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New Assay Method for Allene Oxide Cyclase, an Important Enzyme in Jasmonic Acid Biosynthesis

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Authors' contributions

This work was carried out in collaboration between both authors. Author KO designed the study, performed the experiments and data analysis and wrote the manuscript. Author YS performed the expression and purification of AOC. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: Allene oxide cyclase (AOC) (EC 5.3.99.6) is an important enzyme of jasmonates (JAs) biosynthesis. JAs are important signals that play a pivotal role in defense response of plants to environmental cues. Regulation JA biosynthesis is believed useful for elucidating the mechanism of plant defense system. Despite the high potential of AOC as a target for JA biosynthesis inhibitors, an efficient assay method suitable for screening AOC inhibitors is still not available. The aim of this work is to develop an efficient AOC assay method.
Study Design: Using excess amount of purified recombinant allene oxide synthase (AOS) combined with 13(*S*)-hydroperxy-9(*Z*), 11(*E*), 15(*Z*)-octadecatrienoic acid (13-HPOT), we established an efficient method to generate (12,13*S*)-epoxyoctadecatrienoic acid (EOT), the substrate of AOC. The AOS produced EOT was subsequently converted to (9*S*,13*S*)-12-oxo-(10,15*Z*)-phytodienoic acid (OPDA) by using purified recombinant AOC in a real time manner and the amount of OPDA was determined by HPLC.
Place and Duration of Study: All the experiments were conducted from October 2009 to March 2013 at Akita Prefectural University, Japan.
Methodology: The recombinant AOS and AOC were expressed in *E. coli*. The target proteins were

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purified using affinity chromatography, respectively. The unstable EOT was generated by using excess AOS combined with 13(*S*)-hydroperxy-9(*Z*), 11(*E*), 15(*Z*)-octadecatrienoic acid. The AOC synthesized OPDA was characterized by the comparison of HPLC retention time with the OPDA standard. AOC activity was calculated by determine the amount of OPDA in the assay system.

Results: We found in the presence of 50 nmol of purified AOS together with 20 μM 13-HPOT, the synthesis of OPDA was saturated when using 5 nmol of purified AOC in the enzyme reaction for 30 min. Our results indicated that the AOC activity can be determined by dual enzyme system.

Conclusion: We established an efficient assay method for AOC which may be applied for screening of AOC inhibitors.

Keywords: Allene oxide cyclase; allene oxide synthase; jasmonic acid; plant defense response; plant hormone.

1. INTRODUCTION

Allene oxide cyclase (EC 5.3.99.6) catalyzes the stereospecific cyclization of (12,13*S*)-epoxyoctadecatrienoic acid (EOT), an unstable allene oxide, to (9*S*,13*S*)-12-oxo-(10,15*Z*)-phytodienoic acid (OPDA) in the biosynthetic pathway of jasmonic acid (JA)(Fig. 1). JA and methyl jasmonate (MeJA), collectively named jasmonates (JAs) are important plant hormones that regulate gene expression in plant stress response and development. JAs involve in the defense response of plants against herbivore attack and infection of some pathogens [1-3]. JAs also are implicated in the control of plant responses to abiotic stimuli such as mechanical stress [4], cold stress [5], salt stress [6], and UV irradiation [7]. Exogenous JAs exert numerous inductive and inhibitory effects on plant developmental processes [2,8], correlations between endogenous JAs levels in specific tissues and the effects of the applied hormone have provided evidence that JAs have a role in fruit ripening, embryo development [8], promoting senescence [9] and the accumulation of storage proteins [10].

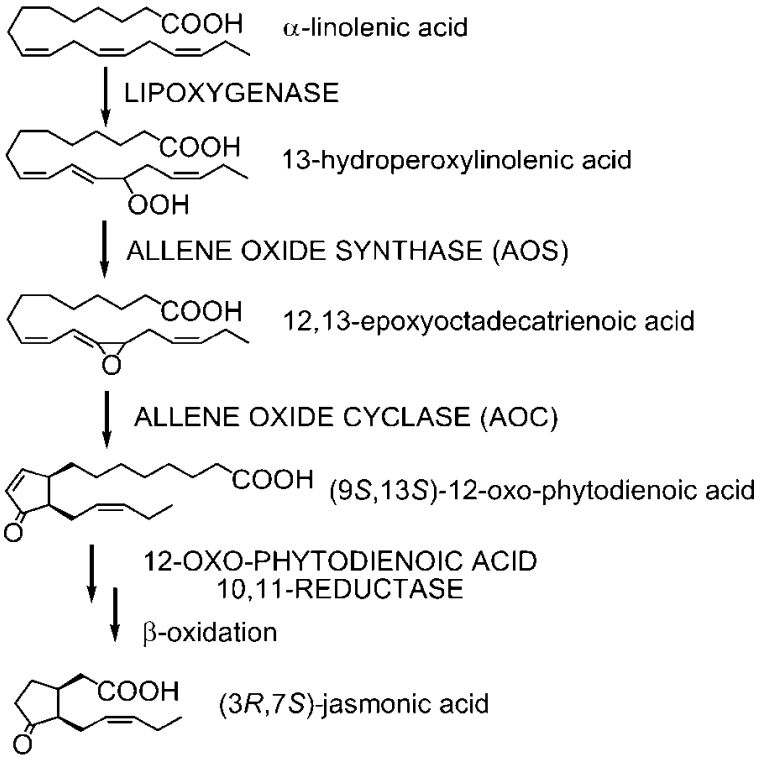

Fig. 1. A simplified biosynthetic pathway of jasmonate

The importance of JAs as signals for plant stress responses and development have promoted interest in understanding the mechanisms of these biological processes by which JA biosynthesis is regulated [8]. Analysis JA-deficient mutants of Arabidopsis has provided evidence that JAs play a key role in flower development. The critical requirement of JAs in male fertility was established by the characterization of an Arabidopsis mutant that fails to produce linolenic acid, the fatty acid precursor of JA [11]. Subsequently, mutations that disrupt other steps in the JA biosynthetic pathway were also shown to cause male sterility [12-14].

An alternative method for regulating JA biosynthesis in plant tissues is the use of specific inhibitors targeting the enzymes involved in JA biosynthesis. This method has advantages over the use of JA-deficient mutants, as it can be used at different stages of plant growth and development [15]. Moreover, inhibitors can easily be applied to different plant species. In this context, the search for potent inhibitors of JA biosynthesis represents a new approach to develop new technologies for manipulating JA levels in plant tissues.

Allene oxide cyclase (EC 5.3.99.6) is a good target for JA biosynthesis inhibitors. AOC produces the key cyclopentanone skeleton in JA biosynthesis (Fig. 1). In some cases, the product of AOC (OPDA) display biological functions in complement to JAs [16]. Thus, design and synthesis specific inhibitor of AOC represent a straightforward approach to regulate the JA biosynthesis.

Development of specific inhibitors of AOC requires an accurate and easy handling assay method for evaluation the inhibitory activity of synthesized compounds. Currently, there are several method for the determination of AOC activity [17,18]. All of these assay methods are not easy to carry out. Therefore, assay method that is suitable for screening AOC inhibitors need to be established. Against this background, we report herein an efficient assay method for determine the AOC activity which using excess allene oxide synthase (AOS) and 13(S)-hydroperxy-9(*Z*), 11(*E*), 15(*Z*)-octadecatrienoic acid to generate EOT, the substrate of AOC in the process of the determination the AOC activity.

2. MATERIALS AND METHODS

2.1 Chemicals

OPDA standard was purchased from Cayman Chemial (Michigan USA). Ampicillin and isopropyl-β-D-thiogalactopyranoside was purchased from Wako Chemical. Inc. (Tokyo, Japan). Linolenic acid and Soybean lipoxygenase was obtained from Sigma (Tokyo, Japan). Other reagents are of the highest quality purchased from Tokyo Kasei. Co. (Tokyo, Japan). Reagents are of the highest grade commercially available. ^{1}H-NMR spectra were recorded with a JEOL ECP-400 spectrometer (Tokyo, Japan), chemical shifts being expressed in ppm downfield from TMS as an internal standard. High resolution electrospray ionization Fourier transform ion cyclotron resonance mass spectra (ESI-FTICR) were recorded on an Exactive MS System (Thermo Fisher Scientific, Waltham, MA, USA).

2.2 Preparation of 13(*S*)-Hydroperxy-9(*Z*), 11(*E*), 15(*Z*)-Octadecatrienoic Acid

13(*S*) HPOT was prepared according to a method as described previously [19], the purity of the compound (>95%) was checked by ^{1}H-NMR and MS. The stock solution of 13(*S*) HPOT was prepared by dissolving 13(*S*) HPOT in methanol (10 mM) and stored at -80°C.

2.3 Expression and Purification of Recombinant *At*AOS

The coding region of *At*AOS cDNA that was restricted by the enzymes *BamH*I and *Kpn*I, was inserted into an *E. Coli* expression vector pQE30 (Qiagen). *E. coli* M15, transformed with this construct, was kindly provided by Prof. E. W. Weiler, Lehrstuhl für Pflanzenphysiologie, Fakultät für Biologie Ruhr-Universität, Germany. An overnight culture of bacteria (2 mL) was inocubated into 1 liter of fresh Luria-Bertani medium supplemented with ampicillin (100 μg/mL), placed in 2-liter culture flasks. Bacteria were grown at 37°C in a shaker at 150 rpm to an OD600 of 0.5. Cultures were cooled to 16°C, and isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM. Induced cultures were incubated for 24 h at 16°C with gentle shaking (100 rmp). Cells were collected by centrifugation. Purification of recombinant *At*AOS was performed as described previously with minor modification [20]. Cells of *E. coli* from a 1-liter culture were pelleted and resuspended in 50

mL of phosphate buffer (50 mM, pH=8.0) plus 0.1% Triton X-100. The cell suspension was sonicated 15 times for 30 sec. each time while on ice. The soluble fraction (supernatant) was prepared by centrifugation at 10000 x g for 1 hr at 4°C. The supernatant fraction was then applied to a His-Tag affinity column, the *At*AOS being purified by use of His-Tag risin (Novagen). The purified *At*AOS was then eluted with a 20% elution buffer according to the Novagen protocol. Eluted sample was dialyzed for 24 hours at 4°C by using an oscillatory dialysis system (Daiichi Pure Chemicals. CO., Ltd. Tokyo, Japan.) against 2 x 300 mL dialysis buffer (50 mM sodium phosphate buffer, pH 7.0). Protein measurements were performed using a Protein Assay Kit (Bio-Red, Herculs, CA, USA) and bovine serum albumin as a standard. The relative purity of recombinant *At*AOS was estimated by SDS-polyacrylamide gel electrophoresis (12% polyacrylamide) and staining of gels with Coomassie Brillant Blue R250.

2.4 Expression and Purification of Recombinant AtAOC2

The overexpression vector of pQE30 with *At*AOC2 cDNA was provided by Professor Wasternack of Leibniz-institut fur Pflanzenbiochemie. Germany [21]. The pQE-30-AOC2 was transformed into the host strain *E. coli*. M15. Expression and purification of *At*AOC2 was carried out in the same condition as AOS described above which gave purified biological active *At*AOC2.

2.5 *In vitro* Assays for *At*AOC Activity

In a mixture contained 50 mM sodium phosphate buffer pH 7.0, *At*AOS, (50 nM) and 5 nM *At*AOC2 was used for determination AOC activity. The enzyme reaction was started by adding 50 μM (13(*S*) HPOT) at 25°C. The enzyme reaction was allowed to be continued for 30 min and was stopped by adding equal volume of MeOH. HPLC separation of the reaction mixture was carried out by using reverse phase HPLC equipped with an UV detector. The mobile phase containing 70% MeOH was used. AOC activity was determined by analysis the amount of OPDA formed in the enzyme reaction.

3. RESULTS AND DISCUSSION

AOC has been cloned from several plant species [22]. Because recombinant AOC2 from *Arabidopsis* displayed high AOC activity [21], the purified recombinant *At*AOC2 was used in the present study. By the way, preparation of AOC substrate is challenging because EOT is extremely unstable [23]. Thus, we used a method by using AOS, an enzyme that catalyze the biochemical conversion of 13(*S*) HPOT to EOT in the assay system to generate EOT in a real time manner in our AOC assay system.

The enzyme activity of purified recombinant AOS and AOC were used in the present study. Separation and detection of enzyme reaction product was achieved by using reverse phase HPLC with a UV detector monitoring at 235 nm. As shown in Fig. 2, we first determined the retention time of the 13(S) HPOT. In a condition when adding the denatured AOS by boiling the purified AOS in the reaction mixture, we found that 13(*S*) HPOT was detected in our HPLC conditions at approximately 9.31 (min) (Fig. 2a). In contrast, when the fresh AOS was added to the enzyme reaction, the amount of 13(*S*) HPOT was significantly decreased (Fig. 2b). This result clearly indicates that AOS display biological activity to metabolize 13(*S*) HPOT. When the purified *At*AOC2 was added to the reaction mixture, a significant peak at a retention time approximately 5.78 (min) was detected. We characterized this peak as OPDA by the comparison of OPDA standard (Fig. 2c). Data obtained from our HPLC analysis of enzyme reactions mixture gave evidences that the dual enzyme system by using excess amount of AOS combined with 13(S) HPOT is useful for determination the AOC activity.

To further explore the condition for determination the AOC activity, we next determined the effect of the amount of the AOC in our assay system. We determined the AOC activity in different amount of AOC and under different incubation time of the enzyme reaction. As shown in Fig 3, the amount of OPDA was increased with the increasing the amount of AOC. We found the efficient amount of AOC concentration for determine the AOC activity is approximately 2 nM.

Next, we determined the effect of incubation time of enzyme reaction on AOC activity. As shown in Fig. 4, the amount of OPDA produced in our enzyme reaction system was increased significantly for approximately 20 min. After 30 min, the reaction progress curve of OPDA produced in our enzyme system is a flat line. Therefore, we choose the incubation time of 30 min as the enzyme reaction time for AOC assay.

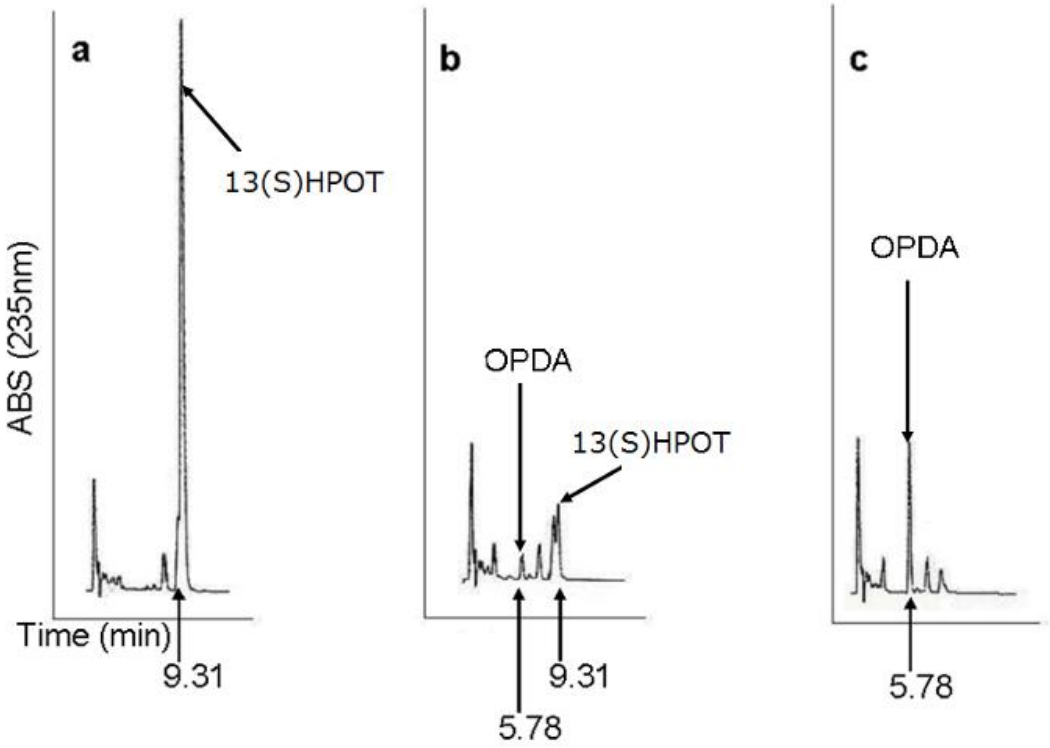

Fig. 2. HPLC determination the products of enzyme reactions

(a) 13(S) HPOT (20 μM, 1 μl injection) was mixed with denatured AOS (50 nM); (b) 13(S) HPOT (20 μM, 1 μl injection) was mixed with purified AOS (50 nM); (c) 13(S) HPOT (20 μM, 1 μl injection) was mixed with purified AOS (50 nM) and AOC2 (2 nM). The condition for enzyme reactrions and HPLC analysis was shown in the experiment section. All the experiment were triplicated to establish the repeatability

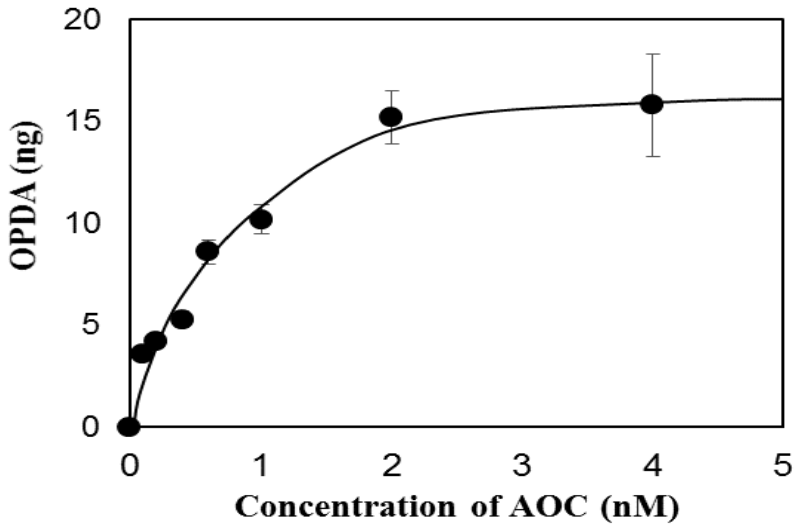

Fig. 3. Effect of the amount of AOC on OPDA production

Enzyme reaction was carried out by incubation the reaction mixture at 25°C for 30 min. Conditions for HPLC analysis were as indicated in the experiment section. The amount of AOC added to the enzyme reaction were 0.2, 0.3, 0.5, 0.8, 1, 2, 4 nM. The amount of OPDA was determined by using a standard curve of commercially available OPDA standard. Data are the means ± s.e. obtained from three independent experiments

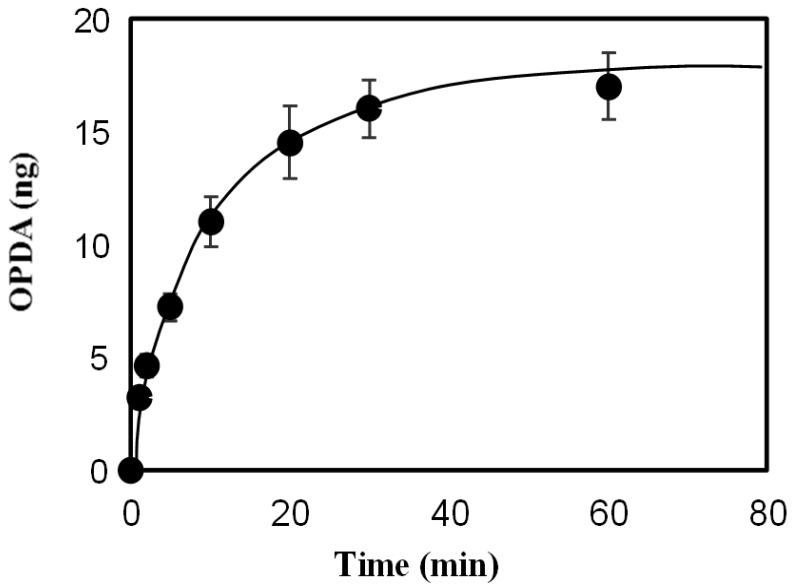

Fig. 4. Effect of the incubation time of AOC reaction on OPDA production

Enzyme reaction was carried out by incubation the reaction mixture with 13(S) HPOT (20 μM) together with purified AOS (50 nM) and AOC2 (5 nM) in a different time. Conditions for HPLC analysis were as indicated in the experiment section. The incubation time were 0, 2, 3, 5, 10, 20, 30, 60 min. The amount of OPDA was determined by using a standard curve of commercially available OPDA standard. Data are the means ± s.e. obtained from three independent experiments

We thus now established an enzyme assay system for the determination of AOC activity. Data obtained from present work indicated that the enzyme system with 50 nM AOS and 20 μM 13(*S*) HPOT for generate EOT is useful for AOC assay. We expect further experimental use of this enzyme system for screening chemicals with inhibitory activity against AOC may lead to the discovery of new jasmonic acid biosynthesis inhibitor targeting AOC.

4. CONCLUSION

In the present work, we used purified AOS and AOC as an enzyme system to produce OPDA thereby determine the enzyme activity of AOC. We found in the presence of 50 nmol of purified AOS together with 20 μM 13-HPOT, the synthesis of OPDA was saturated when using 5 nmol of purified AOC in the enzyme reaction for 30 min. Data obtained from present work indicated that we established an efficient assay method for AOC which may be applied for screening of AOC inhibitors.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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