Biochemical properties of lipoxygenase from opium poppy chloroplasts

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Abstract

Lipoxygenase (LOX) from opium poppy (*Papaver somniferum* L.) chloroplasts was isolated and 126.1-fold purified to electrophoretic homogeneity by combination of ion-exchange chromatography on *HA-Ultragel* column and affinity chromatography on a linoleyl-aminopropyl agarose column. The relative molecular mass of the LOX determined by SDS-PAGE was 92 kDa. Kinetic properties of purified LOX were determined in spectrophotometric assay by using of linoleic acid (K_M = 1.78 mM and V_{max} = 11.4 μmol mg^{-1} min^{-1}) and linolenic acid (K_M = 1.27 mM and V_{max} = 10.2 μmol mg^{-1} min^{-1}). The optimum pH was 6.0 for both linoleic and linolenic acid dioxygenation catalyzed by LOX. HPLC analysis of the products revealed a dual positional specificity of linoleic acid dioxygenation at pH 6.0 with ratio of 9- and 13-hydroperoxide products being about 1:1. The activity of purified LOX was stimulated by Mg^{2+} and Ca^{2+}.

Additional key words: fatty acid dioxygenation, oxylipin biosynthesis, *Papaver somniferum*, stress signaling.

Introduction

Lipoxygenases (LOX, E.C. 1.13.11.12) are non-heme iron containing dioxygenases widely distributed within plant and animal species. LOX are also active in the cells of fungi and also in bacteria. The regio- and stereospecific insertion of molecular oxygen into (1*Z*, 4*Z*)-pentadiene moiety of polyunsaturated fatty acids, catalyzed by LOX, is the first step of the lipoxygenase pathway (Feussner and Wasternack 2002). The suitable physiological substrates of plant LOX are both linoleic (18:2) and α-linolenic (18:3) acid, abundant in plant tissues. By the action of different LOX isoforms, the substrates are converted into corresponding 9- and 13-hydroperoxide derivatives, which are then utilized by the enzymes of the lipoxygenase pathway. In plants, LOX were found in several subcellular compartments. The major evidence for LOX activity is associated with cytoplasm and with chloroplasts.

According to the positional specificity of linoleic acid dioxygenation, LOX are classified as 13-LOX and 9-LOX. However, LOX of some plant species were found to have a dual positional specificity and produce both 9- and 13-hydroperoxides of linoleic acid (Hughes *et al*. 2001, Kim *et al*. 2002, Garbe *et al*. 2006). The most recent classification of plant LOX is based on the relationship in the DNA coding sequences and amino acid sequences respectively. According to this criterion, there are two molecular families known as type 1 and type 2 LOX. Type 1 LOX are soluble enzymes localized in the cytoplasm and share a high degree of homology in nucleic acid sequence. Type 2 LOX, associated with the chloroplasts, share a much lower DNA sequence homology among plant species.

The aim of this work was to isolate and characterize LOX from opium poppy chloroplasts.

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Abbreviations: GrB - grinding buffer; HOD - hydroxyoctadecadienoic acid; HPOD - hydroperoxyoctadecadienoic acid; KPB - potassium phosphate buffer; LA - linoleic acid; LAPA - linoleyl-aminopropyl agarose; LeA - linolenic acid; LOD - limit of detection; LOQ - limit of quantification; LOX - lipoxygenase; PMSF - phenylmethylsulfonyl fluoride; RP HPLC - reversed-phase high-performance liquid chromatography; SP HPLC - straight-phase high-performance liquid chromatography; *TX-114* - *Triton X-114*.
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Materials and methods

The leaves of opium poppy (*Papaver somniferum* L.) cv. Lazur were harvested during flowering and stored on ice or frozen until use. The chemicals used in experiments were purchased from *Sigma*, *Fluka*, *Fermentas*, *Riedel de Haën*, *Cayman Chemical* and *Merck*.

The leaves were cut into pieces (about 1 × 1 cm) and homogenized using a custom grinding buffer (GrB) consisting of Hepes-KOH (50 mM, pH 7.5), sorbitol (330 mM), $MgCl_2$ (1 mM), $MnCl_2$ (1 mM), EDTA sodium salt (1 mM) and cysteine hydrochloride (1 mM). The leaves (about 280 g) were homogenized with 1.9 dm^3 of pre-chilled GrB on ice. The homogenate was filtered through nylon cloth and the filtrate was centrifuged at 4 °C for 1 min at 150 *g* (*JS 24.38 rotor*, *Beckman,* Coulter, USA). The pellet was discarded and the supernatant was subjected to centrifugation 15 min at 2 000 *g*. Partially purified chloroplasts were resuspended in 10 dm^3 of GrB and purified on *Percoll* density gradient (40/80 %) prepared by stacking 8 dm^3 of 40 % *Percoll* (v/v, in GrB) on 4 dm^3 of 80 % *Percoll* in a plastic tube. Chloroplast suspension (2 cm^3) was loaded on the density gradient per tube. Tubes were subjected to centrifugation 20 min at 3000 *g*. Unbroken chloroplasts were concentrated on the interface of 40 and 80 % *Percoll*. Finally, the isolated chloroplasts were collected and refrigerated until use.

The suspension of purified chloroplasts was diluted with GrB (1:1, v/v) and subjected to centrifugation 10 min at 3000 *g* to separate chloroplasts and *Percoll*. The pellet was lysed using 30 cm^3 of 10 mM potassium phosphate buffer (KPB), pH 6.0, containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and lysis was boosted by sonication. Glycerol (30 %, v/v) and *Triton X-114* (*TX-114*, 4 %, v/v) were added to the lysate and it was divided into 2 cm^3 portions in microcentrifuge tubes. Tubes were subjected to temperature-induced phase separation at 37 °C (10 min). During incubation the solution became turbid. The separation of detergent phase (droplets of detergent) and water solution of proteins was achieved by centrifugation (10 min, 10 000 *g*, *Hettich Mikro 200R*, UK). The green pigment of chloroplasts was concentrated in the detergent-rich phase, which was removed by pipette.

The water phase (soluble proteins of chloroplasts) was loaded on a *HA-Ultrogel* column (12 × ø 1.8 cm), equilibrated with three column volumes of 10 mM KPB, pH 6.0. The column was eluted stepwise with 10, 50, 150 and 300 mM KPB at 1 cm^3 min^{-1}. All buffers contained 1 mM PMSF. Active fractions were combined and concentrated using an *Amicon Ultra-4* (*Millipore*, USA) centrifugal filter device with 50 kDa nominal molecular mass limit.

For affinity chromatography, linoleyl-aminopropyl agarose (LAPA) was prepared by binding linoleic acid to ω-aminopropyl agarose using a water-soluble carbodiimide coupling reagent (1-ethyl-3-[3-dimethyl-aminopropyl]carbodiimide hydrochloride). Briefly, 10 cm^3 of ω-aminopropyl agarose was washed with 50 cm^3 of distilled water prior to coupling with linoleic acid. The ligand solution was prepared by solubilizing 0.155 cm^3 of linoleic acid in 50 cm^3 of water with 0.155 cm^3 of *Triton X-100*. The ligand solution was mixed with ω-aminopropyl agarose and the coupling reaction was initiated by the addition of 0.19 g of the coupling reagent. The reaction was carried out under nitrogen for 24 h. The modified carrier was placed in a chromatography column and washed with 100 cm^3 of isopropanol (30 %, v/v) to remove excess linoleic acid. The LAPA column (3.5 × ø 1 cm) was equilibrated with 5 column volumes of 10 mM KPB. The sample obtained by ion exchange chromatography on *HA-Ultrogel* column was loaded onto the LAPA column and the column was eluted stepwise with 10, 150, 300 mM KPB (pH 6.0) and 300 mM KPB (pH 8.5). The elution of proteins was monitored at $\lambda = 280$ nm and the dioxygenase activity of peak fractions was tested spectrophotometrically (*Lambda 35*, *PerkinElmer*, USA). All active fractions were concentrated using *Amicon Ultra-4* centrifugal filter device with 50 kDa nominal molecular mass limit.

The activity of LOX was determined spectrophotometrically (*Lambda 35*) by recording the rate of conversion of linoleic acid to hydroperoxyoctadecadienoic acid (HPOD) at 234 nm (coefficient of absorbance 25000 M^{-1} cm^{-1}). Linoleic acid (10 mM) was used as the substrate. For activity assay, 0.2 cm^3 of enzyme solution was incubated with 0.07 cm^3 of substrate solution and 1.13 cm^3 of KPB (100 mM, pH 6.0). The reaction was initiated by the addition of the substrate. The substrate solution was prepared by dissolving 0.0157 cm^3 of linoleic acid with the same volume of *Tween 20* in 1 cm^3 of distilled water. The solution was clarified by the addition of 0.10 cm^3 of 1 M NaOH. The volume was filled up to 5 cm^3 with distilled water, giving 10 mM substrate solution. The solution of linolenic acid for kinetic assays was prepared by dissolving 0.0151 cm^3 of pure linolenic acid by the same manner as for linoleic acid. For kinetic studies, 0.05, 0.07, 0.09, 0.11 and 0.14 cm^3 of the substrate solution was taken into reaction. K_M and V_{max} were calculated from the Lineweaver-Burk plot.

The concentration of proteins was determined using a bicinchoninic acid assay kit (*QuantiPro BCA* assay kit, *Sigma*, St. Louis, USA) according to manufacturer's instructions. Bovine serum albumin (part of the kit) was used as a standard. The content of chlorophyll *a* was determined according to the method of Porra (2002). Four volumes of cold acetone were mixed with 1 volume of the sample. After centrifugation 5 min at 10 000 *g* (*Hettich Mikro 200R*), absorbance of the supernatant was recorded at 646.6 and 663.6 nm (*Lambda 35*).

SDS electrophoresis was carried out in 8 % polyacrylamide gel according to Laemmli (1970). For determination of relative molecular mass of purified LOX, *Page Ruler™* protein ladder (*Fermentas*, Germany) molecular mass markers were used. The gel was run for 60 min at 160 V (*Mini Protean II* electrophoresis cell, *Bio-Rad,* Hercules, USA) and proteins were stained with silver according to Blum *et al*. (1987).

To resolve the isomeric composition of LOX products, 0.1 cm^3 of purified enzyme was incubated with 0.01 cm^3 of 10 % linoleic acid (v/v, in methanol) and 0.89 cm^3 of KPB (100 mM; pH 6.0) for 30 min at laboratory temperature. Reaction was stopped by the addition of $NaBH_4$ and the solution was acidified with **?**M HCl (0.100 cm^3). Sample was extracted with diethyl ether (2 × 1 cm^3). Diethyl ether was evaporated and sample was reconstituted in 0.2 cm^3 of mobile phase (solvent A/B 85:15, for composition see later) and 0.07 cm^3 was analyzed by RP-HPLC.

RP-HPLC analysis was carried out on *Hewlett–Packard 1050* (Waldbronn, Germany) HPLC system equipped with *MWD*, autosampler and quaternary pump. Chromatograms were recorded simultaneously at 234 and 210 nm. RP-HPLC was carried out on *120-5 Nucleosil* C18 column (*Watrex*, Czech Rebublic; 250 × 4 mm, 5 μm particle size) with a gradient system of: solvent A (methanol/acetic acid 99.9:0.1, v/v) and solvent B (water) and a flow rate 0.2 cm^3 min^{-1}. The program of elution was as follows: 12 min with solvent system A/B 85:15, flow rate 0.2 cm^3 min^{-1}; 10 min with 100 % A, flow rate 0.4 cm^3 min^{-1} and 6 min with solvent system A/B 85:15 at flow rate 0.4 cm^3 min^{-1}. Peak fraction at 234 nm was collected upon elution from the column and the sample was dried under nitrogen.

Sample was reconstituted in 0.15 cm^3 of *n*-hexane and 0.05 cm^3 was injected onto the column. SP-HPLC of hydroxy fatty acid isomers was carried out on *Zorbax Rx SIL* column (*Agilent,* Waldbronn, Germany; 150 × 2.1 mm, 5-μm particle size). The mobile phase mixed of *n*-hexane and acidified 2-propanol (1 cm^3 of acetic acid in 1000 cm^3 of 2-propanol) 99:1 (v/v) has flowed through the system at the rate 0.2 cm^3 min^{-1}. Analyses were performed using the same HPLC system equipped with *MWD* detector. Chromatograms were recorded at 234 nm. The products of LOX were identified using 9(*S*)-hydro-xyoctadeca-10*E*, 12*Z*-dienoic acid (9-HOD, 5 μg cm^{-3}) and 13(*S*)-hydro-xyoctadeca-9*Z*, 11*E*-dienoic acid (13-HOD, 5 μg cm^{-3}) purchased from *Cayman Pharma* (Czech Rebublic). For quantification of LOX products by SP-HPLC, calibration curves for 9- and 13-HOD were obtained in the range 0.1 - 10.0 and 10.0 - 100.0 μg cm^{-3}. LOD and LOQ were determined by analysis at the samples with trace 9- and 13-HOD level (blank samples).

The effect of pH on LOX activity was evaluated in KPB (100 mM, pH 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0) by HPLC as described above. The effect of bivalent ions on linoleic acid dioxygenation catalyzed by LOX was evaluated by HPLC. Incubations were carried out in KPB (100 mM, pH 6.0). Aliquots of $MgCl_2$ (10 mM) were added to the incubation mixtures to obtain final concentrations of Mg^{2+} 0.5, 1.0, 1.5 and 2.0 mM. In the case of Ca^{2+}, aliquots of $CaCl_2$ stock solution (10 mM) were added to the incubations to obtain final concentrations of Ca^{2+} 1.0, 2.0, 3.0, 4.0 and 5.0 mM.

Results and discussion

Purification of LOX: Opium poppy chloroplasts LOX was isolated using a solubilization of chloroplast pellet by sonication in the presence of detergent. The concentration of a detergent exceeding its CMC (critical micellar concentration) is eligible to solubilize hydrophobic and amphipatic molecules into mixed micelles or micellar aggregates (Garavito and Ferguson-Miller 2001). *TX-114* is commonly used in biochemical applications to solubilize and separate proteins due to its low cloud-point temperature being 37 °C (Purkait *et al*. 2009). Its usage in removing chlorophylls, phospholipids, phenols and some hydrophobic proteins from homogenates of chloroplasts has been previously described (Andersen and Møller 1998, Pérez-Gilabert *et al*. 2001). In order to remove chlorophylls from the lysate of chloroplasts, we used 4 % (v/v) *TX-114*. After centrifugation, the green pigment of chloroplasts was concentrated in the detergent-rich phase, which was removed by pipette. To increase the density of

Table 1. Summary of LOX purification.

Stage	Volume [cm^3]	Proteins [μg cm^{-3}]	Specific activity [nkat mg^{-1}]	Chlorophyll *a* [μg cm^{-3}]	Purification [fold]
Lysate of chloroplasts	35	3940	0.79	31.6	1
TX-114 phase partitioning	25	4800	0.43	1.7	0.54
HA Ultrogel	2.6	21.3	18.59	ND	23.7
LAPA	4	4.06	99.10	ND	126.1

the aqueous phase, the concentration of glycerol above 30 % (v/v) is needed, so that the detergent-rich phase overlays the aqueous phase and can be easily removed. The phase partitioning led to a slight decrease in specific activity of LOX (Table 1) but eliminated 96 % of chlorophyll *a* present in the lysate. Loading the protein sample obtained by phase partitioning onto the *HA-Ultrogel* column led to 23.7-fold increase of specific activity of LOX, which was present in protein fraction eluted by 150 mM KPB (Fig. 1). For further purification, active fractions were pooled, concentrated and loaded on a linoleyl-aminopropyl agarose column. LOX activity was present in a sharp peak eluted by increasing the concentration of KPB to 300 mM and by a shift of pH to 8.5 (Fig. 2). According to the soybean LOX (Allen *et al*. 1977), the opium poppy LOX was eluted by turning the pH away from its optimal value. By this step, total 126.1-fold increase of specific activity was achieved.

SDS-PAGE: SDS electrophoresis in 8 % polyacrylamide gel revealed a single protein band in the fraction obtained on linoleyl-aminopropyl agarose column (Fig. 1). The relative molecular mass of opium poppy chloroplast LOX was determined to be 92 kDa. Chloroplast LOX were found to have relative molecular mass ranging from 90 kDa (Rangel *et al*. 2002) to 105 kDa (Wang *et al*. 2008) as determined by SDS-PAGE.

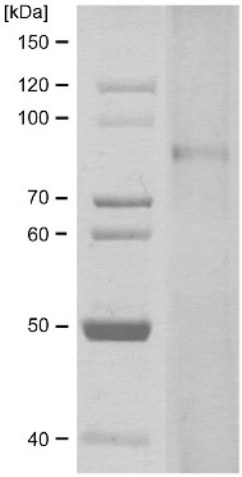

Fig. 1. Electrophoretic analysis of opium poppy chloroplast LOX (SDS-PAGE, 8 % gel, stained with silver). *Lane* 1 - molecular mass marker, *lane* 2 - LOX obtained by linoleyl-aminopropyl agarose.

Kinetic properties of opium poppy chloroplast LOX: Kinetic properties of the purified LOX were investigated in spectrophotometrical assay using sodium linoleate and sodium linolenate. On the basis of Lineweaver-Burk plot, Michaelis constant (K_M) of the purified chloroplast LOX was 1.78 mM and the V_{max} was calculated to be 11.4 μmol mg^{-1} min^{-1} at pH 6.0. For linolenic acid, K_M was found to be 1.27 mM and V_{max} 10.2 μmol mg^{-1} min^{-1}. For other plant LOX, similar values of K_M for linoleic acid were found, but there is a lack of published kinetic data for chloroplast LOX. Michaelis constant of membrane-bound LOX isolated from banana leaf was found to be 0.15 mM (Kuo *et al*. 2006). For plasma membrane-bound LOX from soybean (Fornarolli *et al*. 1999), it was 200 μM. However, LOX from eggplant chloroplasts (López-Nicolas *et al*. 2001) and that from tomato fruit membranes (Bowsher *et al*. 1992) were found to have K_M 1.4 and 6.2 μM, respectively, while olive fruit LOX (Lorenzi *et al*. 2006) was found to have K_M 82.4 μM. In contrast to plastidial and membrane-bound forms, K_M of a soluble LOX from *Cichorium intybus* was 14.9 mM (Daglia *et al*. 2005).

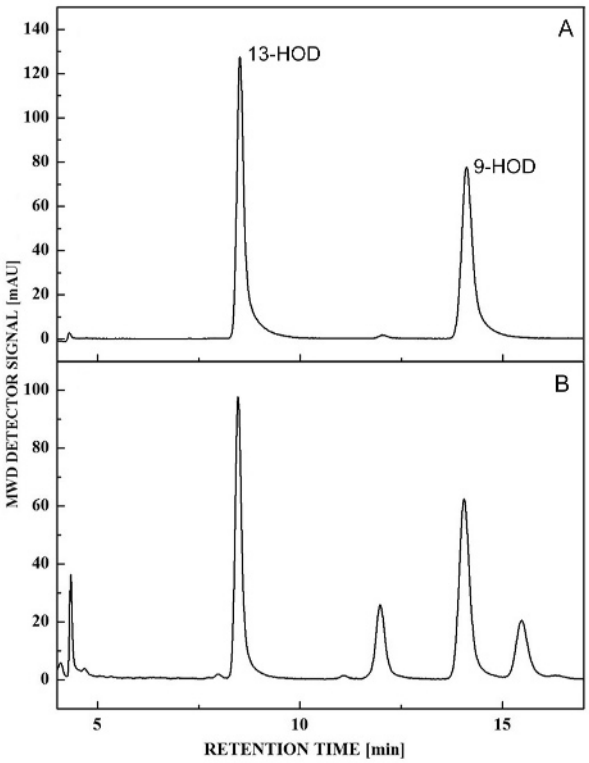

Fig. 2. Determination of the positional specificity of opium poppy chloroplast LOX. Reaction products were separated by RP-HPLC followed by SP-HPLC analysis of hydroperoxide products formed by LOX from linoleic acid. *A* - standards of 13-HOD and 9-HOD (5 μg cm^{-3}), *B* - products of opium poppy chloroplast LOX.

HPLC analysis of the opium poppy chloroplast LOX products showed a dual positional specificity of this enzyme (Fig. 2) with ratio of 9-/13-HOD being about 1:1, when linoleic acid was used as the substrate at pH 6.0. Based on calibration curves, LOD of linoleic acid hydroperoxides in the incubations were calculated to be 87.1 ng cm^{-3} for 9-HOD and 24.9 ng cm^{-3} for 13-HOD. Also LOQ were calculated for both standards to be 96.0 ng cm^{-3} for 9-HOD and 36.3 ng cm^{-3} in the case of 13-HOD.

In agreement with other chloroplast LOX, the optimum of linoleic acid dioxygenation was found at pH 6.0 (Fig. 3). The ratio 9-/13-HOD was not affected by the pH in contrast to some other LOX (Lang and Feussner 2007, Hornung *et al*. 2008). Recently, LOX with dual positional specificity have been described, including that from rice seeds (Wang *et al*. 2008) and olive fruits (Palmieri-Thiers *et al*. 2009). Dual specific LOX are supposed to be able to supply hydroperoxides both for jasmonate and plant leafy volatiles biosynthesis.

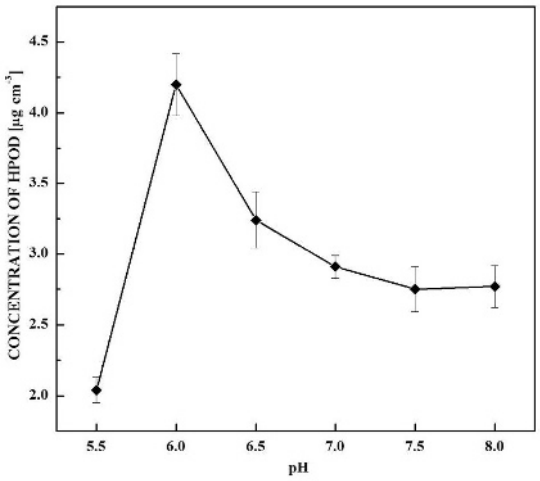

Fig. 3. HPLC analysis of LOX activity at different pH. Analysis of total HPOD formed in the reaction at different pH showed optimum for linoleic acid dioxygenation at pH 6.0.

Chloroplasts have been shown to contain high concentrations of magnesium and calcium ions (Portis and Heldt 1976), which may be able to influence the activity of many plastidial proteins and enzymes. In order to resolve the impact of Ca^{2+} and Mg^{2+}, the effect of $MgCl_2$ and $CaCl_2$ on linoleic acid dioxygenation was analyzed. We found that Mg^{2+} stimulate LOX catalyzed production of linoleic acid hydroperoxides having maximum at 1.0 mM concentration (data not shown). Ca^{2+} also strongly activated linoleic acid hydroperoxide accumulation (data not shown), but showed no maximum in the tested range.

Conclusions: A dual specific LOX, as determined by HPLC analysis of its products, was isolated and purified from opium poppy chloroplasts. Although its activity is elevated in the presence of bivalent cations, its positional specificity is affected neither by Ca^{2+} nor by Mg^{2+} present in plastids. The relative molecular mass (92 kDa) determined by SDS-PAGE corresponds with other known plastidial LOX. However, its physiological role remains still unknown.

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