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Preparative enzymatic solid phase synthesis of *cis*(+)-12-oxo-phytodienoic acid – physical interaction of AOS and AOC is not necessary

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Abstract

The pathway of jasmonic acid (JA) biosynthesis was established in the 1980s by Vick and Zimmerman but, until now, the preparative biosynthesis of the jasmonic acid precursors 12-oxo-phytodienoic acid (OPDA) and 3-oxo-2-[2′-pentenyl]-cyclopentan-1-octanoic acid (OPC-8:0) in their endogenous and biologically relevant *cis*(+)-configuration was only possible in small amounts and had to put up with high costs. This was mainly due to the lack of high amounts of pure and enzymatically active allene oxide cyclase (AOC), which is a key enzyme in the biosynthesis of jasmonates in that it releases, in a coupled reaction with allene oxide synthase (AOS), the first cyclic and biological active metabolite – OPDA. We describe here the expression and purification of AOS and AOC and their subsequent coupling to solid matrices to produce an enantioselective, reusable bioreactor for octadecanoid production. With the method described here it is possible to produce optically pure enantiomers of octadecanoids in high amounts in a cost- and time-efficient manner. Furthermore, it could be demonstrated that a physical interaction of AOS and AOC, hitherto postulated to be required for substrate channeling from AOS to AOC, is not necessary for the *in vitro* cyclization of the unstable epoxide generated by the AOS reaction.

Keywords: Allene oxide cyclase; Allene oxide synthase; Jasmonates; Jasmonic acid; 12-Oxo-phytodienoic acid; Octadecanoid pathway; Oxylipins; Plant hormones

1. Introduction

Since the initial discovery of methyl jasmonate (MeJA) as a secondary metabolite in essential oils of jasmin in 1962 (Demole et al., 1962), jasmonates have become known as a new class of plant hormones. In the early 1980s, their widespread occurrence throughout the plant kingdom (Meyer et al., 1984) and their growth-inhibitory (Dathe et al., 1981) and senescence-promoting activities (Ueda and Kato, 1980) have been established.

A role in plant defense was first shown by Farmer and Ryan who demonstrated the induction of proteinase inhibitors by MeJA and JA as part of the defense response against herbivorous insects (Farmer and Ryan, 1990; Farmer et al., 1991). The function in plant defense reactions was unequivocally confirmed by the analysis of mutants compromised in either the synthesis or the perception of jasmonate signals (for a review, see Schaller et al., 2005). It became increasingly clear, however, that biological activity is not limited to JA but extends to, and even differs between its many metabolites and conjugates as well as its cyclopentenone precursors (Blechert et al., 1995; Kramell et al., 1997; Stintzi et al., 2001; Taki et al., 2005).

The pathway of JA biosynthesis is shown in Fig. 1. Biosynthesis is believed to start with the release of α-linolenic acid (LA) trough the action of (a) lipase(s) possibly triggered by various local and systemic signals (Narváez-Vásquez et al., 1999; Farmer and Ryan, 1992; Mueller et al., 1993; Conconi et al., 1996). The free fatty acid is then

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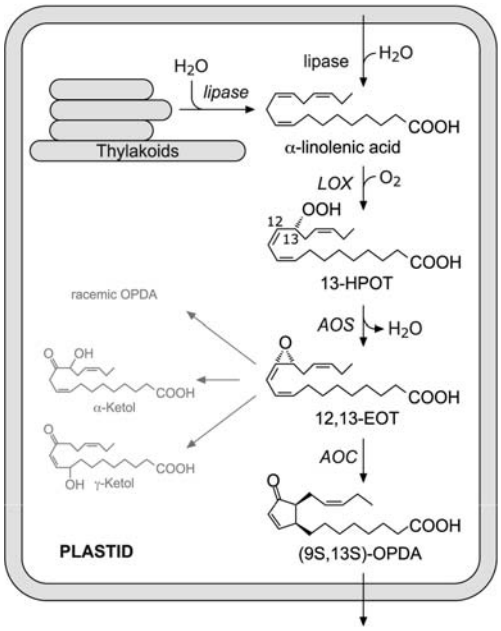

Fig. 1. The first part of the jasmonic acid biosynthetic pathway takes place in the chloroplasts leading to the first intermediate with biological activity, OPDA. LOX: 13-lipoxygenase, AOS: allene oxide synthase, AOC: allene oxide cyclase, 13-HPOT: 13-hydroperoxy-octadecatrienoic acid, 12,13-EOT: 12,13-epoxy-octadecatrienoic acid, OPDA: *cis*(+)-12-oxophytodienoic acid [(9*S*,13*S*)-12-oxo-phytodienoic acid].

oxygenated to 13-hydroperoxy-octadecatrienoic acid (HPOT) in a reaction catalyzed by a 13-lipoygenase (LOX).

AOS catalyzes the dehydration of the hydroperoxides to form an unstable epoxide (12,13-epoxy-octadecatrienoic acid, EOT) which either spontaneously dissociates to a mixture of α- and γ-ketols (80% and 10%, respectively) and racemic *cis*-OPDA (10%) or, in a concerted action with AOC, is converted into enantiomerically pure 9*S*,13*S*-OPDA (i.e. *cis*(+)-OPDA) (Hamberg and Fahlstadius, 1990; Laudert et al., 1997), the first cyclic and biologically active compound of the pathway. Reduction of the 10,11-double bond by a NADPH-dependent OPDA-reductase yields 3-oxo-2-(2′-pentenyl)-cyclopentane-1-octanoic acid (OPC-8:0) which undergoes three cycles of β-oxidation to yield the end product of the pathway, i.e. JA with an acyl-CoA oxidase (ACX1) and a thiolase (KAT2) being involved in the β-oxidation reaction (Cruz Castillo et al., 2004; Li et al., 2005).

The conversion of LA to OPDA is localized in the chloroplasts (Vick and Zimmerman, 1987; Froehlich et al., 2001; Stenzel et al., 2003), while the reduction of OPDA to OPC-8:0 (Schaller et al., 2000; Strassner et al., 2002) and the three steps of β-oxidation, i.e. conversion of OPC-8:0 to JA, occur in peroxisomes (Gerhardt, 1983; Vick and Zimmerman, 1984; Li et al., 2005).

An interesting aspect of the AOC reaction in vitro is the apparent competition between the enzyme-catalyzed formation of optically pure *cis*(+)-OPDA, and the spontaneous decomposition of its substrate, the unstable allene oxide, to form α- and γ-ketols, which can also be found in planta (Theodoulou et al., 2005), and racemic *cis*-OPDA (Fig. 1). The extremely short half-life of allene oxides ($t_{1/2}$ less than 30 s in water; Brash et al., 1988) and the optical purity of natural OPDA suggest a tight coupling of the AOS and AOC reactions possibly in a synthase-cyclase complex in vivo. Functional coupling of the two reactions is also observed in vitro: AOC from potato or recombinant *Arabidopsis* AOC2 in combination with recombinant *Arabidopsis* AOS produced highly asymmetrical *cis*-OPDA consisting almost exclusively of the (9*S*,13*S*)-enantiomer (Laudert et al., 1997).

Although research in recent years generally confirmed the Vick and Zimmerman pathway of JA biosynthesis (the octadecanoid pathway) and brought considerable progress with respect to the biochemistry of the enzymes involved as well as the molecular organization and regulation of the pathway, the preparative biosynthesis of large amounts of optically pure octadecanoid metabolites was possible in only a few laboratories and was expensive, labor-intensive, and time consuming. Here we describe an efficient expression system for AOC2 and the coupling of AOS and AOC to agarose, allowing for the large-scale preparation of optically pure OPDA from unpurified linolenic acid as starting material.

2. Results and discussion

Biological activity is not limited to jasmonic acid but extends to its many conjugates and its biosynthetic precursor, 12-oxo-phytodienoic acid (OPDA). OPDA can either substitute for jasmonic acid (JA) in JA-deficient plants as a signal for the induction of defense responses (Stintzi et al., 2001), or else, may itself be the primary signal as it has been shown for e.g. the mechanotransduction in *Bryonia dioica* (Falkenstein et al., 1991; Weiler et al., 1993). The synthesis of optically pure *cis*(+)-OPDA as the biologically relevant isomer in preparative amounts becomes more and more important since it allows researchers to address long-standing open questions like (i) how OPDA is transported from plastids into the microbodies (ii) if biological activity in different assays is really limited to *cis*(+)-OPDA, and (iii) if OPDA, like JA, can be further derivatized for modulation of its activity (for review, see Schaller et al., 2005).

Aiming to establish a preparative method for "on-column"-synthesis of optically pure *cis*(+)-OPDA, constructs of *Arabidopsis thaliana* allene oxide synthase (AOS) and allene oxide cyclase 2 (AOC2) for heterologous expression in *E.coli* were prepared according to Laudert et al. (1996) and Stenzel et al. (2003) (for details see Section 4). Recombinant AOC2 was purified to homogeneity yielding up to 10 mg/l culture (data not shown). AOS expressed in *E. coli* was solubilized from the bacterial membrane fraction with 0.1% (v/v) Triton X-100, and further purified to homogeneity via Ni-NTA affinity chromatography with a maximum yield of 15 mg/l of culture (data not shown). Enzymatic activity was demonstrated for both purified

proteins using 13-hydroperoxy-octadecatrienoic acid (HPOT) as the substrate. AOC2-activity was assayed by coupling the AOS and AOC reactions, because in the absence of AOC, the product of the AOS reaction, the unstable epoxide 12,13-epoxy-octadecatrienoic acid (EOT), undergoes formation of α- and γ-ketols as well as spontaneous cyclization to racemic OPDA. With increasing amounts of AOC2 the enantioselectivity of the OPDA formation from HPOT increased. At optimum concentrations of AOC2, the *cis*(+)-enantiomer of OPDA was produced almost exclusively, and a significant increase in the total amount of synthesized OPDA was observed (Fig. 2).

The preparation of HPOT as the substrate for AOS/AOC2 from LA using commercially available LOX (e.g. Sigma) is straightforward and well established (Graff et al., 1990). For the *in vitro* production of OPDA from HPOT we first optimized the conditions for the AOS reaction testing a pH range from 4.0 to 11.0, temperatures between 4 and 45 °C and different buffer compositions and ionic strength (PP_i, NaP_i, MES, Tris; 10–100 mM). Highest activity of AOS was observed at a reaction temperature of 23 °C and pH 7.0–8.0. The highest rate of OPDA formation in the coupled reaction of AOS and AOC2 was observed in PP_i- or NaP_i-buffers at an ionic strength of 10 mM and pH 7.0. Additionally, the influence of Triton X-100 on the enzymatic activity of AOS and AOC2 was analyzed. Although a slight decrease in specific enzymatic activity was detected, we used Triton X-100 solubilized AOS for our experiments because yield and purity of the enzyme after Ni-NTA agarose chromatography was much higher as compared to non-solubilized protein. Assays were performed in a total volume of 1 ml with 10 μg recombinant AOS, 20 μg AOC2 in case of the coupled activity assay and 100 μg HPOT as substrate for 15 min under various conditions. After acidification of the reaction mixtures the reaction products were extracted with ethyl acetate and the amount of synthesized OPDA was quantified by GC-MS analysis (see Section 4).

With the intent to create a solid-phase enzyme reactor for OPDA-biosynthesis, the two recombinant enzymes were bound to different matrices and the activity of the immobilized enzymes was measured by quantification of the formed OPDA using GC-MS. We did OPDA-synthesis using the immobilized enzymes in 10 mM PP_i-buffer, pH 7.0, at temperature-values from 14 to 24 °C, because the chosen temperature should assure both the stability of the HPOT as well as the activity of AOS and AOC2. For AOS immobilized on Ni-NTA agarose, phenyl sepharose or HQ-anion exchanger, highest activity was observed with AOS bound to Ni-agarose (Fig. 3).

The spontaneous cyclization of the AOS product (EOT) to racemic OPDA which occurs in solution was also observed for AOS bound to Ni-NTA agarose (Fig. 4A). Therefore, AOC activity can be measured in a coupled AOS/AOC reaction by following the shift in OPDA formation, from the racemic mixture to the *cis*(+) enantiomer. When 0.5 ml of Ni-NTA agarose saturated with purified AOC2 was employed and incubated with soluble AOS, nearly all accumulated OPDA had the (+)-configuration (Fig. 4B). The same shift towards the (+)-enantiomer of OPDA was observed when immobilized AOS was incubated with soluble AOC (Fig. 4C) and, most importantly, when both enzymes were bound to Ni-NTA agarose (Fig. 4D).

The immobilization of AOS and AOC allowed us to address the question of whether or not the close physical interaction of AOS and AOC2 in form of an AOS–AOC-complex is necessary for the formation of *cis*(+)-OPDA. AOS and AOC2 were coupled individually to Ni-NTA agarose. The substituted gels (ratio of 1:2 for AOS and AOC2, respectively) were transferred to a column but separated by a cushion of protein free Ni-NTA agarose. Therefore, the substrate HPOT when applied to the column has to first pass the AOS fraction where it is converted into EOT. EOT continues through the column and is converted to OPDA when it passes the AOC2 fraction. In this arrangement, a direct interaction of AOS and AOC is avoided. If a complex of both proteins was indispensable, the formation of racemic OPDA would be expected using this experimental setup.

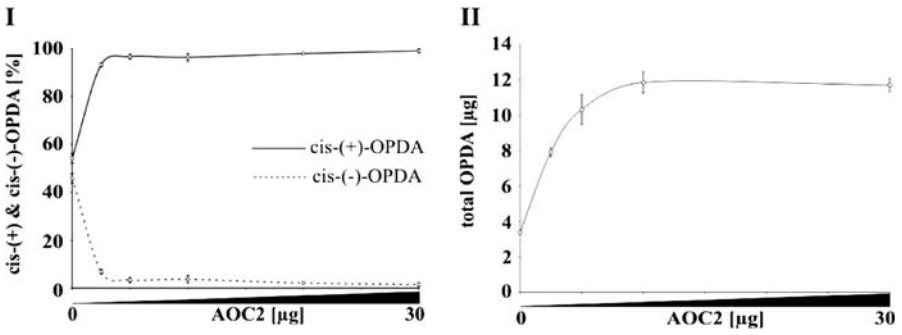

Fig. 2. OPDA formation using 5 μg/ml AOS and increasing amounts of AOC2 (0–30 μg/ml). The enzymatic reaction was carried out in 1 ml of 10 mM PP_i-buffer, pH 7.0, at room temperature for 15 min using 100 μg HPOT as substrate. After 15 min, the reaction mixture was acidified and OPDA extracted using ethyl acetate. Synthesized OPDA was analyzed and quantified by chiral GC- and GC-MS-analysis, respectively. (I) chiral GC-analysis of the relative amounts of the *cis*(+)- and the *cis*(−)-enantiomer of OPDA being formed as a function of the AOC2 concentration in the assay. (II) GC-MS quantification of the total amount of synthesized OPDA with $[^2H]_5$-OPDA as internal standard.

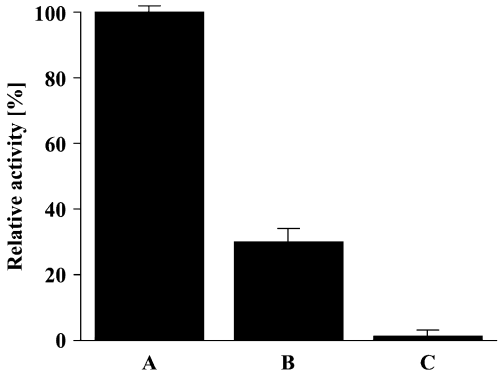

Fig. 3. OPDA formation by AOS bound to different chromatographic matrices. An aliquot of 0.5 ml of each matrix was washed with 50 mM PP_i, pH 7.5, and afterwards saturated with purified AOS. After washing of non-bound protein, 150 μg HPOT in 1 ml PP_i-buffer were passed through the matrix in a mini column by gravitational flow. Residual reaction mixture was washed off with one further ml of buffer. After acidifying the flow through and extraction with ether, OPDA formation was quantitated by GC-MS. (A) Ni-NTA agarose; (B) P11-cation exchanger; (C) HQ-anion exchanger.

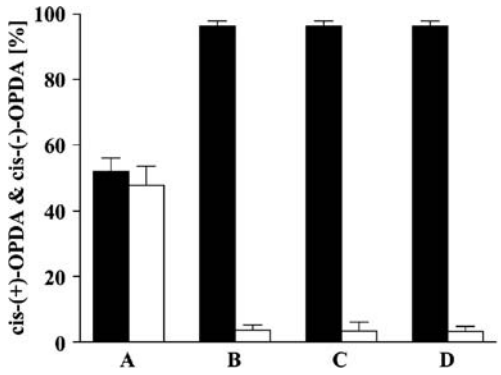

Fig. 4. Enantiomeric composition of OPDA formed by immobilized AOS or immobilized AOC2 with soluble AOC2 or AOS, respectively, and both enzymes bound to Ni-NTA agarose. The reaction of AOS without AOC2 led to the formation of racemic OPDA (A), while the coupled enzymatic reactions with AOC2 and AOS produce almost exclusively the *cis*(+)-enantiomer of OPDA (B–D). (A) AOS bound to Ni-NTA agarose; (B) AOC2 bound to Ni-NTA agarose and soluble AOS; (C) AOS bound to Ni-NTA agarose and soluble AOC2; (D) AOS and AOC2 immobilized on Ni-NTA agarose. Solid bars: *cis*(+)-OPDA; open bars: *cis*(−)-OPDA.

Comparing the activity of AOS and AOC2 coupled to Ni-NTA agarose separated from each other (Fig. 5I C) and coupled to Ni-NTA agarose after preincubation (Fig. 5I D), the ratio of *cis*(+)- to *cis*(−)-OPDA was found to be slightly smaller and a more pronounced decrease in the total amount of produced OPDA (Fig. 5II D) was observed when a direct contact of AOS and AOC was prohibited. However, there is still significantly more *cis*(+)-OPDA being produced than *cis*(−)-OPDA, demonstrating unequivocally that the formation of a complex between AOS and AOC2 is not required for the formation of the *cis*(+)-enantiomer in vitro. Nevertheless, it became clear that the total amount of OPDA produced can be augmented when AOS and AOC are bound together to the agarose beads so that they are spatially associated (Fig. 5II C).

Finally, the conditions of the system were modified in order to reach an optimal efficiency of OPDA formation. Therefore, AOS:AOC2-ratios from 1:1 up to 1:4 and a methanol-concentration from 0 to 30% (v/v) as well as a HPOT-concentration up to 100 μg/ml and flow-rates from 0.5 ml/min up to 7 ml/min were tested. For an optimal preparative on-column synthesis of OPDA, 8 mg AOS and 16 mg AOC2 were preincubated in 5 ml of 10 mM PP_i-buffer, pH 7.0, for 10 min, coupled to 12 ml Ni-NTA agarose and transferred to a column with an inner diameter of 1 cm. As substrate, 10 mg HPOT (50 mg/ml) was dissolved consecutively drop by drop in 10 mM PP_i-buffer, pH 7.0, up to a final concentration of 25 μg/ml and applied to the column at a flow rate of 3 ml/min or higher. To enhance the solubility of HPOT, up to 20% (v/v) methanol can be added to the buffer. After the passage through the column, the flow-through was acidified with conc. HCl to a pH of 2.0–3.0 and extracted twice with 2 volumes of diethyl ether. The organic phase was decanted, dried by addition of anhydrous sodium sulfate, filtered and taken to dryness using a rotary evaporator. The recovered OPDA was further purified on a C18-column as described in Section 4. This procedure resulted in 2 mg *cis*(+)-OPDA corresponding to a yield of 20%. Capillary chiral GC-analysis demonstrated optical purity of the *cis*(+)-enantiomer, exceeding 95%. The overall purity of synthesized OPDA was analyzed via TLC, HPLC and GC and found to be comparable to the purity of OPDA synthesized by previous standard procedures (>95%).

We checked activity of the OPDA-bioreactor up to 6 times over a period of two months. When stored at 4 °C in 10 mM PP_i, pH 7.0 + 10% (v/v) methanol, no significant decrease in overall enzymatic activity and no loss in enantio-selectivity could be detected. Furthermore, we tested if LOX immobilized on chromatographic matrices is active as well. LOX bound to DE52 anion exchanger showed highest activity (data not shown) and therefore may be used as the first step in solid phase production of OPDA directly fom LA especially if one is interested in the production of only small amounts of, e.g., radioactively labelled octadecanoids. For mass production of OPDA, it turned out that the prior synthesis of HPOT is more feasible.

3. Conclusion

(1) With the work presented here we provide the "jasmonate community" with a cheap, quick and simple method for the production of enantiomerically pure *cis*(+)-OPDA. Especially those who need large amounts of unlabelled OPDA or isotopically labelled compounds, can now synthesize these substances economically. Enzyme reactors are active over a period of month. Therefore, huge amounts of enantiomerically pure *cis*(+)-OPDA may be synthesized by repetitive usage of the column. Not reported

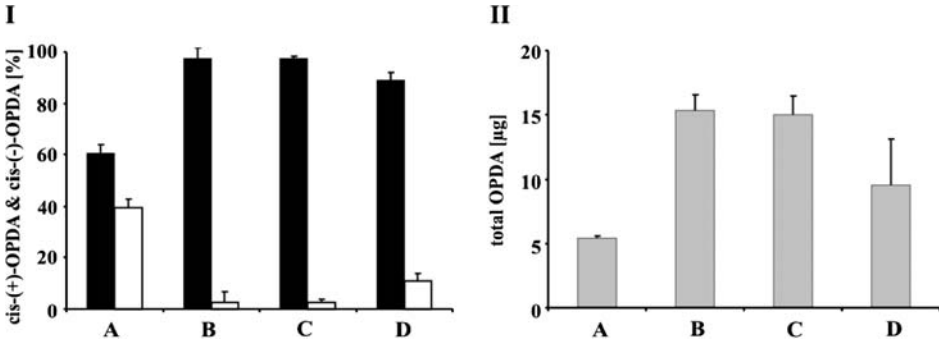

Fig. 5. Analysis wether a complex of AOS and AOC2 is needed for OPDA synthesis in vitro or not. (A) AOS bound to Ni-NTA agarose without AOC; (B) AOS and AOC in solution; (C) AOS and AOC bound to Ni-NTA agarose together after preincubation of both proteins; (D) AOS and AOC2 each coupled to Ni-NTA agarose individually and separated from each other by 200 µl of protein free Ni-NTA agarose. HPOT was dissolved in 10 mM PP_i-buffer, pH 7.0, up to a final concentration of 25 µg/ml and passed through the column For activity assays in solution, AOS and AOC were mixed in 1 ml of 10 mM PP_i-buffer, pH 7.0, and 20 µg HPOT was added as substrate. Samples were acidified and extracted with ethyl acetate. Activity was calculated from the formation of OPDA using either (I) capillary chiral GC (solid bars: *cis*(+)-OPDA; open bars: *cis*(−)-OPDA) or (II) GC-MS analysis.

here is the conversion of OPDA to enantiomerically pure OPC-8:0, which can be done quantitatively on a preparative scale with recombinant OPR3 (F. Schaller unpublished; Schaller et al., 2000).

(2) It was postulated that AOS and AOC act sequentially in a complex, because the lipophilic product of the AOS reaction is extremely unstable in aqueous solutions. We demonstrate here that a direct interaction of both proteins is not necessary for the stereospecific cyclization in vitro. Nevertheless, a small distance between both proteins enhances OPDA formation and optical purity in vitro. In vivo, this situation may be given just because both proteins are located within the chloroplasts, with AOC in excess over AOS.

Clones for expression of AOS, AOC and OPR3 are available to interested researchers from the authors department.

4. Experimental

4.1. Materials

Ni-NTA agarose was purchased from Qiagen (Hilden, Germany), HQ-anion exchanger (Macro-Prep High Q Support) from Bio-Rad (Munich, Germany), P11-cation exchanger and DE52-anion exchanger from Whatmann (Dassel, Germany). 13-Hydroperoxy-octadecatrienoic acid (HPOT) was synthesized from α-linolenic acid (Fluka, Buchs, Germany) using a lipoxygenase from soybean (Sigma, Munich, Germany). $[^2H]_5$-OPDA was synthesized from [17,17,17,18,18-^{2}H]-linolenic acid ethyl ester (Promochem, Wesel, Germany) according to Müller et al. (2002).

4.2. Cloning and heterologous expression of allene oxide synthase

AOS was cloned as described in Laudert et al. (1997). The pQE30-AOS construct was transformed into the host strain *E. coli* M15 and the recombinant AOS was expressed according to Oh and Murofushi (2002). Typically, a 4 ml overnight culture was transferred to 500 ml of Terrific Broth (Ausubel et al., 1995) with 100 µg/ml ampicillin. Bacteria were grown to an optical density (OD_{600}) of ~0.6 at 37 °C, when protein expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM. Induced cultures were grown over night at 16 °C with orbital shaking at 150 rpm.

4.3. cDNA preparation and cloning of allene oxide cyclase 2

For the generation of an AOC2 cDNA, RNA was extracted from rosette leaves of 4 weeks old *Arabidopsis thaliana* plants (ecotype Columbia 0) two hours after wounding of the leaves using the "Aurum™-Total-RNA"-kit (BioRad, Munich, Germany). Subsequently, RT-PCR was used for the amplification of cDNA fragments coding for the amino acids 78–253 of AOC2 using the 5′-primer TATGGATCCCCAAGCAAAGTTCAAGAACTG (*Bam*HI restriction site underlined) and the 3′-primer TATGTCGACTTAGTTGGTATAGTTACTTATAAC (*Sal*I restriction site underlined). PCR fragments were inserted into the vector pGEM®-T (Promega, Heidelberg, Germany), sequenced and further cloned into the expression vector pQE30 (Qiagen, Hilden, Germany).

4.4. Heterologous expression of allene oxide cyclase2

Expression of AOC2 was performed in 500 ml cultures of *E. coli* M15 in 2YT medium (Ausubel et al., 1995) containing 100 µg/ml ampicillin. Cultures were inoculated at 1:100 with an overnight culture of pQE30-AOC2 and grown at 37 °C and 220 rpm to an OD_{600} of 0.5–0.6. Protein expression was then induced by addition of IPTG to a concentration of 0.2 mM and after 5 h, bacterial cells were harvested by centrifugation.

4.5. Purification of recombinant allene oxide synthase and allene oxide cyclase

Cells from 1 to 2 l medium were harvested at 4000*g* for 15 min and resuspended in 20 ml of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0). For purification of AOS, Triton X-100 was added to a final concentration of 0.1% (v/v) to solubilize the AOS from membrane fragments. Bacteria were lyzed by freezing and thawing as well as by sonication (4×1 min on ice). The lysate was centrifuged at 17,000*g* for 30 min and the resulting soluble fraction was applied to a Ni-NTA agarose column (10 ml bed volume) with a flow rate of 1 ml/min. After washing the column with 30 mM of imidazole in lysis buffer for 20 min, recombinant proteins were eluted in 250 mM of imidazole in lysis buffer and desalted by gel filtration over PD10 columns (GE Healthcare, Munich, Germany) according to the manufacturer's protocol. Recombinant AOS was eluted in the presence of 0.1% (v/v) Triton X-100. Purified proteins were analyzed by SDS–PAGE (Laemmli, 1970), Western blot (Towbin et al., 1979) and immunodetection using a monoclonal α-$(His)_5$-mouse-IgG (Novagen, Madison, USA) or a polyclonal α-AOC2-rabbit-IgG (F. Schaller and P. Zerbe, unpublished). To analyze the influence of Triton X-100 on the enzymatic activity of AOS, the protein was purified alternatively without adding Triton X-100 to lysis-, washing- and elution buffers.

4.6. Immobilization of purified proteins

In order to establish an ''on-column'' synthesis of enantiomerically pure *cis*(+)-12-oxo-phytodienoic acid (OPDA) AOS and AOC2 had to be coupled to a reusable solid phase.

For that purpose AOS or AOS in combination with AOC2 were bound to Ni-NTA agarose, cation exchanger, or anion exchangers, respectively.

Matrices were washed with two volumes of 10 mM PP_i buffer, pH 7.0, and then saturated with purified protein in a batch technique for 15 min at 4 °C under constant agitation. The chromatographic material was then transferred to a column and non-bound protein was removed by washing with 2 volumes of 10 mM PP_i buffer, pH 7.5. The protein content of the flow-through and washing fractions was analyzed using the Bradford assay (Bradford, 1976) to determine the amount of protein retained on the column.

4.7. Synthesis of 13-hydroperoxy-octadecatrienoic acid

13-Hydroperoxy-octadecatrienoic acid (HPOT) was prepared from α-linolenic acid (70%, Fluka, Buchs, Germany) following the procedure described in Graff et al. (1990) using soybean lipoxygenase (Sigma, Munich, Germany). α-Linolenic acid (56 mg) was dissolved in 16 ml of O_2-free ethanol and added to 1 l O_2-saturated 100 mM $Na_2B_4O_7$, 0.5 mM EDTA, pH 10.0. 13 mg Lipoxygenase (70800 U/mg, Sigma, Munich, Germany) was resuspended in 20 ml O_2-free 100 mM $Na_2B_4O_7$, 0.5 mM EDTA, pH 10.0, and added to start the reaction. The reaction was allowed to proceed at 4 °C until the absorption maximum at 235 nm was reached, when it was stopped by acidification to pH 2.0–3.0 with conc. HCl. Synthesized HPOT was extracted twice with 2 volumes of diethylether. Anhydrous sodium sulphate was added to remove residual water. The ether phase was taken to dryness using a rotary evaporator. For further purification, HPOT was resuspended in methanol and mixed with 4 volumes of acidic water (pH 3.0). Aliquots of 5 mg acidified HPOT-solutions were applied to C18 columns (Chromabond C18, 500 mg, Macherey & Nagel, Düren, Germany) after equilibration of the matrix with one volume of ethanol and water. After washing with one volume of 20% (v/v) ethanol and iso-hexane, the HPOT was eluted with 1 ml of methanol. Quantification was carried out spectrometrically at 235 nm using the molar coefficient of extinction (ε_{235}) of $23{,}299 \times 10^3$ $cm^2\,mol^{-1}$.

4.8. Activity assay of recombinant AOS

For activity assays, 5–20 μg of purified AOS was added to 1 ml of 10 mM PP_i buffer, pH 7.0. The enzyme reaction proceeded over 15 min at room temperature with 100 μg of HPOT as substrate. After acidification to pH 3.0 with 1 M HCl, the OPDA which had formed as a result of the spontaneous cyclization of the reaction product EOT was extracted twice with 2 volumes of ethyl acetate and taken to dryness. For GC-MS analysis, 1 nmol of $[^2H]_5$-OPDA was added before extraction as an internal standard. To optimize reaction conditions, temperature, pH, ions, ion strength, protein amount and HPOT concentration were varied. Analysis of OPDA was performed using either thin layer chromatography, or GC-MS analysis. For thin layer chromatography, OPDA was applied to a ''Polygram®SIL G''-solid phase (Macherey & Nagel, Düren, Germany) with *n*-hexane:ethyl acetate:methanol (60:40:2.5 (v/v/v)) as the mobile phase. Separated substances were visualized in an iodine vapor chamber. For GC-MS analysis, OPDA was dissolved in methanol and methylated with ethereal diazomethane (Hamberg and Fahlstadius, 1990). The dried fractions were then redissolved in 50–100 μl of chloroform and 1 μl of the sample was injected into a Varian GC 3400 gas chromatograph (Varian, Darmstadt, Germany) in splitless mode in direct connection to a MAT Magnum ion trap mass spectrometer (Finnigan, Bremen, Germany) using a chemical ionization mode with methanol as reactand gas. OPDA was separated on a ZB-35 fused silica capillary column (Phenomenex, Aschaffenburg, Germany, 30 m × 0.25 mm × 0.25 μm film thickness) with He carrier gas at 1 ml/min (gas pre-pressure of 80 kPa) using the following temperature program: injector temperature of 260 °C, 1 min isothermally at 50 °C, with 20 °C/min up to 250 °C, 10 min isothermally at 250 °C, transferline temperature 260 °C.

4.9. Activity assay of AOC2

Activity of recombinant AOC2 was calculated from the ratio of the enzymatically produced (+)- and (−)-enantiomer of OPDA. Activity assays were carried out in a coupled reaction with AOS as described above using various amounts of purified AOC2. For separation of the both enantiomeric forms of OPDA via the β-cyclodextrin GC-column, synthesized OPDA had to be converted into its *trans*-configuration by treatment with 100 mM KOH for 1 h followed by a second acidification and extraction step. Samples were redissolved in 20 μl of chloroform and subsequently analyzed by chiral capillary gas chromatography using a GC-14A gaschromatograph (Shimadzu, Duisburg, Germany) in combination with a β-DEX 120 column (Supelco, Bellefonte, USA; 20% permethyl-β-cyclodextrin in SPB-35; 30 m × 0.25 mm × 0.15 μm film thickness). OPDA-enantiomers were separated after injection of 1 μl using He carrier gas and hydrogen and argon as combustion gases with the following temperature programm: injector temperature of 250 °C, 1 min at 50 °C, with 10 °C/min up to 180 °C and 80 min isothermally at 180 °C. For activity analysis of immobilized protein, AOS and AOC2 were coupled to the matrices as described. Protein-loaded matrices were transferred to a column and excess protein was removed by washing with 10–50 mM PP_i buffer, pH 7.0. HPOT was dissolved in 1 ml of the same buffer up to a final concentration of 25 μg/ml and directly passed through the column. For optimization of OPDA synthesis, e.g., HPOT-concentration, protein amount and flow rate were varied. OPDA was synthesized while passing through the matrix and extracted and analyzed as described.

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