Mycoscience (2002) 43:391–395 © The Mycological Society of Japan and Springer-Verlag Tokyo 2002

FULL PAPER

Kenji Okamoto · Hideshi Yanase

Aryl alcohol oxidases from the white-rot basidiomycete *Pleurotus ostreatus*

Received: May 30, 2002 / Accepted: July 10, 2002

Abstract Three aryl alcohol oxidases (AAOs; EC 1.1.3.7) I, II, and III from the culture filtrate of a strain of white-rot fungus *Pleurotus ostreatus* were purified by multistep chromatography. Each of the purified AAOs I, II, and III had the same molecular masses of 70 kDa and 72 kDa on gel filtration chromatography and sodium dodecyl sulfate-polyacrylamide gel electrophoresis, respectively. Their optimum temperature was 40°C, but their optimum pHs differed slightly. The N-terminal amino acid sequence of AAOs I, II, and III was determined to be Ala-Asp-Lys-Asp-Tyr-Ile-Val-Val-Gly-Ala, which showed significant similarity to those of *Pleurotus eryngii* (80% identity) and *Pleurotus ostreatus* Florida (60% identity).

Key words Aryl alcohol oxidase · Basidiomycete · Isozyme · *p*-Anisaldehyde · *Pleurotus ostreatus*

Introduction

The lignin degradation system of white-rot basidiomycetes is mainly composed of laccase, lignin peroxidase, and manganese peroxidase. It is considered that these lignin-degrading enzymes do not function independently but mutually interact with each other as well as with other oxidases, such as aryl alcohol oxidase (AAO). In our previous study, we found that *Pleurotus ostreatus* (Jacq.: Fr.) Kummer produced a flavor compound, *p*-anisaldehyde, and that its production tended to be closely associated with AAO and manganese peroxidase (Okamoto et al. 2002). Similar results were obtained by Guillén and colleagues (1990, 1992, 1994) and Gutiérrez et al. (1994). These findings suggest that *p*-anisaldehyde produced by *Pleurotus* species plays an important role in generating H_2O_2-activated peroxidase and that this feature is closely associated with the lignin degradation system. Some AAOs as H_2O_2-generating enzymes in basidiomycetes, such as *Pleurotus sajor-caju* (Fr.) Singer (Bourbonnais and Paice 1988), *Pleurotus ostreatus* Florida-strain (Sannia et al. 1991), *Pleurotus eryngii* (DC.) Gillet (Guillén et al. 1992), and *Phanerochaete chrysosporium* Burds. (Asada et al. 1995), have been purified and their properties characterized, but to our knowledge there is no information concerning isozymes except for those from *P. sajor-caju* (Bourbonnais and Paice 1988). In this article, we describe the purification and major properties of AAO isozymes obtained from the culture filtrate of *P. ostreatus* as part of the study on the biosynthetic mechanism of *p*-anisaldehyde.

K. Okamoto (✉) · H. Yanase
Department of Biotechnology, Faculty of Engineering, Tottori University, 4-101 Koyamacho-Minami, Tottori, Tottori 680-8552, Japan
Tel. +81-857-31-5276; Fax +81-857-31-0881
e-mail: okamoto@bio.tottori-u.ac.jp

Materials and methods

Organism and culture condition

Pleurotus ostreatus K16-2 was cultured statically in a 500-ml Erlenmeyer flask containing 80 ml of culture medium (pH 6.3) composed of 1% malt extract (Difco, Detroit, MI, USA), 0.4% yeast extract (Difco), 0.4% glucose, and 5 mM L-tyrosine; addition of L-tyrosine markedly enhanced AAO production (Okamoto et al. 2002) at 30°C for 18 days.

Enzyme assay

Aryl alcohol oxidase activity was assayed spectrophotometrically using veratryl alcohol as a substrate. The reaction mixture of the standard assay contained 1 mM veratryl alcohol, 50 mM potassium phosphate buffer (pH 6.0) (buffer A), and a suitable enzyme in a total volume of 1.0 ml. Oxidation of the substrate at 30°C was monitored based on an absorbance increase at 310 nm resulting from the formation of veratraldehyde (ε_{310} = 9300 $M^{-1}cm^{-1}$). One unit of the enzyme activity was defined as the amount of enzyme required to produce 1 μmol of veratraldehyde per minute under the assay conditions.

Purification of the enzymes

Purification of aryl alcohol oxidase was conducted at 4°C. Proteins in 4.6 l of culture filtrate were precipitated by adding ammonium sulfate up to 80% saturation. The precipitate was collected by centrifugation (51 200 *g* × 20 min) and dissolved in buffer A. The solution was dialyzed against buffer A overnight and loaded onto a diethylaminoethyl (DEAE)-Toyopearl 650M column (3.2 × 43 cm; Tosoh, Tokyo, Japan) previously equilibrated with buffer A. The column was washed with buffer A and eluted with 1000 ml of a linear gradient of 0–1.0 M NaCl in buffer A at a flow rate of 45 ml/h. Two peaks exhibiting enzyme activity (D1 and D2) were separated in this step. Each fraction exhibiting AAO activity was collected.

First, ammonium sulfate was added to active fraction D1 to a final concentration of 30% saturation, and then the solution was loaded onto a butyl-Toyopearl 650M column (1.6 cm × 25 cm; Tosoh) previously equilibrated with buffer A, containing 30% saturation of ammonium sulfate (buffer B). The column was washed with buffer B and eluted with 400 ml of a linear gradient of 30%–0% ammonium sulfate in buffer A at a flow rate of 30 ml/h. Active fractions were pooled and concentrated to 5 ml by ultrafiltration with Centriprep YM-3 (3-kDa cutoff; Millipore, Bedford, MA, USA). The concentrated supernatant was loaded onto a Sephacryl S-100 column (1.6 × 60 cm; Amersham Pharmacia, Uppsala, Sweden), previously equilibrated with buffer A containing 100 mM NaCl in a Pharmacia standard liquid chromatography (P.S.L.C.) system (Amersham Pharmacia). The elution with buffer A was carried out repeatedly at a flow rate of 30 ml/h. A total of 40 ml of active fractions was collected and was then concentrated to 2 ml with Centricon YM-3 (3-kDa cutoff; Millipore). This purified enzyme was named AAO I.

Second, ammonium sulfate was added to active fraction D2 to a final concentration of 30% saturation, and then the solution was loaded onto a butyl-Toyopearl 650M column (1.6 × 25 cm) previously equilibrated with buffer A, containing 30% saturation of ammonium sulfate (buffer B). The column was washed with buffer B and eluted with 400 ml of a linear gradient of 30%–0% ammonium sulfate in buffer A at a flow rate of 30 ml/h. This step resolved the activity into two peaks, D2-1 and D2-2. Active fractions corresponding to D2-1 were pooled and dialyzed against buffer A for 24 h and then loaded onto a DEAE-Toyopearl 650M column (2 × 44 cm; Tosoh) previously equilibrated with buffer A. The column was washed with buffer A and eluted with 200 ml of a linear gradient of 0–1.0 M NaCl in buffer A at a flow rate of 25 ml/h, and 19 ml of active fractions was collected; this was concentrated to 5 ml by ultrafiltration with Centriprep YM-3. The concentrated supernatant was loaded onto a Sephacryl S-100 column that was previously equilibrated with buffer A containing 100 mM NaCl in a P.S.L.C system. The elution with buffer A was carried out repeatedly at a flow rate of 30 ml/h. A total of 15 ml of active fractions was collected and then concentrated to 2 ml with Centricon YM-3. This purified enzyme was named AAO II. Next, active fractions corresponding to D2-2 were pooled and dialyzed against buffer A for 24 h and then loaded onto a DEAE-Toyopearl 650M column (2 × 44 cm) previously equilibrated with buffer A. The column was washed with buffer A and eluted with 200 ml of a linear gradient of 0–1.0 M NaCl in buffer A at a flow rate of 25 ml/h, and 17 ml of active fractions was collected; this was concentrated to 5 ml by ultrafiltration with Centriprep YM-3. The concentrated supernatant was loaded onto a Sephacryl S-100 column that was previously equilibrated with buffer A containing 100 mM NaCl in a P.S.L.C system. The elution with buffer A was carried out repeatedly at a flow rate of 30 ml/h. A total of 12 ml active fractions was collected and concentrated to 2 ml with Centricon YM-3. This purified enzyme was named AAO III.

Protein assay

Protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as the standard. During aryl alcohol oxidase purification after the first anion-exchange chromatography step, protein concentration was monitored based on the absorbance at 280 nm.

Molecular mass measurement

The molecular mass of the purified enzymes was estimated by gel filtration chromatography and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gel filtration chromatography was performed on a Sephacryl S-100 column (P.S.L.C. system; Amersham Pharmacia) using a low molecular weight protein standard calibration kit (Amersham Pharmacia). SDS-PAGE was carried out with a 10% gel, as described by Laemmli (1970). Proteins were stained with Quick CBB (Wako, Osaka, Japan).

Isoelectric focusing

The isoelectric point (pI) of the purified enzymes was estimated by isoelectric focusing using an Ampholine PAG plate, pH 4.0–6.5 (Amersham Pharmacia).

N-terminal amino acid sequence

The N-terminal amino acid sequence of the purified enzymes was determined using a protein sequencer PPSQ-10 (Shimadzu, Kyoto, Japan).

Results and discussion

Purification and some properties of AAOs

AAO was purified from 4.6 l of filtrate of an 18-day-old culture with the maximum activity. The first anion-exchange chromatography resolved the activity into two peaks. Moreover, the second anion-exchange chromatogra-

Table 1. Summary of purification of aryl alcohol oxidase (AAO) I (A), AAO II (B), and AAO III (C) from *Pleurotus ostreatus* K16-2

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (-fold)	Yield (%)
AAO I					
Culture filtrate	1440	853	1.7	–	100
Ammonium sulfate	1070	382	2.8	1.6	74.3
1st DEAE-Toyopearl active fraction D1	280	12.4	22.6	13.3	19.4
Butyl-Toyopearl	172	2.9	59.3	34.9	11.9
Sephacryl	30.1	0.5	60.2	35.4	2.1
AAO II					
Culture filtrate	1440	853	1.7	–	100
Ammonium sulfate	1070	382	2.8	1.6	74.3
1st DEAE-Toyopearl active fraction D2	545	107	5.1	3.0	37.8
Butyl-Toyopearl	409	14.6	28.0	16.5	28.4
2nd DEAE-Toyopearl active fraction D2-1	220	3.5	62.9	37.0	15.2
Sephacryl	31.8	0.5	63.6	37.4	2.2
AAO III					
Culture filtrate	1440	853	1.7	–	100
Ammonium sulfate	1070	382	2.8	1.6	74.3
1st DEAE-Toyopearl active fraction D2	545	107	5.1	3.0	37.8
Butyl-Toyopearl	409	14.6	28.0	16.5	28.4
2nd DEAE-Toyopearl active fraction D2-2	66.6	3.0	22.2	13.1	4.6
Sephacryl	8.3	0.2	41.5	24.4	0.6

U, units; DEAE, diethylaminoethyl

phy resolved the activity into two more peaks. Three enzymes, AAOs I, II, and III, were obtained in these purification steps. Bourbonnais and Paice (1988) also reported that veratryl alcohol oxidase (VAO) from *P. sajor-caju* produced two isozymes that slightly differed in a few properties. Taken together, the results clearly suggest that several AAO isozymes from *P. ostreatus* are excreted extracellularly. Table 1 summarizes the purification steps. The specific activities of the purified AAOs I, II, and III were 60.2, 63.6, and 41.5 U/mg of protein, respectively. It is assumed that both AAOs I and II differ from AAO from *P. eryngii* (79.5 U/mg), whereas AAO III is relatively similar to VAOs I and II from *P. sajor-caju* (39.8 and 36.4 U/mg, respectively). The molecular mass of each native AAO was estimated to be 70 kDa by gel filtration chromatography. On SDS-PAGE, each purified AAO yielded a single protein band (Fig. 1), and the molecular mass of each AAO was estimated to be 72 kDa. These results indicate that AAOs I, II, and III from *P. ostreatus* are monomeric in structure. AAOs I, II, and III showed absorption peaks at 370 and 450 nm, indicating that these enzymes contain flavin as the prosthetic group, similarly to others (Bourbonnais and Paice 1988; Guillén et al. 1992; Asada et al. 1995).

The enzyme reaction for investigating the optimum pH was conducted at various pHs (using 50 mM McIlvaine's buffer for pH 3–6 and 50 mM potassium phosphate buffer for pH 6–8) for 30 min. On the other hand, the enzyme reaction for investigating optimum temperature was performed using 50 mM potassium phosphate buffer (pH 6.0) at various temperatures for 30 min. The optimum temperature was 50°C, but their optimum pHs differed slightly, i.e., pH 5 for AAOs I and II and pH 6 for AAO III (Fig. 2). It was considered that this observation may be related to their isoelectric points. Then, the isoelectric points were examined by isoelectric focusing polyacrylamide gel electrophoresis (IEF-PAGE) using an Ampholine PAG plate (pH 3.5–9.5; Pharmacia). The isoelectric points tended to differ from each other; i.e., the values of AAOs I, II, and III decreased slightly in that order near pI 3.5 (data not shown). Although their isoelectric points have not been determined

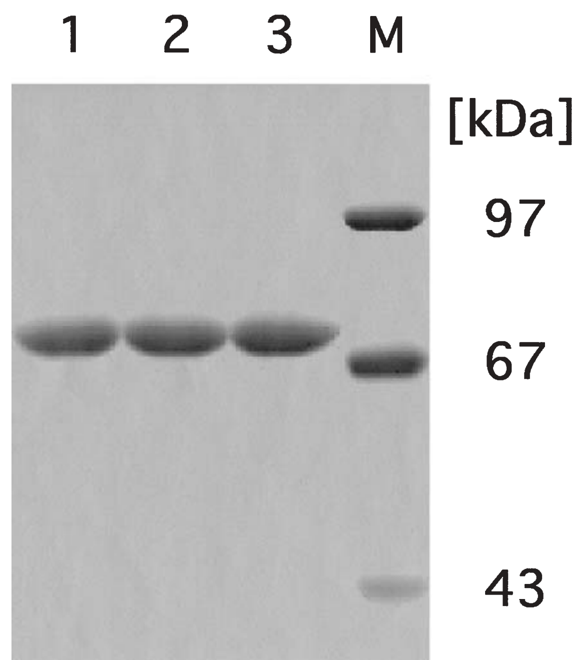

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the purified aryl alcohol oxidases (AAOs) I, II, and III. Purified AAOs I, II, and III were subjected to SDS-PAGE, followed by staining with Coomassie brilliant blue R-250. *Lane 1*, AAO I; *lane 2*, AAO II; *lane 3*, AAO III; *M*, molecular weight markers (phosphorylase, 97 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa

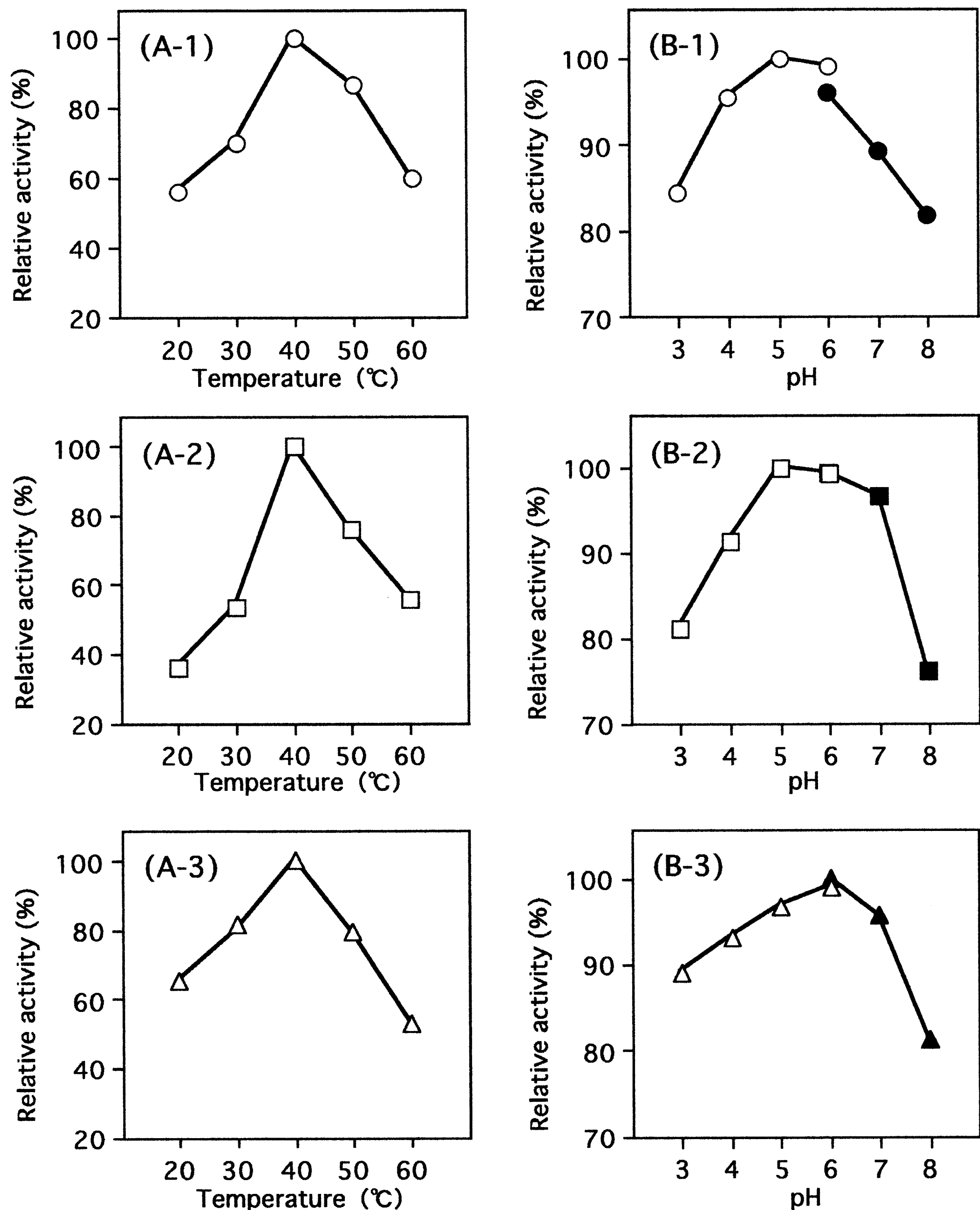

Fig. 2. Effects of temperature (**A**) and pH (**B**) on AAO activity. **A** AAO activity was measured at the indicated temperatures at pH 6.0. *A-1*, AAO I; *A-2*, AAO II; *A-3*, AAO III. **B** The activity was measured at the indicated pH at 30°C. *B-1*, AAO I; *B-2*, AAO II; *B-3*, AAO III. *Open symbols* and *closed symbols* indicate 50 mM McIlvaine's buffer (pH 3.0–6.0) and 50 mM potassium phosphate buffer (pH 6.0–8.0), respectively. The highest activity was designated 100% for both **A** and **B**

Table 2. Comparison of major properties of AAOs from basidiomycetes

Strain	Molecular weight (kDa)	Optimum temperature (°C)	Optimum pH	N-terminal amino acid sequence
Pleurotus ostreatus K16-2				
AAO I	72	40	5.0	Ala-Asp-Lys-Asp-Tyr-Ile-Val-Val-Gly-Ala
AAO II	72	40	5.0	Ala-Asp-Lys-Asp-Tyr-Ile-Val-Val-Gly-Ala
AAO III	72	40	6.0	Ala-Asp-Lys-Asp-Tyr-Ile-Val-Val-Gly-Ala
P. sajor-caju[a]				
VAO I	71	–	5.5	–
VAO II	71	–	5.0	–
P. ostreatus Florida[b]				
VAO	72.5	–	6.5	Lys-Pro-Thr-Ala-Asp-Phe-Asp-Tyr-Ile-Val
P. eryngii[c]				
AAO	72.6	55	5.0	Ala-Asp-Phe-Asp-Tyr-Val-Val-Val-Gly-Ala
Phanerochaete chrysosporium[d]				
AAO	78	45	6.0–7.0	–

[a] Bourbonnais and Paice (1988)
[b] Sannia et al. (1991)
[c] Guillén et al. (1992)
[d] Asada et al. (1995)

accurately to date, this result on IEF-PAGE helps account for the separation of AAOs I, II, and III on anion-exchange chromatography.

N-terminal analysis and comparison with other AAOs

In Table 2, the major properties of AAOs from basidiomycetes are compared. The properties of AAOs I, II, and III purified from *P. ostreatus* differed slightly from other AAOs in a few points. Comparison of the N-terminal amino acid sequence of the *P. ostreatus* AAOs I, II, and III with those of *P. eryngii* (Guillén et al. 1992) and *P. ostreatus* Florida (Sannia et al. 1991) showed 80% and 60% similarity, respectively, in the first ten residues.

Here, we have described the purification and the major properties of AAO isozymes. This is the first report on the production of AAO isozymes by *P. ostreatus*. No difference in the N-terminal amino acid sequence among AAOs I, II, and III was found. The differences in their elution pattern in anion-exchange chromatography and their optimum pH may be due to some charged amino acid residues locating in a position other than N-terminal. There is also room for further investigation of some properties, such as substrate specificity and kinetic constants, of AAOs I, II, and III. We are now cloning a gene encoding AAO isozymes from *P. ostreatus* as the next step to clarify the biosynthetic mechanism of *p*-anisaldehyde.

Acknowledgment This work was supported in part by a Grant-in-Aid for the Encouragement of Young Scientists (no. 12760117) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

References

Asada Y, Watanabe A, Ohtsu Y, Kuwahara M (1995) Purification and characterization of an aryl-alcohol oxidase from lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Biosci Biotechnol Biochem 59:1339–1341

Bourbonnais R, Paice MG (1988) Veratryl alcohol oxidases from the lignin-degrading basidiomycete *Pleurotus sajor-caju*. Biochem J 255:445–450

Guillén F, Martínez AT, Martínez MJ (1990) Production of hydrogen peroxide by aryl-alcohol oxidase from the ligninolytic fungus *Pleurotus eryngii*. Appl Microbiol Biotechnol 32:465–469

Guillén F, Martínez AT, Martínez MJ (1992) Substrate specificity and properties of aryl-alcohol oxidase from the ligninolytic fungus *Pleurotus eryngii*. Eur J Biochem 209:603–611

Guillén F, Martínez AT, Martínez MJ, Evans CS (1994) Hydrogen-peroxide-producing system of *Pleurotus eryngii* involving the extracellular enzyme aryl-alcohol oxidase. Appl Microbiol Biotechnol 41:465–470

Gutiérrez A, Caramelo L, Prieto A, Martínez MJ, Martínez AT (1994) Anisaldehyde production and aryl-alcohol oxidase and dehydrogenase activities in ligninolytic fungi of the genus *Pleurotus*. Appl Environ Microbiol 60:1783–1788

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond) 227:680–685

Lowry OH, Rosebrough NJ, Farr AL, Randall RL (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275

Okamoto K, Narayama S, Katsuo A, Shigematsu I, Yanase H (2002) Biosynthesis of *p*-anisaldehyde by the white-rot basidiomycete *Pleurotus ostreatus*. J Biosci Bioeng 93:207–210

Sannia G, Limongi P, Cocca E, Buonocore F, Nitti G, Giardina P (1991) Purification and characterization of a veratryl alcohol oxidase enzyme from the lignin degrading basidiomycete *Pleurotus ostreatus*. Biochim Biophys Acta 1073:114–119