Short Communication

cDNA cloning of bilirubin oxidase from *Pleurotus ostreatus* strain Shinshu and its expression in *Aspergillus sojae*: an efficient screening of transformants, using the laccase activity of bilirubin oxidase

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Bilirubin oxidase (BOX; EC1.3.3.5) catalyzes the oxidation of bilirubin to biliverdin. It is used to measure the concentration of bilirubin in the serum to diagnose the degree of jaundice. It is also useful to remove bilirubin for the exact measurement of many clinical markers. Filamentous fungus *Myrothecium verrucaria* (Murao and Tanaka, 1982) and many basidiomycetes (Hiromi et al., 1992; Matsui et al., 1984; Uwajima and Hamamatsu, 1984) have been known as bilirubin oxidase producing fungi. Recently, BOX was purified and also characterized from *Penicillium janthinellum* (Seki et al., 1996). From *Myrothecium*, a BOX gene was cloned and expressed in yeast (Koikeda et al., 1993) and in *Aspergillus* (Xu et al., 1996).

Enzyme stable during a long-term storage in solution is generally required for a commercial diagnostic enzyme. We found that BOX from the *Pleurotus ostreatus* strain Shinshu was more stable than the other previously reported BOXs from other fungi during storage in solution. Since *P. ostreatus* strain Shinshu produced a small amount of BOX (1 U/ml in 7-day culture), we cloned the cDNA of BOX from *P. ostreatus* strain Shinshu and tried to express it in *Aspergillus sojae*.

P. ostreatus strain Shinshu was aerobically cultured at 25°C in the BOX production medium (6.0% soluble starch, 0.5% yeast extract, 0.1% sodium citrate, 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0005% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, and

0.008% CaCl_2) for 5 days. After centrifugation, the supernatant was adjusted to pH 9.2 and applied to a DEAE-cellulose column equilibrated with 0.01 M carbonic buffer (pH 9.2). The enzyme was eluted by using a linear gradient of 0 M to 0.6 M ammonium sulfate. The fractions containing the bilirubin oxidase activities were concentrated and dialyzed against 0.01 M carbonic buffer (pH 9.2). The concentrated fraction was applied to a Sephadex G-75 column equilibrated with 0.01 M carbonic buffer (pH 9.2). Purified enzyme was obtained by concentrating and dialyzing the peak fractions. The molecular weight of the purified enzyme was 60,000 by SDS-PAGE (data not shown). The standard assay of BOX activity was performed as follows. A standard assay solution was prepared by dissolving 5 mg of crystallized bilirubin in 2 ml of 0.2 M NaOH and diluted to 200 ml by 0.1 M phosphate-buffer (pH 7.5). The reaction was performed at 37°C by adding 0.5 ml of enzyme solution to 2 ml of standard assay solution. The decrease in absorbance at 440 nm was measured. One unit is defined as the amount of enzyme that oxidizes 1 μmol of bilirubin per min (the millimolar extinction coefficient of bilirubin under this assay condition is 40.93).

N-terminal amino acid sequence of the purified enzyme was AIGPTGNMYIVNED. The purified BOX stored in the ammonium sulfate solution for a long period was applied to SDS-PAGE after removing the ammonium sulfate by ultrafiltration. We could not observe the band at the position of BOX, 60,000. Instead, we found a few bands at lower molecular weights (Fig. 1A). Long-term storage, the removal of ammonium

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Fig. 1. SDS-PAGE and the partial amino acid sequences of the BOX protein.
A: SDS-PAGE of the BOX after a long-term storage in ammonium sulfate solution. B: The similarities of the partial amino acid sequences of the BOX from strain Shinshu to those of the protein deduced from the *pox2* and *pox1* genes from strain Florida. The protein bands were eluted from the SDS-PAGE gel, and the partial amino acid sequences were determined. Florida POX2, the *pox2* gene product from strain Florida; Florida POX1, the amino acid sequence deduced from the *pox1* gene from strain Florida. X indicates the amino acid that could not be determined during the peptide sequencing. Numbers in parentheses show those of the amino acid residue starting from the amino terminus.

sulfate from the BOX solution, or contamination of proteases might be responsible for the degradation of the BOX protein. The three protein bands were eluted from the SDS-PAGE gel, and the amino acid sequences were determined by the method of Kurth and Stoffel (1990). In a search of PIR and SWISS-PROT databases, a very high degree of homology was observed with the *pox2* gene product (identified as laccase) and the protein sequence deduced from the *pox1* gene (the protein is not identified) from *P. ostreatus* strain Florida (Giardina et al., 1995, 1996) (Fig. 1B). Therefore we examined whether the purified BOX had laccase activity by using the method of Giardina et al. (1995) with 2,2-azinobis-3-ethylbenzthiazoline-6-sulfonic acid or syringaldazine as the substrate. Laccase activity was detected. We also detected the laccase activity of BOX by the method of de Vries et al. (1986), using 4-amino-2,6-dibromophenol as the substrate. Consequently, we considered that BOX was the product of the *pox1* or *pox2* gene.

To identify whether this BOX protein was the product of the *pox1* or *pox2* gene, we tried to clone these genes and determine the partial DNA sequences. The N-terminals and central parts of the *pox1* and *pox2* genes of *P. ostreatus* strain Florida showed a high similarity. However, the C-terminals were not conserved. We designed PCR primers, a common sense primer (5'-GCGTACTGCTACACCTACCACATATGTTTCCAGGCGCACGGATTCT-3'), from the N-terminal sequence, and respective antisense primers (5'-TA-

AGTAGGTAGACATATGCTAAGCTATGCC-3' and 5'-GAAGTAGGAATAAGAAGAGGCATATGTTAGGACGGAACGATGCCTC-3') from the C-terminal sequences of the *pox1* gene and *pox2* gene. The *pox1* and *pox2* genes were amplified by PCR (94°C for 30 s, 60°C for 2 min, and 72°C for 2 min, 30 cycles), using genomic DNA prepared from *P. ostreatus* strain Shinshu. We have determined approximately 300 bp DNA sequences coding the N-terminal regions of these PCR products. The deduced N-terminal amino acid sequences of the *pox1* and *pox2* genes are shown in Fig. 2. By comparing the deduced and experimentally determined N-terminal amino acid sequences of BOX, we concluded that BOX was encoded by the *pox2* gene.

BOX cDNA was cloned by RT-PCR, using the primers that were used to clone the genomic DNA. *P. ostreatus* strain Shinshu was cultured in the medium above described, and the total RNA was extracted with guanidinium thiocyanate, followed by centrifugation in cesium chloride solution. We performed RT-PCR by using 1 µg of total RNA as a template. A reverse extension reaction was done for 60 min at 42°C. After 50 PCR cycles (94°C for 30 s, 60°C for 2 min, and 72°C for 2 min), *pox2* cDNA was amplified, but the *pox1* cDNA was not obtained. We considered that *pox1* might not be expressed under this culture condition.

We determined the *pox2* cDNA sequence (DDBJ/EMBL/GenBank accession number AB020026), which

Shinshu POX2	1	MFPGARILATLTALHLLHGTHAAIGPTGNMYIVNEDVSPDGFARS	50
Shinshu POX1	1	MFPGARILATLTALHLLHGTHAAIGPTGDMYIVNEDVSPDGFNRSA	50
	51	RSVPATDPTPATVSI	97
		PGVLVQGNKGNFQLNVNQLSDTTMLKTTSI...	
	51	RDVPATXAA-ASVT--GVLVQGNKGNFELNVNQLSDTTMLKTTSI...	94

Fig. 2. Comparison of the deduced amino acid sequences of the *pox2* and *pox1* genes from *P. ostreatus* strain Shinshu. Shinshu POX2, the deduced N-terminal amino acid sequence of the *pox2* gene; Shinshu POX1, the deduced N-terminal amino acid sequence of the *pox1* gene. The experimentally determined sequences are underlined.

encodes a protein consisting of 533 amino acids. We compared the deduced amino acid sequence of BOX to those of the other BOX and laccase (Fig. 3). A 97% homology was found between BOX from *P. ostreatus* strain Shinshu and laccase from strain Florida (Giardina et al., 1996). The homology among *P. ostreatus* BOX, *Trachyderma tsunodae* BOX (Iwamoto, 1997), and *Schizophyllum commune* laccase (Hatamoto et al., 1999) was relatively high (58–60%). The homology between *P. ostreatus* BOX and *M. verrucaria* BOX (Koikeda et al., 1993) was relatively low (30%).

Since we could not succeed in the BOX expression in *Escherichia coli*, we chose *A. sojae* as the host to express and secrete the BOX protein. The expression plasmid was constructed as follows. The cDNA that encoded the mature BOX protein was amplified by PCR. The PCR product was inserted into pTPT/pUC19 (Hatamoto et al., 1999), which has a tannase promoter, tannase signal, and tannase terminator. *A. sojae* 1860 (*niaD*[−] *pro*⁺), which has no laccase activity (Hatamoto et al., 1999), was cotransformed with this expression plasmid and *niaD* plasmid (Hatamoto et al., 1996) by the method of Campbell et al. (1989). The *niaD*⁺ transformants were transferred to the assay plates containing gallic acid (1.2% (NH₄)₂HPO₄, 0.2% KH₂PO₄, 0.1% MgSO₄·7H₂O, 5.3% glucose, 1.0% gallic acid, 0.005% CuSO₄·5H₂O, pH 6.0). If the transformant produced and secreted BOX into the medium, gallic acid would be oxidized and turn dark brown because of the laccase activity of BOX. The expression levels of the laccase activity of the transformants could be estimated by the intensity of the color.

Thirty-six transformants of 59 *niaD*⁺ transformants showed a brown color. Four that showed an intense brown color were selected, and a single colony isolation was performed. We selected the transformant, B222, which showed the darkest color (Fig. 4A). Southern-blot analysis of the chromosomal DNA from the transformant B222 indicated that at least 3 copies of the BOX gene were integrated into the chromosomes (Fig. 4B). B222 was cultured in the BOX production medium containing 2% tannic acid as an inducer. The BOX activity of B222 was 0.6 U/ml after 6 days.

In this report, we succeeded in cloning the BOX

cDNA from *P. ostreatus* strain Shinshu and its expression in *A. sojae*. Since a very high degree of homology was observed between the partial amino acid sequences of BOX and laccase, we found that the BOX from *P. ostreatus* strain Shinshu had laccase activity. It is already known that BOX from filamentous fungi *M. verrucaria* has laccase activity (Xu et al., 1996). On the other hand, it was reported that laccase from basidiomycete *Polyporus* has no BOX activity (Tanaka and Murao, 1983). Although many reports have been made about laccases from basidiomycetes (Coll et al., 1993; Heinzkill et al., 1998; Kojima et al., 1990; Sannia et al., 1986), no information about the BOX activity has been presented, and this is first report that the laccase from basidiomycete has BOX activity. In *Coprinus cinereus* (Ducros et al., 1998; Uwajima and Hamamatsu, 1984), *Schizophyllum commune* (de Vries et al., 1986; Matsui et al., 1984), and *Trametes versicolor* (Bourbonnais et al., 1995; Uwajima and Hamamatsu, 1984), laccase and BOX were separately reported. It would be very interesting to examine whether these laccases have BOX activities. We recently succeeded in cloning the laccase cDNA from *S. commune* and expressed it in *A. sojae* (Hatamoto et al., 1999). *S. commune* laccase had no BOX activity, though the deduced amino acid sequence showed a high degree of homology (58–60%) with those of *P. ostreatus* BOX and *Trachyderma tsunodae* BOX. Because some basidiomycetes produce several laccases, the BOX of *S. commune* may be encoded in other laccase genes. Amino acid residues that are conserved in *P. ostreatus* BOX and *T. tsunodae* BOX but not in *S. commune* laccase may be involved in substrate specificity, or the active sites for bilirubin and polyphenols may be different.

Transformants producing BOX were successfully obtained by the screening of transformants, using laccase activity of BOX as a selection marker. Further studies, such as improving culture conditions and increasing copy numbers of the BOX gene in transformants, will enable us to obtain a higher productivity of BOX at a commercial level.

Fig. 3. Alignment of the BOX from *P. ostreatus* strain Shinshu with the other BOXs and laccases.

P. o. Shinshu BOX, BOX from the *Pleurotus ostreatus* strain Shinshu (AB020026); *P. o. Florida laccase*, laccase from the *Pleurotus ostreatus* strain Florida (Z34848); *T. t. BOX*, BOX from *Trachyderma tsunodae* (AB006824); *S. c. laccase*, laccase from *Schizophyllum commune* (AB015758); *M. v. BOX*, BOX from *Myrothecium verrucaria* (D12579). The accession numbers in DDBJ/EMBL/GenBank databases are shown in parentheses.

Fig. 4. Plate assay and Southern-blot analysis of the transformant B222.

A: Plate assay of the transformant by laccase activity. 1860, *A. sojae* 1860 (the host strain); B222, a transformant. B: Southern-blot analysis of B222. Chromosomal DNAs digested with *EcoRI* were loaded onto each lane, and the 1.2 kb *BamHI-NdeI* fragment of the BOX cDNA labeled with digoxigenin was used as a probe. 1860, *A. sojae* 1860 (the host strain); B222, the transformant.

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