12-Oxo-phytodienoic acid (OPDA) is an important metabolite on the octadecanoid pathway of plants. This study shows an efficient in vitro synthesis of (+)-cis-OPDA by using a flaxseed extract and an allene oxide cyclase. The OPDA yield in the reaction in this study was almost 7-fold higher than that in the conventional reaction with the flaxseed extract.

Key words: allene oxide cyclase; flaxseed extract; (+)-cis-12-oxo-phytodienoic acid

12-Oxo-phytodienoic acid (OPDA), an intermediate of jasmonic acid (JA) biosynthesis, has been shown to exert JA-independent biological activities. A transcriptome analysis has shown that more than 150 genes were induced by OPDA, but not JA or methyl jasmonate. The biological functions and signaling mechanism of JA have been intensively studied, although details of the mechanism for OPDA-dependent physiological events in plants remain to be elucidated.

OPDA is biosynthesized by the octadecanoid pathway (Fig. 1). The octadecanoid pathway begins with the peroxidation of ω-linolenic acid at C-13 by lipoxygenase (LOX). The resulting hydroperoxide, (5S)-13-hydroperoxyoctadecatrienoic acid (13-HPOT), is changed into the unstable intermediate, (5S)-12,13-epoxyoctadecatrienoic acid (12,13-EOT), by allene oxide synthase (AOS). 12,13-EOT is cyclized by allene oxide cyclase (AOC) to produce (+)-cis-OPDA, the naturally occurring form.

Zimmerman and Feng have synthesized racemic cis-OPDA in a reaction mixture containing a flaxseed extract and ω-linolenic acid, most OPDA samples used for biological experiments having been produced by this method. Although an enantioselective total synthesis of (+)-cis-OPDA has been achieved, several chemical steps using water-reactive substances were required for this synthesis. Zerbe et al. have achieved a preparative solid-phase synthesis of (+)-cis-OPDA in a yield of 20%, using 13-HPOT as a substrate. The synthesis of (+)-cis-OPDA is not easy, as previously explained, and the limited supply of (+)-cis-OPDA is one reason why progress in OPDA research on plants has slowed; however, OPDA is an important compound in plant physiology. The synthesis of racemic cis-OPDA by a flaxseed extract has probably been due to biologically active LOX and AOS, but not AOC. 12,13-EOT, which is produced by AOS, is non-enzymatically converted into racemic cis-OPDA, ω-ketol and γ-ketol (Fig. 1).

The addition of AOC to the reaction mixture prepared from the flaxseed extract and ω-linolenic acid has been speculated to produce a good yield (+)-cis-OPDA, because the undesirable reaction leading to racemic OPDA, ω-ketol and γ-ketol would be suppressed.

The flaxseed extract was prepared according to the method of Zimmerman and Feng. We had already obtained recombinant PpAOC2 of Physcomitrella patens for an analysis of enzymatic activity, and PpAOC2 was therefore used for this study. To produce recombinant PpAOC2, total RNA was extracted from P. patens, and reverse-transcription (M-MLV reverse transcriptase, Invitrogen, USA) was carried out according to the manufacturer’s instructions to yield cDNA. PCR used cDNA with a forward primer (PpAOC2-F1, 5′-GAG-ATGGGGAAATAAGGTAGACAAG-3′) and a reverse primer (PpAOC2-R1, 5′-CTCTAATTGGTGAAATTTGGGG-3′) which were designed according to the sequence of the PpAOC2 gene (accession no. XM_001751202). The PCR product was ligated into cloning vector pBluescript SK II (+) (Stratagene, USA) to obtain the pSK-PpAOC2 plasmid. After a sequence analysis of pSK-PpAOC2, PCR was carried out, using pSK-PpAOC2 as a template, and primers containing the restriction enzyme site for an analysis of enzymatic activity, and PpAOC2 for a sequence analysis of the restriction enzyme site, a forward primer (PpAOC2-F2 with the BamHI site, 5′-GGATCCCGAATAAGGTTGACAAG-3′) and a reverse primer (PpAOC2-R2 with the HindIII site, 5′-AAGCTTCTAATTGGTGAAATTTGGGG-3′). The PCR product was digested with BamHI and HindIII (Takara, Japan), and the gene fragment was ligated into expression vector pQE30 (Qiagen, USA) which was digested by the same enzymes to obtain the pQE30-PpAOC2 plasmid. It was transformed into E. coli M15 after a sequence analysis. A single colony was grown overnight at 37°C in 10 mL of an LB medium containing 100μg/mL of ampicillin, and a one-step IPTG induction method was used to produce (+)-cis-OPDA. It was purified by reverse-phase HPLC.

Note
Efficient Synthesis of (+)-cis-12-Oxo-phytodienoic Acid by an in Vitro Enzymatic Reaction
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Abbreviations: AOC, allene oxide cyclase; AOS, allene oxide synthase; 12,13-EOT, (5S)-12,13-epoxyoctadecatrienoic acid; GC-MS, gas chromatography mass spectrometry; HREI-MS, high-resolution electron ionization mass spectrometry; 13-HPOT, (5S)-13-hydroperoxyoctadecatrienoic acid; IPTG, isopropyl β-D-thiogalactopyranoside; JA, jasmonic acid; LOX, lipoxygenase; OPC-8, 3-oxo-2-(cis-2-pentenyl)-cyclopentane-1-octanoic acid; OPDA, 12-oxo-phytodienoic acid; UPLC-MS/MS, ultra-performance liquid chromatography tandem mass spectrometry
ampicillin. A 10-mL aliquot of the culture was inoculated into 1 L of the LB medium containing 100 μg/mL of ampicillin and incubated at 37°C. After the culture had grown to a cell density of OD600 = 0.6, the recombinant protein synthesis was induced by adding IPTG at 0.2 mM. The cells were collected by centrifugation at 5,000 × g for 5 min after further incubation at 25°C for 3 h, and then washed with a basal buffer of 0.3 M NaCl and 20 mM imidazole, and then disrupted by ultrasonication. The cell debris was removed by centrifugation at 20,000 × g for 10 min, and then the supernatant was subjected to Ni-NTAagarose column chromatography (2 mL, GE Healthcare, USA). PpAOC2 fused with the His-tag was eluted with the basal buffer containing 50 mM sodium phosphate at pH 7.8. The cells were resuspended in the basal buffer containing 0.3 M NaCl and 20 mM imidazole, and then disrupted by ultrasonication.

**Fig. 1.** The Octadecanoid Pathway.

![Diagram of the Octadecanoid Pathway](image)

cis-OPDA, was converted into trans-OPDA by an alkaline treatment, methylated by ethereal diazomethane, and then analyzed by chiral GC-MS.[10,11] The molecular ion peak of the OPDA methyl ester at m/z 306 [M]+ was monitored to evaluate the stereochemistry of OPDA (Fig. 2). The product of the reaction carried out according to the conventional method[10] gave two molecular ion peaks of the racemic trans-OPDA methyl ester; racemic cis-OPDA was synthesized by the reaction of the flaxseed extract and α-linolenic acid. In contrast, single peak corresponding to the (+)- and (−)-trans-OPDA methyl ester in the reaction mixture prepared by the flaxseed extract, α-linolenic acid, and PpAOC2, and then more than 95% of OPDA in this reaction was formed as (+)-cis-OPDA (Fig. 2). The addition of PpAOC2 to the reaction mixture composed of the flaxseed extract and α-linolenic acid was accordingly shown to enantioselectively produce (+)-cis-OPDA.

To examine the optimum pH value for this reaction, 230 μg of α-linolenic acid and 2.5 μg of PpAOC2 were dissolved in the supernatant of the flaxseed extract in a 50 mM Tris–HCl buffer (pH 7.0–9.0 at 0.5 intervals), or in the 50 mM sodium phosphate buffer (pH 6.0–8.0 at 0.5 intervals). The enzymatic reactions were performed at 25°C for 3 h, and then the amount of OPDA in the reaction mixture was analyzed by UPLC-MS/MS according to Sato et al.[12] [1–2H9]OPC-8 was used as an internal standard.[13] The yield of OPDA tended to gradually rise from pH 7.0 to pH 8.0 in the 50 mM Tris–HCl buffer (Fig. 3A). In contrast, the minimum yield of OPDA was observed at pH 7.0 in the 50 mM sodium phosphate buffer, and varying the pH value from pH 7.0 increased the yield of OPDA (Fig. 3A). The typical pH curve was not apparent in this reaction for OPDA synthesis in either buffer, because it was a combined reaction of LOX, AOS, and AOC. The Tris–HCl buffer...
was better than the phosphate buffer for the yield of OPDA (Fig. 3A); the pH value for the OPDA synthetic reaction in this study was therefore set at pH 8.0 in 50 mM Tris–HCl buffer.

To check the relationship between the amount of PpAOC2 and the yield of OPDA, OPDA was synthesized in a reaction mixture of α-linolenic acid (230 μg) and PpAOC2 (2.5 μg) in 50 μL of a 50 mM Tris–HCl buffer (pH 7.0–9.0), or 50 mM sodium phosphate buffer (pH 6.0–8.0) at 25 °C. The yield of OPDA was analyzed by UPLC-MS/MS (Waters Micromass Quattro Premier tandem quadrupole mass spectrometer). Each value shows the mean ± SD (n = 3). B. The reactions were performed using α-linolenic acid as a substrate in a 50 mM Tris–HCl buffer (pH 8.0) at 25 °C. The concentration of PpAOC2 was varied from 0 to 1,000 μg/mL in 50 μL of the reaction solution. The yield of OPDA was analyzed by UPLC-MS/MS. Each value shows the mean ± SD (n = 3).

In conclusion, the synthetic method for producing (+)-cis-OPDA more easily and efficiently than other reported methods.

Next applied our method to the synthesis of deuterium-labeled (+)-cis-OPDA. A reaction mixture (1 mg of α-[17,17,18,18,18-2H]linolenic acid 13 and 125 μg of PpAOC2 in 250 μL of the supernatant of the flaxseed extract) was incubated for 3 h at 25 °C in an atmosphere of H2. The resulting solution was injected into a reverse-phase HPLC (Capcellpak C18 UG80, 4.6 mm i.d. × 250 mm; Shiseido, Japan). The reaction products were eluted from the column with a mixed solution of methanol and water, using a mixture of 0:100 from 0 min to 5 min, then linearly changing from 0:100 to 100:0 from 5 min to 20 min, and finally maintaining the mixture of 100:0 from 20 min to 25 min. A 0.33 mg amount of (+)-cis-[17,17,18,18,18-2H2]OPDA was afforded (32%); HREI-MS: 297.23523 (found), 297.23463 (calculated for C18H28D18O3). 13H-NMR (500 MHz, CDCl3) δ: 1.04–1.18 (m, 2 H), 2.13 (m, 1 H), 2.35 (m, 1 H), 2.42 (m, 1 H), 2.48 (m, 1 H), 2.96 (br.s, 1 H), 5.29–5.45 (m, 2 H), 6.16 (dd, J = 5.5 Hz, 1 H), 7.72 (dd, J = 5.5, 2.5 Hz, 1 H); 13H-NMR (76.5 MHz, CHCl3) δ: 2.00 (br.s), 0.90 (br.s). Stelmach et al. have synthesized racemic cis-[17,17,18,18,18-2H2]OPDA from α-[17,17,18,18,18-2H]linolenic acid by means of LOX and AOS reactions, the total yield of this synthesis of racemic cis-[17,17,18,18,18-2H2]OPDA being 8%. Stable isotopic-labeled compounds are useful as standard substances for analysis. An isotopic precursor is valuable, and there is therefore a need for the efficient synthesis of isotopic-labeled compounds. The other type of deuterium labeled α-linolenic acid has also been produced. The OPDA synthetic method used in this study was able to prepare several deuterium-labeled OPDA samples, which is useful for an analysis of OPDA metabolism in plants, in a good yield.

In conclusion, the synthetic method for producing (+)-cis-OPDA developed by this study can provide (+)-cis-OPDA more easily and efficiently than other methods, and will certainly contribute to the research of OPDA in plants.

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