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Calcium modulates membrane association, positional specificity, and product distribution in dual positional specific maize lipoxygenase-1

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ABSTRACT

This study investigates how calcium modulates the properties of dual positional specific maize lipoxygenase-1, including its interaction with substrate, association with subcellular membrane and alteration of product distribution. Bioinformatic analyses identified Asp^{38}, Glu^{127} and Glu^{201} as putative calcium binding residues and Leu^{37} as a flanking hydrophobic residue also potentially involved in calcium-mediated binding of the enzyme to subcellular membranes. Asp^{38} and Leu^{37} were shown to be important but not essential for calcium-mediated association of maize lipoxygenase-1 to subcellular membranes *in vitro*. Kinetic studies demonstrate that catalytic efficiency (V_{max}/K_m) shows a bell-shaped dependence on log of the molar ratio of substrate to unbound calcium. Calcium also modulates product distribution of the maize lipoxygenase-1 reaction, favoring 13-positional specificity and increasing the relative amount of (*E*,*Z*)-isomeric products. The results suggest that calcium regulates the maize lipoxygenase-1 reaction by binding to substrate, and by promoting binding of substrate to enzyme and association of maize lipoxygenase-1 to subcellular membranes.

1. Introduction

Lipoxygenases (LOXs) (EC 1.13.14.12) catalyze the hydroperoxidation of polyunsaturated fatty acids (PUFA) with one or more (1*Z*, 4*Z*)-pentadienes. Most LOX-catalyzed reactions are regioselective and stereoselective. For example, linoleic acid (LA) and linolenic acid (LNA) are oxygenated by 9-LOX at C-9 or 13-LOX at C-13 of the hydrocarbon backbone, generating (9*S*)-(10*E*,12*Z*) hydroperoxyoctadecadienoic (trienoic) acid (HPOD(T)E) or (13*S*)-(9*Z*,11*E*)HPOD(T)E, respectively [1,2]. However, the positional specificity of some LOX enzymes is dual specific and does not agree with the prediction based on specificity motifs [3,4]. Four of those LOX enzymes identified to date are expressed in monocot plants and might not be regarded as classical LOX [3–5]. One of these, maize lipoxygenase-1 (ZmLOX1) has dual positional specificity [6] and its pre-steady state kinetic behavior has been extensively characterized [7]. Although the catalytic mechanism of dual positional specific LOX enzymes remains controversial [8], we provided regiochemical and stereochemical evidence that ZmLOX1 acts by a unique enzyme-initiated catalytic mechanism [5,6].

The specific functions of calcium in LOX have been thoroughly investigated in animals and calcium was shown to bind to LOX, mediate its association with membranes, and activate the LOX activity [9–11]. The crystal structure of a calcium-dependent LOX revealed that it is composed of two domains: a C-terminal domain containing the catalytic site and an N-terminal C2-like domain known as PLAT (Polycystin-1, Lipoxygenases and Alpha Toxin) domain. Molecular basis for the interaction of calcium regulating membrane binding and stimulation of activity has been proposed [9,11]. In plants, calcium is known to play roles in senescence and defense against pathogens, and a possible role for calcium in oxidation of PUFA has been proposed [12]. Although the activity of plant LOX enzymes was initially thought to be calcium-independent, it has been reported that calcium regulates association of soybean LOX to subcellular membranes [13] through an interaction with the N-terminal domain of LOX, which also regulates catalytic activity [14,15]. ZmLOX1 is involved in pathogen defense and in activating responses to oxidative stress [16]. Here, we propose that calcium may play important roles in regulating the activity of

Abbreviations: HOT(D)E, hydroxyoctadecatri(di)enoic acid; HPOT(D)E, hydroperoxyoctadecatri(di)enoic acid; KC, kinetically-controlled; KODE, keto-octadecadienoic acid; LA, linoleic acid; LNA, linolenic acid; LOX, lipoxygenase; PLAT, Polycystin-1, Lipoxygenases and Alpha Toxin; PUFA, polyunsaturated fatty acid; TC, thermodynamically controlled; ZmLOX1, maize lipoxygenase-1..

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ZmLOX1. In fact, we previously reported that ZmLOX1, when overexpressed in the cytoplasm of transgenic rice cells, associated with the chloroplast membrane in a calcium-dependent manner [17]. In this paper, evidence is provided that calcium facilitates the subcellular membrane association of ZmLOX1, promotes binding to LA, and alters the product distribution of reaction products during oxidation of LA.

2. Materials and methods

2.1. Bioinformatic analysis: modeling, structural analysis, and identification of calcium binding site of ZmLOX1

Three-dimensional structure of ZmLOX1 was modeled using the SWISS-MODEL ExPASy server (http://swissmodel.expasy.org) in automated mode. From known X-ray coordinates of LOXs [5], soybean LOX1 (Protein Data Bank accession code: 1yge, 1.4 Å resolution) was identified as a suitable template for the structure of ZmLOX1 (Gene bank accession code: AAF76207). Energy minimization was carried out with GROMOS. The quality of the ZmLOX1 structural model was evaluated using PROCHEK (SWISS-MODEL ExPASy server). Sequences of soybean LOX1, soybean LOX3, and ZmLOX1 were aligned and the secondary structures were analyzed using *i*MolTalk v3.1 (http://i.moltalk.org). PyMOL v0.99 (http://pymol.sourceforge.net) was used to superimpose ZmLOX1 and soybean LOX1 models, and calcium binding residues of soybean LOX1 were identified according to the previously reported method [13]. Putative calcium-binding residues in ZmLOX1 were predicted based on structural homology and alignment of the two proteins [17,18] and Glu^{21}, Glu^{106} and Glu^{179} residues of soybean LOX1 that compose calcium binding site I were used to predict corresponding amino acid residues in ZmLOX1.

2.2. Overexpression and purification of ZmLOX1 and N-terminal truncated ZmLOX1

ZmLOX1 and N-terminal truncated ZmLOX1 (lacking Met^1 to Cys^{60}) were expressed in *E. coli*, purified as described previously [17]. Briefly, recombinant pRSETB-ZmLOX1 and pRSETB-N-terminal truncated ZmLOX1 plasmids were transformed into BL21(DE3)pLysS, induced using IPTG, and cell extracts were prepared for enzyme purification by Q-Sepharose chromatography. Purified enzymes were analyzed by SDS–PAGE.

2.3. Preparation of subcellular membrane fraction and analysis of membrane association

A subcellular membrane fraction was isolated from leaves of one month-old rice plants (*Oryza sativa* L. *Japonica* cv. Nakdong) by the previously reported method as follows [19]. Rice leaves (5 g) were homogenized at 10,000 g for 2 min in 10 mM KH_2PO_4 buffer (20 mL), pH 7.8, with 0.5 M sucrose and 1 mM EDTA. The homogenate was filtered through four layers of gauze and centrifuged at 1,000 g for 30 min. The supernatant was centrifuged again at 12,000 g for 1 h. The pellet was washed 6 times with 1 mM EDTA to remove any metal species and washed again with PBS buffer several times and stored at 4 °C. The affinity of ZmLOX1 for the subcellular membrane fraction was determined as follows [20]. Subcellular membranes (1 mg/mL, Fresh weight) were pelleted by centrifugation at 12,000 g for 30 min and resuspended in 0.5 mL of 10 mM KH_2PO_4 buffer, pH 7.8, containing 1 mM, 10 μM, 100 nM, or 1 nM calcium chloride. The membrane suspension (180 μL) was mixed with 13.8 μg of ZmLOX1 (or N-terminal truncated ZmLOX1) and incubated for 5 min at room temperature. The supernatant was separated from the pellet by centrifugation at 12,000 g and the pellet resuspended in an equal volume of the same buffer. An aliquot (20 μL) of the supernatant and solubilized pellet was analyzed by 10% SDS–PAGE followed by immunoblot as described previously [17].

2.4. Kinetic studies of ZmLOX1 in the presence and absence of calcium

Spectral analysis of the oxidation of LA by ZmLOX1 in the presence of calcium was performed as follows. ZmLOX1 reaction was initiated by adding purified ZmLOX1 (6 μg) to reaction buffer, containing 50 mM Tris–HCl (2.5 mL), pH7.2, 0.5 mM LA, 0.05% Tween 20, 100 μM EGTA without or with 200 μM $CaCl_2$. UV spectra (210–350 nm) were recorded every 2 min for 20 min. The concentrations of HPODE and KODE were calculated using the extinction coefficients of 25,000 $M^{-1}cm^{-1}$ at 234 nm and 22,000 M^{-1} cm^{-1} at 280 nm, respectively.

Kinetic studies of ZmLOX1 in the presence and absence of calcium were performed as follows. The enzymatic activity of purified ZmLOX1 was quantified by monitoring absorbance at 234 nm in variable concentrations of LA (0.05, 0.1, 0.25, 0.5, 0.75 and 1 mM) and calcium chloride (0, 1, 10, 25, 50, 75, 100, 120, 150, 200, 400, and 600 μM). Assays were conducted as described [7] with minor modifications. The reaction solution contained 50 mM Tris–HCl (2.5 mL), pH 7.2, 100 μM EGTA, and various concentrations of LA/Tween 20 (with molar ratio of 3.07) and calcium chloride. The reaction mixture was pre-incubated for 5 min at 25 °C and then the reaction was initiated by adding 6 μg purified ZmLOX1. The concentration of free calcium in the reaction mixture was calculated using a Ca^{2+}/EGTA calculator (http://entropy.brneurosci.org/egta.html). Curves were fitted using the Boltzmann equation in OriginPro 7.5 program and initial rates were calculated from the linear portion of the curves. ZmLOX1 activity was plotted against concentration of free calcium at various concentrations of LA; plots of $\log[LA]/[Ca^{2+}]_{free}$ were analyzed using LAB Fit Curve Fitting software v7.2.37 (http://zeus.df.ufcg.edu.br/labfit/), which determined the best fit equation to be $[y = (a + x)/(b + cx^2)]$. Kinetic parameters (K_m, V_{max}) were obtained by Hanes-Woolf plots, where the ratio of LA to free calcium was 0.3 to 10,000, at various concentrations of LA (0.05–1 mM).

3. Results

3.1. Putative calcium binding residues in ZmLOX1 and calcium-dependence of membrane association

Sequence alignment, homology search and structural modeling were used to identify two protein domains in ZmLOX1, both of which are typical domains in the LOX family (Fig. 1). The N-terminal domain (residues 23–170) is a conserved eight-stranded β-barrel known as a PLAT domain. The C-terminal catalytic domain (residues 171–873) of ZmLOX1 contains 21 α-helixes, as does the C-terminal domain of soybean LOX1 and LOX3. Lysine (Lys^{121}) in β5 of the ZmLOX1 structural model corresponds to Lys^{100} in β5 of soybean LOX1, a residue thought to be involved in membrane association [18,21]. Superimposition of ZmLOX1 and soybean LOX1 suggests that Asp^{38}, Glu^{127} and Glu^{201} residues of ZmLOX1 are structurally equivalent to Glu^{21}, Glu^{106} and Glu^{179} residues of soybean LOX1 that play a role in calcium binding in soybean LOX1 (Fig. 2, Fig. S1). Calcium-binding residues in soybean LOX1, LOX3, VLX-B and VLX-D, are invariably flanked to the N-terminal side by a hydrophobic residue (*i.e.*, leucine), of which side chain intercalates into the membrane [13]. The equivalent residue in ZmLOX1 is Leu^{37}.

To examine the functional roles of the proposed residues in ZmLOX1, an expression construct was prepared to overexpress

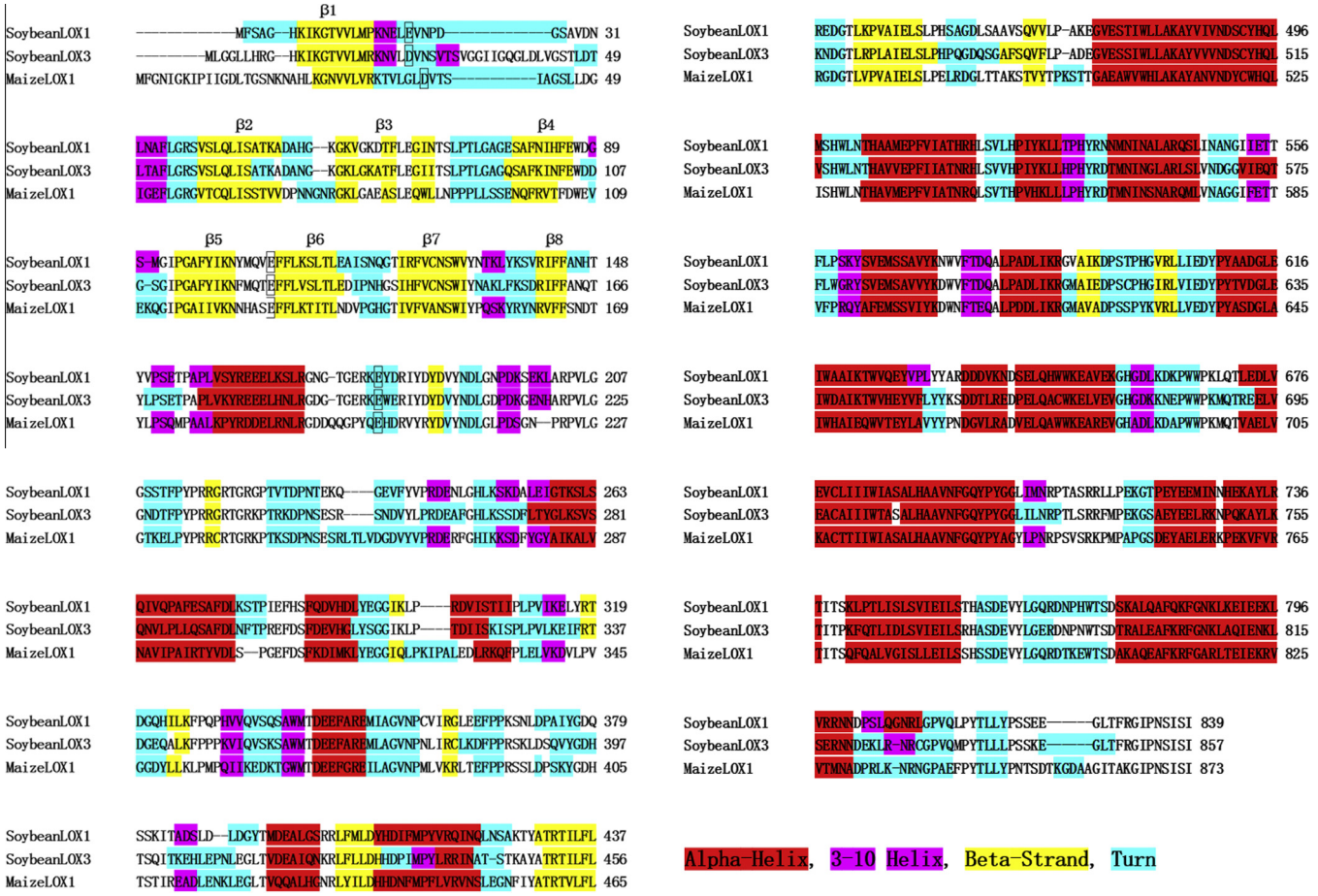

Fig. 1. Sequence alignment and secondary structure of ZmLOX1. The alignment of sequences of ZmLOX1 (GenBank accession no. Af271894), soybean LOX1 (PDB code no: 1YGE, 1.4 Å resolution) and soybean LOX3 (1RRL, 2.09 Å) were automatically performed in SWISS Model Server (http://swissmodel.expasy.org/). The secondary structure of modeled ZmLOX1 was analyzed by iMolTalk v3.1 (http://i.moltalk.org/).

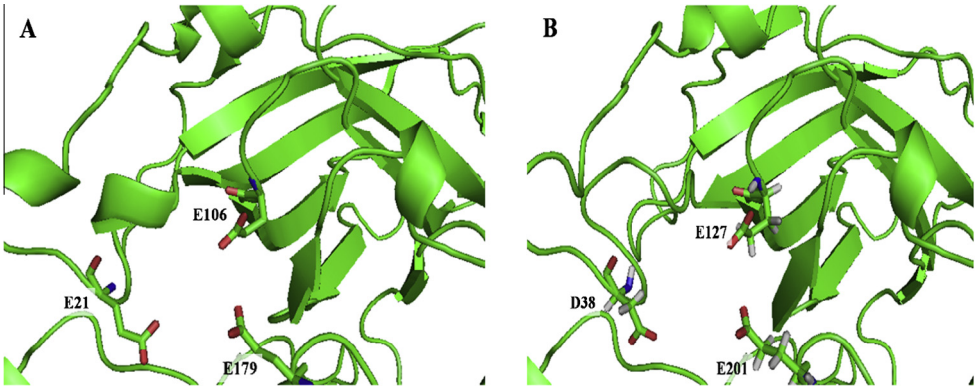

Fig. 2. Calcium-binding sites of ZmLOX1 and soybean LOX1 revealed by three-dimensional superimposition. Three dimensional structure of ZmLOX1 was modeled by an automated mode of SWISS-MODEL sever (http://swissmodel.expasy.org) using known X-ray coordinates of soybean LOX1 (Protein Data Bank accession code: 1yge, 1.4 Å resolution) as the best template, secondary structures of soybean LOX1 and ZmLOX1 were analyzed by *i*MolTalk v3.1 (http://i.moltalk.org), and superimposed to reveal residues within the calcium binding site as described in Materials and methods. (A) The calcium-binding residues (Glu^{21}, $Glu1^{106}$, Glu^{179}) in soybean LOX1. (B) The calcium-binding residues (Asp^{38}, Glu^{127} and Glu^{201}) in ZmLOX1.

N-terminally truncated ZmLOX1 (amino acid 61 to 873), which lacks Asp^{38} and Leu^{37}, but retained a normal level of catalytic activity as reported previously [17]. The affinity of the purified proteins for a subcellular membrane fraction was then evaluated *in vitro* in the presence of various concentrations of calcium. The results show that calcium promotes dose-dependent association of ZmLOX1 to a purified subcellular membrane fraction *in vitro*, but the affinity of N-terminal truncated ZmLOX1 for the membrane fraction was weaker than the affinity of full-length ZmLOX1, especially at low concentrations of calcium (Fig. 3). Similar result was obtained with His-tagged LOX (Fig. S2). This result demonstrates that calcium promotes association of ZmLOX1 to subcellular membranes *via* its N-terminal PLAT domain *in vitro*. This result is consistent with our previous observation that calcium mediates translocation of recombinant ZmLOX1 from cytoplasm to chloroplast, when it is overexpressed in transgenic rice [17].

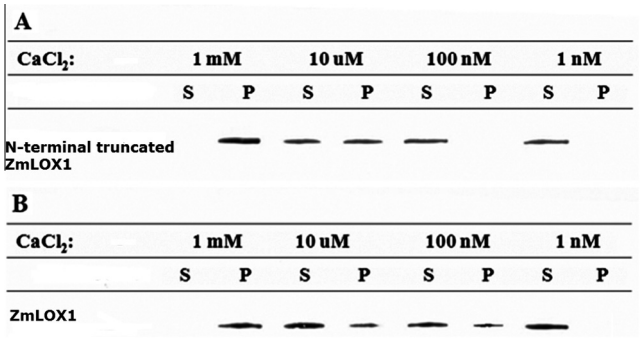

Fig. 3. Dependence of membrane association on calcium of ZmLOX1 and N-terminal truncated LOX1. Subcellular membranes were prepared from rice leaves and mixed with enzymes, and the degree of membrane association was analyzed by immunoblot as described in Materials and methods. (A) N-terminal truncated ZmLOX1 and (B) ZmLOX1.

3.2. Effect of calcium on kinetics of oxidation of LA by ZmLOX1

The oxidation of LA by ZmLOX1 was examined in the presence and absence of calcium, and reaction kinetics were analyzed. Results indicate that calcium stimulates production of HPODE and KODE (keto-octadecadienoic acid) from LA (Fig. 4A and Fig. 4B) [22,23], and that the induction period of ZmLOX1 [7] is altered by calcium (Fig. S3). Although stimulation of ZmLOX1 activity by calcium increases with increasing concentration of LA (Fig. 5A), the reaction reached a plateau and then declined slightly, indicating inhibition by high concentration of free calcium. This suggests that the effect of calcium on the oxidation of LA by ZmLOX1 depends on the concentrations of both calcium and LA. To explore this further, ZmLOX1 activity was analyzed as a function of log $[LA]/[Ca^{2+}]_{free}$ (Fig. 5B). This plot reveals that ZmLOX1 activity is maximal when log $[LA]/[Ca^{2+}]_{free}$ is in the range 1 to 3 (depending on concentration of LA). A similar analysis of V_{max} and K_m as a function of log $[LA]/[Ca^{2+}]_{free}$ (Fig. 6A) shows that V_{max} of ZmLOX1 decreases as $[LA]/[Ca^{2+}]_{free}$ increases with an inverted bell-shape curve of K_m, while a plot of catalytic efficiency (V_{max}/K_m) vs log $[LA]/[Ca^{2+}]_{free}$ results in a bell-shaped curve (Fig. 6B).

3.3. Effect of calcium on positional specificity and product distribution

The dual positional specificity and lack of stereochemical specificity of ZmLOX1-catalyzed oxidation of LA has been explained by invoking an enzyme-initiated catalytic mechanism [5,6]. Data presented above (Fig. 6) suggests that calcium interacts with both ZmLOX1 and LA, which implied that calcium might modulate the positional specificity of ZmLOX1. Table 1 demonstrate that calcium increased relative proportions of 13-isomers and kinetically controlled (KC) products. Therefore, calcium modulates the positional specificity and product distribution during ZmLOX1-catalyzed oxidation of LA.

4. Discussion

Calcium can stimulate LOX activity and promotes interaction with membrane lipids by forming salt bridges between acidic protein residues of LOX and negatively-charged lipids in the membrane [13,24]. The PLAT domain is conserved in LOXs and known to promote protein-lipid interactions in a calcium-dependent manner [21]. The PLAT domain of ZmLOX1 encompasses putative amino acid residues for binding to calcium (Asp^{38}, Glu^{127} and Glu^{201}) and membrane lipids (Lys^{121}) (Fig. 2). However, results in Fig. 3 indicate that the N-terminal truncated ZmLOX1 lacking Met^1 to Cys^{60} still interacts with subcellular membrane fractions *in vitro*, albeit with reduced affinity (Fig. 3 and Fig. S2). We conclude that Leu^{37} and Asp^{38} play important but non-essential roles in calcium-mediated binding of ZmLOX1 to subcellular membranes. This in turn suggests that calcium-binding residues Glu^{127} and Glu^{201} and Lys^{121} in the PLAT domain may play a dominant role in promoting association between ZmLOX1 and subcellular membranes.

The ability of calcium to stimulate LOX activity has been reported previously [25–28]. Evidence provided here and elsewhere demonstrates that calcium may regulate ZmLOX1-catalyzed oxidation of LA by more than one mechanism. Calcium interacts with fatty acid substrates of LOX, modulating their physical state in solution [29], and increasing accessibility of substrate to enzyme. In addition, calcium can activate enzymes by forming an Enzyme-Metal-Substrate or Enzyme-Substrate-Metal bridge [30]. Our data indicate that calcium-mediated stimulation of oxidation of LA by ZmLOX1 is strongly dependent on log $[LA]/[Ca^{2+}]_{free}$ (Fig. 5). Calcium reduced the induction period of the ZmLOX1 reaction (Fig. S3, which could reflect oxidation of Fe(II) to Fe(III) in the active site of the enzyme by hydroperoxy fatty acid [31]. In addition, V_{max} and K_m of ZmLOX1 strongly depend on log $[LA]/[Ca^{2+}]_{free}$ (Fig. 6A) and a plot of catalytic efficiency (V_{max}/K_m) *vs* log $[LA]/[Ca^{2+}]_{free}$ generates a bell-shaped curve (Fig. 6B), while K_m vs log $[LA]/[Ca^{2+}]_{free}$ generates an inverted bell-shaped curve (Fig. 6A). Therefore, we propose that calcium could bind the HPODE reaction product and promote reoxidation of Fe(II), which could in turn enhance the enzyme efficiency.

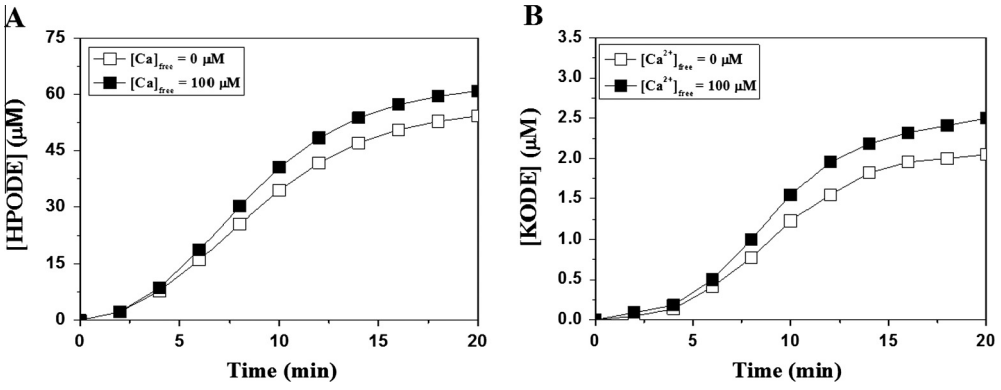

Fig. 4. Effect of calcium on the oxidation of LA by ZmLOX1. ZmLOX1 reaction was initiated by adding 6 μg purified ZmLOX1 in 50 mM Tris–HCl (pH 7.2), 0.5 mM LA, 0.05% Tween 20, 100 μM EGTA. Spectra of ZmLOX1 reaction mixtures were recorded every 2 min for 20 min in the presence of 200 μM $CaCl_2$. HPODE and KODE were quantified using the extinction coefficients 25,000 M^{-1} cm^{-1} at 234 nm and 22,000 M^{-1} cm^{-1} at 280 nm, respectively. (A) Quantification of HPODE and (B) quantification of KODE.

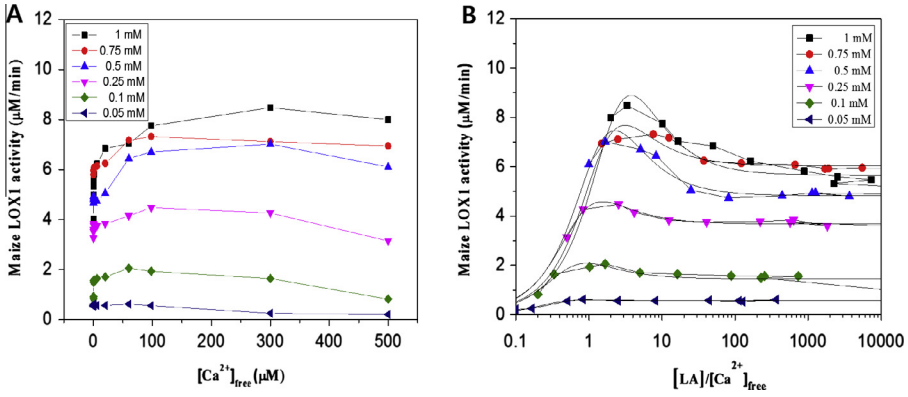

Fig. 5. Dependence of ZmLOX activity on concentrations of LA and calcium. (A) Dependence of ZmLOX1 activity on free calcium. Absorbance of product was monitored at 234 nm in the presence of the indicated concentration of LA (0.05, 0.1, 0.25, 0.5, 0.75 and 1 mM) and $CaCl_2$ (0, 1, 10, 25, 50, 75, 100, 120, 150, 200, 400, 600 μM). The activity of ZmLOX1 was measured and the concentration of free free calcium was calculated using on-line Ca^{2+}/EGTA calculator (http://entropy.brneurosci.org/egta.htmL) as described in Materials and methods. (B) Dependence of ZmLOX1 activity on log $[LA]/[Ca^{2+}]_{free}$. The data points were analyzed using the equation $[y = (a + x)/(b + cx^2)]$ and LAB Fit Curve Fitting software v7.2.37 (http://zeus.df.ufcg.edu.br/labfit/).

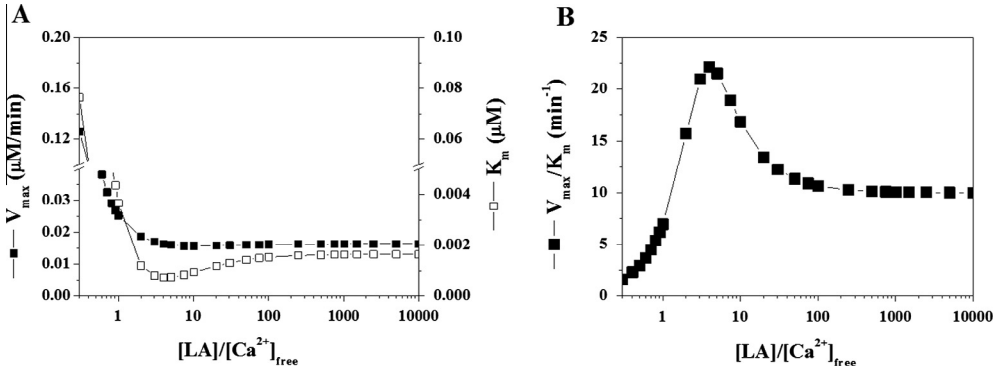

Fig. 6. Dependence of ZmLOX1 kinetic parameters on log $[LA]/[Ca^{2+}]_{free}$. Kinetic parameters (K_m, V_{max}) were obtained by Hanes-Woolf plot using ratios of 0.3 to 10,000 LA to free calcium at the indicated concentrations of LA (0.05–1 mM). (A) Dependence of V_{max} and K_m on log $[LA]/[Ca^{2+}]_{free}$. (B) Dependence V_{max}/K_m on log $[LA]/[Ca^{2+}]_{free}$.

Table 1
Effect of calcium on product distribution and positional specificity.[a]

$[Ca^{2+}]_{free}$ (mM)	Product distribution				Positional specificity		KC[b] vs TC[c]	
	13-(9E,11Z)HOD (1)	13-(9E,11E)HOD (2)	9-(10E,12Z)HOD (3)	9-(10E,12E)HOD (4)	13-HOD (1 + 2)	9-HOD (3 + 4)	KC (1 + 3)	TC (2 + 4)
0	35.5 ± 0.3	13.0 ± 0.1	34.4 ± 0.3	17.2 ± 0.1	48.5 ± 0.4	51.6 ± 0.4	69.9 ± 0.6	30.2 ± 0.2
1.5	43.8 ± 0.4	8.8 ± 0.1	38.3 ± 0.3	9.1 ± 0.1	52.6 ± 0.5	47.4 ± 0.4	82.1 ± 0.7	17.9 ± 0.2
31.0	39.0 ± 0.3	16.0 ± 0.1	36.0 ± 0.3	9.0 ± 0.1	55.0 ± 0.4	45.0 ± 0.4	75.0 ± 0.6	25.0 ± 0.2

[a] The value is the percentage of each regioisomeric form to total HOD products (average and standard error of two replications).
[b] KC: kinetically controlled products.
[c] TC: thermodynamically controlled products.

The positional specificity of other dual positional specific LOX enzymes (pea 9/13 LOX and potato 13/9-LOX) depends on the degree of penetration of the methyl terminus of LA into the enzyme active site [32,33]. In the case of ZmLOX1, we previously reported that ZmLOX1 generates four regioisomeric products of LA, which has been explained by postulating an enzyme-initiated catalytic mechanism [6]. Here, we provide evidence that calcium modulates the positional specificity and distribution of products of the ZmLOX1 reaction (Table 1). Most classical 9-LOX and 13-LOX enzymes produce only KC products because the (1*Z*, 4*Z*)-configuration of substrate is retained in the active site during the catalytic cycle. The enzyme-initiated catalytic mechanism of ZmLOX1 predicts that calcium would influence the distribution of between KC and thermodynamically-controlled (TC) products. In particular, the presence of calcium favors kinetically-controlled (*E*,*Z*)-isomeric products over thermodynamically-controlled (*E*,*E*)-isomers (Table 1). In addition, the accessibility of substrate to ZmLOX1 is sensitive to the ratio of LA to free calcium, which results in the bell-shaped dependence of catalytic efficiency on log $[LA]/[Ca^{2+}]_{free}$ (Fig. 6B). Accessibility of substrate to the enzyme active site might be enhanced through an electrostatic interaction between cationic calcium ion and anionic fatty acid substrate of LOX1, although the order of interaction between enzyme, calcium and substrate remains unknown.

In summary, the results presented here suggest that calcium binds to and promotes binding of LA to ZmLOX1, activates the enzyme, and may participate in an Enzyme-Metal-Substrate bridge. The bell-shaped (or inverted bell-shaped) relationship between kinetic parameters of ZmLOX1 and log $[LA]/[Ca^{2+}]_{free}$ are likely to reflect the combined influence of calcium on the above-mentioned properties of ZmLOX1, and the possibility that calcium may also interact with ZmLOX1 reaction products.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bioorg.2015.04.001.

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