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Regiochemical and Stereochemical Evidence for Enzyme-Initiated Catalysis in Dual Positional Specific Maize Lipoxygenase-1

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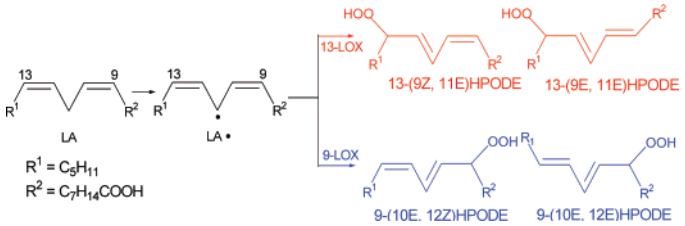
ABSTRACT



Dual positional specific maize lipoxygenase-1 catalyzed the formation of racemic mixtures of four possible regioisomers and was strongly inhibited by the radical scavenger, 4-hydroxy-2,2,6,6-tetramethyl-1-piperidinoxy radical. Molecular modeling studies indicated that the oxygen-binding cavity is segregated from the substrate-binding cavity. The data suggest that a bis-allylic radical reaction intermediate is generated enzymatically, released from the enzyme active site, and subsequently oxygenated outside of the enzyme active site by a nonenzymatic mechanism.

Lipoxygenases (LOXs) catalyze the hydroperoxidation of polyunsaturated fatty acids with one or more (1*Z*,4*Z*)-pentadiene structures in the presence of molecular oxygen. These enzymes play important functions in host defense systems in plants.[1] Theoretically, four regioisomers, 13-(9*Z*,11*E*)-hydroperoxyoctadecadienoic acid (HPODE) (**1**), 13-(9*E*,11*E*)-HPODE (**2**), 9-(10*E*,12*Z*)-HPODE (**3**), and 9-(10*E*,12*E*)-HPODE (**4**), can be produced from linoleic acid (LA), if the W-conformation of the substrate is maintained throughout the LOX reaction (Scheme 1). However, most LOX-catalyzed reactions are highly regioselective and stereoselective; for example, in plants, 9(*R*)-(10*E*,12*Z*)-HPODE is formed from LA by 9-LOX, and 13(*S*)-(9*Z*,11*E*)-HPODE is formed from LA by 13-LOX.[2] The regioselectivity of 9-LOX and 13-LOX has been explained by one of two models: the orientation-determined model (orientation of substrate insertion) or the space-determined model (frame-shift realignment of substrate).[2b] Evidence indicates that regioselectivity and stereoselectivity might be linked in LOX reactions,[3] such that oxygenation occurs on the opposite face (antarafacial) of the initially abstracted prochiral bis-allylic hydrogen at one or another end of the bis-allylic radical. However, a few naturally occurring plant LOXs, including maize LOX1, exhibit dual positional specificity; these enzymes are clas-

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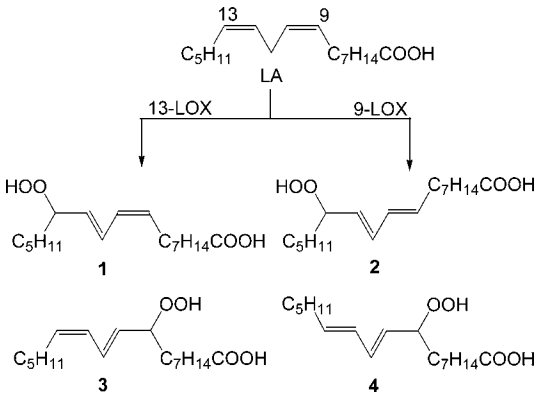
Scheme 1



sified as nontraditional LOXs, because their patterns of regio- and stereoselectivity are distinct from traditional LOX enzymes.[4] Even though issues regarding regioselectivity and stereoselectivity of the LOX reaction have been extensively addressed by many groups,[5] the detailed catalytic mechanism of the nontraditional dual positional LOX reaction remains controversial.[6] This study analyzes the regio- and stereoselectivity of maize LOX1 and the structure and distribution of its reaction products. Product analysis and molecular modeling studies were conducted, and their implications with regard to an enzyme-initiated catalytic mechanism are discussed.

Recombinant maize LOX1 (free epitope or affinity tags) was expressed in *E. coli* and purified by Q-Sepharose chromatography.[4a] Purified maize LOX1 was incubated with LA, the reaction products reduced with TPP and isolated as reported previously.[5] Structures of all regioisomers were identified by GC-MS and NMR. The regioselectivity of maize LOX1 was estimated from straight phase HPLC (SP-HPLC), and enantiomeric ratios of each regioisomer were determined by chiral phase HPLC (CP-HPLC).[4a,5] As seen in Figure 1, maize LOX1 produced all four regioisomers, **1**, **2**, **3**, and **4**, as racemic mixtures. Product formation was not significant with the boiled maize LOX1 enzyme under the employed enzymatic incubation condition. The positional specificity was 13-HPODE:9-HPODE = 53.2:46.8, which is consistent with our previous report regarding the dual positional specificity of maize LOX1 with linolenic acid (LNA) as a substrate.[4a]

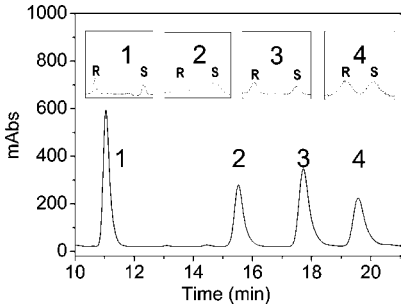


Figure 1. Regiochemical and stereochemical analysis of maize LOX1 reaction products by SP-HPLC and CP-HPLC.

This is unusual and interesting, because the products of most LOX reactions, even when carried out by a dual positional specific LOX, are primarily **1** and **3**.[2c,4b] A similar product distribution is seen during autoxidation of methyl linoleate in benzene.[7] Formation of compounds **1** and **3** is considered to be kinetically controlled while compounds **2** and **4** are thermodynamically controlled products. Figure 1 shows that maize LOX1 generates compounds **2** and **4** as well as compounds **1** and **3** from LA. Particularly, the nonstereoselective nature of this reaction is unique to dual positional specific maize LOX1, and it implies that oxygenation may occur outside the enzyme active site by a nonenzymatic mechanism. Therefore, product distribution was compared for the maize LOX1 reaction and autoxidation of linoleic acid at different temperatures (Table 1). The results showed that compounds **1** and **3** were the major products (78%) at 0 °C (kinetically controlled); in contrast, all four regiosiomers were formed in more comparable amounts when the reaction was carried out at 50 °C (thermodynamically controlled). More than 90% of the products were kinetically controlled compounds **1** and **3** when autoxidation was performed in the frozen state at −20 °C. Therefore, the product distribution pattern in the maize LOX1-catalyzed reaction is similar to that of thermodynamically controlled autoxidation. The first and rate-determining chemical step of the LOX1 reaction involves abstracting the prochiral hydrogen from C-11.[6a,8] According to the two-step model,[9] the abstraction of hydrogen from the substrate by catalytically active Fe(III) produces a bis-allylic radical intermediate with concurrent reduction of the iron cofactor to catalytically inactive Fe(II). The second step is insertion of dioxygen, which is accompanied by reoxidation of Fe(II) to Fe(III), allowing LOX1 to recycle to an active state. The two-step model is consistent with the nonstereoselective outcome of the maize LOX1 reaction, because with such a mechanism, the allyl radical intermediate could dissociate from the enzyme active site after the first reaction step. Thus, oxygenation of the bis-allylic radical intermediate could subsequently occur outside of the enzyme active site by a nonenzymatic mechanism.

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Table 1. Comparison of Product Distributions of the Maize LOX1 Reaction with Autoxidation

maize LOX1 (μg)	temp (°C)	**1** (S:R) (%)	**3** (S:R) (%)	KC **1** + **3** (%)	**2** (S:R) (%)	**4** (S:R) (%)	TC **2** + **4** (%)
72	25	29.0 (47.5:52.5)	26.0 (45.6:54.4)	55.0	23.2 (46.2:53.8)	21.8 (46.1:53.9)	45.0
0	50	16.6	16.5	33.1	31.4	35.5	66.9
0	0	43.5	34.7	78.2	9.3	12.5	21.8
0	−20	49.4	44.0	93.4	3.4	3.2	6.6

The regiochemical and stereochemical outcome of the maize LOX1 reaction in Figure 1 and Table 1 strongly suggest that the bis-allylic radical intermediate could be exported from the active site to the surface of the enzyme. If this is in fact the case, it is predicted that the reaction will be inhibited by a free radical scavenger such as 4-hydroxy-TEMPO (4-hydroxy-2,2,6,6-tetramethyl-1-piperidinoxy radical).[5] As shown in Figure 2, 4-hydroxy-TEMPO srongly

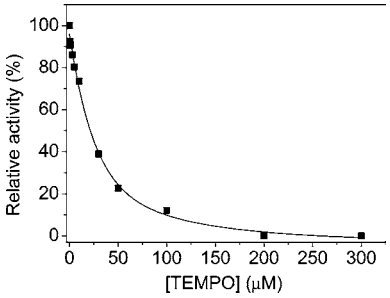

Figure 2. Inhibition of maize LOX1 by 4-hydroxy TEMPO.

inhibited maize LOX1, such that 0.1 mM 4-hydroxy-TEMPO reduced the relative enzyme activity by more than 5-fold. These data strongly support a model in which the bis-allylic radical reaction intermediate is released from the maize LOX1 active site prior to the oxygenation step. However, we cannot exclude the possibility that the maize LOX1 active site is accessible to 4-hydroxy-TEMPO, which quenches the bis-allylic radical intermediate before it is released from the enzyme active site. The latter situation has been reported for the manganese-containing LOX.[10]

Molecular modeling was used to explore the possible structural basis for the regiochemical and stereochemical outcome of LOX1. To this end, the three-dimensional structure of maize LOX1 was modeled by using an automated mode of the SWISS-MODEL server. Cavities in maize LOX1 were compared with those of soybean LOX1, a typical 13-LOX, by superimposition of the maize and soybean LOX1 structures. The modeling studies revealed that nine residues (Q524, L525, W529, A571, L575, V576, F582, F593, L783) in the oxygen-binding cavity of maize LOX1 had corresponding residues in soybean LOX1 (i.e., Q495, L496, W500, A524, L546, L547, I553, V564, and L754).

However, modeling also revealed a striking difference between the two structures: the oxygen-binding cavity is disconnected from the substrate-binding cavity in maize LOX1 as seen in Figure 3, while the oxygen-binding cavity

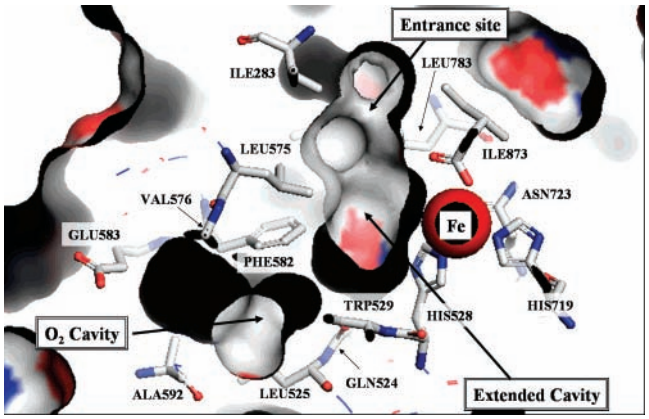

Figure 3. Substrate channel and oxygen cavity of the maize LOX1 shown as a white surface. The substrate channel is composed of entrance site and extended cavity.

is cross-connected to the substrate-binding cavity in soybean LOX1. Previous studies showed that the oxygen-binding cavity of soybean LOX1 intersects the substrate-binding cavity near the point of C-13 for 13-LOX, and the point where the two cavities intersect determines the regioselectivity of oxygenation in the reaction.[6a] The crystal structure of the dual positional-specific soybean LOX3 indicated that the oxygen cavity and the substrate-binding cavity intersect over a relatively broad area.[11] This is in marked contrast to the isolation of the oxygen-binding cavity in maize LOX1, as reported here. This observation is consistent with a high probability of dissociation of the bis-allylic radical intermediate from the enzyme active site of maize LOX1. Therefore, the separation of the oxygen and substrate binding cavities in maize LOX1 could provide a structural explanation for release of the bis-allylic radical intermediate from the enzyme active site prior to its oxygenation.

Maize LOX1 is, to our knowledge, the first example of a naturally occurring nonstereoselective plant LOX in monocots. Thus, it may be a prototype for a two-step enzyme-

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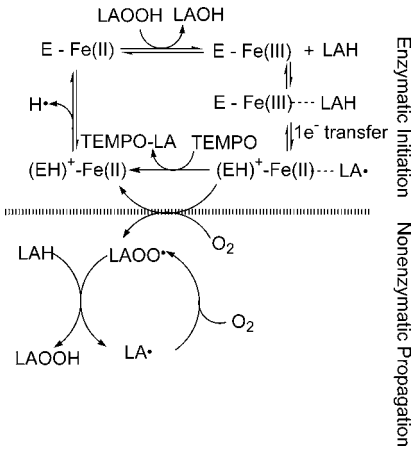
Scheme 2



initiated catalytic mechanism for the oxidation of unsaturated fatty acids. As stated above, our model proposes that the enzyme-initiated first step of the reaction involves hydrogen abstraction from the substrate, followed by dissociation of the bis-allylic radical intermediate, which is subsequently oxygenated at the surface of the enzyme (Scheme 2). This model is consistent with the dual positional specificity and nonstereoselectivity of maize LOX1.

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Supporting Information Available: Experimental details, SDS-PAGE of the maize LOX1, structural characterization of the products, and modeled structures of maize LOX1 and soybean LOX1. This material is available free of charge via the Internet at http://pubs.acs.org.

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