

# Purification and Characterization of Allene Oxide Cyclase from Dry Corn Seeds<sup>1</sup>

Jörg Ziegler\*, Mats Hamberg, Otto Miersch, and Benno Parthier

Department of Hormone Research, Institute of Plant Biochemistry, Weinberg 3, D-06120 Halle, Germany (J.Z., O.M., B.P.); and Department of Medical Biochemistry and Biophysics, Division of Physiological Chemistry II, Karolinska Institute, S-17177 Stockholm, Sweden (M.H.)

Allene oxide cyclase (AOC; EC 5.3.99.6) catalyzes the cyclization of 12,13(*S*)-epoxy-9(*Z*),11,15(*Z*)-octadecatrienoic acid to 12-oxo-10,15(*Z*)-phytyldienoic acid, the precursor of jasmonic acid (JA). This soluble enzyme was purified 2000-fold from dry corn (*Zea mays* L.) kernels to apparent homogeneity. The dimeric protein has a molecular mass of 47 kD. Allene oxide cyclase activity was not affected by divalent ions and was not feedback-regulated by its product, 12-oxo-10,15(*Z*)-phytyldienoic acid, or by JA. ( $\pm$ )-*cis*-12,13-Epoxy-9(*Z*)-octadecenoic acid, a substrate analog, strongly inhibited the enzyme, with 50% inhibition at 20  $\mu$ M. Modification of the inhibitor, such as methylation of the carboxyl group or a shift in the position of the epoxy group, abolished the inhibitory effect, indicating that both structural elements and their position are essential for binding to AOC. Nonsteroidal anti-inflammatory drugs, which are often used to interfere with JA biosynthesis, did not influence AOC activity. The purified enzyme catalyzed the cyclization of 12,13(*S*)-epoxy-9(*Z*),11,15(*Z*)-octadecatrienoic acid derived from linolenic acid, but not that of 12,13(*S*)-epoxy-9(*Z*),11-octadecadienoic acid derived from linoleic acid.

JA and its volatile methyl ester have been detected in numerous plant species (Meyer et al., 1984) and are involved in many physiological and developmental processes in plants. They promote senescence, inhibit seedling growth, delay seed germination, induce tuberization and tendrill coiling, and mimic stress responses observed after wounding and desiccation when applied exogenously to plants (Sembdner and Parthier, 1993; Creelman and Mullet, 1995). Concomitantly with these effects, changes in gene expression and protein turnover take place. Among these changes are the repression of chloroplast proteins such as Rubisco (Reinbothe et al., 1993; Roloff et al., 1994), or the induction of vegetative storage proteins (Franceschi and Grimes, 1991), late embryogenesis-abundant proteins (Reinbothe et al., 1994), proteinase inhibitors (Farmer and Ryan, 1990), and various other jasmonate-induced proteins (Weidhase et al., 1987), as well as enzymes involved in the metabolism of phenylpropanoids (Kauss et al., 1994) or alkaloids (Kutchan, 1993).

<sup>1</sup> Funding for this work was provided by the Deutsche Forschungsgemeinschaft through grant no. SFB 363 and by the Swedish Medical Research Council (project no. 03X-05170).

\* Corresponding author; e-mail jziegler@ipb.uni-halle.de; fax 49-345-5582-166.

Several of the effects inducible by JA or methyl jasmonate are also observed in osmotically stressed barley leaf segments (Lehmann et al., 1995), as well as after wounding of tomato leaves (Farmer and Ryan, 1992), elicitation of cell cultures (Gundlach et al., 1992; Nojiri et al., 1996), and mechanical stimulation of the tactile organs in *Bryonia dioica* Jacq. (Falkenstein et al., 1991). Moreover, shortly after plants are exposed to these stimuli and before the respective genes are activated, the endogenous JA concentration increases (Gundlach et al., 1992; Weiler et al., 1993; Doares et al., 1995b; Lehmann et al., 1995; Nojiri et al., 1996), indicating that JA can serve as a signal molecule between plant tissues or as a signal transducer within cells (Parthier, 1991).

The biosynthesis of JA takes place via the oxylipin pathway (Vick and Zimmerman, 1983; Hamberg and Gardner, 1992) (Fig. 1). This pathway starts with the LOX-catalyzed oxygenation of fatty acids containing a *cis-cis* pentadiene moiety to hydroperoxy fatty acids. The major substrates of LOX within plant cells are linoleic and linolenic acids, which can be oxidized in either the 9- or the 13-position, resulting in 9- or 13-hydroperoxy derivatives, respectively. The hydroperoxy compounds can serve as substrates for several enzymes. Hydroperoxide lyase cleaves the carbon chain at the site of hydroperoxidation, resulting in the generation of aldehydes (Gardner, 1991; Shibata et al., 1995), whereas divinyl ether synthase rearranges the hydroperoxide to form a divinyl ether (Galliard and Phillips, 1972; Grechkin and Hamberg, 1996). A peroxygenase utilizes the terminal hydroperoxide oxygen from the hydroperoxides to epoxidize other unsaturated fatty acids, which are further hydrolyzed to polyalcohols (Blée and Schuber, 1990; Hamberg and Hamberg, 1990). AOS dehydrates the hydroperoxide to form an allene oxide derivative (Song and Brash, 1991).

These epoxides are prone to hydrolysis and decay into  $\alpha$ - and  $\gamma$ -ketols with a half-life of 25 s in aqueous media. In the

Abbreviations: AOC, allene oxide cyclase; AOS, allene oxide synthase; 12,13-EOT, 12,13(*S*)-epoxy-9(*Z*),11,15(*Z*)-octadecatrienoic acid; 13-HPOD, 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid; 13-HPOT, 13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid; JA, jasmonic acid;  $\alpha$ -ketol, 12-oxo-13-hydroxy-9(*Z*),15(*Z*)-octadecadienoic acid;  $\gamma$ -ketol, 12-oxo-9-hydroxy-10(*E*),15(*Z*)-octadecadienoic acid; LOX, lipoxygenase; 12-oxo-PDA, 12-oxo-10,15(*Z*)-phytyldienoic acid; tetracyclis, 5-(4-chlorophenyl)-3,4,5,10-pentaazatetracyclo [5.4.10<sup>2,6,0,8,11</sup>]-dodeca-3,9-diene.

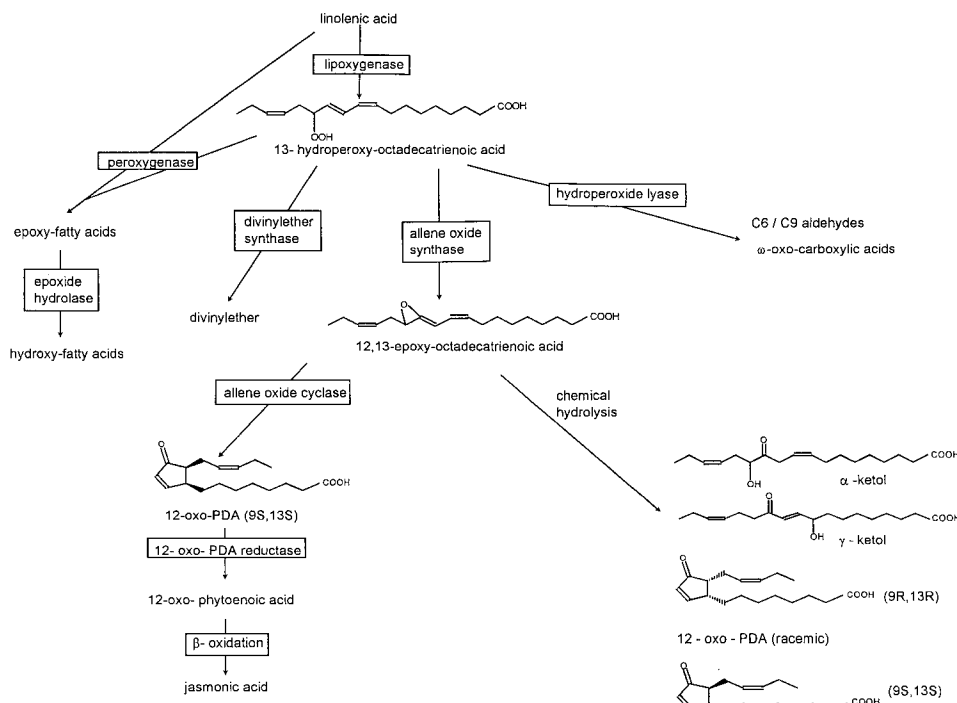


Figure 1. Oxylinpin pathway with emphasis on JA biosynthesis.

case of 12,13-EOT, which is produced from 13-HPOT, spontaneous degradation not only results in the formation of ketols but also in cyclopentanones, i.e. racemic 12-oxo-PDA (Crombie and Morgan, 1988; Hamberg, 1989). In contrast to the chemical rearrangement, 12,13-EOT is cyclized to stereochemically pure 12-oxo-PDA by AOC. The ring double bond is then reduced in an NADPH-dependent reaction by 12-oxo-PDA reductase, and thereafter the side chain with the carboxyl group is shortened by three rounds of  $\beta$ -oxidation to yield JA.

With the exception of LOX, which has been purified and cloned from several plant species and the properties of which are fairly well known (Siedow, 1991), little is known about the other enzymes. AOS has been cloned from flax, guayule, and *Arabidopsis* (Song et al., 1993; Pan et al., 1995; Laudert et al., 1996), and 12-oxo-PDA reductase activity has been partially characterized from corn (*Zea mays* L.) (Vick and Zimmerman, 1986). The enzyme involved in the  $\beta$ -oxidation steps is as yet unknown.

In the early days of LOX pathway research, the conversion of 13-HPOT to 12-oxo-PDA was believed to be the result of one enzyme, which was called hydroperoxide cyclase (Vick and Zimmerman, 1981). In 1990 Hamberg and Fahlstadius showed that corn seed membranes containing AOS were able to convert 13-HPOT to  $\alpha$ - and  $\gamma$ -ketols and 12-oxo-PDA via an unstable intermediate, 12,13-EOT. The soluble fraction of the corn homogenate did not metabolize 13-HPOT, but incubation of 13-HPOT with corn membranes together with the soluble fraction resulted in a large increase in the amount of 12-oxo-PDA compared with incubation with membranes alone. Furthermore, they showed that the 12-oxo-PDA generated by the addition of

supernatant was highly enriched in the 9(*S*),13(*S*) configuration in contrast to that produced by the membranes, which consisted of a 1:1 mixture of the 9(*S*),13(*S*) and 9(*R*),13(*R*) stereoisomers. This indicated the presence of an enzyme activity in the supernatant, which specifically generated the 9(*S*),13(*S*)-12-oxo-PDA enantiomer. This enzyme was named AOC. These results also showed that the generation of 12-oxo-PDA from 13-HPOT occurs via two enzymes; AOS (also called hydroperoxide dehydratase, EC 4.2.1.92) is associated with membranes, whereas AOC (EC 5.3.99.6) is soluble (Hamberg and Fahlstadius, 1990). The present paper describes the purification of AOC as one important step in the elucidation of the JA biosynthetic pathway.

## MATERIALS AND METHODS

### Chemicals

Linoleic and linolenic acids were obtained from Nu-Chek Prep (Elysian, MN) and [ $^{14}\text{C}$ ]linoleic and [ $^{14}\text{C}$ ]linolenic acid were purchased from DuPont NEN. 13(*S*)-HPOD and 13(*S*)-HPOT were prepared by incubation of the respective fatty acids with soybean LOX (Sigma) (Hamberg and Gotthammar, 1973). ( $\pm$ )-*cis*-12,13-Epoxy-9(*Z*)-octadecenoic acid and ( $\pm$ )-*cis*-9,10-epoxy-12(*Z*)-octadecenoic acid were prepared by epoxidation of methyl linoleate with *m*-chloroperbenzoic acid (Hamberg and Fahlstadius, 1990). JA was obtained by alkaline hydrolysis of methyl jasmonate (Firmenich, Geneva, Switzerland). 12-Oxo-10,15(*Z*)-phytodienoic acid was kindly provided by Dr. Rainer Atzorn (from our laboratory).

### Enzyme Purification

All steps were performed at 4°C. Dry corn (*Zea mays* L. cv Boss, RAGT Saaten, Herford, Germany) seeds (600 g) were ground in a coffee mill and the resulting powder was defatted with *n*-hexane. After drying, the hexane-extracted powder was homogenized in 50 mM potassium phosphate buffer, pH 7 (1:5, w/v) (Ultra-Turrax homogenizer, Janke & Kunkel, Staufen, Germany). The homogenate was passed through cheesecloth and centrifuged at 10,000g for 10 min. The supernatant was recentrifuged at 100,000g for 65 min to separate the membrane fraction from the soluble proteins, and AOC activity was precipitated from the 100,000g supernatant by the addition of cold acetone between 45 and 70% saturation. The acetone pellets were dissolved in 20 mM Tris-HCl, pH 7.5, containing 750 mM ammonium sulfate, and subjected to ultrafiltration through a membrane (XM 50, Amicon, Witten, Germany) with a molecular cutoff of 50 kD.

The concentrate was brought to 1 M ammonium sulfate, divided into two portions, and applied to an Octyl-Sepharose CL-4B column (70 × 26 mm; Pharmacia) equilibrated in 20 mM Tris-HCl, pH 7.5, 1 M ammonium sulfate, and 5% (v/v) ethanol. AOC activity was eluted from the column with a decreasing 150-mL linear gradient of 1 to 0 M ammonium sulfate in 20 mM Tris-HCl, pH 7.5, 5% (v/v) ethanol at a flow rate of 2 mL min<sup>-1</sup>. Active fractions from both runs were combined and the buffer was exchanged to 20 mM potassium phosphate, pH 6.5, by ultrafiltration through a membrane (XM 50). The concentrate was loaded at a flow rate of 0.3 mL min<sup>-1</sup> on a hydroxyapatite column (52 × 7 mm; CHT 2-I, Bio-Rad), which was equilibrated in 20 mM potassium phosphate, pH 6.5. Bound proteins were eluted at a flow rate of 1 mL min<sup>-1</sup> by a linear increase in phosphate concentration up to 0.3 M in a volume of 30 mL.

Active fractions were pooled, brought to 1 M ammonium sulfate, and separated on a Phenyl-Superose 5/5 column (50 × 5 mm; Pharmacia) at a flow rate of 0.5 mL min<sup>-1</sup>. The column was equilibrated in 20 mM Tris-HCl, pH 7.5, 1 M ammonium sulfate. AOC activity was eluted with a 25-mL linear gradient from 1 to 0 M ammonium sulfate in 20 mM Tris-HCl, pH 7.5. Fractions containing enzyme activity were combined, diluted with 20 mM Tris-HCl, pH 7.5, to decrease the ionic strength, and injected on a Mono-Q HR 5/5 column (50 × 5 mm; Pharmacia) equilibrated in 20 mM Tris-HCl, pH 7.5. The column was washed thoroughly with equilibration buffer and AOC activity was eluted by a linear gradient of 0 to 0.3 M potassium chloride in 20 mM Tris-HCl, pH 7.5, over 15 mL. Active fractions were stored at -70°C.

### Enzyme Assays

AOC activity was determined as described by Hamberg and Fahlstadius (1990) with some minor modifications. The reaction mixture included the 100,000g pellet of the corn homogenate with an AOS activity of about 7 nkat resuspended in 50 mM Tris-HCl, pH 7.5, and the solution to be tested for AOC activity in a final volume of 625 μL. The reaction was initiated by the addition of [1-<sup>14</sup>C]13(S)-HPOT

(0.7 mCi mmol<sup>-1</sup>) in 2.6 μL of ethanol to a final substrate concentration of 40 μM. After 10 min the assay was terminated by the addition of 625 μL of methanol, and the solution was acidified to pH 3 with 2 N HCl and extracted with 4 mL of diethyl ether. The organic phase was evaporated and the reaction products were taken up in acetonitrile:water:acetic acid (55:45:0.02, v/v/v) and separated isocratically with the same mixture of solvents by HPLC (Jasco, Gross Zimmern, Germany) using a C18 column (5 μM, 200 × 4.6 mm; Eurospher 100, Knauer, Berlin, Germany) at a flow rate of 1 mL min<sup>-1</sup>. Quantitation of α-ketols, γ-ketols, and 12-oxo-PDA was performed by scintillation counting, and the AOC activity was calculated using the equation:

$$\left[ \frac{\left( \frac{12\text{-oxo-PDA}}{\alpha\text{-ketol}} \right) - 0.17}{\frac{12\text{-oxo-PDA}}{\alpha\text{-ketol}}} \cdot \frac{12\text{-oxo-PDA}}{\text{total}} \right] \cdot X$$

where total means all of the radioactivity seen in the chromatogram and X is the amount of 13-HPOT (in nanomoles) added to the enzyme assay.

AOS activity was measured by the conversion of 13-HPOT to α- and γ-ketol and 12-oxo-PDA. The reaction mixture contained the resuspended corn membranes in 50 mM Tris-HCl, pH 7.5, in a total volume of 200 μL. The reaction was initiated by the addition of [1-<sup>14</sup>C]13(S)-HPOT (0.7 mCi mmol<sup>-1</sup>) to a final concentration of 45 μM. The assay was terminated by reducing the hydroperoxy fatty acid to the hydroxide with 0.5 M SnCl<sub>2</sub> in acetone (100 μL). The reaction products were extracted with diethyl ether and separated and quantified by HPLC under the conditions described for the AOC assay. Substances tested for their effect on AOC and AOS activity were added to the enzyme assay as a 10 mM aqueous or ethanolic solution.

### Electrophoresis

Enzyme purity was assessed by SDS-PAGE using 12% separating gels according to the procedure of Laemmli (1970).

### Protein Determination

The protein content was determined by the Coomassie blue dye-binding technique described by Bradford (1976).

### Estimation of the Native Molecular Mass

After hydrophobic-interaction chromatography on Octyl-Sepharose CL 4B, the fractions enriched in AOC activity were concentrated over an ultrafiltration membrane (XM 50) and loaded onto a Superdex column (60 × 16 mm; TM 75 Hi-Load, Pharmacia) equilibrated with 20 mM Tris-HCl, pH 7.5, 50 mM potassium chloride. Size-exclusion chromatography was carried out at a flow rate of 0.5 mL min<sup>-1</sup>. The column was calibrated using BSA (67 kD), ovalbumin (45 kD), and α-chymotrypsinogen (25 kD).

**Table 1.** Purification of AOC from dry corn seeds  
Typical data for 600 g of starting material.

Step	Total Protein	Total Activity	Specific Activity	Purification	Yield
	mg	nmol PDA	mol PDA mg <sup>-1</sup>	-fold	%
100,000g Supernatant	1372	16,098	11.7	1	100
Acetone precipitation	178	10,450	58.5	5	65
Octyl-Sepharose	12.3	8,200	666	56	50
Hydroxyapatite	1.05	2,133	2,032	173	13
Phenyl-Superose	0.095	1,110	11,684	998	6.8
Mono-Q	0.020	498	24,900	2128	3.1

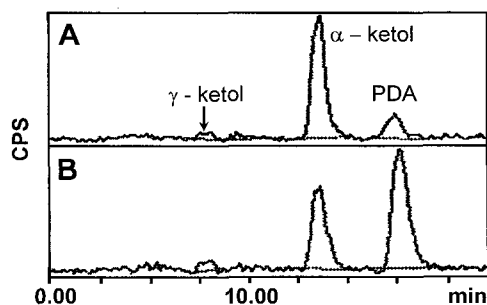
### Determination of the pI

The pI of AOC was determined by preparative IEF within a pH range of 3 to 10 using a Rotofor chamber (Bio-Rad) following the directions of the manufacturer.

## RESULTS

### Enzyme Assay

AOC activity was measured by incubation of 13-HPOT with membranes containing AOS activity and simultaneous addition of the extracts to be tested for AOC activity. The inclusion of AOS in the enzyme assay is necessary to circumvent the problems imposed by the instability of the substrate for AOC, which is now provided during the course of the test by the conversion of 13-HPOT to 12,13-EOT. In the absence of AOC, chemical hydrolysis of this compound occurs and  $\alpha$ - and  $\gamma$ -ketols and 12-oxo-PDA are formed (Fig. 1). In the presence of AOC, the allene oxide is converted solely to 12-oxo-PDA. Since the chemical and the enzymatic reaction yield the same product, one has to discriminate between the 12-oxo-PDA derived from the chemical and the enzymatic cyclization. This can be done by chiral analysis of 12-oxo-PDA. The chemical cyclization always gives an enantiomeric mixture of 50% (*R/R*)- and 50% (*S/S*)-12-oxo-PDA, whereas the enzymatically derived product possesses only the *S/S* configuration (Hamberg and Fahlstadius 1990). Therefore, one can calculate the amount of 12-oxo-PDA that is enzymatically formed by AOC by estimating the amount of 12-oxo-PDA in the *S/S*



**Figure 2.** Radiochromatograms of the products formed after incubation of [1-<sup>14</sup>C]13(*S*)-HPOT with corn membranes containing AOS and a proteinase-digested AOC preparation (A) and [1-<sup>14</sup>C]13(*S*)-HPOT with corn membranes containing AOS and active AOC (B).

configuration in addition to the 50% observed after chemical hydrolysis.

Since chiral analysis is a rather laborious and time-consuming procedure, it is not very convenient for enzyme purification, and therefore determination of the ratio of 12-oxo-PDA to  $\alpha$ -ketol was chosen to calculate AOC activity. The chemical hydrolysis of 12,13-EOT always gives a constant ratio of 0.17 (12-oxo-PDA to  $\alpha$ -ketol) (Fig. 2A). However, the presence of AOC in the test mixture results in an increase in the amount of 12-oxo-PDA and, therefore, in a changed ratio of 12-oxo-PDA to  $\alpha$ -ketol in favor of 12-oxo-PDA (Fig. 2B). The amount of 12-oxo-PDA exceeding the ratio of 0.17 is the 12-oxo-PDA that is enzymatically formed by AOC. An estimate of the total enzymic 12-oxo-PDA product can be made by determining the proportion of 12-oxo-PDA to all of the products formed and calculating the AOC activity by the equation:

$$\left[ \frac{\left( \frac{12\text{-oxo-PDA}}{\alpha\text{-ketol}} \right) - 0.17}{\frac{12\text{-oxo-PDA}}{\alpha\text{-ketol}}} \cdot \frac{12\text{-oxo-PDA}}{\text{total}} \right] \cdot X$$

where X is the amount of 13-HPOT (in nanomoles) added to the reaction mixture. The result of this calculation is the amount of 12-oxo-PDA enzymatically formed, which is also expressed in nanomoles.

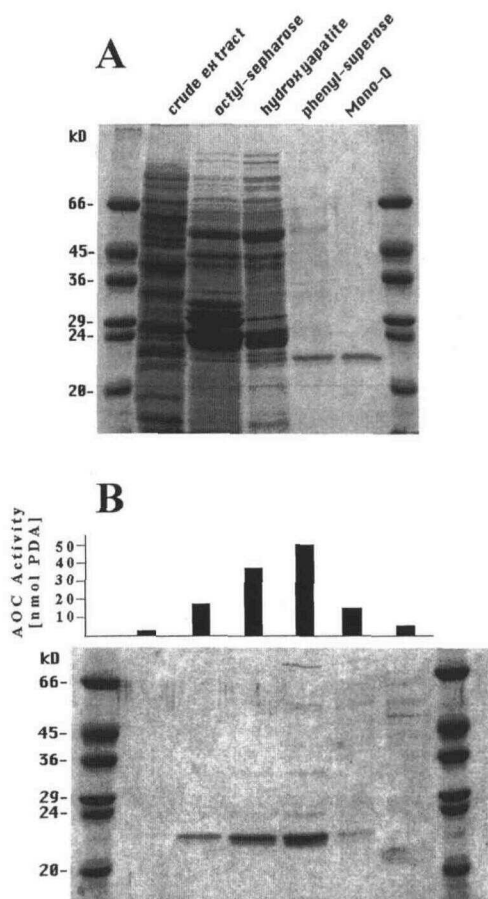
The generation of 12-oxo-PDA by the nonenzymatic reaction, and the competition of the enzyme reaction with the chemical hydrolysis of the substrate make this enzyme assay rather insensitive. For example, by reducing the amount of enzyme added to the test shown in Figure 2B to 20%, the activity can no longer be detected. Therefore, a rather high amount of enzyme is necessary to follow the activity during enzyme purification, and a tissue with a rather high AOC content is essential as starting material. We decided to start with dry corn seeds for purification of AOC, since this tissue contains the highest AOC activity examined so far (Hamberg and Fahlstadius, 1990).

### Enzyme Purification

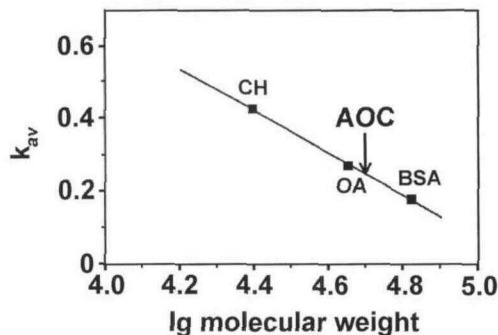
AOC could be enriched about 2000-fold with a recovery of 3% (Table 1). The most efficient steps during this purification were chromatography on resins based on hydrophobic interactions. Octyl-Sepharose, from which this pro-

tein can be eluted only with the addition of 5% ethanol in all buffers, led to a 10-fold enrichment and 70% recovery with respect to the former step. Similar results could be achieved by Phenyl-Superose. The importance of the Phenyl-Superose column for purification of AOC is illustrated in the protein pattern on SDS-PAGE (Fig. 3A). Almost all impurities were removed by this step, which is similar to results obtained by affinity chromatography.

Chromatographic media based on ionic interaction proved less valuable due to the elution of AOC in rather broad peaks on both hydroxyapatite and anion-exchange chromatography, thereby decreasing the purification and recoveries. Nevertheless, both columns were indispensable to obtain the highly purified protein shown in Figure 3A. To identify this protein as AOC we applied the activity profile obtained by Mono-Q on SDS-PAGE. As Figure 3B



**Figure 3.** A, Twelve percent SDS-PAGE of the single purification steps of AOC. With the exception of the crude extract all lanes contain the same amount of AOC activity. The amount of protein applied to the gel was 36, 36, 12, 2, and 2  $\mu\text{g}/\text{lane}$  (from left to right). B, Top, elution pattern of AOC from Mono-Q column; bottom, 12% SDS-PAGE of the fractions obtained by chromatography on Mono-Q exhibiting AOC activity. The amount of protein applied in each lane corresponds to the AOC activity seen in the top. A protein weight marker was applied on the left and right side of each gel; the molecular mass range is indicated on the left. In both gels the proteins were detected by Coomassie blue stain (CBB G-250, Serva, Heidelberg, Germany).



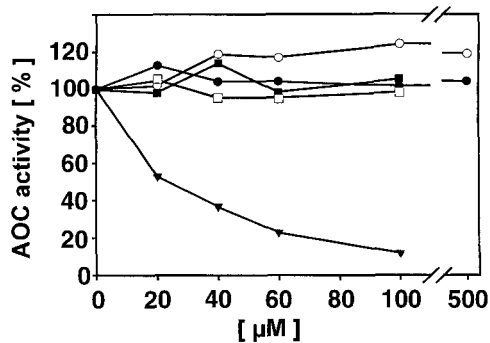
**Figure 4.** Determination of the molecular mass of AOC by size-exclusion chromatography. A fraction 50-fold enriched in AOC activity obtained after chromatography on Octyl-Sepharose was subjected to gel filtration on Superdex TM 75 (Pharmacia) at a flow rate of 0.5  $\text{mL min}^{-1}$ . The column was equilibrated with  $\alpha$ -chymotrypsinogen A (CH, 25 kD), ovalbumin (OA 45 kD), and BSA (67 kD). The elution time of AOC is indicated by an arrow.

illustrates, there is only one prominent protein band at 20 kD, which corresponded in intensity to the activity of the respective fractions. This was also the only protein that we always observed during our efforts to improve the purification scheme using other chromatographic techniques such as gel filtration or IEF (data not shown), which caused very high losses in total activity. However, we could determine the native molecular mass by size-exclusion chromatography on Superdex TM 75 (Fig. 4). A native molecular mass of 47 kD was calculated. Compared with the migration distance on SDS-PAGE, gel filtration indicated a dimeric structure for AOC. The dimerization is not due to disulfide bridges, since omission of reducing agents during electrophoresis resulted in the same electrophoretic mobility (data not shown).

As with the Mono-Q column, we observed a broad and asymmetric activity peak using preparative IEF at pH values from 5.5 to 6.5 (data not shown), which may indicate the existence of one or more isoforms of AOC with differing pI values.

### Properties of AOC

The design of the enzyme assay also requires some additional considerations when characterizing AOC. Since the first enzyme in the reaction, AOS, provides the substrate, any altered reaction condition also affected the turnover rate of this enzyme. This could lead to different substrate supplies to AOC, which would result in a changed concentration of 12,13-EOT. For example, under the conditions described in "Materials and Methods" 13-HPOT is converted within 5 s to the AOC substrate 12,13-EOT, which chemically hydrolyzes within about 4 min. This means that AOC has less than 4 min to carry out its catalytic activity. Inhibition of AOS would slow down the generation of the allene oxide, thereby increasing the time until all of the substrate is hydrolyzed and prolonging the time for AOC to act on the allene oxide. The result is an increase of the 12-oxo-PDA to  $\alpha$ -ketol ratio and an apparent



**Figure 5.** Influence of oxylipins on AOC activity. The enzyme assay was preincubated for 5 min with the indicated compounds before the reaction was initiated by the addition of [ $1\text{-}^{14}\text{C}$ ]13(*S*)-HPOT. ●, 12-Oxo-PDA; ○, JA; ▼, ( $\pm$ )-*cis*-12,13-epoxy-9(*Z*)-octadecenoic acid; □, methyl ( $\pm$ )-*cis*-12,13-epoxy-9(*Z*)-octadecenoate; and ■, ( $\pm$ )-*cis*-9,10-epoxy-12(*Z*)-octadecenoic acid.

higher AOC activity. Stimulation of AOS activity would have the reverse effect.

This effect was most evident in estimating the pH optimum for AOC. The activity was highest around pH 7. Acidic conditions of about pH 4 to 5 also showed a distinct increase in AOC activity, which might be explained by the low activity of AOS at this pH (data not shown).

Divalent ions such as calcium, magnesium, and zinc were tested for their effect on AOC. None of them showed any significant effect on AOC activity up to a concentration of 1 mM (data not shown).

To evaluate whether the enzyme is feedback-regulated, AOC was incubated with 12-oxo-PDA, the immediate product, and with JA, the end product of this branch of the oxylipin pathway (Fig. 5). Neither compound influenced enzyme activity when added at concentrations up to 500  $\mu\text{M}$ . However, another oxygenated fatty acid, ( $\pm$ )-*cis*-12,13-epoxy-9(*Z*)-octadecenoic acid, which is derived from the peroxygenase branch of the oxylipin pathway, exhibited a strong inhibitory effect. This compound reduced the AOC activity by 50% at a concentration of 20  $\mu\text{M}$ . This inhibition could be alleviated by increasing the 13-HPOT concentration, indicating a competitive inhibition. Methylation of the free carboxy group abolished the inhibitory effect of ( $\pm$ )-*cis*-12,13-epoxy-9(*Z*)-octadecenoic acid. The positional isomer ( $\pm$ )-*cis*-9,10-epoxy-12(*Z*)-octadecenoic acid showed no influence on AOC activity up to 100  $\mu\text{M}$ . Neither the divalent ions nor the oxylipins affected AOS activity.

In several systems endogenous JA accumulation after stress was prevented by the addition of different compounds to the plants, e.g. ibuprofen, induprofen, mefenamic acid, *n*-propyl gallate, phenylbutazone, tetcyclacis, ursolic acid, salicylic acid, and aspirin (Peña-Cortés et al., 1993; Wasternack, 1994; Wasternack et al., 1994; Doares et al., 1995a). In our experiments the addition of these substances at 100  $\mu\text{M}$  or 1 mM did not result in any significant inhibitory effect on AOC activity (data not shown). Aspirin and tetcyclacis stimulated AOC activity by 50 and 30%, respectively, at a concentration of 1  $\mu\text{M}$ . Tetcyclacis also inhibited AOS activity about 50% at a concentration of 100  $\mu\text{M}$  (data not shown).

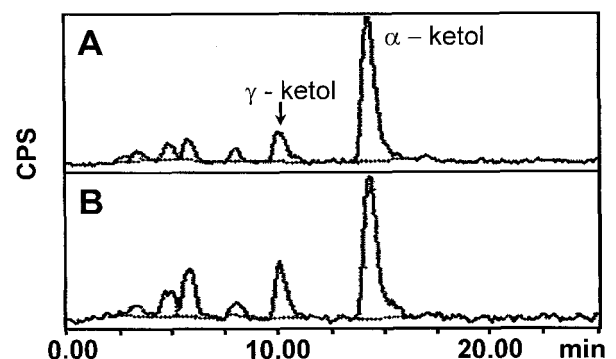
AOC was also incubated with 13-HPOT to test whether the enzyme could catalyze ring formation from allene oxides derived from linoleic acid, which lacks the 15(*Z*) double bond of linolenic acid. In the absence of AOC we observed the conversion of 13-HPOT to  $\alpha$ - and  $\gamma$ -ketols, which are derived from the chemical hydrolysis of the intermediate 12,13(*S*)-epoxy-9(*Z*),11-octadecadienoic acid (Fig. 6A). When we performed the same test in the presence of highly purified AOC, no difference in the chromatogram was detectable, indicating that no cyclization occurred with linoleic acid derivatives (Fig. 6B).

## DISCUSSION

AOC was purified to near homogeneity, as shown by Coomassie blue-stained SDS-PAGE, in which it migrated as a protein of 20 kD. Comparison of these data with the native molecular mass obtained by gel filtration, which gave a size of about 47 kD, indicates a dimeric structure for AOC. Whether it is a homo- or heterodimer awaits further analysis.

One of the interesting properties of AOC we observed during purification was its high affinity to hydrophobic-interaction chromatography matrices. For instance, the enzyme activity could only be eluted from octyl-Sepharose by inclusion of 5% ethanol to all of the buffers. Elution of the protein from Phenyl-Superose was dependent on salt-free buffers. The precipitation of AOC at a rather high acetone concentration of 45 to 70% also reveals a high hydrophobicity of this protein (Scopes, 1994). Since most of the other proteins of the 100,000g supernatant precipitated between 20 and 40% acetone saturation, this step already led to a 5-fold purification and was therefore superior to ammonium sulfate precipitation, which gave lower enrichment and higher losses of activity.

AOC was purified from the supernatant after ultracentrifugation at 100,000g. Since the homogenization buffer did not contain any salt, all peripheral and integral membrane proteins should sediment (Scopes, 1994). This implies that AOC is a soluble protein, although the precise cellular localization of this enzyme was not determined. Other enzymes of the oxylipin pathway, such as LOX, peroxygenase, and AOS, were shown to reside in the outer



**Figure 6.** Radiochromatograms of the products formed after incubation of [ $1\text{-}^{14}\text{C}$ ]13(*S*)-HPOT with corn membranes containing AOS in the absence (A) or presence (B) of highly purified AOC.

chloroplast envelope (Blée and Joyard, 1996). Since 12,13-EOT, the product of AOS and the substrate for AOC, has a half-life of only 25 s in vitro at 0°C, it would be reasonable to assume that both enzymes should be located in close proximity. This would reduce the diffusion time for 12,13-EOT from AOS to AOC and thereby diminish its chemical hydrolysis and favor the enzymatic conversion to 12-oxo-PDA. Although soluble in vitro, AOC, because of its hydrophobicity, may be loosely attached to membranes in vivo.

AOC activity was strongly inhibited by ( $\pm$ )-*cis*-12,13-epoxy-9(Z)-octadecenoic acid. The high similarity to the natural substrate suggests a competitive inhibition. This suggestion is further supported by the reversal of inhibition at higher substrate concentrations. Methylation of the carboxyl group of the inhibitor completely abolished the inhibition. This may be the reason why we did not succeed in purification of AOC by affinity chromatography, where the inhibitor was coupled to the resin via the carboxy group. The positional isomer ( $\pm$ )-*cis*-9,10-epoxy-12(Z)-octadecenoic acid did not affect AOC activity. Therefore, we conclude that the free carboxyl group of ( $\pm$ )-*cis*-12,13-epoxy-9(Z)-octadecenoic acid, as well as the location of the epoxy group in the 12,13-position, is essential for binding to AOC. The high degree of similarity between the inhibitor and the natural substrate suggests that both structural elements are essential for binding of the substrate to AOC.

In various systems accumulation of endogenous JA is observed after stress. One hour after wounding of tomato plants the JA content is elevated about 5-fold (Conconi et al., 1996). In barley leaf segments floated on 1 M sorbitol, the endogenous JA content starts to increase after 2 to 4 h and proceeds up to 10-fold (Lehmann et al., 1995). These observations support a role for JA as a signal molecule in plants. There is strong evidence that the increase in JA proceeds via de novo synthesis, as shown by the application of inhibitors of JA biosynthesis (Peña-Cortés et al., 1993; Wasternack, 1994).

How JA biosynthesis is regulated on the enzymatic level remains an open question. AOC is situated downstream of LOX and AOS in the biosynthesis of JA. In contrast to the former enzymes, the products of which can be metabolized by a variety of chemicals and enzymes, the product of the AOC reaction ultimately leads to JA (Hamberg, 1993). Therefore, AOC might be a candidate for regulation of JA biosynthesis. Neither 12-oxo-PDA nor JA, both products of this enzyme and pathway, respectively, altered AOC activity up to a concentration of 500  $\mu$ M. This rules out feedback regulation. Calcium and magnesium had no effect on AOC activity up to 1 mM, suggesting that AOC is not regulated by divalent cations. The same was found for AOS activity, which we always tested to exclude the possibility of a change in AOC activity caused by an alteration in AOS activity.

Several inhibitors have been reported to prevent JA accumulation after stress (Peña-Cortés et al., 1993; Wasternack, 1994; Wasternack et al., 1994; Doares et al., 1995a). Most of these substances were shown to inhibit fatty acid signaling in mammalian systems by inhibition of cycloo-

xigenase, the key enzyme in the formation of prostaglandins, prostacyclins, or thromboxanes (Smith and Marrett, 1991). But for most of these inhibitors the mode and the place of action in plants are not known. Except for tetacyclacis and aspirin none of these compounds had any effect on AOC or AOS. Tetacyclacis and aspirin seemed to stimulate AOC activity. In the case of tetacyclacis the stimulating effect can be explained by the inhibition of AOS (data not shown), which would result in a delayed supply of substrate for AOC. This increases the time for AOC to bind and metabolize its substrate, resulting in an apparently higher AOC activity.

A strong inhibitor of AOC was the substrate analog ( $\pm$ )-*cis*-12,13-epoxy-9(Z)-octadecenoic acid. This compound can be synthesized in vivo by the peroxygenase branch of the oxylipin pathway (Blée and Schuber, 1990; Hamberg and Hamberg, 1990). This branch uses the hydroperoxide generated by LOX to epoxidize another unsaturated fatty acid molecule. The inhibition of AOC by the product of another branch within the same pathway raises the possibility that the different branches of the oxylipin pathway can influence each other. Therefore, one might speculate that JA biosynthesis can also be regulated by a change in the carbon flow through the oxylipin pathway.

When we incubated the highly purified enzyme with 13-HPOD instead of 13-HPOT, we could not detect any cyclization product. Therefore, the double bond at position 15 is essential for the cyclization. Grechkin (1994) proposed a mechanism for the chemical cyclization whereby the 15(Z) double bond facilitates the opening of the oxirane ring of the allene oxide. The resulting C-13 carbocation is stabilized by the  $\pi$ -electrons of the 15(Z) double bond, and the cyclization is accomplished by ring closure involving the carbanion at C-9 and the carbocation at C-13. This mechanism could also apply to the enzymatic cyclization.

Recently, Blechert et al. (1995) reported the identification of 15,16-dihydro-12-oxo-phytodienoic acid and proposed a biosynthetic route for dihydroJA starting with linoleic acid. This would include the hydroperoxidation of linoleic acid to 13-HPOD and its dehydration to the allene oxide 12,13(5)-epoxy-9(Z),11-octadecadienoic acid, followed by cyclization of this compound to 15,16-dihydro-12-oxo-PDA. It is well known that LOX and AOS make use of linoleic and linolenic acid derivatives (Siedow, 1991; Song and Brash, 1991; Pan et al., 1995; Laudert et al., 1996). However, according to our results with highly purified AOC from corn kernels, the allene oxide derived from 13(5)-HPOD is not converted enzymatically to 15,16-dihydro-12-oxo-PDA. This is corroborated by other studies using crude extracts from the same tissue or from other sources such as potato tubers, cotton seedlings, and flax seed (Vick et al., 1980; Vick and Zimmerman, 1981; Hamberg and Fahlstadius, 1990). Although formation of 15,16-dihydro-12-oxo-PDA by chemical or enzymatic cyclization of the allene oxide derived from 13(5)-HPOD does not occur, or occurs to a very low extent, it is noteworthy that the allene oxide can be cyclized in the presence of albumins from several species (Hamberg and Hughes, 1988). In contrast to 12-oxo-PDA, the cyclopentanone produced in this

way was found to have the *trans* relationship between its two side chains attached to C-9 and C-13.

The presented results give us the basis for further work on the cloning and expression of AOC from corn kernels, which may be a valuable tool to elucidate the biosynthesis and regulation of JA accumulation.

#### ACKNOWLEDGMENTS

The authors thank Dr. Thomas Vogt for his excellent advice and for critically reading the manuscript, Ms. G. Hamberg for technical assistance, and Dr. Rainer Atzorn for the gift of 12-oxo-PDA.

Received January 23, 1997; accepted March 10, 1997.

Copyright Clearance Center: 0032-0889/97/114/0565/09.

#### LITERATURE CITED

- Blechert S, Brodschelm W, Hölder S, Kammerer L, Kutchan TM, Müller JM, Xia ZQ, Zenk MH (1995) The octadecanoic pathway: signal molecules for the regulation of secondary pathways. *Proc Natl Acad Sci USA* **92**: 4099–4105
- Blée E, Joyard J (1996) Envelope membranes from spinach chloroplasts are a site of metabolism of fatty acid hydroperoxides. *Plant Physiol* **110**: 445–454
- Blée E, Schuber F (1990) Efficient epoxidation of unsaturated fatty acids by a hydroperoxide-dependent oxygenase. *J Biol Chem* **265**: 12887–12894
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254
- Conconi A, Miquel M, Browse JA, Ryan CA (1996) Intracellular levels of free linolenic and linoleic acids increase in tomato leaves in response to wounding. *Plant Physiol* **111**: 797–803
- Creelman RA, Mullet JE (1995) Jasmonic acid distribution and actions in plants: regulation during development and response to biotic and abiotic stress. *Proc Natl Acad Sci USA* **92**: 4114–4119
- Crombie L, Morgan DO (1988) Formation of acyclic  $\alpha$ - and  $\gamma$ -ketols and 12-oxo-phytodienoic acid from linolenic acid 13-hydroperoxide by a flax enzyme preparation. Evidence for a single enzyme leading to a common allene epoxide intermediate. *J Chem Soc Chem Commun* **8**: 558–560
- Doares SH, Narváez-Vásquez J, Conconi A, Ryan CA (1995a) Salicylic acid inhibits synthesis of proteinase inhibitors in tomato leaves induced by systemin and jasmonic acid. *Plant Physiol* **108**: 1741–1746
- Doares SH, Syrovets T, Weiler EW, Ryan CA (1995b) Oligogalacturonides and chitosan activate plant defensive genes through the octadecanoic pathway. *Proc Natl Acad Sci USA* **92**: 4095–4098
- Falkenstein E, Groth B, Mithöfer A, Weiler EW (1991) Methyl jasmonate and  $\alpha$ -linolenic acid are potent inducers of tendrils coiling. *Planta* **185**: 316–322
- Farmer EE, Ryan CA (1990) Interplant communication: airborne methyl jasmonate induces synthesis of proteinase inhibitors in plant leaves. *Proc Natl Acad Sci USA* **87**: 7713–7716
- Farmer EE, Ryan CA (1992) Octadecanoic precursors of jasmonic acid activate the synthesis of wound-inducible proteinase inhibitors. *Plant Cell* **4**: 129–134
- Franceschi VR, Grimes HD (1991) Induction of soybean vegetative storage proteins and anthocyanins by low-level atmospheric methyl jasmonate. *Proc Natl Acad Sci USA* **88**: 6745–6749
- Galliard T, Phillips DR (1972) The enzymic conversion of linoleic acid into 9-(nona-1',3'-dienoxy)non-8-enoic acid, a novel unsaturated ether derivative isolated from homogenates of *Solanum tuberosum* tubers. *Biochem J* **129**: 743–753
- Gardner HW (1991) Recent investigations into the lipoxygenase pathway of plants. *Biochim Biophys Acta* **1084**: 221–239
- Grechkin AN (1994) Cyclization of natural allene oxide fatty acids: the anchimeric assistance of  $\beta$ , $\gamma$ -double bond beside the oxirane and the reaction mechanism. *Biochim Biophys Acta* **1213**: 199–206
- Grechkin AN, Hamberg M (1996) Divinyl ether synthase from garlic (*Allium sativum* L.) bulbs: sub-cellular localization and substrate regio- and stereospecificity. *FEBS Lett* **388**: 112–114
- Gundlach H, Müller MJ, Kutchan TM, Zenk MH (1992) Jasmonic acid is a signal transducer in elicitor-induced plant cell cultures. *Proc Natl Acad Sci USA* **89**: 2389–2393
- Hamberg M (1989) Fatty acid allene oxides. *J Am Oil Chem Soc* **66**: 1445–1449
- Hamberg M (1993) Pathways in the biosynthesis of oxylipins in plants. *J Lipid Med* **6**: 375–384
- Hamberg M, Fahlstadius P (1990) Allene oxide cyclase: a new enzyme in plant lipid metabolism. *Arch Biochem Biophys* **276**: 518–526
- Hamberg M, Gardner HW (1992) Oxylipin pathway to jasmonates: biochemistry and biological significance. *Biochim Biophys Acta* **1165**: 1–18
- Hamberg M, Gotthammar B (1973) A new reaction of unsaturated fatty acid hydroperoxides: formation of 11-hydroxy-12,13-epoxy-9-octadecenoic acid from 13-hydroperoxy-9,11-octadecadienoic acid. *Lipids* **8**: 737–744
- Hamberg M, Hamberg G (1990) Hydroperoxide-dependent epoxidation of unsaturated fatty acids in the broad bean (*Vicia faba* L.). *Arch Biochem Biophys* **283**: 409–416
- Hamberg M, Hughes M (1988) Fatty allene oxides. III. Albumin-induced cyclization of 12,13(S)-epoxy-9(Z),11-octadecadienoic acid. *Lipids* **23**: 469–475
- Kauss H, Jeblick W, Ziegler J, Krabler W (1994) Pretreatment of parsley (*Petroselinum crispum* L.) suspension cultures with methyl jasmonate enhances elicitation of activated oxygen species. *Plant Physiol* **105**: 89–94
- Kutchan TM (1993) 12-oxo-Phytodienoic acid induces accumulation of berberine bridge enzyme transcript in a manner analogous to methyl jasmonate. *J Plant Physiol* **142**: 502–505
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685
- Laudert D, Pfannschmidt U, Lottspeich F, Holländer-Czytko H, Weiler EW (1996) Cloning, molecular and functional characterization of *Arabidopsis thaliana* allene oxide synthase (CYP 74), the first enzyme of the octadecanoic pathway to jasmonates. *Plant Mol Biol* **31**: 323–335
- Lehmann J, Atzorn R, Brückner C, Reinbothe S, Leopold J, Wasternack C, Parthier B (1995) Accumulation of jasmonate, abscisic acid, specific transcripts and proteins in osmotically stressed barley leaf segments. *Planta* **197**: 156–162
- Meyer A, Miersch O, Büttner C, Dathe W, Sembdner G (1984) Occurrence of the plant growth regulator jasmonic acid in plants. *J Plant Growth Regul* **3**: 1–8
- Nojiri H, Sugimori M, Yamane H, Nishimura Y, Yamada A, Shibuya N, Kodama O, Murofushi N, Omori T (1996) Involvement of jasmonic acid in elicitor-induced phytoalexin production in suspension-cultured rice cells. *Plant Physiol* **110**: 387–392
- Pan Z, Durst F, Werck-Reichhart D, Gardner HW, Camara B, Cornish K, Backhaus RA (1995) The major protein of guayule rubber particles is a cytochrome P450. *J Biol Chem* **270**: 8487–8494
- Parthier B (1991) Jasmonates, new regulators of plant growth and development: many facts and few hypotheses on their actions. *Bot Acta* **104**: 446–454
- Peña-Cortés H, Albrecht T, Prat S, Weiler EW, Willmitzer L (1993) Aspirin prevents wound-induced gene expression in tomato leaves by blocking jasmonic acid biosynthesis. *Planta* **191**: 123–128
- Reinbothe C, Tewes A, Lehmann J, Parthier B, Reinbothe S (1994) Induction by methyl jasmonate of embryogenesis-related proteins and mRNAs in *Nicotiana plumbaginifolia*. *Plant Sci* **104**: 59–70
- Reinbothe S, Reinbothe C, Parthier B (1993) Methyl jasmonate represses translation initiation of a specific set of mRNAs in barley. *Plant J* **4**: 459–467



- Roloff A, Parthier B, Wasternack C** (1994) Relationship between degradation of ribulose-bisphosphate carboxylase/oxygenase and synthesis of an abundant protein of 23 kDa of barley leaves (*Hordeum vulgare* cv. Salome) induced by jasmonates. *J Plant Physiol* **143**: 39–46
- Scopes RK** (1994) *Protein Purification: Principles and Practice*, Ed 3. Springer-Verlag, Berlin
- Sembdner G, Parthier B** (1993) The biochemistry and the physiological and molecular actions of jasmonates. *Annu Rev Plant Physiol Plant Mol Biol* **44**: 569–589
- Shibata Y, Matsui K, Kajiwara T, Hatanaka A** (1995) Purification and properties of fatty acid hydroperoxide lyase from green bell pepper fruits. *Plant Cell Physiol* **36**: 147–156
- Siedow JN** (1991) Plant lipoxygenase: structure and function. *Annu Rev Plant Physiol Plant Mol Biol* **42**: 145–188
- Smith WL, Marnett LJ** (1991) Prostaglandin endoperoxide synthase: structure and catalysis. *Biochim Biophys Acta* **1083**: 1–17
- Song WC, Brash AR** (1991) Purification of an allene oxide synthase and identification of the enzyme as a cytochrome P-450. *Science* **253**: 781–784
- Song WC, Funk CD, Brash AR** (1993) Molecular cloning of an allene oxide synthase: a cytochrome P450 specialized for the metabolism of fatty acid hydroperoxides. *Proc Natl Acad Sci USA* **90**: 8519–8523
- Vick BA, Feng P, Zimmerman DC** (1980) Formation of 12-<sup>[18O]</sup>oxo-*cis*-10,*cis*-15-phytodienoic acid from 13-<sup>[18O]</sup>hydroperoxylinolenic acid by hydroperoxide cyclase. *Lipids* **15**: 468–471
- Vick BA, Zimmerman DC** (1981) Lipoxygenase, hydroperoxide isomerase, and hydroperoxide cyclase in young cotton seedlings. *Plant Physiol* **67**: 92–97
- Vick BA, Zimmerman DC** (1983) The biosynthesis of jasmonic acid: a physiological role for plant lipoxygenase. *Biochem Biophys Res Commun* **111**: 470–477
- Vick BA, Zimmerman DC** (1986) Characterization of 12-oxo-phytodienoic acid reductase in corn. *Plant Physiol* **80**: 202–205
- Wasternack C** (1994) Jasmonate-induced alteration of gene expression in barley. *Trends Comparative Biochem Physiol* **1**: 1255–1268
- Wasternack C, Atzorn R, Blume B, Leopold J, Parthier B** (1994) Ursolic acid inhibits synthesis of jasmonate-induced proteins in barley leaves. *Phytochemistry* **35**: 49–54
- Weidhase RA, Kramell HM, Lehmann J, Liebisch HW, Lerbs W, Parthier B** (1987) Methyl jasmonate-induced changes in the polypeptide pattern of senescing barley leaf segments. *Plant Sci* **51**: 177–186
- Weiler EW, Albrecht T, Groth B, Xia ZQ, Luxem M, Liss H, Andert L, Spengler P** (1993) Evidence for the involvement of jasmonates and their octadecanoid precursors in the tendrill coiling response of *Bryonia dioica*. *Phytochemistry* **32**: 591–600