Purification and properties of lipoxygenase from opium poppy seedlings
(Papaver somniferum L.)

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Abstract: Lipoxygenase (linoleate:oxygen oxidoreductase; EC 1.13.11.12; LOX) is a key enzyme in the signaling pathway leading to the production of biologically active compounds involved in plant growth development and stress responses. In this study the novel LOX enzyme from opium poppy (Papaver somniferum L.) seedlings was purified to electrophoretic homogeneity and characterized. LOX activity during the germination of opium poppy seeds was analyzed, and the highest LOX activity was determined on the fourth day of germination. Opium poppy LOX was purified from 4-day-old poppy seedlings after ammonium sulfate precipitation followed by hydrophobic chromatography (Phenyl-Sepharose CL-4B), ion exchange chromatography (Q-Sepharose), and affinity chromatography (linoleyl-aminopropyl agarose) for the first time. The relative molecular weight of purified LOX was estimated to be 78 kDa by immunoblotting. The highest enzyme activity occurred at pH 6.5 and 0.75 mM calcium ion concentration. LOX showed preferential activity towards linoleic acid followed by linolenic acid as a substrate. To investigate the positional specificity of the LOX reaction, purified LOX was incubated with linoleic acid, and the products were analyzed by high-performance liquid chromatography. Opium poppy LOX converted linoleic acid to 13-hydroperoxy-(9Z,11E)-octadecadienoic acid (63.5%) and, to a lesser extent, 9-hydroperoxy-(10E,12Z)-octadecadienoic acid (36.5%).

Key words: Lipoxygenase, enzyme purification, biochemical characterization, positional specificity, Papaver somniferum

1. Introduction
Lipoxygenases (LOXs; linoleate:oxygen oxidoreductases, EC 1.13.11.12) belong to a class of widespread fatty acid dioxygenases. This group of enzymes catalyzes the regio- and stereospecific oxygenation of polyunsaturated fatty acids containing a cis, cis-1,4-pentadiene system of double bonds to form conjugated hydroperoxy fatty acids. Linoleic and linolenic acids are the best substrates for lipoxygenases in plants (Liaonanchka and Feussner, 2006). According to the positional specificity of linoleic acid dioxygenation, plant LOXs are classified as 13-LOX and 9-LOX; 13-LOX catalyzes the addition of oxygen at carbon atom 13 and 9-LOX at carbon atom 9 of polyunsaturated fatty acids (Liaonanchka and Feussner, 2006). The corresponding 9-hydroperoxy or 13-hydroperoxy derivatives are further metabolized by the action of other enzymes via several different biochemical routes into biologically active substances such as signaling molecules (jasmonates), molecules with antimicrobial or antifungal function (Mosblech et al., 2009). LOX is a key enzyme in this octadecanoid (lipoxygenase) pathway. The final products of the lipoxygenase pathway have a role in plant growth and development and in the responses of plants to biotic or abiotic stress (Feussner and Wasternack, 2002; Porta-Rocha Sosa, 2002; Liptáková et al., 2013).

Based on their primary structure and overall sequence similarity, plant LOXs are divided into two gene subfamilies. Type-1 LOXs have no plastidic transit peptide, share a high sequence similarity (>75%) to one another, and consist of both 9- and 13-LOXs. Type-2 LOXs carry a plastidic transit peptide sequence and show a much lower sequence homology (~35%) among plant species. To date, type-2 LOXs all belong to the subfamily of 13-LOXs (Liaonanchka and Feussner, 2006; Andreou and Feussner, 2009). LOX enzyme biochemical studies, as well as cloning, expression, and functional analysis of genes encoding LOXs allow for clarification of the function of specific LOX isoenzymes. High LOX activities are often found in newly developing tissues. LOXs were also determined in mature tissues, where their levels
increased during stress such as wounding or pathogen infection. The intracellular location of LOXs may be cytosolic or organelle-associated (Porta and Rocha-Sosa, 2002). LOX activity has been reported during germination in cytoplasm and on the phospholipid monolayer of oil bodies. LOX-catalyzed oxygenation of polyenoic fatty acids preceded the degradation of storage lipids during seed germination in certain oilseeds (Feussner et al., 2001; Gerhardt et al., 2005; Yadav and Bhatla, 2011).

Opium poppy (Papaver somniferum L.) is one of the most important medicinal plants, producing a large number of benzylisoquinoline alkaloids. The accumulation of morphinan alkaloids is almost exclusively restricted to the opium poppy. It remains the only commercial source for the narcotic analgesic morphine, codeine, and semisynthetic derivatives of thebaine (Ziegler et al., 2009; Beaudoin and Facchini, 2014). The ability to produce alkaloids is also connected with germinating and developing poppy seedlings, where the alkaloids can be detected after a few days postimbibition (Zichová et al., 1996; Huang and Kutsch, 2000). To date, information about LOX in opium poppy has been limited. The amount of total lipids and the distribution of free fatty acids and lipoxygenase activity were determined during poppy seedling development by Bezáková et al. (1994). LOX isoenzyme from chloroplasts of opium poppy leaves was purified and characterized (Vanko et al., 2012). In our previous work, we studied the relationship between LOX activity and secondary metabolite biosynthesis after elicitor treatment in opium poppy cultures (Holková et al., 2010). Considering the possible role of LOX in the signal transduction process leading to pharmacologically utilizable secondary metabolite biosynthesis, this enzyme is an interesting subject of study.

The aim of the present work was to purify and characterize a novel LOX enzyme from opium poppy seedlings, including determination of optimum pH, kinetic parameters, the relative molecular mass, and the effect of calcium ion concentration on activity. In addition, the regioselectivity of substrate dioxygenation of purified opium poppy LOX was evaluated. Characterization of LOX from opium poppy could increase scientific knowledge about LOX in plants and might help to elucidate its physiological function in opium poppy seedlings.

2. Materials and methods

2.1. Plant material

Opium poppy seeds (Papaver somniferum L. ‘Lazur’) were obtained from the Garden of Medicinal Plants, Faculty of Pharmacy, Comenius University in Bratislava. The seedlings were grown on a polyurethane foam layer covered by a nylon cloth and moistened with distilled water in petri dishes. Poppy seeds were germinated in darkness at 25 °C and 75%–80% relative humidity.

2.2. Preparation of crude extracts from opium poppy seedlings

After different periods of germination (1st–6th day) poppy seedlings were harvested, separated from endosperms, and 5 g was ground to a fine powder using liquid nitrogen. The homogenate was prepared with 10 mL of 25 mM potassium phosphate buffer (pH 6.0) containing 0.5 mM ethylenediaminetetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, 1 mM cysteine hydrochloride, 10 mM sodium thiosulfate, and 0.4% (v/v) Polyclar AT (Serva, Heidelberg, Germany). Homogenates were centrifuged for 15 min at 12,000 × g and 4 °C, and the resulting supernatants were assayed for LOX activity (according to the procedure described in 2.4.).

2.3. Enzyme purification

First 80 g of 4-day-old P. somniferum seedlings were homogenized in 160 mL of 25 mM potassium phosphate buffer (pH 6.0) containing 0.5 mM ethylenediaminetetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, 1 mM cysteine hydrochloride, 10 mM sodium thiosulfate, and 0.4% (v/v) Polyclar AT. The homogenate was filtered through two layers of cheesecloth and centrifuged for 15 min at 12,000 × g. Then the supernatant fluid was centrifuged at 100,000 × g for 30 min (JS 24.38 rotor; Beckman Coulter, USA), in order to separate the unsolubilized membranes (pellet) from the solubilized fraction. Both fractions were assayed for LOX activity. The activity of LOX in the membrane fraction was determined after adding 25 mM potassium phosphate buffer (pH 6.0) containing 0.1% (v/v) Triton X-100.

All extraction and purification procedures were performed at 4 °C. The proteins in the supernatant were precipitated with ammonium sulfate to 60% (w/v) saturation. The proteins precipitated were collected by centrifugation (30 min, 15,000 × g) and dissolved in 3 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 1 M ammonium sulfate and applied to a Phenyl-Sepharose CL-4B column (Ø 1.5 × 15 cm; Sigma-Aldrich, St Louis, MO, USA). The resin was washed with 50 mM potassium phosphate buffer (pH 7.0) containing 1 M ammonium sulfate, and LOX was eluted with 10 mM potassium phosphate buffer (pH 6.5) containing 0.5 mM glutathione and 0.04% (v/v) Tween 20. Fractions of 1.5 mL were collected at the flow rate of 1 mL/min. The protein elution profile was measured spectrophotometrically at 280 nm, and LOX activity was determined at 234 nm.

Active fractions were pooled, freeze-dried, and dissolved in 3 mL of 50 mM potassium phosphate buffer (pH 6.8) and applied to a Q-Sepharose column (Ø 2 × 15 cm; Sigma-Aldrich) previously equilibrated with the same buffer. The resin was washed with 50 mM potassium phosphate buffer (pH 6.8), and 2-mL fractions were collected at the flow rate of 1 mL/min. The protein elution profile was measured spectrophotometrically at 280 nm, and LOX activity was determined at 234 nm.
collected. The eluate was monitored at 280 nm. After the absorbance was returned to baseline, elution of absorbed proteins was carried out with a discontinuous salt gradient of NaCl (0.25 M and 0.5 M) at the flow rate 1 mL/min. Fractions with LOX activity were collected, freeze-dried, and loaded onto a linoyleyl-aminopropyl agarose (LAPA) column (Ø 1.0 × 10 cm).

For affinity chromatography, LAPA (Sigma-Aldrich) was prepared by binding linoleic acid to ω-aminopropyl agarose using a water-soluble carbodiimide coupling reagent [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride], according to Vanko et al. (2012). The LAPA column was eluted stepwise with 10 mM potassium phosphate buffer (pH 6.0), 0.3 M potassium phosphate buffer (pH 6.0), and 0.3 M potassium phosphate buffer (pH 8.5). Fractions of 1.5 mL were collected, and absorbance at 280 nm (protein content) was monitored. Each fraction was assayed for LOX activity. The LOX enzyme preparation was then stored at −20 °C until further analysis.

2.4. Measurement of LOX activity and protein determination

The activity of LOX was determined spectrophotometrically at room temperature by measuring the increase of absorbance at 234 nm. An UV/VIS spectrometer (Lambda 35, Perkin Elmer, USA) was used. The substrate, linoleic acid, was prepared according to Chen and Whitaker (1986). The reaction mixture contained 1.24 mL of 100 mM potassium phosphate buffer (pH 6.0), 140 µL of substrate solution (10 mM), and 20 µL of LOX enzyme preparation. The LOX activity was expressed in kats.

Protein concentrations were determined by the Bradford method using bovine serum albumin as a standard (Bradford, 1976).

2.5. Kinetic study and effects of pH and calcium ion concentration on lipoxygenase activity

For enzyme kinetic assays, reaction velocities of LOX-catalyzed reaction were measured by spectrophotometric method. Linoleic acid or linolenic acid stock solutions (10 mM) were prepared according to Chen and Whitaker (1986) and used to determine the Michaelis constant (Km) and the maximum velocity (Vmax) of the LOX reaction. A substrate concentration range of 0–3 mM was used. The kinetic parameters were evaluated by Lineweaver–Burk’s method (Ritchie and Prvan, 1996).

The optimum pH of LOX was determined by measuring enzyme activity in 100 mM potassium phosphate buffer in a range from 5.0 to 8.5 with linoleic acid as a substrate.

The effect of calcium ion concentration on the purified enzyme was determined by measuring LOX activity in 100 mM potassium phosphate buffer (pH 6.5) and various CaCl₂ concentrations in a range between 0 and 2 mM. The reaction was initiated by adding linoleic acid as a substrate.

2.6. Electrophoresis and immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a Mini-PROTEAN 3 CELL vertical electrophoresis apparatus (Bio-Rad, Richmond, CA, USA) in 8% polyacrylamide gel, according to the method of Laemmli (1970). Standard molecular weight markers (10–225 kDa) from Novagen (USA) were used as reference. Gel was stained for proteins using PageBlue solution (contains Coomassie Brilliant Blue G-250, Fermentas, Germany). After electrophoresis, proteins were transferred to the nitrocellulose membrane using Trans-Blot SD Semi-Dry Transfer Cell (Bio Rad), according to manufacturer's instructions. The LOX was detected by immunoblot method with anti-LOX serum. The secondary antibody reaction was carried out using goat anti-rabbit IgG secondary antibody conjugated to peroxidase (Scintilla, Czech Republic). The reaction was visualized with 3,3',5,5'-tetramethylbenzidine (TMB-stabilized substrate for horse radish peroxidase; Promega, Madison, WI, USA). Polyclonal anti-LOX serum was prepared against soybean LOX, according to the procedure described in Holková et al. (2010).

2.7. Analysis of LOX reaction products

For product analysis, 100 µL of purified poppy LOX was added to 900 µL of 100 mM potassium phosphate buffer (pH 6.5) and incubated with 10 µL of the substrate (10% methanol solution of linoleic acid, v/v). Soybean LOX (Sigma) product was analyzed using 100 mM potassium phosphate buffer (pH 9.0). The reaction was allowed to proceed for 30 min at room temperature. Then it was stopped by acidification with 100 µL of concentrated HCl, and the hydroperoxides were reduced to their corresponding hydroxides with 100 mg of NaBH₄. Hydroxoyctadecadienoic acids (HODEs) were extracted with diethyl ether (2 × 1 mL) and evaporated to dryness in the nitrogen stream. After removing the organic solvent, the residue was reconstituted in 0.2 mL of mobile phase (methanol/water/acetate acid, 85:15:0.1, v/v/v). Aliquots of 70 µL were analyzed by reverse-phase high-performance liquid chromatography (RP-HPLC), according to Vanko et al. (2012).

High-performance liquid chromatography (HPLC) analysis was performed with an Agilent Technologies 1050 series HPLC system (Waldbronn, Germany) coupled to an UV detector. Hydroxy fatty acids were separated from fatty acids by RP-HPLC using column 120-5 Nucleosil C18 (250 × 4 mm; Wartex, Czech Republic) with a gradient system of solvent A (methanol/acetate acid, 85:0.1, v/v) and solvent B (water). The program of elution was as follows: 10 min with solvent system of 85% A and 15% B at a flow rate of 0.2 mL/min; 12 min with 100% A, flow rate 0.4 mL/min; and 5 min with 85% A and 15% B, flow rate 0.4 mL/min. The eluate containing hydroxy fatty acids (peak fraction at
234 nm) was collected and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 150 µL of hexane, and aliquots of 50 µL were analyzed by straight phase high-performance liquid chromatography (SP-HPLC).

SP-HPLC of hydroxy fatty acid isomers was carried out on a Zorbax Rx-SIL column (150 × 2.1 mm, 5 µm particle size; Agilent Technologies, Waldbronn, Germany) eluted with a solvent system of hexane/2-propanol/acetic acid (99:1:0.1, v/v/v) at a flow rate of 0.2 mL/min. The absorbance at 234 nm (conjugated diene system of the hydroxy fatty acids) was recorded simultaneously during all chromatographic steps. The identity of products was confirmed by co-chromatography with the authentic standards 9(S)-hydroxy-(10E,12Z)-octadecadienoic acid (9-HODE, 5 µg/mL) and 13(S)-hydroxy-(9Z,11E)-octadecadienoic acid (13-HODE, 5 µg/mL) purchased from Cayman Pharma (Czech Republic). For quantification of LOX products by SP-HPLC, calibration curves for 9- and 13-HODE were obtained in the range 0.1–10.0 µg/mL and 10.0–100.0 µg/mL.

3. Results and discussion

3.1. LOX activity during germination of poppy seeds and enzyme purification

Changes in LOX activity during growth of poppy seedlings (Papaver somniferum L.) were analyzed. LOX activity was measured in the extracts from poppy seedlings grown in the dark and separated from endosperms at various stages of development. The highest LOX activity (96.8 ± 1.7 nkat/mg) was detected on the fourth day of postimbibition (Figure 1). Based on these results, 4-day-old seedlings were chosen for further purification of LOX.

Most newly characterized enzymes are prepared as recombinant proteins, but it is also beneficial to obtain the protein from the original organism in its most authentic form. In the present paper, we describe the purification and characterization of a wild-type LOX from opium poppy seedlings. LOX purification consisted of several steps, as summarized in the Table. First, the plant material was fractionated into a 100,000 × g soluble fraction and a membrane fraction. LOX activity was found predominantly in the soluble fraction. The activity of LOX in the membrane fraction reached only 4% (1.28 nkat/mg), in comparison with LOX activity in the soluble fraction (32 nkat/mg). For further purification, the proteins in the supernatant were precipitated with ammonium sulfate to 60% saturation and loaded on a Phenyl-Sepharose CL-4B column. The elution profile is shown in Figure 2A. Fractions containing LOX activity were further purified by anion exchange chromatography (Q-Sepharose; Figure 2B) to a specific activity approximately 412 nkat/mg (Table). Chromatography on a Q-Sepharose column resulted in elimination of the majority of non-LOX proteins. The remaining contaminants were separated using affinity chromatography on a LAPA column (Figure 2C). A summary of the purification steps is given in the Table. An overall 47-fold purification was achieved. The specific activity of purified opium poppy LOX reached 1150 nkat/mg when linoleic acid was used as a substrate. SDS-PAGE analysis of the eluted fractions showed that LOX was purified to electrophoretic homogeneity (Figure 3A, lane 2). The identity of the purified LOX was confirmed by immunoblot analysis where a single intense band was obtained, as shown in Figure 3B (lane 2). The purified LOX enzyme preparation was used for further biochemical characterization.

Results of our purification procedures correspond with the results obtained by other authors. LOX enzyme from pea seeds was purified 47.2-fold (Szymanowska et al., 2009). Lorenzi et al. (2006) obtained 65-fold purification of LOX from olives using differential centrifugation and hydrophobic chromatography. Three isoenzymes of LOX from pearl millet seedlings were purified using ammonium sulfate fractionation, DEAE-Sephadex A-50, and Sephadex G-200 gel-filtration (about 46.5-, 73.6-, and 115.7-fold, respectively) (Babitha et al., 2004).

3.2. Determination of the relative molecular mass

SDS electrophoresis of purified opium poppy LOX in 8% polyacrylamide gel revealed a band that corresponded to a protein with a relative molecular mass of 78 kDa (Figure 3A, lane 2). After Western blotting with anti-soybean LOX

Table. Purification summary of LOX from opium poppy seedlings.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Activity (nkat/mL)</th>
<th>Proteins (mg/mL)</th>
<th>Specific activity (nkat/mg)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>1417</td>
<td>57.5</td>
<td>24.6</td>
<td>1.0</td>
</tr>
<tr>
<td>100,000 × g soluble fraction</td>
<td>1380</td>
<td>43.1</td>
<td>32.0</td>
<td>1.3</td>
</tr>
<tr>
<td>Phenyl-Sepharose CL-4B</td>
<td>482.5</td>
<td>11.1</td>
<td>43.5</td>
<td>1.8</td>
</tr>
<tr>
<td>Q-Sepharose</td>
<td>64.3</td>
<td>0.156</td>
<td>412.2</td>
<td>16.8</td>
</tr>
<tr>
<td>LAPA</td>
<td>23.0</td>
<td>0.020</td>
<td>1150</td>
<td>46.7</td>
</tr>
</tbody>
</table>
antibodies, one band was identified showing the same molecular mass (Figure 3B, lane 2). As a comparison, the relative molecular mass of commercial soybean LOX (97 kDa) was determined (Figures 3A and 3B, lane 3). Plant species typically have multiple LOX isoforms that differ in biological properties. In the present work, one LOX enzyme was purified from the cytosolic fraction of opium poppy seedlings. In our previous work, we characterized a different LOX isoenzyme (Vanko et al., 2012). This isoenzyme was isolated from chloroplasts of opium poppy leaves and had 92 kDa due to the presence of N-terminal chloroplast transit peptide sequence (Vanko et al., 2012).

Most plant LOX have a relative molecular mass ranging from 94 to 104 kDa (Siedow, 1991). The relative molecular mass of LOX from olive fruit is 98 kDa (Lorenzi et al., 2006), and LOX from pea seeds is 93 kDa (Szymanowska et al., 2009). However, several publications reported relative molecular masses of 85 kDa for LOX purified from banana leaves (Kuo et al., 2006) and 74 kDa for LOX from chicory (Daglia et al., 2005). Isoenzymes of LOX purified from pearl millet seedlings had the relative molecular masses 83 kDa, 77 kDa, and 73 kDa (Babitha et al., 2004).

3.3. Effect of pH on LOX activity
The optimal pH for activity of purified opium poppy LOX was determined using linoleic acid as a substrate. LOX activity was at a maximum at pH 6.5. The enzyme exhibited 70% activity at pH 6.0 and 50% activity at pH 5.5 and 7.0; the enzyme was almost inactive above pH 7.5 (Figure 4). The optimum pH of other plant LOXs varies and is related to the type of LOX isoenzyme. A similar optimum pH was reported for LOX from avocado (Jacobo-Velázquez et al., 2010) and LOX from tomato fruit (Bowsher et al., 1992). The partially purified isoenzymes of LOX from lentil seedlings showed optimum pH around pH 6.0 and 6.5, and one isoenzyme had maximal activity at pH 9.0 (Hilbers et al., 1995).

Figure 1. Changes of LOX-specific activity in crude extracts of developing opium poppy seedlings (1st–6th day after germination). Data present means ± SD from three experiments.

Figure 2. Purification of LOX from opium poppy seedlings. (A) Elution profile of opium poppy LOX on a Phenyl-Sepharose CL-4B column. (B) Purification on a Q-Sepharose column. (C) Purification on a linoleyl-aminopropyl agarose (LAPA) column. Proteins were measured at 280 nm. Triangles represent LOX activity determined at 234 nm. The arrows indicate the point at which elution buffers were changed. PB: potassium phosphate buffer.
3.4. Kinetic study and the effect of calcium ion concentration on LOX activity

The Lineweaver–Burk method was used to determine the effect of substrate concentration on the initial reaction velocities catalyzed by purified opium poppy LOX. Activity assays using either linoleic or linolenic acid were carried out in order to determine LOX substrate specificity. The values of both kinetic parameters, Michaelis constant ($K_m$) and maximum reaction velocity ($V_{max}$), for each type of substrate were calculated. The values represent the mean values of three independent experiments. The $K_m$ value of the purified LOX was $4.9 \pm 0.3 \text{ mM}$, whereas the $V_{max}$ was $100.15 \pm 0.9 \mu\text{mol/min/mg}$ for linoleic acid. For linolenic acid, $K_m$ value was $14.7 \pm 1.6 \text{ mM}$, and $V_{max}$ was $772.4 \pm 0.6 \mu\text{mol/min/mg}$. Although a higher reaction rate ($V_{max}$) value was achieved for linolenic acid, the lower $K_m$ value was determined using linoleic acid as a substrate. The kinetic parameters determined indicate that linoleic acid binds with higher affinity to the active site of purified LOX than linolenic acid, while the reaction rate ($V_{max}$) of the enzyme was higher for linolenic acid. The majority of LOXs isolated from plants are most active toward linoleic acid. The $K_m$ value of purified opium poppy LOX determined using linoleic acid as a substrate was similar to the $K_m$ value of commercial soybean LOX (Sigma).

Figure 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis of purified opium poppy LOX. (A) SDS-PAGE polyacrylamide gel (8%). (B) Immunoblot analysis of a gel loaded with the same samples. Lane 1: marker proteins (25–225 kDa), lane 2: purified enzyme after affinity chromatography, lane 3: commercial soybean LOX (Sigma).

Figure 4. Optimum pH of purified LOX from opium poppy seedlings. Linoleic acid was used as a substrate. Buffer system consists of 100 mM potassium phosphate buffer ranging from pH 5.0 to 8.5. Values are averages ± SD from three experiments.

Figure 5. The effect of calcium ion concentration on the activity of purified LOX from opium poppy seedlings. Linoleic acid was used as a substrate. Values are averages ± SD from three experiments.

Figure 6. The concentration of reaction products formed by purified opium poppy LOX (A) and commercial soybean LOX (B) analyzed using HPLC. Enzymes were incubated with linoleic acid. The reaction products are in their reduced forms, 13-hydroxy-(9Z,11E)-octadecadienoic acid (13-HODE) and 9-hydroxy-(10E,12Z)-octadecadienoic acid (9-HODE). Values are averages ± SD from three experiments.
value of soluble LOX from tomato fruits (Suurmeijer et al., 1998). The $K_m$ value of LOX from pea seeds was 0.44 mM (Szymanowska et al., 2009) and was 0.15 mM in LOX purified from banana leaves (Kuo et al., 2006) and soluble LOX from chicory (Daglia et al., 2005). Other plant LOXs display $K_m$ values in the lower micromolar range (Fornaroli et al., 1999; Lorenzi et al., 2006; Sellhorn et al., 2011).

The activity of several LOX isoenzymes is calcium dependent (Jacobo-Velázquez et al., 2010). Therefore, the effect of calcium ion concentration on the activity of purified opium poppy LOX was evaluated. Calcium ion concentration influenced the activity of purified LOX. The highest activity of opium poppy LOX was observed when $\text{Ca}^{2+}$ concentration reached 0.75 mM. The activity of LOX showed an approximately 2-fold increase (Figure 5). Cho et al. (2011) provided evidence for calcium-mediated translocation and membrane association of dual-positional–specific maize LOX without a chloroplast-targeting sequence from cytoplasm to chloroplast. Previous studies also identified $\text{Ca}^{2+}$-binding domain in LOX1 from soybean, which mediated the interaction of the enzyme with lipid membranes (Tatulian et al., 1998). Soluble LOX can be transported from cytoplasm to membranes, depending on the physiological state of the cells. For example, the increase in cytoplasmic calcium during stress responses could also induce the translocation of soluble LOX to membranes for interaction with its fatty acid substrate and stimulate its activity (Braidot et al., 2003).

### 3.5. Characterization of LOX reaction products

LOX isoenzymes differ in the proportion of hydroperoxy-octadecadienoic acid (HPODE) products formed during the reaction. To investigate the positional specificity of the LOX reaction, HPLC analysis was performed. The hydroperoxides obtained after incubation of purified opium poppy LOX with linoleic acid as a substrate were reduced with NaBH$_4$, and the hydroxides formed were analyzed using HPLC. The reaction products were separated by RP-HPLC followed by SP-HPLC to resolve the hydroxy isomers. Based on calibration curves prepared using 9-HODE or 13-HODE standards, the amount of LOX products formed during the reaction was determined (Figure 6). The major reaction product was 13-hydroperoxy-(9Z,11E)-octadecadienoic acid (13-HPODE, 63.5%), while 9-hydroperoxy-(10E,12Z)-octadecadienoic acid (9-HPODE) was formed at 36.5%. As a comparison, commercial soybean LOX was analyzed. Optimal pH for its activity was pH 9.0. Soybean LOX converted linoleic acid into 82.0% 13-HPODE and 18% 9-HPODE (Figure 6). This enzyme is known to preferentially produce 13-HPODE with a small amount of 9-isomer at pH 9.0 (Gardner, 1989).

The major product reported, for other plant LOXs such as soluble LOX purified from tomato fruits, was 9-HPODE (96%), while only 1% 13-HPODE was formed (Suurmeijer et al., 1998); olive LOX converted linoleic acid only to 13-HPODE (Lorenzi et al., 2006); and LOX from developing rice seedlings had dual positional specificity, releasing both C-13 and C-9 oxidized products (Wang et al., 2008). The 9- or 13-selective positional specificity of LOXs allows for production of specific metabolites with diverse roles in plant growth, development, or defense. For example, 13-LOX is reportedly localized in chloroplasts of photosynthesizing tissues with a function in jasmonic acid biosynthesis. Only 13-LOX activity, not 9-LOX activity, leads to the biosynthesis of jasmonic acid (Feussner and Wasternack, 2002; Farmaki et al., 2007). The functions of 9-LOX products are less well known. However, Christensen and Kolomiets (2011) suggest that 9-LOX derivatives have hormone-like activities and may regulate growth, development, and defense against pathogens and pests.

Most LOX enzymes catalyze the formation of one particular regiospecific isomer. Several plant LOXs exhibiting dual positional specificity are classified as nontraditional LOX enzymes. These LOXs can produce a wider range of final products in a lipoxygenase pathway than strictly 13-LOX or 9-LOX and might have more diverse physiological functions (Kim et al., 2003; Wang et al., 2003; Cho et al., 2011). Knowledge of the biochemical features of LOXs in different plant species is necessary for classification of these enzymes and might help to elucidate their corresponding cellular roles. LOX characterized in maize seedlings with dual positional specificity had a role in pathogen and wound stress responses (Kim et al., 2003). In cucumber seedlings 13-LOX, a special linoleate that is involved in the initial stages of lipid mobilization during germination, was detected. This enzyme originally appears in the cytosol but is then transferred to the lipid body membrane, where it is attached to the phospholipid monolayer and oxygenates esterified linoleate residues (Feussner et al., 2001). LOX characterized from developing rice seedlings had dual positional specificity, releasing both C-13 and C-9 oxidized products (Wang et al., 2008). LOX purified from poppy seedlings had dual positional specificity and it was involved in the germination process. However, it may also have another function, for example, in stress responses. The microarray data gained by Mishra et al. (2013) for opium poppy demonstrated that LOX expression increased after wound stress. The role of opium poppy LOX in stress responses requires further investigation.

In conclusion, LOX from opium poppy (Papaver somniferum L.) seedlings was purified to electrophoretic homogeneity, and biochemical properties of the purified
enzyme were analyzed. It belongs to the group of nontraditional plant LOXs exhibiting dual positional specificity, with 63.5% 13-HPODE and 36.5% 9-HPODE formed during the reaction of purified LOX with linoleic acid.

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References


