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Purification and inactivation by substrate of an allene oxide synthase (CYP74) from corn (*Zea mays* L.) seeds

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Abstract

The allene oxide synthase (AOS) was purified from corn (*Zea mays*) seeds to homogeneity and characterized partially. The corn AOS was a hemoprotein cytochrome P450 with a molecular weight and p*I* of 53,000 and 6.0, respectively. The corn AOS was found to be irreversibly inactivated by a substrate, 13-hydroperoxyoctadienoic acid. The rate of the enzyme inactivation was higher at low pHs. © 2000 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Allene oxide synthase (AOS; EC 4.2.1.92) in plants is a cytochrome P450 specifically catalyzing dehydration of lipid hydroperoxides to chiral allene oxides (Song & Brash, 1991; Song, Funk & Brash 1993; Brash & Song, 1995). The hydroperoxide substrates are formed by the lipoxygenase-catalyzed oxygenation of linoleic and linolenic acids. The AOS is the first enzyme which directs, in plants, α-linolenic acid metabolism toward the octadecanoid pathway to jasmonates that are plant growth regulators (Gardner, 1991; Laudert, Pfannschmidt, Lottspeich, Holländer-Czytko & Weiler 1996): the product of the AOS-catalyzed reaction from 13-hydroperoxyoctadecatrienoic acid derived from α-linolenic acid undergoes non-enzymatic or stereospecific enzymatic conversion to cyclopentenone acids, which are then metabolized through reduction and subsequent β-oxidations to jasmonates. It has recently been proposed that linoleic acid can also serve as a precursor for the octadecanoid pathway to jasmonates parallel to that of α-linolenic acid (Laudert et al., 1996).

In 1991, Song and Brash purified the AOS from flaxseed (*Linum usitatissimum*) and showed on the basis of its spectral characteristics that it is a member of the cytochrome P450 family (Song & Brash, 1991). They cloned and sequenced the AOS *c*DNA to show that the flax AOS has also sequences which are conserved around carboxy terminal regions of known P450s, but with some unusual amino acid substitutions (Song et al., 1993). The entire amino acid sequence of the flax enzyme was less than 25%, identical to those of known P450s. The enzyme thus represents a novel class of cytochrome P450s, designated CYP74 (Nelson et al., 1996). The AOS has been identified and characterized from several other plants, such as tulip (*Tulipa gesneriana* cv. Red Apeldoorn) bulbs (Lau, Harder & O'Keefe, 1993), corn (*Zea mays*) seeds (Gardner, Klei-

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Table 1
Purification of AOS from corn seeds

Purification	Total protein (mg)	Total activity (nkatal)	Specific activity (nkatal/mg)	Recovery (%)	Purification (-fold)
1. Corn seed extract	5100	132,600	26	100	1
2. Ammonium sulfate fractionation	1800	86,400	48	65	2
3. Q-Sepharose FF	536	97,600	182	74	7
4. Sephacryl S-200	263	79,700	303	60	12
5. Hi-prep Q	114	43,800	384	33	15
6. Heat treatment	27	22,500	835	17	32
7. PENTAX SH-0710F	0.9	2280	2530	1.7	97

man, Christianson & Weisleder, 1975), guayule (*Parthenium argentatum*) rubber particles (Pan et al., 1995), and *Arabidopsis thaliana* (Laudert et al., 1996). The corn seeds are known as good sources of the AOS activity. Gardner et al. (1975) analyzed the substrate specificity of the corn AOS and showed that it can act equally on both 9- and 13-hydroperoxyoctadienoic acids, in contrast with the flaxseed enzyme which exhibits a preference for the 13-hydroperoxide. However, the physicochemical properties of the corn AOS remain to be clarified. Here, we describe the purification and some molecular properties of allene oxide synthase from corn seeds to show that (13*S*, 9*Z*, 11*E*)-13-hydroperoxy-9,11-octadecadienoic acid (13-HPOD), a substrate, acts as an effective inactivator of the enzyme.

2. Results and discussion

The allene oxide synthase was purified from the acetone powder of corn seeds by purification procedures including the ion-exchange, hydrophobic-interaction, and hydroxyapatite chromatographies (Table 1). At the final purification step, a fraction of the purified enzyme (0.9 mg) gave a single protein band corresponding to a molecular weight of 53,000, as judged by staining with Coomassie Brilliant Blue R after the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 1). The purified enzyme had an isoelectric point of 6.0 as analyzed by isoelectric focusing (Miyazaki & Saito, 1994) in the presence of 0.2% Emulgen 911 with 4% polyacrylamide gel containing 2% Ampholine (pH 3.5–10, Pharmacia Biotechnology). The specific activity of the purified enzyme was 2530 nkat mg^{-1} at 30°C, and thus, the purification was 97-fold with an activity yield of 1.7%. The specific activity corresponds to a turnover number of 134.2 s^{-1}. The turnover number of the purified AOS and the actual degree of purification might have been considerably higher, given the instability of this enzyme, during the purification procedures; for comparison, reported turnover numbers are 6200 s^{-1} for the partially purified corn AOS (microsomal fraction, AOS concentrations estimated on the basis of the difference spectrum of the ferrocytochrome–CO complex in saturating CO; Lau et al., 1993), 1000 s^{-1} for flaxseed AOS (Song & Brash, 1991), and 3000 s^{-1} for guayule AOS (Pan et al., 1995). Nonlinear regression analysis of the dependence of the initial velocities on substrate concentrations showed that the enzyme had a K_m for 13-HPOD of 63 ± 21 μM at pH 7.0 and 30°C. This value was comparable with that (70 μM) reported previously (Gardner, 1970). The purified enzyme exhibited spectra with absorption maxima at 394, 514, and 640 nm, which are characteristic of a hemoprotein cytochrome P450. The identity of the corn AOS as the P450 is further confirmed by the CO-binding spectrum of the reduced form of the enzyme; reduction of the enzyme with sodium dithionite followed by bubbling with CO gas caused the appearance of a differential absorption maximum at 450 nm, which is slowly replaced by that centered at 420 nm. Potassium cya-

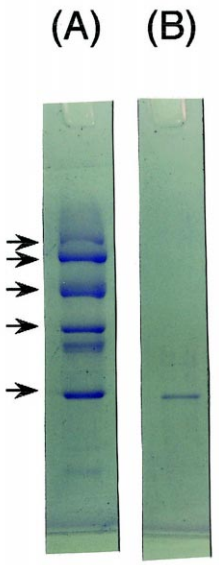

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified AOS from corn seeds: (A) marker proteins (BDH, England) with arrows indicating positions of protein bands with a molecular weight of 52,000, 76,000, 116,000, 170,000, 212,000 (bottom to top) and (B) Purified AOS.

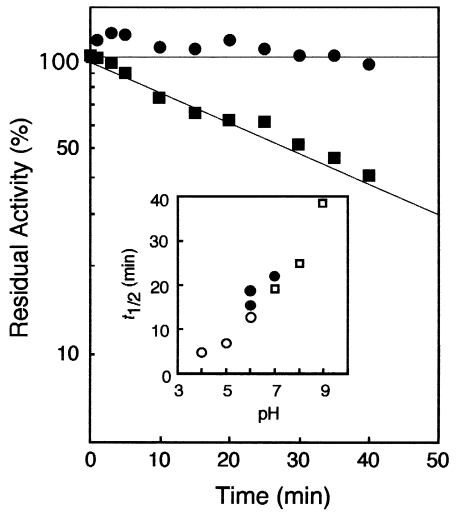

Fig. 2. Pseudo-first order inactivation of corn AOS by 13-HPOD. The 13-HPOD concentrations are ●, 0 mM and ■, 0.42 mM. For experimental details, see the text. (Inset) pH dependence of $t_{1/2}$ values of the corn AOS inactivation by 13-HPOD. The corn AOS was incubated with 0.42 mM 13-HPOD at different pHs in buffers [○, 0.1 M sodium acetate (pH 4–6); ●, 0.1 M potassium phosphate (pH 6–7); and □, 0.1 M Tris–HCl (pH 7–9)]. The 13-HPOD concentration was maintained at $100 \pm 20\%$ of the initial level during incubation. The $t_{1/2}$ values of the enzyme inactivation were plotted against pH.

nide (1 mM) did not significantly inhibit the corn AOS under the assay conditions (remaining activity, 83%). Also, the following (final concentration, 1 mM) were not inhibitors of the corn AOS: ethylenediaminetetraacetic acid, sodium azide, 2-mercaptoethanol, $ZnCl_2$, $MnCl_2$, $NiCl_2$, and $CoCl_2$. However, 13-HPOD, a substrate, served as an effective inactivator of the corn AOS. The inactivation was irreversible; removal of the residual 13-HPOD from the reaction mixture by dialysis against 0.1 M potassium phosphate buffer, pH 7.0, containing 0.2% Emulgen 911 at 4°C did not restore enzyme activity. Therefore, product inhibition did not account for the observed loss of enzyme activity during incubation with the substrate. Also, treatment of the inactivated enzyme with reducing agents (10 mM), such as dithiothreitol, 2-mercaptoethanol, ascorbic acid, and sodium dithionite did not restore the activity. The inactivation followed pseudo-first order kinetics (Fig. 2), and the rate of inactivation by 13-HPOD was higher at acidic pH than at alkaline pH (Fig. 2, inset). It is known that hydroperoxides can oxidize cysteine and methionine residues (Rapoport, Härtel & Hausdor, 1984) and that the rate of oxidation of the cysteine residue is greater at higher pH, whereas that of the methionine residue is higher at lower pH (Toennies & Callan, 1939). This provides one of the possibilities that the inactivation may be related to the oxidation of methionine residue(s) by 13-HPOD. Other possible causes which may also be considered for the AOS inactivation include noncovalent modification, radical formation, and nonspecific protein modification by 13-HPOD as well as the related species generated during the reaction.

It has been pointed out that the AOS reaction shares several parallels with the reactions catalyzed by the thromboxane A_2 and prostaglandin I_2 synthases (Brash & Song, 1995); like AOS, these hemoproteins use neither O_2 nor NADPH but use natural peroxide substrates. Importantly, the thromboxane A_2 and prostaglandin I_2 synthases are inactivated by their substrates during catalysis (Jones & Fitzpatrick, 1991; Wade, Voelkel & Fitzpatrick, 1995), and the thromboxane A_2 synthase is also inactivated by lipid hydroperoxides, e.g., 15-hydroperoxyeicosatetraenoic acid, which is not a substrate (Jones & Fitzpatrick, 1991). The inactivation of the thromboxane A_2 and prostaglandin I_2 synthases by substrates was proposed to proceed by a mechanism-based process and involve non-covalent tight modification of the prosthetic heme group (Jones & Fitzpatrick, 1991; Wade et al., 1995), whereas, inactivation by hydroperoxides appeared to arise from apoenzyme modification (Jones & Fitzpatrick, 1991). These examples provide important implications for mechanistic considerations of the AOS inactivation by 13-HPOD.

The observed irreversible inactivation by substrate of the corn AOS may have a contribution to restraint of jasmonate overproduction and could have implication for a reguratory role of the AOS in the jasmonate pathway. Attempts are currently underway to elucidate the kinetic and molecular mechanism as well as the physiological significance of the inactivation of the corn AOS by 13-HPOD.

3. Experimental

3.1. Materials

The 13-HPOD was prepared by the enzymatic oxygenation of linoleic acid (Nacalai Tesque, Kyoto, Japan) with the purified soybean lipoxygenase (type 1; Serva, Heidelberg, Germany) at pH 9.0 and was isolated by using reversed-phase HPLC with a Bakerbond octadecyl column (JT Baker, Phillipsburg, NJ, USA) as previously described (Brash & Song, 1996). The identity of the isolated product as 13-HPOD was confirmed by GC–MS analysis with a Shimadzu GC-14B/GCMS-QP1100EX apparatus after reduction with $NaBH_4$ and esterification with trimethylsilyldiazomethane (Tokyo Kasei Kogyo, Tokyo, Japan) followed by trimethylsilylation with *N*,*O*-bis(trimethylsilyl)acetamide in pyridine.

3.2. Enzyme assay

The enzymatic dehydration of 13-HPOD catalyzed by AOS was monitored by the decrease in absorbance at 234 nm at 30°C (Brash & Song, 1996). The decrease in the 234 nm absorbance is due to the disappearance of a conjugated diene system, caused by the rapid hydrolysis of the product allene oxide to ketols (Brash & Song, 1996). The assay mixture contained 50 μM of 13-HPOD, 0.01 M Tris–HCl, pH 7.0, and 0.025% Tween20 in a final volume of 3.0 ml and was preincubated at 30°C. The reaction was started by the addition of the enzyme (up to 30 μl) and the changes in absorbance at 234 nm were recorded at 30°C with a Shimadzu UV-160 spectrophotometer (kinetic mode) equipped with a temperature-controlled cell positioner CPS-240A. AOS activity was estimated from the absorbance changes during 60 s immediately after the addition of the enzyme. The extinction coefficient, ε_{234}, for the conjugated diene of 25,000 M^{-1} cm^{-1} (Siedow, 1991) was used for the determination of the 13-HPOD concentration. The protein concentration was determined by the method of Bradford (1976).

3.3. Purification

Purification of the corn seed AOS was completed at 0–5°C, unless otherwise stated, as follows: Buffer A was 0.01 M potassium phosphate buffer, pH 7.0, containing 0.1 mM phenylmethylsulfonyl fluoride and 0.4% (v/v) Emulgen 911 (Kao, Tokyo, Japan). The corn seeds (Canberra 90, Takii Seeds, Kyoto, Japan) were ground in a mortar with acetone that was chilled at −20°C. The suspension was filtered through filter paper (Advantec Toyo, No. 2), and the debris was again defatted in the same way. The resultant acetone powder (239 g) was homogenized in a Waring blender with 4 l of buffer A which was chilled at 0°C. The homogenate was centrifuged at 8000 rpm for 20 min. To the supernatant solution, solid ammonium sulfate was added to 30% saturation. After the mixture was left for 30 min, the precipitate was removed by centrifugation. To the supernatant solution, solid ammonium sulfate was slowly added to 50% saturation. The precipitate was collected by centrifugation and dissolved in a minimum volume of buffer A and dialyzed against buffer A. The enzyme solution (approximately 400 ml) was placed on a column of Q-Sepharose Fast Flow (220 ml) equilibrated with buffer A. The column was washed with 5 column volumes of this buffer. The enzyme was eluted with a linear gradient of NaCl (0–0.6 M) in the same buffer (600 ml each). Active fractions were combined and concentrated to 30 ml using an Amicon 8200 ultrafiltration unit with a PM10 membrane. A 5 ml portion of the concentrate was placed on a Sephacryl S-200 column (2.6 × 80 cm) equilibrated with buffer A containing 0.15 M NaCl and eluted. The other aliquots were chromatographed in the same way. The active fractions were combined and dialyzed against the buffer A. Fast protein liquid chromatography (FPLC) was then performed with an FPLC apparatus equipped with a Hi-Prep Q column (Pharmacia Biotech). The enzyme solution (96 ml) was put on the column equilibrated with buffer A. The enzyme was eluted at a flow rate of 1.0 ml/min with a linear gradient of NaCl (0–0.5 M in 120 min) in the same buffer. The active fractions were collected and dialyzed at 4°C against 0.01 M sodium acetate buffer, pH 4.0, containing 0.4% Emulgen 911. The 5 ml aliquots of the enzyme solution in glass test tubes (16 × 160 mm) were incubated at 45°C for 30 min and then chilled on ice. The precipitate formed during the heat treatment was removed by centrifugation, and the supernatant was dialyzed against buffer A containing 0.3 mM $CaCl_2$. The enzyme solution was then subjected to a Shimadzu LC9A HPLC system equipped with a PENTAX SH-0710F hydroxyapatite column (7.5 × 100 mm, PENTAX, Tokyo, Japan) that is equilibrated with buffer A. The enzyme activity was eluted with a linear gradient of potassium phosphate (at pH 7.0, 0.01–0.6 M in 40 min) at a flow rate of 0.7 ml/min. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis was completed with 10% gels according to the procedures of Laemmli (1970).

3.4. Inactivation studies

The enzyme (protein concentration, 1.1 mg/ml; specific activity, 294 nkatal/mg) was incubated at 30°C with 0.42 mM of 13-HPOD in 0.1 M potassium phosphate, pH 7.0, containing 0.2% Emulgen 911. At appropriate time intervals, an aliquot was removed from the inactivation mixture and assayed for the enzyme activity. In addition, in order to keep the 13-HPOD concentrations constant during incubation, 13-HPOD (prepared as stock solutions in methanol) was supplemented intermittently to the inactivation mixture; timing and amounts of the 13-HPOD addition were determined by preliminary experiments. Thus, we could maintain the 13-HPOD concentration at 100 ± 20% of the initial levels throughout the incubation. Although these procedures also caused an increase in the methanol concentrations to 23% (v/v), control experiments showed that the AOS was fully active under the identical conditions without 13-HPOD. The assay mixture also contained dilute concentrations of methanol (the maximum methanol concentration, 1.15% (v/v)), whose effect on enzyme activity was negligible. It should also be noted that the protein concentrations of the mixture were set where the possible dilution-dependent inactivation was negligible. The logarithm of the

remaining activities, which were corrected for dilution, was plotted against incubation time.

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