP	nDyP
Substrate specificity (%)[a]			
Dyes			
Reactive blue 5	100	100	100
Reactive blue 19	119	102	101
Reactive blue 21	26	32	25
Reactive blue 114	4.4	2.4	1.8
Reactive black 5	1.0	0.9	1.0
Reactive red 33	2.3	1.8	2.2
Reactive red 120	0.96	0.61	0
Reactive orange 13	0.22	0	0
General substrates			
Veratryl alcohol	0	0	0
Guaiacol	9.2	10	6.0
2,6-Dimethoxyphenol	9.3	9.0	8.1
Optimum pH[b]	3.2	3.2	3.2
Thermostability (%)[c] at:			
40 °C	99	106	99
50 °C	83	100	92
60 °C	42	44	92
Molecular mass (kDa)[d]	47	58	60

[a] Substrate specificity was defined as relative activity toward Reactive blue 5.
[b] Reactive blue 5 was used as the substrate.
[c] Remaining activity at 30 °C after 60 min treatment at each temperature.
[d] All molecular masses were estimated from SDS–PAGE.

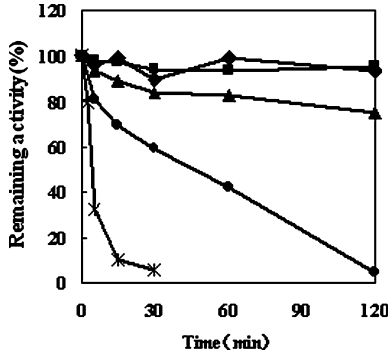

Fig. 2. Thermostability of eDyP. The enzyme activity remaining after heating at 30 °C (diamond), 40 °C (square), 50 °C (triangle), 60 °C (circle), or 70 °C (star) is shown.

as nDyP and aDyP except the thermostability at 60 °C, we conclude that eDyP is useful for research of the structure and the reaction mechanism of DyP.

Characterization of mutants H164A and H166A

The expression of H164A and H166A was confirmed by SDS–PAGE and Western blotting after purification according to the previous purification method as shown in Fig. 3. Molecular masses of both proteins were the same as that of eDyP on SDS–PAGE and gel filtration chromatography. Absorbance spectra of eDyP and H164A are shown in Fig. 4. The specific activity and RZ value of the purified H164A were 1.52 U/mg

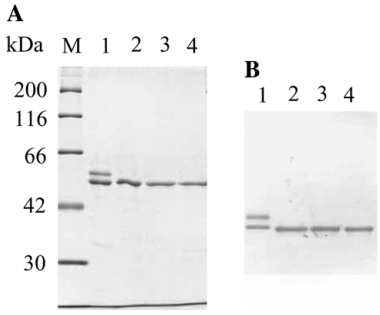

Fig. 3. (A) SDS–PAGE of purified aDyP, eDyP, H164A, and H166A. The enzymes were subjected to electrophoresis on a 10% polyacrylamide gel at pH 8.0 in a Tris–glycine buffer, and the protein was stained with Coomassie brilliant blue R-250. Myosin (200 kDa), β-galactosidase (116 kDa), bovine serum albumin (66 kDa), aldolase (42 kDa), and carbonic anhydrase (30 kDa) were used as standard molecular markers. Top and bottom bands of aDyP are glycosylated and deglycosylated proteins, respectively. Lanes: M, molecular mass standards; 1, 1 μg aDyP; 2, 1 μg eDyP; 3, 1 μg H164A; and 4, 1 μg H166A. (B) Western blot of purified aDyP, eDyP, H164A, and H166A. Anti-DyP antibody was prepared from rabbit serum. Lanes: 1, 0.1 μg aDyP; 2, 0.1 μg eDyP; 3, 0.1 μg H164A; and 4, 0.1 μg H166A.

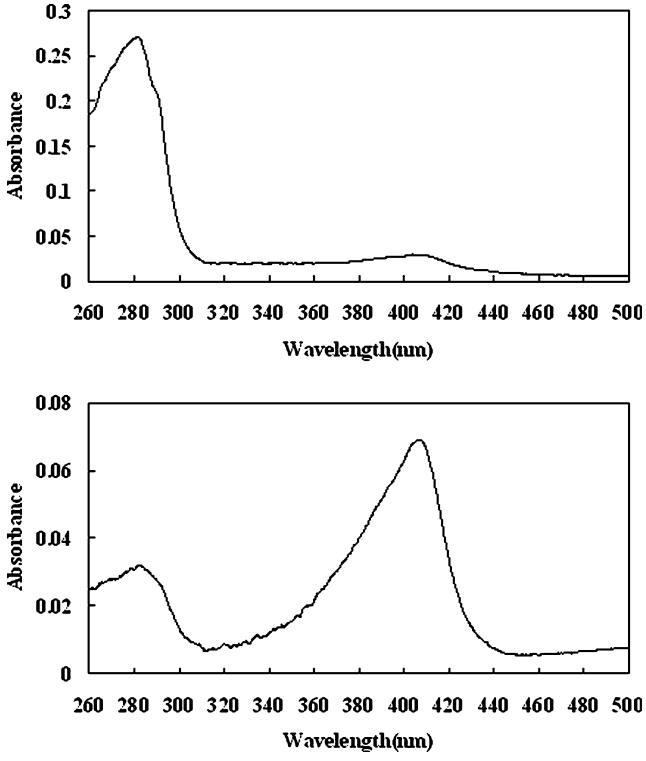

Fig. 4. Absorbance spectra of H164A and eDyP. (Top) 0.35 mg/ml H164A; (bottom) 0.04 mg/ml eDyP.

and 0.11, respectively, which were 99.8% and 95% lower than those of eDyP, respectively. This suggests that heme was not fixed in the enzyme, resulting in no activity in H164A. Actually, in case of horseradish peroxidase, which is a representative plant peroxidase, the site-directed mutation of proximal histidine to alanine lowered the enzyme activity to 10^{-5} [29]. The specific activity and RZ value of H166A were 496 U/mg and 2.16, respectively, which were similar to those of eDyP. In a previous report, H164 was suggested to be the proximal histidine based on the preliminary X-ray crystallographic analysis of DyP [5], and the present data confirmed this and H166 near H164 showed no influence on DyP activity. Generally, proximal histidine corresponds to the fifth ligand of the heme. Therefore, H164 of DyP is also considered to correspond to the fifth ligand of heme although its surrounding amino acid residues are low homologous to those of other peroxidases as described in the previous report [5]. On the other hand, the distal site of the heme in DyP, which is important for H_2O_2 binding [30–34], was very different from those of general peroxidases such as LiP and MnP from our previous study [5]. In case of plant peroxidase superfamily [6], one distal histidine and one essential arginine are necessary to bind H_2O_2 without exception. Furthermore, apart from the H_2O_2 binding site, some substrate binding sites have been proposed from several peroxidases [35–38]. Those sites have been proposed by site-directed mutagenesis. DyP is also expected to have active site(s) for substrate binding apart from H_2O_2 binding site. To find out the active site, both site-directed mutagenesis and X-ray crystallographic analysis are in progress.

When the crystal was constructed by aDyP, it was necessary to remove saccharides from aDyP to raise the homogeneity of DyP [5]. Actually, it was difficult to remove saccharides perfectly, and thus, the low level homogeneity affects crystallization and results in the poor resolution maximum of X-ray diffraction. Therefore, eDyP is expected to be a good material for its crystallization. The purity of eDyP is very high. Moreover, the homogeneity among molecular level of DyP is high compared with that of aDyP because eDyP has never been modified with any saccharides.

In conclusion, we succeeded in the expression of DyP in *E. coli*. This expression method must accelerate the finding novel knowledge for DyP because several mutant DyP without glycosylation is easy to construct.

References

[1] S.J. Kim, K. Ishikawa, M. Hirai, M. Shoda, Characteristics of a newly isolated fungus, *Geotrichum candidum* Dec1, which decolorizes various dyes, J. Ferment. Bioeng. 79 (1995) 601–607.

[2] S.J. Kim, M. Shoda, Purification and characterization of a novel peroxidase from *Geotrichum candidum* Dec1 involved in decolorization of dyes, Appl. Environ. Microbiol. 65 (1999) 1029–1035.

[3] Y. Sugano, K. Sasaki, M. Shoda, cDNA cloning and genetic analysis of a novel decolorizing enzyme, peroxidase gene *dyp* from *Geotrichum candidum* Dec1, J. Biosci. Bioeng. 87 (1999) 411–417.

[4] Y. Sugano, R. Nakano, K. Sasaki, M. Shoda, Efficient heterologous expression in *Aspergillus oryzae* of a unique dye-decolorizing peroxidase, DyP, of *Geotrichum candidum* Dec 1, Appl. Environ. Micobiol. 66 (2000) 1754–1758.

[5] T. Sato, S. Hara, T. Matsui, G. Sazaki, S. Saijo, T. Ganbe, N. Tanaka, Y. Sugano, M. Shoda, A unique dye-decolorizing peroxidase, DyP, from *Thanatephorus cucumeris* Dec 1: heterologous expression, crystallization and preliminary X-ray analysis, Acta Crystallogr. D 60 (2004) 149–152.

[6] K.G. Welinder, Superfamily of plant, fungal, and bacterial peroxidases, Curr. Opin. Struct. Biol. 2 (1992) 388–393.

[7] M.H. Gold, M. Kuwahara, A.A. Chju, J.K. Glen, Purification and characterization of an extracellular H_2O_2-requiring diarylpropane oxygenase from the white rot basidiomycete, *Phanerochaete chrysosporium*, Arch. Biochem. Biophys. 234 (1984) 353–362.

[8] J.K Glenn, M.H. Gold, Purification and characterization of an extracellular Mn(II)-dependent peroxidase from the lignin-degrading Basidiomycete, *Phanerochaete chrysosporium*, Arch. Biochem. Biophys. 242 (1985) 329–341.

[9] T. Johjima, M. Ohkuma, T. Kudo, Isolation and cDNA cloning of novel hydrogen peroxide-dependent phenol oxidase from the basidiomycete *Termitomyces albuminosus*, Appl. Microbiol. Biotechnol. 61 (2003) 220–225.

[10] S.F. Altschul, T.L. Madden, A.A. Shaffer, J. Zang, Z. Zhang, W. Miller, D.J. Lipman, Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, Nucleic Acid Res. 25 (1997) 3389–3402.

[11] P. Ollikka, K. Alhonmaki, V.M. Leppanen, T. Glimoff, T. Rainola, I. Suominen, Decolorization of azo triphenyl methane, heterocyclic, and polymeric dyes by lignin peroxidase isozymes from *Phanerochaete chrysosporium*, Appl. Environ. Microbiol. 59 (1993) 4010–4016.

[12] C. Cripps, J.S. Bumpus, S.D. Aust, Biodegradation of azo and heterocyclic dyes by *Phanerochaete chrysosporium*, Appl. Environ. Microbiol. 56 (1990) 1114–1118.

[13] B.R.M. Vyas, H.P. Molitoris, Involvement of an extracellular H_2O_2-dependent ligninolytic activity of the white rot fungus *Pleurotus ostreatus* in the decolorization of remazol brilliant blue R, Appl. Environ. Microbiol. 61 (1995) 3919–3927.

[14] M. Chivukula, J.T. Spadaro, V. Renganathan, Lignin peroxidase catalyzed oxidation of sulfonated azo dyes generates novel sulfophenyl hydroperoxidase, Biochemistry 34 (1995) 7765–7772.

[15] S. Goszcynski, A.P. Pasczynski, M.B. Grigsby, R.L. Crawford, D.L. Crawford, New pathway for degradation of sulfonated azo dye by microbial peroxidases of *Phanerochaete chrysosporium* and *Streptomyces chromofuscus*, J. Bacterial. 172 (1994) 1339–1347.

[16] S. Camareo, S. Sarker, F.J. Ruiz-Dueas, M.J. Martínez, A.T. Martínez, Description of a versatile peroxidase involved in the natural degradation of lignin that has both manganese peroxidase and lignin peroxidase substrate interaction sites, J. Biol. Chem. 274 (1999) 10324–10330.

[17] V. Gómez-Toribio, A.T. Martínez, M.J. Martínez, Oxidation of hydroquinones by the versatile ligninolytic peroxidase from *Pleurotus eryngii*, Eur. J. Biochem. 268 (2001) 4787–4793.

[18] F.J. Ruiz-Dueñas, S. Camarero, M. Pérez-Boada, M.J. Martínez, A.T. Martínez, A new versatile peroxidase from *Pleurotus*, Biochem. Soc. Trans. 29 (2001) 116–122.

[19] A. Heinfling, M.J. Martinez, A.T. Martinez, M. Bergbauer, U. Szewzyk, Purification and characterization of peroxidases from the dye-decolorizing fungus *Bjerkandera adusta*, FEMS Microbiol. Lett. 165 (1998) 43–50.

[20] A. Heinfling, F.J. Ruiz-Duenas, M.J. Martinez, A.T. Martinez, M. Bergbauer, U. Szewzyk, A study on reducing substrates of

manganese-oxidizing peroxidases from *Pleurotus eryngii* and *Bjerkandera adusta*, FEBS Lett. 428 (1998) 141–146.
[21] F.J. Ruiz-Dueńas, M.J. Martínez, A.T. Martínez, Heterologous expression of *Pleurotus eryngii* peroxidase confirms its ability to oxidize Mn^{2+} and different aromatic substrates, Appl. Environ. Microbiol. 65 (1999) 4705–4707.
[22] A. Heinfling, M.J. Martinez, A.T. Martinez, M. Bergbauer, U. Szewzyk, Transformation of industrial dyes by manganese peroxidases from *Bjerkandera adusta* and *Pleurotus eryngii* in a manganese-independent reaction, Appl. Environ. Microbiol. 64 (1998) 2788–2793.
[23] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, second ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.
[24] F. Sanger, S. Nicklen, A.R. Coulson, DNA sequencing with chain-terminating inhibitors, Proc. Natl. Acad. Sci. USA 74 (1977) 5463–5467.
[25] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[26] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature (London) 277 (1970) 680–685.
[27] P. Edman, A method for the determination of the amino acid sequence in peptides, Arch. Biochem. Biophys. 22 (1949) 475–476.
[28] G. Nie, N.S. Reading, S.D. Aust, Relative stability of recombinant versus native peroxidases from *Phanerochaete chrysosporium*, Arch. Biochem. Biophys. 365 (1999) 328–334.
[29] S.L. Newmyer, J. Sun, T.M. Loehr, P.R. Oritz, O. Montellano, Rescue of the horseradish peroxidase His-170 → Ala mutant activity by imidazole: importance of proximal ligand tethering, Biochemistry 35 (1996) 12788–12795.
[30] S.L. Newmyer, P.R.O. de Montellano, Rescue of the catalytic activity of an H42A mutant of horseradish peroxidase by exogenous imidazoles, J. Biol. Chem. 271 (1996) 14891–14896.
[31] S. Nagano, M. Tanaka, K. Ishimori, Y. Watanabe, I. Morishima, Catalytic roles of the distal site asparagines–histidine couple in peroxidases, Biochemistry 35 (1996) 14251–14258.
[32] M.I. Savenokova, S.L. Newmyer, P.R.O. de Montellano, Rescue of His-42 → Ala horseradish peroxidase by a Phe-41 → His mutation, J. Biol. Chem. 271 (1996) 24598–24603.
[33] M. Tanaka, K. Ishimori, M. Mukai, T. Kitazawa, I. Morishima, Catalytic activities and structural properties of horseradish peroxidase distal His42 → Glu or Gln mutant, Biochemistry 36 (1997) 9889–9898.
[34] M. Tanaka, S. Nagano, K. Ishimori, I. Morishima, Hydrogen bond network in the distal site of peroxidases: spectroscopic properties of Asn70 → Asp horseradish peroxidase mutant, Biochemistry 36 (1997) 9791–9798.
[35] H. Itakura, Y. Oda, K. Fukuyama, Binding mode of benzhydroxamic acid to *Arthromyces ramosus* peroxidase shown by X-ray crystallographic analysis of the complex at 1.6 Å resolution, FEBS Lett. 412 (1997) 107–110.
[36] W.A. Doyle, W. Blodig, N.C. Veitch, K. Piontek, A.T. Smith, Two substrate interaction sites in lignin peroxidase revealed by site-directed mutagenesis, Biochemistry 37 (1998) 15097–15105.
[37] A. Henriksen, D.J. Schuller, K. Meno, K.G. Welinder, A.T. Smith, M. Gajhede, Structural interactions between horseradish peroxidase C and the substrate benzhydroxamic acid determined by X-ray crystallography, Biochemistry 37 (1998) 8054–8060.
[38] M.D.S. Gelpke, J. Lee, M.H. Gold, Lignin peroxidase oxidation of veratryl alcohol: effects of the mutants H82A, Q222A, W171A, and F267L, Biochemistry 41 (2002) 3498–3506.