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Purification and Properties of Flavin- and Molybdenum-Containing Aldehyde Oxidase from Coleoptiles of Maize[1]

Tomokazu Koshiba*, Eriko Saito, Naoki Ono, Naoki Yamamoto, and Mitsuhiko Satô

Department of Biology, Tokyo Metropolitan University, Hachioji-shi, Tokyo 192–03, Japan (T.K., E.S., N.O., M.S.); and National Institute of Agrobiological Resources, Tsukuba 305, Japan (N.Y.)

Aldehyde oxidase (AO; EC 1.2.3.1) that could oxidize indole-3-acetaldehyde into indole-3-acetic acid was purified approximately 2000-fold from coleoptiles of 3-d-old maize (*Zea mays* L.) seedlings. The apparent molecular mass of the native enzyme was about 300 kD as estimated by gel-filtration column chromatography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed that the enzyme was composed of 150-kD subunits. It contained flavin adenine dinucleotide, iron, and molybdenum as prosthetic groups and had absorption peaks in the visible region (300–600 nm). To our knowledge, this is the first demonstration of the presence of flavin adenine dinucleotide and metals in plant AO. Other aromatic aldehydes such as indole-3-aldehyde and benzaldehyde also served as good substrates, but *N*-methylnicotinamide, a good substrate for animal AO, was not oxidized. 2-Mercaptoethanol, *p*-chloromercuribenzoate, and iodoacetate partially inhibited the activity, but well-known inhibitors of animal AO, such as menadione and estradiol, caused no reduction in activity. These results indicate that, although maize AO is similar to animal enzymes in molecular mass and cofactor components, it differs in substrate specificity and susceptibility to inhibitors. Immunoblotting analysis with mouse polyclonal antibodies raised against the purified maize AO showed that the enzyme was relatively rich in the apical region of maize coleoptiles. The possible role of this enzyme is discussed in relation to phytohormone biosynthesis in plants.

AO (EC 1.2.3.1) has been extensively investigated in animals and microorganisms. The enzyme catalyzes the oxidation of a variety of aldehydes and N_2-containing heterocycles in the presence of O_2 or certain redox dyes (Rajagopalan and Handler, 1966; Hall and Krenitsky, 1986). The enzyme has also been reported to reduce diphenyl sulfoxides (Yoshihara and Tatsumi, 1986), aromatic heterocyclic compounds (Bauer and Howard, 1991), and oximes (Tatsumi and Ishigai, 1987). AO is similar to xanthine dehydrogenase (oxidase) in being a multicomponent enzyme that contains a molybdenum cofactor, nonheme iron, and FAD as prosthetic groups. The enzyme is found in the small intestine and liver of animals and has been implicated in the detoxification of various xenobiotics, including certain cancer chemotherapeutic agents (Bauer and Howard, 1991; Stoddart and Levine, 1992; Hirao et al., 1994). The enzyme may also play a role in retinoic acid synthesis (Tomita et al., 1993; Huang and Ichikawa, 1994) and in the degradation of aromatic aldehydes formed as a result of lignin breakdown by a soil bacterium (Crawford et al., 1982) and snails (Large and Connock, 1994).

However, in plants only a limited amount of information has been published concerning this enzyme, including studies of *Avena* coleoptiles (Rajagopal, 1971), potato tubers (Rothe, 1974), cucumber seedlings (Bower et al., 1978), and pea seedlings (Miyata et al., 1981). Much attention has been focused on this enzyme because of its possible involvement in IAA synthesis. An AO, tentatively designated IAAld oxidase, could catalyze the oxidation of IAAld to form IAA (Rajagopal, 1971; Bower et al., 1978; Miyata et al., 1981), but the actual function of the enzyme in IAA biosynthesis is not currently known. In our previous study using [^{14}C]Trp as a tracer, we suggested that in maize (*Zea mays* L.) coleoptiles a major portion of IAA was synthesized from Trp (Koshiba et al., 1995). We also found that an in vitro system of maize coleoptile extracts could catalyze the production of IAA from Trp and the IAA-forming activity was co-purified with an IAAld oxidase (Koshiba and Matsuyama, 1993). These results suggested that IAAld oxidase was probably involved in IAA biosynthesis in maize coleoptiles, but this remains to be demonstrated more conclusively. A better understanding of the function and properties of this enzyme requires experiments with highly purified enzymes. In this paper, we describe the molecular properties and kinetics of AO that was highly purified from maize coleoptiles.

MATERIALS AND METHODS

Plant Material

Seeds of maize (*Zea mays* L. cv Golden Cross Bantam 70) were soaked in running tap water overnight. The seeds were germinated on layers of wet paper towels at 25°C under red light (0.8 W m^{-2}) for 2 d and then in darkness for

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* Corresponding author; e-mail koshiba-tomokazu@c.metro-u.ac.jp; fax 81–426–2559.

Abbreviations: AO, aldehyde oxidase; DCIP, 2,6-dichloroindophenol; IAAld, indole-3-acetaldehyde; mAMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide; MTT, 3(4, 5-dimethylthiazolyl-2)2,5-diphenyltetrazolium-bromide.

an additional 1 d. Coleoptiles (about 1.5 cm long) were harvested under a green safety light, frozen with liquid N_2, and stored at −30°C. The red and green lights were as described previously (Koshiba et al., 1995).

Enzyme Extraction and Purification

Extraction and purification of the enzyme were carried out at 0 to 4°C. The frozen coleoptile tips (about 200 g for one series of purification) were homogenized in 400 mL of 0.1 M potassium phosphate buffer, pH 7.4, with a Physcotron (equipped with a 20-mm-diameter knife; Nichion-Irika, Funabashi, Japan) operated at about 20,000 rpm with intermittent pauses to avoid heating the samples. After centrifugation of the homogenate at 12,000*g* for 30 min, proteins in the supernatant (crude extract) were fractionated with ammonium sulfate. The fraction that precipitated between 30 and 50% saturation was dialyzed against 20 mM Tris-HCl buffer, pH 8.0. The dialyzed sample was centrifuged at 20,000*g* for 20 min, and the supernatant was passed through a cellulose acetate filter (0.45-μm pore size; Advantec, Tokyo, Japan). The filtrate was applied to a DEAE column (DEAE-Toyopearl 650M, 23 mm i.d. × 200 mm; Tosoh, Tokyo, Japan) equilibrated with the Tris-HCl buffer. After the column had been washed with 150 mL of the buffer, the adsorbed proteins were eluted with a linear gradient of KCl from 0 to 1 M in the Tris-HCl buffer at a flow rate of 5 mL min^{-1}. The elution of proteins was monitored at 280 nm, and the activity of AO was assayed with IAAld as a substrate by determining the amount of IAA formed as described below. One major peak of activity was eluted at approximately 0.3 M KCl. The active fractions were pooled and the enzyme was precipitated with 60% saturation of ammonium sulfate. The precipitate was dissolved in a small volume of 50 mM potassium phosphate buffer, pH 7.5, and the solution was dialyzed against the phosphate buffer containing 12.5% (w/v) ammonium sulfate for 60 min. The enzyme solution was centrifuged at 25,000*g* for 15 min, and the supernatant was applied to a hydrophobic column (Phenyl-5PW, 8 mm i.d. × 75 mm; Tosoh) equilibrated with 50 mM phosphate buffer containing 12.5% ammonium sulfate. After the column had been washed with the salt-containing buffer, the adsorbed proteins were eluted with a linear gradient of ammonium sulfate from 12.5 to 0% in the phosphate buffer at a flow rate of 1 mL min^{-1}. Fractions having IAAld oxidase activity were pooled. The combined fractions were concentrated by ultrafiltration (UFC-C3LGC; Millipore), and the buffer was changed to 10 mM potassium phosphate buffer, pH 6.8, containing 1 mM $MgCl_2$. The sample was then applied to a hydroxyapatite column (HA-1000, 7.8 mm i.d. × 75 mm; Tosoh) equilibrated with the phosphate buffer. A linear gradient of potassium phosphate from 10 to 500 mM was applied to the column at a flow rate of 0.8 mL min^{-1}. A major peak and a small shoulder of AO activity were eluted (Fig. 1A). The fractions of the major activity peak were concentrated, and the buffer was changed to 20 mM Tris-HCl buffer, pH 8.0, by ultrafiltration as described above. The enzyme was applied to a DEAE column (DEAE-5PW, 6 mm i.d. × 50 mm; Tosoh) equilibrated with the Tris buffer. The adsorbed proteins were eluted with a linear gradient of KCl from 0 to 0.5 M in the buffer at a flow rate of 0.7 mL min^{-1}. The AO activity was recovered in the second major protein fraction eluted at approximately 0.3 M KCl (Fig. 1B). In experiments in which FAD or metal content were to be measured and antibodies were to be prepared, the enzyme was further purified by gel-filtration column chromatography (G3000SWXL, 8 mm i.d. × 300 mm; Tosoh) with an isocratic solvent system of 50 mM potassium phosphate buffer, pH 6.8, containing 0.25 M KCl at a flow rate of 0.8 mL.min^{-1}. One major protein peak having AO activity was eluted with a molecular mass of approximately 300 kD, determined by reference to a calibration based on proteins of known molecular mass (Fig. 1C).

The protein concentration of samples from each step was measured by using a protein assay kit from Bio-Rad or estimating the A_{280}.

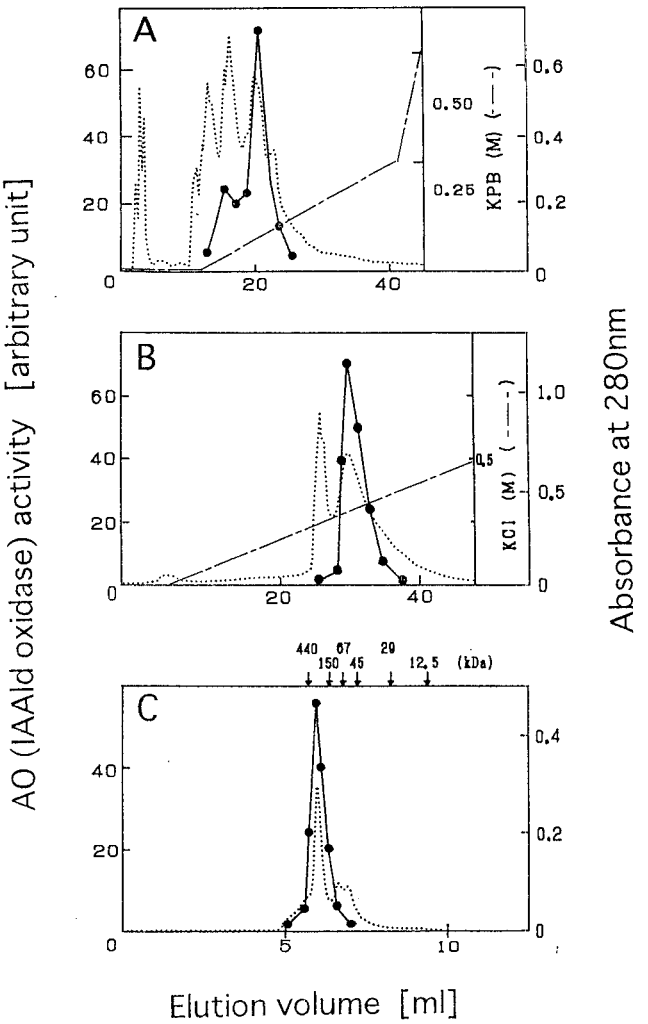

Figure 1. Elution profiles of proteins and AO (IAAld oxidase) activity from HA-1000 (A) and DEAE-5PW (B) columns. In some experiments, AO was further purified by G3000SWXL column chromatography (C). AO activity (●) was assayed by determining the amount of IAA formed from IAAld by using HPLC, and proteins (·····) were monitored by A_{280}. Numbers with arrows in C indicate the positions of molecular markers: 440 kD, ferritin; 150 kD, alcohol dehydrogenase; 67 kD, BSA; 45 kD, ovalbumin; 29 kD, carbonic anhydrase; and 12.5 kD, Cyt *c*. KPB, Potassium phosphate buffer.

Enzyme Assay

The substrate specificity of AO was investigated by using several aromatic aldehydes and determining the amount of reaction product formed in the presence of O_2 as an electron donor. Each product from the corresponding aldehyde (indole-3-aldehyde, indole-3-carboxylic acid; IAAld, IAA; benzaldehyde, benzoic acid; and protocatechualdehyde, protocatechuic acid) was detected and quantified with reverse-phased HPLC. Reaction mixtures (100 μL) contained 5 to 50 μL of enzyme solution, 0.1 mM substrate, and 0.1 M potassium phosphate buffer, pH 7.4. The reaction was performed at 30°C for 1 to 30 min (a 30-min reaction period was routinely used for an approximate estimation of activity in the fractions from column chromatography; whereas a shorter reaction time was used for other studies). After incubation, the reaction was stopped by adding 8 μL of 1 N HCl and 50 μL of 100% methanol, successively. When IAAld was used as a substrate, 5 μL of 2 M $NaHSO_3$ were added before the addition of methanol, and the mixture was left for 2 min at room temperature. This treatment resulted in the formation of a bisulfite salt of IAAld, which can be completely separated from IAA by HPLC (Koshiba and Matsuyama, 1993). The mixture was then centrifuged, and a portion of the supernatant (100 μL) was subjected to HPLC with an ODS C_{18} column by essentially the same method described previously (Koshiba and Matsuyama, 1993). The indole compounds were monitored at 280 nm, and the amount of the product was determined from its peak area with a calibration curve for each product. One unit of activity was defined as the activity required to produce 1 nmol product min^{-1}.

The substrate specificity of AO for aromatic and aliphatic aldehydes and for other substrates was measured using DCIP as an electron donor according to the method of Courtright (1967), by monitoring the decrease in A_{600} (16.1 mM^{-1} cm^{-1}) with a double-beam photometer (U-2000; Hitachi, Tokyo, Japan). The reaction mixture (150 μL) contained 5 to 10 μL of an enzyme solution, 0.1 M phosphate buffer, pH 7.4, 0.002% DCIP, 0.1 mM phenazine methosulfate, and a desired amount of substrate. One unit of activity was defined as the activity required to reduce 1 μmol DCIP min^{-1}. This assay method cannot be used for crude extracts or ammonium sulfate fractions.

For determination of kinetic parameters, assays were conducted with 1 μM to 500 mM substrate for 1 to 5 min, during which time the reaction rates remained constant.

FAD and Metal Analysis

The isolation and identification of flavin from purified AO were performed by the method of Stuehr et al. (1991) with some modifications. A portion of the purified AO was boiled for 7 min to release noncovalently bound FAD, and the sample was deproteinized by filtration through an Ultra-Filter UFC3-LGC (5000 NMWL; Millipore). FAD was analyzed by HPLC (ODS-C_{18} column) equipped with an Hitachi 650–10S flow-through cell fluorometer with an isocratic system of 5 mM ammonium acetate buffer, pH 6.0, in 25% methanol at a flow rate of 1 mL min^{-1}. The fluorometer was set at 460 nm for excitation, and the relative fluorescence at 530 nm was measured. Under these conditions, FAD and FMN standards were completely resolved. The amount of FAD was determined by measuring the peak area relative to standard curves of authentic FAD. Further confirmation of the identity of HPLC-purified fluorophore was carried out by analyzing its excitation-emission spectra with an RF-5000 fluorometer (Shimazu, Kyoto, Japan).

The molybdenum and iron contents were determined according to the method described by Triplett et al. (1982) on a Perkin-Elmer model 4100ZL atomic absorption spectrophotometer. Samples of purified AO were directly injected into the graphite furnace, and the amounts of molybdenum and iron were estimated using the standard curves of authentic molybdenum and iron. The wavelengths for molybdenum and iron were set at 313.3 and 248.3 nm, respectively.

Preparation of Anti-AO Mouse Antibodies

Since it was difficult to obtain the purified enzyme in large quantities, we prepared antibodies from mice. About 15 μg of purified enzyme were emulsified with Freund's complete adjuvant and injected into the abdominal cavity of a mouse (BALB/c AnNCrj, female). Three additional injections were made at 2-week intervals. One week after the last injection, antiserum was collected and the production of anti-AO antibodies was checked by immunoblotting. The binding of the antibodies to AO was tested by immunoprecipitation using protein-A-Sepharose CL-4B (Pharmacia). Almost all of the activity in the enzyme solution was removed when the antibodies and Sepharose were added, whereas all of the activity was left in the supernatant when nonimmune mouse serum was used.

Native PAGE and Activity Stain

Native PAGE was performed with a 7.5% acrylamide gel in Laemmli's systems (Laemmli, 1970) in the absence of SDS at 4°C. After electrophoresis, the gel was immersed in 0.1 M potassium phosphate buffer, pH 7.5, for 5 min, and then the activity of AO was developed in a reaction mixture containing 0.1 M phosphate buffer, pH 7.4, 1 mM substrate, 0.1 mM phenazine methosulfate, and 0.4 mM MTT at room temperature (about 25°C). When IAAld was used as a substrate, the gel instantly became dark purple. Therefore, we used benzaldehyde for this experiment.

SDS-PAGE and Immunoblotting

SDS-PAGE was carried out by the method of Laemmli (1970) in a 5 to 20% polyacrylamide gradient gel. Immunoblotting was performed using a Vectastain Elite ABC kit (Vector, Burlingame, CA) with primary antibodies (anti-AO mouse serum) diluted 1000-fold in PBS and secondary antibody (biotinylated horse anti-mouse IgG; Funakoshi, Tokyo, Japan) also diluted 1000-fold in PBS. The secondary antibody had previously been conjugated with horseradish peroxidase bound to avidin. Peroxidase activity was visu-

alized by staining with an immunostain HRP-1000 kit (Konica, Tokyo, Japan).

Chemicals

Free IAAld was prepared from IAAld bisulfite (Sigma) according to the method described by Bower et al. (1978). mAMSA was kindly supplied by the National Cancer Institute Chemotherapeutic Agents Repository (Bethesda, MD).

RESULTS

AO Purification

AO was purified from 200 g of maize coleoptiles for each purification series. Typical results are summarized in Table I. The activity was assayed by measuring the oxidation of IAAld to IAA using HPLC. During purification only one major peak of activity was detected following DEAE and Phenyl-5PW column chromatography, but a second, small peak of activity was found in the eluate from the hydroxyapatite column (Fig. 1A). Native PAGE and immunoblotting showed that the enzyme of the second peak was a modified type of major AO artificially formed during purification (data not shown). After chromatography on the DEAE-5PW column (Fig. 1B), 90 μg of purified AO at a purification factor of 1950 were obtained. The purified enzyme had a specific activity of 137 nmol min^{-1} mg^{-1} protein. The activity of this enzyme preparation was stable in 20 mM Tris-HCl buffer, pH 8.0, at −30°C.

Molecular Properties

Gel-filtration column chromatography of the purified enzyme following DEAE-5PW column chromatography indicated that the apparent molecular mass of the native enzyme was about 300 kD (Figs. 1–3). After native PAGE was performed with the DEAE-5PW column-purified sample, only a single band with activity was detected (Fig. 2A), which corresponded to one protein band detected by silver stains (Fig. 2B). After SDS-PAGE (Fig. 2C), a major polypeptide band of 150 kD (band a) was observed along with an 85-kD polypeptide (band b) and some other faint bands. These minor bands appeared even when the single protein band detected on a native polyacrylamide gel was cut out and subjected to SDS-PAGE. They could not be removed by further gel-filtration chromatography or by rechromatography on DEAE-5PW or HA-1000 columns. However, the amounts of these polypeptides differed depending on the purification procedures followed. In particular, the 85-kD polypeptide appeared in larger amounts in preparations that had lost some activity during storage at 4°C. It is thus likely that the purified AO preparation had undergone proteolytic degradation during purification. A similar type of proteolysis was found for AO from pig liver (Yoshihara and Tatsumi, 1985) and rabbit liver (Stell et al., 1989) and also for xanthine oxidase from human liver (Krenitsky et al., 1986).

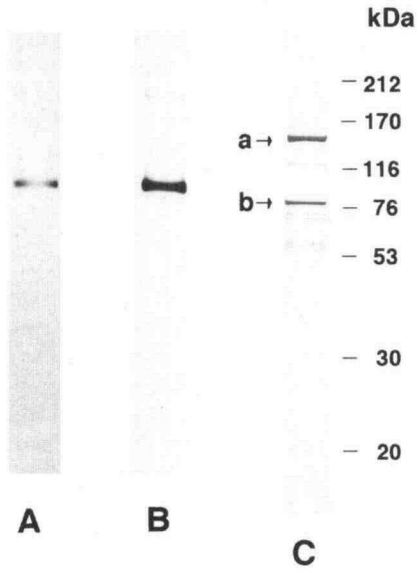

Figure 2. Native and SDS-polyacrylamide gels loaded with purified maize AO. Following electrophoresis, activity or silver staining was carried out as described in "Materials and Methods." A, Activity stain of a native polyacrylamide gel with benzaldehyde as a substrate. B, Silver stain of a native polyacrylamide gel. C, Silver stain of SDS-polyacrylamide gel with positions of molecular markers.

The absorption spectra of the purified maize AO are shown in Figure 3. The oxidized enzyme had a broad absorption peak between 400 and 500 nm and shoulders at approximately 340 and 540 nm, in addition to the peak due to protein at 280 nm. The ratio of A_{280}/A_{450}, which indicates the ratio of protein to flavin, was 6.0. Similar ratios were reported for hog liver AO (5.3–5.8) (Felsted et al.,

Table I. *Purification of AO (IAAld oxidase) from maize coleoptiles*

The results presented are for a typical purification starting from 200 g (fresh weight) of maize coleoptiles. The activity was assayed by determining the amount of IAA formed from IAAld with HPLC.

Purification Step	Protein	IAAld Oxidase Activity	Specific Activity	Purification
	mg	*units*[a] (%)	*units* mg^{-1} *protein*	*-fold*
Crude extract	1873	138 (100)	0.07	1
30–50% $(NH_4)_2SO_4$	735	153 (111)	0.21	3
DEAE-Toyopearl	52.0	97.7 (70.8)	1.88	26.9
Phenyl-5PW	15.7	75.3 (54.6)	4.80	68.6
HA-1000	0.40	23.4 (17.0)	58.5	836
DEAE-5PW	0.09	12.3 (8.9)	137	1950

[a] One unit of enzyme activity was defined as the activity required to produce 1 nmol IAA min^{-1}.

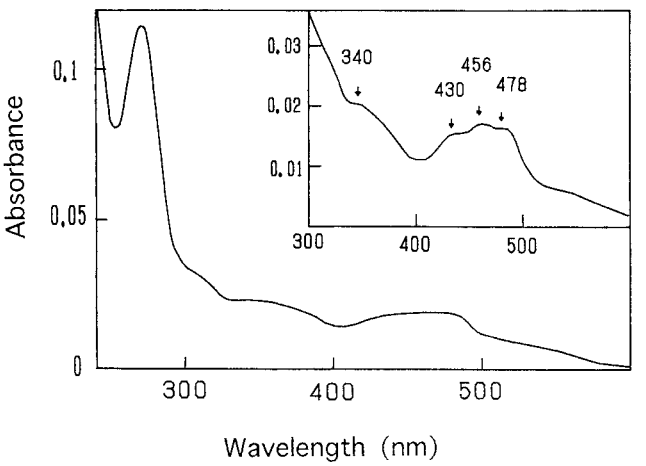

Figure 3. Absorption spectrum of the purified maize AO. The inset shows an expanded view of the absorption spectrum from between 300 and 600 nm. Arrows and numbers indicate the positions and wavelengths of peak or shoulder.

1973) and guinea pig liver AO (5.88) (Yoshihara and Tatsumi, 1985). The A_{450}/A_{550} ratio of 2.9 observed for maize AO was similar to that reported for guinea pig AO (2.55). Upon heat denaturation, AO released FAD, which had the same retention time and elution profile on HPLC as authentic FAD. In addition, the fluorescence excitation-emission spectra of the peak fractions of FAD released from AO were the same as those for authentic FAD (data not shown). FAD content was estimated in two different enzyme preparations with similar results of about 5.0 ng mg^{-1} protein, which is approximately what would be expected if the enzyme contained 2 mol FAD mol^{-1} native enzyme. This value is in good agreement with that of animal AOs. The presence of iron and molybdenum in purified enzyme preparations was confirmed using atomic absorption spectrophotometry. Obtaining an accurate determination of the metal content of the enzyme was difficult because of the requirement for a large amount of pure enzyme in an intact state. In addition, quantitation of the purified enzyme is unreliable using 280-nm absorption methods. However, there was at least 0.67 ng iron and 0.15 ng molybdenum mg^{-1} protein (two determinations from one protein preparation). This approximates a molecular ratio of iron:molybdenum of 7:1. In animal AO, a ratio of 8:2 is expected, suggesting the possibility of molybdenum being removed from maize AO during purification. No attempt was made to detect molybdopterine in the purified enzyme preparation.

These molecular properties are similar to those reported for several animal AOs (Rajagopalan et al., 1962; Felsted et al., 1973; Andres, 1976; Yoshihara and Tatsumi, 1985; Stell et al., 1989), animal xanthine dehydrogenases (oxidases) (Andres, 1976; Krenitsky et al., 1986), and soybean nodule xanthine dehydrogenase (Triplett et al., 1982).

Substrate Specificity

The enzyme had a relatively broad substrate specificity with respect to aldehydes. The kinetics of the reaction between maize AO and five aromatic aldehydes, three aliphatic aldehydes, *N*-methylnicotinamide, and xanthine were studied (Table II). Of these substrates, IAAld, indole-3-aldehyde, benzaldehyde, protocatechualdehyde, and butyraldehyde were used very efficiently by the enzyme. However, the enzyme was less active with either phenylacetaldehyde or acetaldehyde, and *N*-methylnicotinamide, a good substrate for animal AO, was not oxidized. The fact that the purine does not serve as a substrate indicates that maize AO has some differences from animal AOs. Similar results have been reported for a bacterial (Turner et al., 1987) and a gastropod (Large and Connock, 1994) AO. Xanthine, a good substrate of xanthine dehydrogenase (oxidase), was not used by maize AO, indicating that the enzyme purified here is not xanthine dehydrogenase (oxidase).

The enzyme was not active under anaerobic conditions. The aldehydes were oxidized at similar rates when either DCIP or molecular O_2 were used as an electron acceptor (Table II). MTT, ferricyanide, and Cyt *c* could also be used as electron acceptors, although the latter two were less efficient.

Inhibitors

Several agents were tested for their effects on maize AO (IAAld oxidase) activity and the results are presented in Table III. Treatment with 10 mM 2-mercaptoethanol gave 45% inhibition, but DTT had no inhibitory effect even at a concentration of 5 mM. Thiol reagents, such as *p*-chloromercuribenzoate and iodoacetate, gave 60 to 70% reduction in AO activity. Cyanide and azide did not affect the enzyme. Menadione, estradiol, Triton X-100, and mAMSA,

Table II. *Kinetic properties of maize AO with various substrates*

The enzyme sample used for the activity assay was the protein eluted following hydroxyapatite column chromatography (Table I). This AO preparation gave one activity band on a native polyacrylamide gel.

Substrate	Electron Acceptor			
	O_2		DCIP	
	K_m[a]	V_{max}[b]	K_m[c]	V_{max}[b]
	μM		μM	
IAAld	3.2	76	5	28
Indole-3-aldehyde	4.5	69	14	190
Benzaldehyde	1.5	453	5	247
Protocatechualdehyde	2.9	175	26	316
Phenylacetaldehyde	–[d]	–	250	120
Butyraldehyde	–	–	26	113
Propionaldehyde	–	–	74	70
Acetaldehyde	–	–	345	57
N-Methylnicotinamide	–	–	N.D.[e]	–
Xanthine	–	–	N.D.	–

[a] The enzyme activity was assayed by determining the amount of the reaction product formed in the presence of O_2 as an electron acceptor. [b] Nanomoles of product formed or DCIP reduced min^{-1} mg^{-1} protein. [c] The enzyme activity was assayed by monitoring the reduction of DCIP as an electron acceptor. [d] Not determined. [e] Not detected under the specified conditions.

Table III. *Effect of various agents on maize AO (IAAld oxidase)*

The enzyme used was the same as in Table II. The activity was assayed by using HPLC to determine the amount of IAA formed from IAAld in the presence of the agents.

Agent	Concentration	Activity
	mM	%
Control		100
2-Mercaptoethanol[a]	5.0	60
	10.0	45
DTT[a]	1.0	105
	5.0	107
p-Chloromercuribenzoate[a]	0.05	39
Iodoacetate[a]	1.0	69
	5.0	32
KCN[a]	1.0	111
NaN_3[a]	3.0	104
Menadione	0.1	95
Estradiol	0.1	100
mAMSA	0.01	97
Allopurinol	0.25	93
2,4-D[a]	1.0	102
Triton X-100[a]	0.01%[b]	95
Methanol	2.0%[b]	27
Ethanol	2.0%[b]	102
Isopropanol	2.0%[b]	100

[a] The enzyme was incubated with the agent for 3 min before the reactions started. [b] (v/v).

which are known as potent inhibitors of animal AO (Rajagopalan et al., 1962; Gormley et al., 1983; Yoshihara and Tatsumi, 1985), did not have any significant effects, and this was also the case for allopurinol, an inhibitor of xanthine dehydrogenase (oxidase). 2,4-D was shown to have some inhibitory effects on cucumber IAAld oxidase (Bower et al., 1978), but such inhibition was not detected when maize AO was treated with 2,4-D. Of the three alcohols tested (methanol, ethanol, and isopropanol), methanol reduced enzyme activity to 30% but the two others did not inhibit the enzyme.

Other Properties

The enzyme had a pH optimum of between 7.0 and 8.0. The enzyme lost all activity after boiling for 5 min but was relatively stable at 60 to 70°C, with catalytic activity being detected at 60°C (Koshiba and Matsuyama, 1993). Addition of NAD(P) or NAD(P)H produced no change in the activity.

We previously reported that partially purified preparations of AO can produce IAA from Trp without the formation of intermediates as detected by HPLC (Koshiba and Matsuyama, 1993). The AO purified in this study could also catalyze the formation of IAA from Trp, although the activity was very low (data not shown). The mechanism of this reaction is still obscure, but one possibility involves the FAD molecules in AO, which could nonenzymatically catalyze the formation of IAAld from Trp (Koshiba et al., 1993).

Immunological Analysis

On native polyacrylamide gels, four bands (Fig. 4A, lanes 1 and 2; bands a–d) with benzaldehyde oxidase activity were detected in crude extracts of apical and subapical sections of coleoptiles. Of these bands, the purified enzyme corresponded to band d (Fig. 4A, lane 3). Band a appeared to be xanthine oxidase because an intense band was observed at the same position when the activity stain was performed with xanthine instead of benzaldehyde (data not shown). Polyclonal mouse antibodies raised against the purified maize AO reacted with only one protein (band d) in the crude extracts (Fig. 4B). These results suggested that the purified AO is one of three isozymes of benzaldehyde oxidase in maize coleoptile tissues. The properties of the other two isozymes (bands b and c) remain to be studied.

Comparisons of the amount of AO in the apical and subapical regions of maize coleoptiles were carried out. The intensities of both the protein with activity and those recognized by the antibodies following native PAGE were stronger in the extracts from the apical region (Fig. 4, A and B). This difference was also observed when the same samples were used for SDS-PAGE followed by immunoblotting (Fig. 4C). Control experiments were carried out by using specific antibodies to detect cytosolic ascorbate peroxidase in the same extracts. In this case the

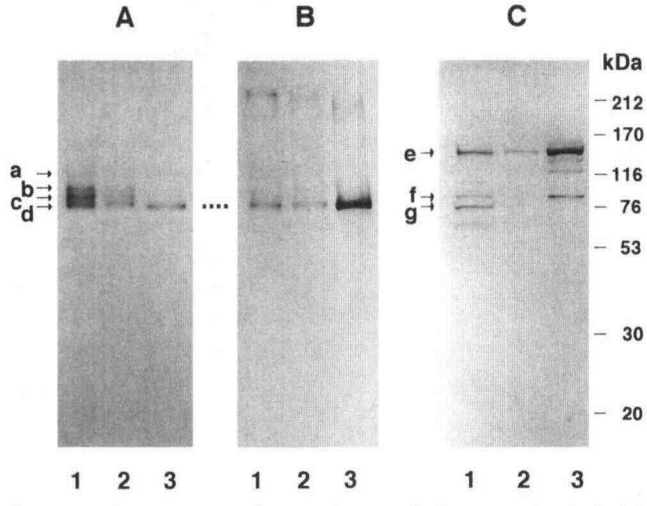

Figure 4. Zymogram and immunoblot made from gels loaded with crude extracts of different sections from maize coleoptiles and purified enzyme. Sections from apical (2.5 mm long) and subapical (3 mm long, 4.5–7.5 mm from the top) regions of coleoptiles were collected separately. The sections (2–3 g fresh weight) were homogenized with 0.1 M potassium phosphate buffer, pH 7.4, and the extracts were subjected to ammonium sulfate fractionation (0–80% saturation). The protein fraction was dialyzed against 50 mM phosphate buffer, and the dialyzed samples were subjected to native and SDS-PAGE. Activity staining with benzaldehyde as a substrate and immunoblotting were carried out as described in "Materials and Methods." A, Activity stain after native PAGE. B, Immunoblot after native PAGE. C, Immunoblot after SDS-PAGE. Lane 1, Crude extract of apical region; lane 2, crude extract of subapical region; lane 3, purified AO. About 50 μg of total protein were loaded in both lanes 1 and 2, and 1 μg of purified AO was loaded in lane 3.

intensities of the immunostained bands were the same in both extracts (data not shown). This also confirms the even distribution of the ascorbate peroxidase in apical and subapical regions, which had previously been reported (Koshiba, 1993). The results presented here indicate that AO is present in larger amounts in the apical regions of maize coleoptiles than in the subapical parts.

Following immunoblotting after SDS-PAGE, two polypeptides with molecular masses different from that of the 150-kD subunit of AO (Fig. 4C, band e) were detected. These polypeptides had molecular masses of 72 kD (band g) and 85 kD (band f). The 85-kD polypeptide may be a degraded form of the 150-kD subunit formed during purification as described before. However, because the 72-kD peptide was not observed in the purified AO preparation, it was probably the result of a polypeptide in the tissues cross-reacting with anti-AO antibodies or modification of AO in the tissue prior to extraction. The 72-kD polypeptide was also detected in extracts from roots in a relatively high quantity (data not shown).

DISCUSSION

Comparison with Animal AO

Maize AO has very similar properties to animal AOs with respect to molecular mass, subunit structure, prosthetic groups, pH optimum, heat stability, and use of electron acceptors (Rajagopalan et al., 1962; Felsted et al., 1973; Andres, 1976; Yoshihara and Tatsumi, 1985; Stell et al., 1989). Because maize AO must be purified by a complex series of steps, it is unlikely that all of the purified enzymes have fully intact prosthetic groups. Confirmation of its FAD and metal content as well as the structure of its prosthetic groups must await detailed spectroscopic studies or production of the recombinant enzyme in an overexpression system. However, our qualitative estimation of FAD, iron, and molybdenum suggests that maize AO has similar prosthetic groups to the better-characterized animal enzymes (Mahler et al., 1954; Rajagopalan et al., 1962; Felsted et al., 1973; Andres, 1976; Yoshihara and Tatsumi, 1985).

The purified maize AO had a V_{max} of 137 nmol min^{-1} mg^{-1} protein as measured by the production of IAA from IAAld (Table I). However, the value shown in Table II is lower (76 nmol) because samples from a less pure enzyme preparation were used for these experiments (after HA-1000 column chromatography; Table I). In animals, different V_{max} values have been reported within the relatively wide range of 60 nmol to 5 μmol min^{-1} mg^{-1} protein for AOs from several sources. These include hog liver AO, which was shown to have a V_{max} of 100 nmol min^{-1} mg^{-1} protein (Felsted et al., 1973), rat liver cytosolic AO, which had V_{max} values between 60 and 440 nmol min^{-1} mg^{-1} protein (Tomita et al., 1993), and rabbit liver AO, with V_{max} values between 1 and 4.8 μmol min^{-1} mg^{-1} protein (Felsted et al., 1973; Hall and Krenitsky, 1986; Stell et al., 1989).

Maize AO and animal AOs differ in substrate specificity and susceptibility to inhibitors. *N*-Methylnicotinamide, a good substrate for animal AO, was not a substrate for maize AO. Menadione, estradiol, and mAMSA, which are all potent and specific inhibitors of animal AO, did not inhibit maize AO. Like maize AO, AO from a bacterium and a gastropod has a low affinity for purines (Turner et al., 1987; Large and Connock, 1994). However, at present the reason for the differences in substrate specificity remains unclear because of lack of sequence data for this class of enzyme. To date, amino acid sequence data have been reported for only bovine AO (EMBL accession number S46980).

Comparison with AOs in Plants

Although no extensive purification and detailed characterization of AO from other plants has been carried out, enzyme preparations have been obtained from several tis sues, including *Avena* coleoptiles (Rajagopal, 1971), potato tubers (Rothe, 1974), cucumber seedlings (Bower et al., 1978), and pea seedlings (Miyata et al., 1981). The kinetic properties of some of these enzymes have been investigated. Two AO isozymes from potato tubers have similar properties to both animal liver and maize AO with respect to pH optimum, wide substrate specificity, and electron acceptor usage. However, the two isozymes from potato tubers have a preference for aliphatic aldehydes, in contrast to maize AO, which has an affinity for aromatic aldehydes. Maize AO has a high affinity for IAAld, and AOs from *Avena* and cucumber have also been shown to oxidize IAAld to produce IAA (Rajagopal, 1971; Bower et al., 1978). *Avena* AO is also an enzyme with a relatively high molecular weight and similar substrate specificity compared to maize AO. However, the enzyme from *Avena* has a lower pH optimum (pH 4.4) and a K_m value (345 μM for IAAld) of 2 orders higher than that of maize AO (3.2–5 μM, Table III). We showed that maize AO contains FAD and molybdenum. Cucumber oxidase (named IAAld oxidase) is also thought to be a metalloflavoprotein, but the only evidence for this is based on some results obtained from experiments with inhibitors. Cucumber AO can also use a number of the substrates used by maize AO, including IAAld, indole-3-aldehyde, benzaldehyde, and phenylacetaldehyde. In addition, the enzyme from cucumber is strongly inhibited by a synthetic auxin, 2,4-D, and activated by brief heating or treatment with 2-mercaptoethanol, but this was not the case for maize AO. Comparison of AO enzymes from maize and pea shows that both enzymes require molecular O_2, are inhibited by *p*-hydroxymercuribenzoate, and have high pH optima. However, pea AO has a high K_m value (1.4 mM for IAAld), a low molecular weight, and no affinity for benzaldehyde and propionaldehyde, in contrast to maize AO. Therefore, at present, little is known about the properties of plant AOs and it is difficult to generalize about the common features of this enzyme from plants.

Possible Role of AO in Plants

It is well known that IAA plays important roles in a variety of physiological processes in plants, but the biosyn-

thetic pathway and the related enzymes are not very well understood despite extensive investigations (Nonhebel et al., 1993; Bandurski et al., 1995; Normanly et al., 1995, and refs. cited therein). IAAld oxidase is thought to catalyze the final step in the pathway where IAA is produced from IAAld, and therefore this enzyme may be a key enzyme for IAA biosynthesis. However, this has only been studied in a few plants (Rajagopal, 1971; Bower et al., 1978; Miyata et al., 1981). Among these studies, only results from experiments with *Avena* coleoptiles suggest that AO may function in IAA biosynthesis (Rajagopal, 1971). These studies showed that there was a concentration gradient of the enzyme along the length of the coleoptiles and that this might in turn form a concentration gradient of IAA. This supports the results presented here, which showed that larger amounts of AO were present in the apical regions of maize coleoptiles. Furthermore, in our previous in vivo experiments, the conversion of [^{14}C]Trp into [^{14}C]IAA was observed only in the apical sections of maize coleoptile (Koshiba et al., 1995). In addition, maize AO had a high affinity for IAAld (K_m 3–5 μM), indicating that even if a low concentration of IAAld was present in cells of the apical regions, the aldehyde could be converted into IAA. IAAld has been found in several plant species (Rajagopal, 1968; Purves and Brown, 1978; Ludwig-Müller and Hilgenberg, 1990). In combination, all of these observations provide evidence to support the suggestion that AO is involved in the biosynthesis of IAA.

Walker-Simmons et al. (1989) found that a molybdenum cofactor-deficient mutant of barley was also deficient in ABA-aldehyde oxidase and xanthine dehydrogenase activities and that this mutant had impaired ABA production. These findings suggest that ABA-aldehyde oxidase is a molybdenum-containing enzyme that is important for ABA biosynthesis. Similar results were also reported for tobacco mutants (Leydecker et al., 1995). However, the properties of ABA-aldehyde oxidase, in particular the presence of molybdenum, have not been studied. To our knowledge, our present report is the first to show the presence of molybdenum in an AO enzyme. Therefore, it will be interesting to study whether the ABA-aldehyde oxidase is an enzyme that has similar properties to the maize AO.

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