A Substrate Serves as a Hydrogen Atom Donor in the Enzyme-Initiated Catalytic Mechanism of Dual Positional Specific Maize Lipoxygenase-1

Thavrak Huon, Sungkuk Jang, Kyoungwon Cho, Randeep Rakwal,† Je Chang Woo,‡ Ilchul Kim,§ Seung-Wook Chi,# and Oksoo Han*

*Department of Molecular Biotechnology, BK21 Globalization of Biotechnology Human Resources, Biotechnology Research Institute, College of Agriculture and Life Sciences, Chonnam National University, Gwangju 500-757, Korea. *E-mail: oshan@chonnam.ac.kr*

†Health Technology Research Center (HTRC), National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba 305-8569, Ibaraki, Japan

‡Department of Biotechnology, College of Natural Sciences, Mokpo National University, Mokpo 534-729, Korea

§Department of Biological Sciences, College of Natural Sciences, Chonnam National University, Gwangju 500-757, Korea

#Medical Proteomics Research Center, KRIBB, Daejeon 305-806, Korea

Received December 17, 2008, Accepted February 23, 2009

The maize lipoxgyenase-1 is a non-traditional dual positional specific enzyme and the reaction proceeds *via* enzyme-initiated catalysis. Bioinformatic analysis indicated that the maize lipoxygenase-1 is structurally more similar to soybean LOX1 than pea LOXN2 in that it has an additional external loop (residues 318-351) in the carboxy-terminal catalytic domain. We analyzed the dependence of product distribution on concentration of linoleic acid and monitored the formation of hydroperoxyoctadecadienoic acid as a function of enzyme concentration. Product distribution was strongly influenced by substrate concentration, such that kinetically-controlled regioisomers were enriched and thermodynamically-controlled regioisomers were depleted at high substrate concentration. Kinetic studies indicated that the formation of hydroperoxyoctadecadienoic acid saturated rapidly in an enzyme concentration-dependent manner, which implied that reactivation by reoxidation of inactive Fe(II) failed to occur. Our results support the previously proposed enzyme-initiated catalytic mechanism of the maize lipoxgyenase-1 and reveals that a substrate molecule serves as a hydrogen atom donor in its enzyme-initiated catalysis.

Key Words: Dual positional lipoxygenase, Enzyme-initiated mechanism, Kinetic control, Thermodynamic control, Radical mechanism

Introduction

Lipoxygenases (LOXs) (EC 1.13.14.12) are iron- or manganese-containing dioxygenases, which are ubiquitous in animals and plants.[1,2] In the presence of molecular oxygen, LOXs catalyze the hydroperoxidation of polyunsaturated fatty acids with one or more (1Z,4Z)-pentadienes. Theoretically, four regioisomers can be produced with linoleic acid (LA) as a substrate if the W-conformation of the substrate is maintained throughout the reaction (Figure 1). These regioisomers can be divided into two groups: (9Z,11E)-13-hydroperoxy-9,11-octadecadienoic acid (13-(9Z,11E)HPODE) and (10E,12Z)-9-hydroperoxy-10,12-octadecadienoic acid (9-(10E,12Z)HPODE) are *cis/trans* isomers and are considered kinetically-controlled products; and (9E,11E)-13-hydroperoxy-9,11-octadecadienoic acid (13-(9E,11E)HPODE) and (10E, 12E)-9-hydroperoxy-10,12-octadecadienoic acid (9-(10E,12E)HPODE) are *trans/trans* isomers and are thermodynamically-controlled products.[3] Most LOX-catalyzed reactions are regioselective and stereoselective, and produce primarily kinetically-controlled products. For example, LA and linolenic acid (LNA) are oxygenated at C-9 or C-13 of the hydrocarbon backbone of the unsaturated fatty acid, and 9(S)-(10E,12Z)-hydroperoxyoctadecadienoic (trienoic) acid (HPOD(T)E) or 13(S)-(9Z,11E)HPOD(T)E are produced by 9-LOX or 13-LOX, respectively.[4,5]

A few naturally-occurring plant LOXs exhibit dual positional specificity; these enzymes are classified as non-traditional LOXs, because their patterns of regio- and stereoselectivity are distinct from traditional LOX enzymes.[6-9] Wound-inducible maize LOX1 is a non-traditional LOX with dual positional specificity,[6,10,11] whose pre-steady state kinetic behavior has been characterized.[12] Although the regioselectivity and stereoselectivity of traditional LOX enzymes have been characterized extensively,[13] the catalytic mechanism of non-traditional dual positional specific LOX enzymes remains controversial.[14] We recently demonstrated that maize LOX1 produced not only kinetically-controlled (KC) products but also thermodynamically-controlled (TC) products, and provided regiochemical/stereochemical evidence that maize LOX1 acts by a unique enzyme-initiated catalytic mechanism.[15] This study compares properties of the maize LOX1 with other non-traditional LOXs, examines the dependence of product distribution on the concentrations of LA, and effect of enzyme concentration on reaction kinetics (*i.e.*, rate of formation of HPODE). The results support the previously proposed enzyme-initiated catalytic mechanism, and reveal that substrate concentration is a critical determinant of product distribution. A possible chemical mechanism for the formation of thermodynamically- and kinetically-controlled products during enzyme-initiated catalysis by maize LOX1 is discussed.

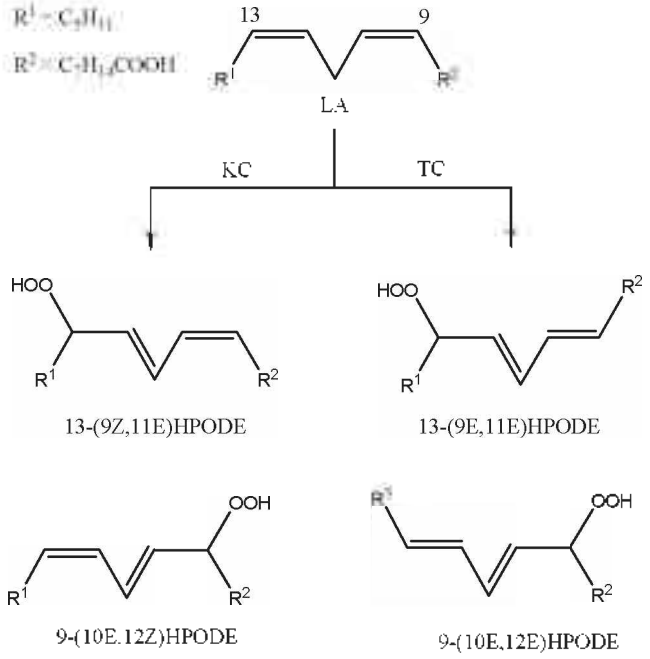

Figure 1. Formation of kinetically-controlled and thermodynamically-controlled products during non-traditional LOX reaction with LA as a substrate.

Experimental Section

Protein purification, separation, and structural identification of regioisomers. The pRSETB/LOX vector[6,15] was transformed into BL21(DE3)pLysS, which was grown in 50 mL of LB containing 50 μg/mL ampicillin and 35 μg/mL chloramphenicol at 37 °C until the cell density reached to OD_{600} = 0.6, and further cultured at 25 °C for 12 hr after addition of isopropy β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 1 mM. Bacterial cells were harvested by centrifugation (3000 xg, 15 min, 4 °C), briefly washed with 50 mM Tris-HCl (pH 7.2), and resuspended in 3 mL of 50 mM Tris-HCl (pH 7.2), 0.1% Tween 20 (v/v) and 0.2 mM phenylmethylsulphonyl fluoride (PMSF). The resuspended cells were disrupted by sonication and cell debris was removed by centrifugation (13,000 xg, 1 hr, 4 °C). Soluble fraction (1 mL) of the maize LOX1 was loaded on a 2 mL of Q-sepharose resin column pre-equilibrated with 50 mM Tris-HCl (pH 7.2). The column was washed with 10 mL of 50 mM Tris-HCl (pH 7.2). The maize LOX1 was eluted with 5 mL of 50 mM NaCl in 50 mM Tris-HCl (pH 7.2) by gravity flow. The homogeneity of purified maize LOX was confirmed by SDS-PAGE.

Enzymatic reaction and structural identification of enzymatic products. Enzymatic reaction and reduction with triphenylphosphine (TPP) was carried out according to a previously described procedure.[13,15] For $NaBH_4$-reduction[6] method, purified maize LOX1 (72 μg) was incubated in 50 mM Tris-HCl (pH 7.2) containing 0.5 mM LA, 0.05% Tween 20 (v/v) for 15 min at room temperature in a final volume of 2.5 mL. The reaction was stopped by addition of $NaBH_4$. HPODEs were reduced by $NaBH_4$ for 15 min at 25 °C. The pH was adjusted to 3 by addition of 1N HCl. The reaction mixture was immediately loaded on a Sep-pak C_{18} solid-phase extraction cartridge pre-washed and pre-equilibrated with distilled water. Reaction product and un-reacted substrate were retained by the cartridge, which was washed with 10 mL of 10% methanol (v/v) in water and 10 mL distilled water. Excess water was removed from the cartridge with a syringe and the adsorbed components were eluted with 3 mL dry 2-propanol.

The solvent was evaporated and positional isomers of oxygenated fatty acids were separated by SP-HPLC as described previously.[15] The mixture of HODEs was loaded on a Mightysil Si 60 column (250 × 4.6 mm, 5 μm particle size) from Kanto Chemical (Tokyo, Japan) and eluted with isocratic solvent system of *n*-hexane/2-propanol/acetic acid (100/2/0.1, v/v/v) with a flow rate of 1.0 mL/min. Absorbances at 234 nm and 210 nm were recorded simultaneously. Solvent was evaporated and separated positional isomers were subjected to NMR and GC-MS analysis.[6,15] For GC-MS analysis, each HODE separated by SP-HPLC was derivatized with 10 μL of SIGMA-SIL-A at 80 °C for 5 min. Trimethylsilylated HODE (1 μL) was loaded onto HP-5MS (30 m × 0.25 mm; 0.25 μm film thickness). Injector and detector temperatures were set at 260 °C and 300 °C, respectively. The temperature program was 100 °C (hold for 1 min) to 160 °C at 20 °C/min and 4 °C/min to 280 °C (hold for 4 min).

Dependence of product distribution on substrate concentration and quantification of HPODE. Maize LOX1 (72 or 1.0 μg) was incubated in 50 mM Tris-HCl (pH 7.2) containing 0.05% Tween 20 (v/v) and various concentrations of LA (0.03, 0.04, 0.05, 0.1 and 0.5 mM) at room temperature for 15 min in a final volume of 2.5 mL. The reaction was stopped by addition of chilled-methanol/acetic acid and products were extracted with Sep-pak C_{18} solid-phase extraction cartridge. Reaction products were reduced with TPP, and analyzed by SP-HPLC. Production of HPODE was quantified in standard LOX assays containing 0.4, 0.7, 1.8, 3.5, 4.9, 6.0 or 7.0 μg maize LOX1. Standard assay conditions included 0.5 mM LA, 0.05% Tween 20 (v/v) and 50 mM Tris-HCl (pH 7.2) in a final volume of 2.5 mL, with incubation at 25 °C for 15 min. Reactions were monitored spectrophotometrically at 234 nm.

Results

Sequence analysis and structural modeling of maize LOX1. The regio- and stereochemical characteristics of non-traditional plant LOXs, including maize LOX1, are summarized in Table 1. Most non-traditional LOXs show dual positional specificity and loose stereoselectivity. For example, maize LOX1 produces a 1:1 mixture of enantiomers at both C-9 and C-13 and is only example of completely non-stereoselective dual positional specific LOX in monocots. Amino acid sequence comparison showed that maize LOX1 shared a high degree of homology among LOX enzymes from monocot species. The regio- and stereoselectivity of maize LOX1 were similar to those of soybean LOX3 and pea LOXs. Figure 2 shows a protein sequence alignment of maize LOX1, pea LOXN2, and soybean LOX3. The alignment indicates strong

Table 1. Regioselectivity and stereoselectivity of non-traditional plant LOXs

Species		Enzyme	Protein ID	Homology with LOX1-Zm1 (%)	Putative amino acid determinant		Specificity		
					Regio	Stereo	Regio	Stereo	
							13:9	13(S:R)	9(S:R)
Monocot	Maize	LOX1-Zm1	AAF76207	100	TV:R	A	60:40[a]	50:50	50:50
	Rice	LOX1-Os1	CAA45738	86	TV:R	A	89:11[b]	91:9	50:50
		OsLOX1	ABD47523	79	TV:R	A	57:43[c]	S	S
	Barley	LOX1-Hv3	AAB70865	84	TV:K	A	88:12[d]	78:22	80:20
Dicot	Potato	LOX1-St2	CAB65460	72	TV:R	A	50:50[e]	NA	NA
	Cucumber	LOX1-Cs3	CAA63483	71	TH:R	A	84:16[f]	97:3	89:11
	Pea	LOX1-Ps3	CAA55319	70	TF:R	A	33:67[g]	NA	NA
	Pea	LOX1-Ps2	CAA55318	69	SF:R	A	80:20[g]	NA	NA
	Pea	LOXN2	CAG44502	65	TY:R	A	25:75[h]	NA	NA
	Soybean	LOX3	CAA30016	70	TF:R	A	38:62[i]	50:50	50:50

[a,15]. [b,18]. [c,19]. [d,9]. [e,*]. [f,20]. [g,21]. [h,16]. [i,22]. NA : Not available.

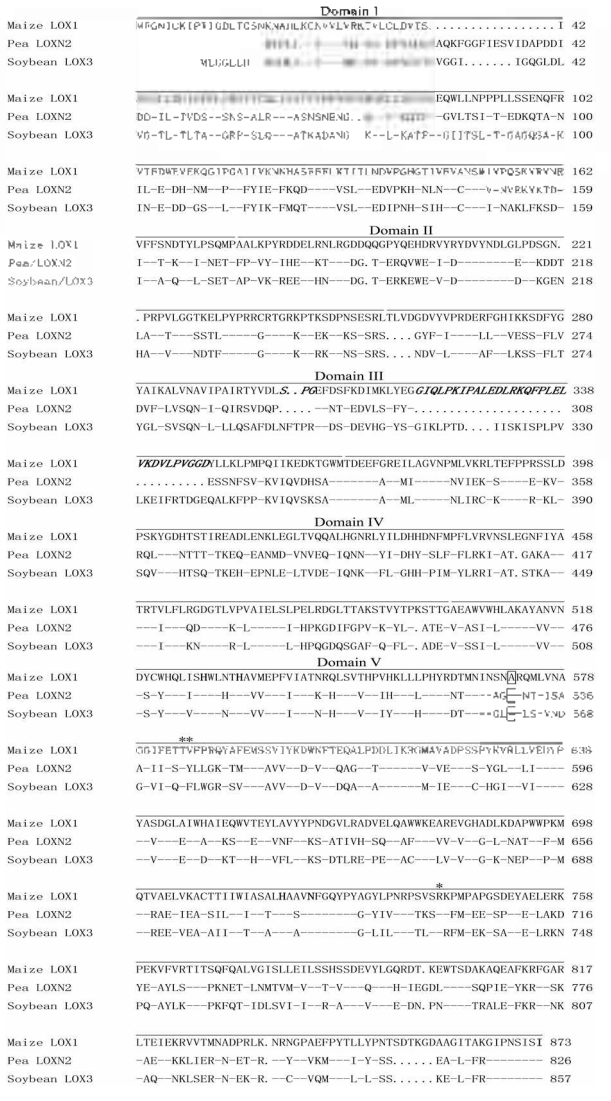

Figure 2. Amino acid sequence alignment of maize LOX1, pea LOXN2, and soybean LOX3. The sequence alignment was performed using CLUSTAL W (v.1.83). Identical residues are indicated by a hyphen (-). Dots (·) indicate gaps introduced to maximize the alignment. Bold letters indicate residues involved in iron binding. Asterisks and boxes indicate motifs in traditional LOXs that are responsible for positional specificity and stereoselectivity, respectively. Bold italics represent the external loop region of domain III (deleted in pea LOXN2). Domains are indicated by lined on the MzLOX amino acid sequence.

conservation of residues involved in iron binding[16] as well as motifs found in traditional LOXs that play roles in positional selectivity and stereoselectivity, so called "Coffa site".[15]

Upon sequence comparison, maize LOX1 (Genbank accession No. AF271894) showed 52% identity and 69% similarity to soybean LOX1 (Genbank accession No. J02795) and 49% identity and 65% similarity to pea LOXN2 (EMBL accession No. AJ749702). The homology modeled structures of maize LOX1 and pea LOXN2 were obtained by program Swiss-Model using the structural template of soybean LOX1 (PDB code: 1F8N). For comparison, the modeled structure of the maize LOX1 was superimposed with crystal structure of soybean LOX1[23] and the homology modeled structure of pea LOXN2.[16] Results are shown in Figure 3A. The residues 1-20 are missing in the modeled maize LOX1 structure because the corresponding region is absent in the structural template. The Ramachandran plots drawn by the program PROCHECK[24] showed that 85.3% of all the residues fall within the most favored regions, 12.9% in additional allowed regions, 1.2% in generously allowed regions, and 0.5% in disallowed regions. As seen in Figure 3A, the maize LOX1 consists of two discrete functional domains, amino-terminal β-barrel PLAT domain (colored cyan in the upper part) and the carboxy-terminal catalytic domain (colored blue in the lower part). The overall structures of the three LOX enzymes are very similar. The structure of maize LOX1 is more similar to that of soybean LOX1 than that of pea LOXN2 in that it has an additional external loop (residues 318-351) in the carboxy-terminal catalytic domain, which is consistent with regiochemical and stereochemical outcome seen in Table 1. The superimposition with structure of soybean LOX1 also reveals five inserted loops in the structure of maize LOX1. The inserted loops include residues 35-49, 140-143 in domain I, 254-257 in domain II, 324-327 in domain III, and 855-860 in domain V. The most noticeable difference between maize LOX1 and the other LOXs is its amino-terminal extension. One of the resulting extra loops may be an external loop composed of residues 35-49 in the domain I. The loop is likely to very flexible as observed in the structure of soybean LOX1. Upon insertion of the five external loops, both the β-barrel domain and the catalytic site undergo no appreciable conformational change. The iron binding residues (His528, His533, His719, Asn723, and Ile873) also remain in the same positions in domain V. Figure 3B shows electrostatic potential

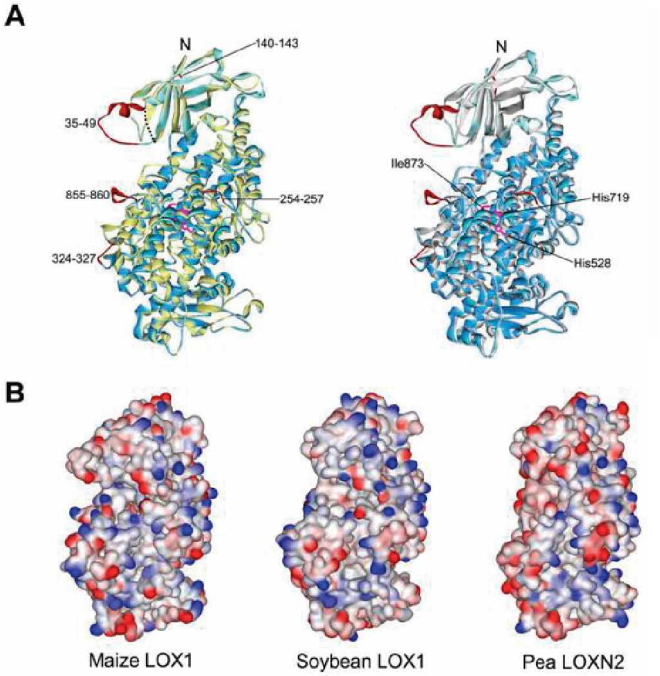

Figure 3. Structural comparison of the maize LOX1 with soybean LOX1 and pea LOXN2. (A) The superimposition of the modeled maize LOX1 structure (blue and cyan) with structures of soybean LOX1 (yellow in the left panel) and pea LOXN2 (gray in the right panel). The amino-terminal β-barrel PLAT domain and the carboxy-terminal catalytic domain are colored cyan and blue, respectively. The regions that are present in maize LOX1 but absent in soybean LOX1 or pea LOXN2 are colored red and iron binding residues are colored pink. The N-termini are labeled with capital letter N. Dotted lines indicate the absence of crystal structure in soybean LOX1 due to little electron density.[23] The homology modeled structure of pea LOXN2 contains residues 43-826. (B) Electrostatic potential distribution of the three LOX enzymes. The molecular surfaces with electrostatic potential were generated by the Discovery Studio software (Accelrys). Positive and negative potentials are colored blue and red, respectively. The molecular orientation is the same as in (A).

distribution of the molecular surface of three LOX enzymes. Although the overall electrostatic potential distribution is similar, pea LOXN2 shows relatively more negatively-charged surface than maize LOX1 and soybean LOX1. Like overall structure, the electrostatic potential distribution of maize LOX1 is more similar to that of soybean LOX1 than that of pea LOXN2. Such subtle difference in surface charge of the catalytic domain may influence the substrate orientation, potentially leading to distinction in positional specificity and stereoselectivity of LOX enzymes.

Enzyme purification and metal analysis. Recombinant maize LOX1 (free of epitope or affinity tag) was expressed in *E. coli* from the pRSETB:LOX plasmid and soluble protein was purified to homogeneity by Q-Sepharose chromatography as described previously.[15] Metal analysis was conducted by inductively coupled plasma (ICP). Purified maize LOX1 (3.5 mg: 7.6 nmole) contained 0.36-0.40 μg iron (6.8-7.2 nmole). Therefore, the maize LOX1 is a non-heme iron-containing LOX with one iron atom per enzyme molecule.

UV-visible spectral analysis of the maize LOX1 catalytic cycle. UV-visible spectral change during the maize LOX1 reaction is shown in Figure 4. HPODE was quantified by measuring absorbance at 234 nm (ε = 25,000 $cm^{-1}M^{-1}$), a characteristic conjugated diene signal.[25] Concomitant formation of oxo-octadecadienoic acid (OXOD) was estimated by measuring absorbance at 268 nm (ε = 22,000 $cm^{-1}M^{-1}$). The ratio of 234 nm to 268 nm absorbance indicated that the molar concentration of OXOD was approximately 4.9% of the molar concentration of HPODE at t = 14 min. The rate of nonenzymatic autooxidation of LA was negligible under the conditions used here. Therefore, maize LOX1 produced HPODE as a major product and OXOD as a minor product. TPP-reduced reaction products were separated by SP-HPLC and unambiguously identified by GC-MS and NMR. As reported previously,[15] maize LOX1 generated four major products (I, II, III, IV): these products were 13-(9Z,11E) HPODE (I), 13-(9E,11E)HPODE(II), 9-(10E,12Z)HPODE(III), and 9-(10E,12E)HPODE (IV).

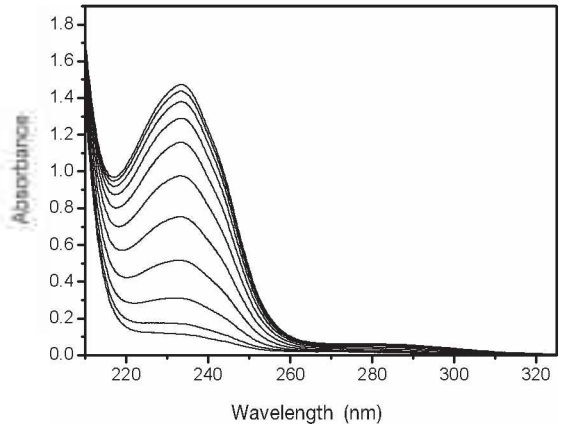

Figure 4. UV-visible spectral changes during oxidation of LA by maize LOX1. The reaction was started by adding 6 μg purified maize LOX1 to 50 mM Tris-HCl (pH 7.2) containing 0.5 mM LA, 0.05 % Tween-20 at 25 °C in a final volume of 2.5 mL. Spectra were recorded at 2 min intervals for 22 min in a Shimadzu UV-2550 spectrophotometer with a resolution of 0.2 nm.

Dependence of product distribution on reducing agents. Reaction products were reduced with TPP or $NaBH_4$ and analyzed by SP-HPLC (Figure 5). Since OXOD is formed by dehydration of HPODE and can be reduced to hydroxyoctadecadienoic acid (HODE) by $NaBH_4$ but not by TPP, the distribution of products was expected to depend on the choice of reducing agent. As shown in Table 2, the relative proportions of 13-(9Z,11E)-HPODE and 9-(10E,12Z)-HPODE were slightly higher when the reducing agent was $NaBH_4$. Therefore, we conclude that maize LOX1 produced 13-(9Z, 11E)-OXOD and 9-(10E,12Z)-OXOD by dehydration of 13-(9Z,11E)-HPODE and 9-(10E,12Z)-HPODE, respectively. To avoid reduction of OXOD to HODEs and simplify product analysis, TPP was used as a reducing agent in all subsequent studies reported here. Analysis of TPP-reduced products of the LOX1 reaction with LA showed that the positional specificity of maize LOX1 was 13-HPODE:9-HPODE = 52.2: 47.8 (Table 2). This result is consistent with our previous report[6] and confirms that maize LOX1 exhibits dual posi-

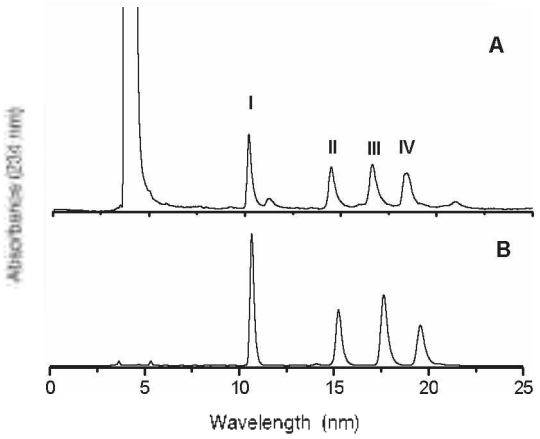

Figure 5. SP-HPLC analysis of regioisomers produced by maize LOX1. Reaction products were reduced with TPP (A) or $NaBH_4$ (B) as described in Experimental Section, loaded on a Mightysil Si 60 column (250 X 4.6 mm, 5 μm particle size) from Kanto Chemical (Tokyo, Japan) and eluted with an isocratic solvent system consisting of *n*-hexane/2-propanol/acetic acid (100/2/0.1, v/v/v) at a flow rate of 1.0 mL/min.

Table 2. Distribution of maize LOX1 reaction products reduced with TPP or $NaBH_4$

Reduction method	Compounds			
	13-(9Z,11E)-HPODE (%)	13-(9E,11E)-HPODE (%)	9-(10E,12Z)-HPODE (%)	9-(10E,12E)-HPODE (%)
TPP	29.0	23.2	26.0	21.8
NaBH4	32.9	20.2	28.9	18.0

tional specificity with both LNA and LA.

Dependence of product distribution on substrate concentration. We previously proposed that the enzyme-initiated first step of the reaction involves abstraction of bis-allylic hydrogen at C-11 from the substrate, followed by dissociation of the delocalized carbon-centered bis-allyllic radical intermediate, which is subsequently oxygenated outside the active site of the enzyme.[15] If this is the case, the bis-allylic radical intermediate needs to be released from the maize LOX1 active site prior to the oxygenation step and kinetically-controlled products will be formed in the initial stage of the reaction, as shown in Figure 6. Thermodynamically-controlled products are also produced when the kinetically-formed peroxyl radical undergoes the bond rotation followed by β-fragmentation, as shown in Figure 6. This mechanism has been proposed previously for the autooxidation of unsaturated fatty acid methyl ester.[3]

Formation of thermodynamically-controlled products requires bond rotation and β-fragmentation of kinetically controlled products. Because the bis-allylic hydrogen of LA is an efficient hydrogen atom donor and the availability of a hydrogen atom donor is critical for the proposed maize LOX1 reaction mechanism, substrate concentration is predicted to be an important determinant of reaction product distribution. Indeed, the product distribution was strongly dependent on the concentration of LA, and more importantly, kinetically-controlled regioisomers formed preferentially at high substrate concentration. The enzyme concentration, however, did not influence product distribution (Table 3). These results clearly support the idea that the carbon-centered bis-allylic radical species is released from the maize LOX1 active site and oxygenated outside the active site to form kinetically-controlled *cis/trans* isomers of HPODE, which are subsequently converted to thermodynamically-controlled products by the bond rotation followed by β-fragmentation.

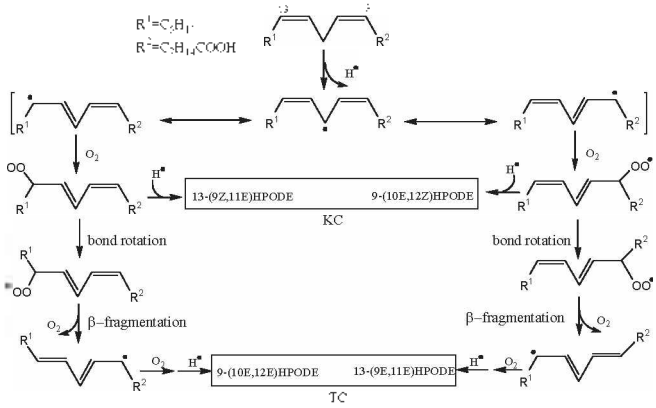

Figure 6. Mechanism for formation of kinetically- and thermodynamically-controlled products by dual positional specific maize LOX1.

Monitoring the formation and final concentrations of HPODEs as a function of enzyme concentration. The active form of LOX contains Fe(III), which is thought to abstract the bis-allylic hydrogen at C-11 of LA and reduced to Fe(II) during the initiation step of enzyme catalysis.[26] Consistent with this hypothesis, hydrogen abstraction by a mononuclear ferric hydroxide complex was demonstrated in a model study.[27] If the bis-allylic radical leaves the LOX1 active site prior to oxygenation, and the enzyme will remain inactive until Fe(II) is reoxidized to Fe(III) by HPODE. This hypothesis was tested by monitoring the formation of HPODE at different enzyme concentrations. As shown in Figure 7, the final concentration of HPODE increased in an enzyme concentration-dependent manner. This indicates that a signi-

Table 3. Dependence of product distribution on the substrate concentration during maize LOX1-catalyzed oxidation of LA

Maize LOX1 (μg)	LA (mM)	13-(9Z,11E)-HPODE (%)	9-(10E,12Z)-HPODE (%)	KC (%)	13-(9E,11E)-HPODE (%)	9-(10E,12E)-HPODE (%)	TC (%)
72	0.03	19.7	19.2	38.9	21.2	39.9	61.1
72	0.04	23.3	19.2	42.5	26.4	31.1	57.5
72	0.05	26.1	20.3	46.4	27.9	25.7	53.6
72	0.10	26.7	19.4	46.1	29.6	24.3	53.9
72	0.50	29.0	26.0	55.0	23.2	21.8	45.0
1.0	0.50	29.3	28.7	58.0	21.0	21.0	42.0

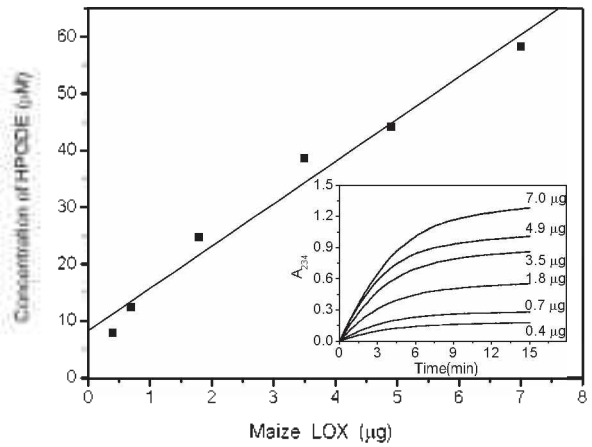

Figure 7. Monitoring the formation and final concentrations of HPODE at different concentrations of maize LOX1. HPODE was quantified in the presence of various amounts of enzyme (0.4, 0.7, 1.8, 3.5, 4.9, 6.0 or 7.0 μg). The standard reaction mixture contained 0.5 mM LA, 0.05% Tween 20 (v/v) and 50 mM Tris-HCl (pH 7.2) in a final volume of 2.5 mL. Reactions were monitored spectrophotometrically at 234 nm at 25 °C for 15 min. Inset shows each time course of the LOX reaction.

ficant fraction of the enzyme is converted into inactive form during the initiation step of catalysis. Since the formation of HPODE is rapidly saturated, the inactivation is not due to a random irreversible inactivation during turnover but Figure 7 suggests HPODE is not readily available to reoxidize the inactive Fe(II) species. Therefore, enzyme kinetic data also supports the proposed enzyme-initiated reaction mechanism for maize LOX1.

Discussion

Even though progress has been made in understanding the substrate specificity, access, orientation, oxygen pathway, regioselectivity, and stereoselectivity of traditional LOXs,[17,28] important questions about the determinants of regio- and stereoslectivity of these enzymes still remain unanswered.[4,7] To our knowledge, maize LOX1 and soybean LOX3 are only examples of completely non-stereoselective dual positional specific LOX in plants as summarized in Table 1. It has been hypothesized that the external loop in domain III may be structurally required for positional specificity and that absence of the external loop, as in pea LOXN2, may be mechanistically responsible for dual positional specificity.[7,16] However, as shown in Figure 2, a putative external loop in domain III is retained in maize LOX1 and soybean LOX3. Therefore, deletion of the external loop may not be the only determinant of dual positional specificity, at least in the case of maize LOX1 and soybean LOX3. On the other hand, the hydrophobic character on the molecular surface of catalytic domain in maize LOX1 may play important roles in orientation of substrate binding and oxygenation of the bis-allylic radical species, which are critical factors to determine regiospecificty and stereospecificity of maize LOX1. The role of hydrophobic surface of maize LOX1 in the enzyme-initiated catalytic mechanism has been proposed.[15]

The proposed mechanism for positional specificity of traditional LOXs implies that enzyme-bound iron is required for oxygen activation and may influence the site of oxygenation. However, it is difficult to reconcile the participation of Fe(II) in oxygen activation with the fact that abstraction of the prochiral hydrogen by Fe(III)-OH should be antarafacial to oxygen insertion. In order to explain this contradiction, Knapp and coworkers[14] suggested the existence of a separate channel specifically for oxygen access, that interfaces with the active site on the face of cavity IIa (*i.e.*, opposite Fe(II)); they also proposed that bulky hydrophobic amino acid residues would play a critical role in controlling access of oxygen to the proposed channel. We recently proposed the existence of a segregated oxygen channel and an enzyme-initiated mechanism for dual positional specific maize LOX1.[15] In the context of this model, we propose here that the bond rotation followed by β-fragmentation, as shown in Figure 6, can explain formation of both kinetically- and thermodynamically-controlled products. This model also predicts that the availability of a hydrogen atom donor would play a critical role in determining the distribution of reaction products. If the hydrogen atom donor is readily available, 13-(9Z,11E) and 9-(10E,12Z) peroxyl radicals will be rapidly converted to kinetically-controlled products, 13-(9Z,11E)HPODE and 9-(10E,12Z)HPODE. Otherwise, the delocalized bis-allylic radicals will undergo a bond rotation followed by β-fragmentation, leading to thermodynamically-controlled products, 9-(10E,12E)HODE and 13-(9E,11E)HODE. Therefore, the results shown in Table 3 further supports the proposed enzyme-initiated mechanism of dual positional specific maize LOX1 and Figure 6 provides a mechanistic rationale for the formation of both kinetically- and thermodynamically-controlled products.

Previous studies also showed that the reaction between the bis-allylic radical and triplet oxygen is diffusion-controlled in aqueous solution.[14] However, oxygen concentration was an important determinant of product distribution during soybean LOX2/LOX3-catalyzed oxidation of LA.[22] Because both thermodynamically- and kinetically-controlled products are generated during maize LOX1-catalyzed oxidation of LA, the oxygen supply to the bis-allylic radical may be limited due to the segregation of oxygen channel and thus, the bis-allylic radical will be easily released from the active site and oxygenated non-enzymatically at the enzyme surface. This produces a racemic mixture of C-9 or C-13 peroxyl radicals, and the peroxyl radicals are subsequently converted into kinetically-controlled products or thermodynamically-controlled products depending on the availability of a hydrogen atom donor. In addition, if the bis-allylic radical is released from the enzyme active site to be oxygenated by a non-enzymatic mechanism at the enzyme surface, the peroxyl radical would not be available for reoxidation of Fe(II) in the active site and the enzyme will remain inactive. This is clearly demonstrated in Figure 7. Alternatively, the catalytically active yellow LOX–Fe(III) might be converted into catalytically inactive purple LOX-Fe(III) by excess HPODE. This alternative possibility, however, is not likely, because the concentration of HPODE at saturation is linearly proportional to

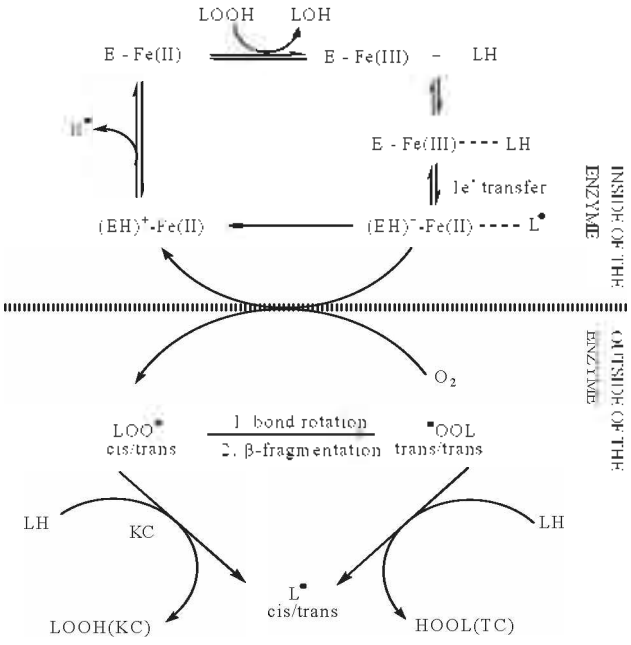

Figure 8. Substrate as a hydrogen atom donor in enzyme-initiated catalysis of maize LOX1.

enzyme concentration, as seen in Figure 7. Therefore, the bis-allylic radical species is generated in the enzyme active site by abstraction of hydrogen at C-11, and this resonance stabilized intermediate is subsequently released from the active site, undergoing oxygenation on the enzyme surface by a non-enzymatic mechanism.

The overall mechanism of dual positional specific maize LOX1 is summarized in Figure 8. In this mechanism, a substrate molecule serves as a hydrogen atom donor for the peroxyl radical species, and therefore, substrate availability plays a critical role in the relative abundance of kinetically- and thermodynamically-controlled reaction products. Together with previous regiochemical and stereochemical analysis, the results presented here strongly support enzyme-initiated catalysis with the bond rotation followed by β-fragmentation for dual positional specific maize LOX1. The regioisomers of hydroperoxy fatty acids produced by maize LOX1 are expected to exert diverse roles in plant host defense, possibly acting as signaling molecules for the biosynthesis of antifungal/antimicrobial phytooxylipins,[29] and/or as precursors for the biosynthesis of cutin monomers.[30] Furthermore, bisallylic or corresponding peroxyl radical species are a potential source of reactive oxygen species (ROS),[31] and thus dual positional specific maize LOX1 may be a prototype for enzymatic production of ROS during peroxidation of unsaturated fatty acids. Possible involvement of dual positional specific LOX in ROS signaling and disease resistance needs to be studied further.[32-35]

Acknowledgments. This work was supported by Korea Research Foundation (2007-313-F00021) and Agricultural Research Promotion Center (106074-03-2-CG000) to O. Han. We thank Dr. Pasqua Veronico for kindly giving us the coordinates of homology modeled structure for pea LOXN2.

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