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Effect of endocannabinoids on soybean lipoxygenase-1 activity

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ABSTRACT

Endocannabinoids appear to be involved in a variety of physiological processes. Lipoxygenase activity has been known to be affected by unsaturated fatty acids or phenolic compounds. In this study, we examined whether endocannabinoids containing both N-acyl group and phenolic group can affect the activity of soybean lipoxygenase (LOX)-1, similar to mammalian 15-lipoxygenase in physicochemical properties. First, N-arachidonoyl dopamine and N-oleoyl dopamine were found to inhibit soybean LOX-1-catalyzed oxygenation of linoleic acid in a non-competitive manner with a K_i value of 3.7 μM and 6.2 μM, respectively. Meanwhile, other endocannabinoids failed to show a remarkable inhibition of soybean LOX-1. Separately, N-arachidonoyl dopamine and N-arachidonoyl serotonin were observed to inactivate soybean LOX-1 with K_{in} value of 27 μM and 24 μM, respectively, and k_3 value of 0.12 min^{-1} and 0.35 min^{-1}, respectively. Furthermore, such an inactivation was enhanced by ascorbic acid, but suppressed by 13(S)-hydroperoxy-9,11-octadecadienoic acid. Taken together, it is proposed that endocannabinoids containing polyunsaturated acyl moiety and phenolic group may be efficient for the inhibition as well as inactivation of 15-lipoxygenase.

1. Introduction

Lipoxygenases (LOXs), a family of non-heme iron-containing dioxygenases, catalyzes the addition of molecular oxygen to polyunsaturated fatty acids containing at least one (*Z*, *Z*)-pentadiene system to produce corresponding hydroperoxides [1–5]. Recently, our recent studies demonstrated that polyunsaturated lysophosphatidylcholines and lysophosphatidylethanolamines containing linoleoyl, arachidonoyl or docosahexaenoyl groups were efficiently oxygenated by reticulocyte LOX, leukocyte LOX or soybean LOX-1 [6–8]. In general, LOXs contain an essential iron atom, which is present as Fe^{2+} in the ground-state inactive form; enzymatic activation occurs through lipid hydroperoxide-driven oxidation of Fe^{2+} to Fe^{3+} [1–3]. LOXs are widely distributed among plants and animals [1–3]. It is well-known that in plant system, LOXs catalyze oxygenation of linoleic acid and linolenic acid to generate cellular regulators, such as jasmonic acid, traumatic acid, and alkenals, responsible for the growth regulation and wound healing [9,10]. In animals, LOXs are known to convert arachidonoic acid to lipid mediators, such as leukotrienes or lipoxins [11,12], which are implicated in various diseases such as atherosclerosis, cancer, inflammation and asthma [13]. Since LOX metabolites of polyunsaturated fatty acids are involved in cellular response under normal or disease conditions [14–17], the regulation of LOX-catalyzed oxygenation of polyunsaturated fatty acids may result in the alteration of a pathophysiological response in animals. In humans, lipoxygenase plays a key role in the biosynthesis of leukotrienes, proinflammatory mediators mainly released from myeloid cells. Thus, inhibitors of lipoxygenases have attracted attention initially as potential agents for the treatment of inflammatory and allergic diseases, but their therapeutic potential has now been expanded to certain types of cancer and cardiovascular diseases [18–21]. Soybean lipoxygenase-1 (soybean LOX-1) is a plant-derived 15-LOX catalyzing efficiently the oxidation of linoleic acid to 13-hydroperoxy-9,11-octadecadienoic acid. Because of structural and functional similarities with mammalian 15-LOX, soybean LOX-1 is commonly used as a model for mammalian 15-LOX for both mechanistic and inhibition studies [1–3,14–17].

Earlier, soybean LOX-1 had been reported to undergo inactivation during the incubation with polyunsaturated fatty acids, and

Abbreviations: 13-HpODE, 13(S)-hydroperoxyoctadecadienoic acid; 15(S)-HpETE, 15(S)-hydroperoxyeicosatatraenoic acid; E_{ox}, active ferric enzyme form; E_{red}, inactive ferrous enzyme form; LOX, lipoxygenase; NA-DA, N-arachidonoyl dopamine; NA-Gly, N-arachidonoyl glycine; NA-Tau, N-arachidonoyl taurine; NA-Ser, N-arachidonoyl serine; NA-5HT, N-arachidonoyl serotonin; NO-DA, N-oleoyl dopamine; NP-DA, N-palmitoyl dopamine.

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the inactivation was suggested to be ascribed to the oxidants from oxygenation products [22]. In support of this, the activities of soybean LOX-1 or potato 5-LOX was observed to decline gradually during preincubation with 5(S)-hydroperoxyeicosatetraenoic, 9(S)-hydroperoxy-10(E), 12(Z), 15(Z)-octadecatrienoic acid or 15(S)-hydroxyperoxyeicosatetraenoic [23–25], supporting the assumption that the unstable intermediates from the respective hydroperoxy product may be responsible for the gradual loss of enzyme activity. Recently, it was observed that polyunsaturated lysophosphatidylcholines, which can be utilized as substrates for lipoxygenase, could inhibit lipoxygenase [26]. Separately, there are many reports about the inhibitory effect of some phenolic compounds on lypoxygenases activity [27–29]. Endocannabinoids are compounds containing fatty acids link to amino acid or neurotransmitter. Most endocannabinoids are differentially distributed in the body and the level of several ones undergoes hormonal regulation in the brain [30]. Endocannabinoids are of great interest as potential tools to probe new binding sites of G protein coupled receptors (GPCRs), transporters or ion channels [31]. Previously, N-arachidonoyl glycine and N-arachidonoyl dopamine had been reported to be substrates for lipoxygenase [32,33]. Nonetheless, the reports concerning the regulation of endocannabinoids on lipoxygenase or cyclooxygenase activity is limited [34,35].

The present study evaluated the inhibitory or inactivating effect of N-acylated endocannabinoids on soybean LOX-1 activity. Here, we demonstrate that N-acyl dopamine inhibited soybean LOX-1 potently and N-arachidonoyl dopamine or N-arachidonoyl serotonin is a strong inactivator of soybean LOX-1. The purpose of this study is to examine the structural requirement of endocannabinoids for the efficient inhibitor of soybean LOX-1, and to provide an idea for the design of a potential inhibitor of mammalian 15-LOX.

2. Material and methods

2.1. Materials

Soybean lipoxygenase-1 (type 1-B), linoleic acid, ascorbic acid, dopamine hydrochloride, serotonin and xylenol orange were purchased from Sigma–Aldrich Company (Alabaster, Alabama, USA). Iron (II) sulfate was from Kanto Chemical Co., Inc (Tokyo, Japan). Arachidonic acid, N-arachidonoyl dopamine (NA-DA), N-oleoyl dopamine (NO-DA), N-palmitoyl dopamine (NP-DA), N-arachidonoyl taurine (NA-Tau), N-arachidonoyl glycine (NA-Gly), N-arachidonoyl serine (NA-Ser), N-arachidonoyl serotonin (NA-5HT) were obtained from Cayman Chemical (Ann Arbor, MI, USA). 13(S)-hydroperoxy-9,11-octadecadienoic acid (13-HpODE, >98%) and 15(S)-hydroperoxy-5,8,11,13-eicosatetraenoic acid (15-HpETE, >98%) were prepared as described before [25]. Briefly, soybean LOX-1 (0.8 unit/mL) was incubated with linoleic acid or arachidonoic acid (50 μM) in borax buffer (50 mM, pH 9.0) at 25 °C for 30 min. Subsequently, the mixture was passed through a C_{18} column (2 cm × 1 cm), and the products were eluted with methanol and concentrated under N_2 gas.

2.2. Determination of kinetic values in soybean LOX-1 – catalyzed oxygenation of endocannabinoids

Oxygenation of NA-DA, NA-Tau, NA-Gly, NA-Ser, arachidonoic acid or linoleic acid by soy-LOX-1 was monitored by measuring the increase of the absorbance at 234 nm (A_{234}) reflecting to the formation of conjugated diene. In kinetic study, endocannabinoids (0–40 μM) were incubated soybean LOX-1 (0.05 units/mL) in 50 mM borax buffer (pH 9.0). One unit is defined as the activity of enzyme forming 1 μmole of oxygenation product per min. The kinetic parameters, km and kcat, were determined by non-linear regression analysis using enzyme kinetic analysis in GraphPad Prism 5 software.

2.3. Determination of LOX activities by UV spectrophotometer and Eadie–Hofstee Plot analysis for inhibition of LOX activities by N-acyl dopamine

Soybean LOX-1 (0.05 units/mL) was incubated with linoleic acid (0–80 μM) in the presence of N-arachidonoyl dopamine or N-oleoyl dopamine in borax buffer (50 mM, pH 9.0) at 25 °C for 1 min. The remaining LOX activity was determined by measuring

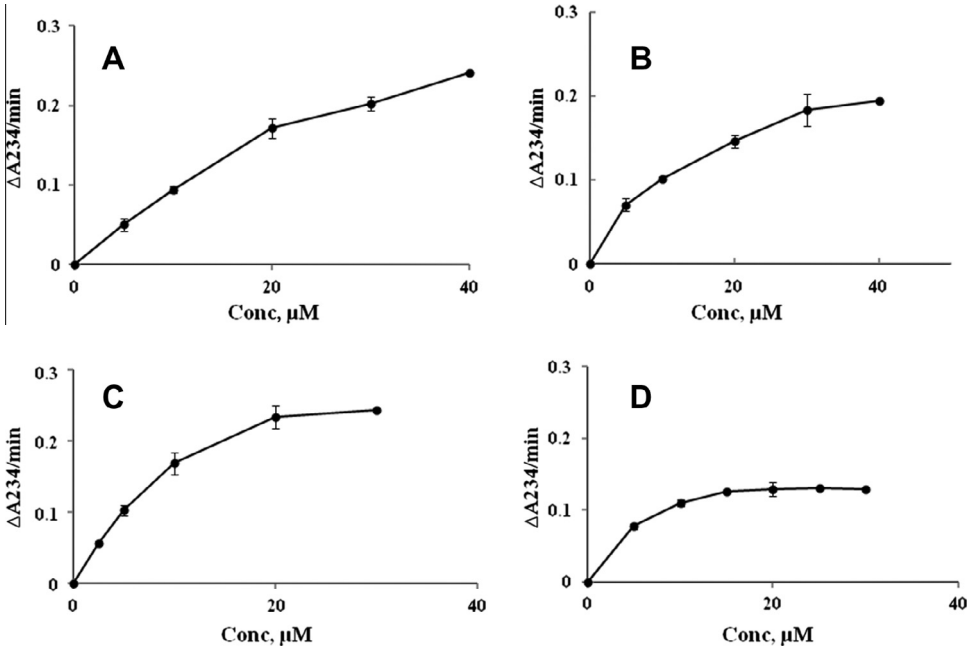


Fig. 1. Effect of substrate concentration on LOX-catalyzed oxygenation of various endocannabinoids. Soybean LOX-1 (0.05 units/mL) was incubated with some endocannabinoids of various concentration (0–40 μM) in 50 mM borax buffer (350 μl, pH 9.0). (A) N-arachidonoyl taurine, (B) N-arachidonoyl dopamine, (C) N-arachidonoyl glycine, (D) N-arachidonoyl serine. Data are expressed as a means ± SD value of triplicate assays.

A_{234}, and the data were analyzed by Eadie–Hofstee plot using enzyme kinetic analysis in Sigma Plot 12 program.

2.4. Inactivation of soybean LOX-1 by N-acyl dopamine or N-arachidonoyl serotonin

Soybean LOX (0.8 units/mL) was incubated with each endocannabinoid in 50 mM borax pH 9 (final volume, 300 μL) at 25 °C. The aliquot (3 μL) was taken at indicated time and used for the assay of remaining activity. The preincubation of lipoxygenases with endocannabinoids in the buffer containing ascorbic acid, 13-HpODE or 15-HpETE was performed as described above, unless described otherwise. The effect of radical scavengers or hydroperoxide was tested as described in figure legends. Experiment was conducted in triplicate.

2.5. Determination of soybean LOX-1 activity by FOX assay

The FOX assay was carried out according to a slight modification of the procedure reported previously [36]. Briefly, the oxygenation reaction was terminated at designed time by the addition of FOX reagent mixture (5 volumes) containing perchloric acid (110 mM), xylenol orange (150 μM) and ferrous sulfate heptanhydrate (2 mM) in methanol:water (9:1). After 10 min of reaction, the absorbance at 550 nm, due to the formation of the complex Fe^{3+}/xylenol orange, was measured using UV–Vis spectrophotometer (UVmini 1240, Shimadzu). For the blank, each substrate was incubated with endocannabinoid for designated time, and then, to the mixture was added FOX reagent and soybean LOX-1. A standard curve of peroxide was prepared using H_2O_2 as standard peroxide.

2.6. RP-HPLC analysis of determination of soybean LOX-1 activity by NADA

Soybean LOX-1 was incubated with LA (50 μM) in the presence or absence of NADA (10 μM) in 500 μL borax buffer (50 mM, pH 9.0) at 25 °C. After 30 min of oxygenation, an aliquot was taken and injected into RP-HPLC system (Shimadzu, Kyoto, Japan), equipped with XBridge C18 column (3.5 μm, 3.0 mm × 100 mm, Waters, Ireland) containing a C18 precolumn, which was eluted with mobile phase (Methanol/H_2O/formic acid; 70:30:0.1) as described before [26]. The flow rate was 0.4 mL/min, and the elution was monitored by SPD-20A UV/Vis detector at 234 nm.

Table 1
Kinetic values in oxygenation of various endocannabinoids by soybean-LOX-1. Each endocannabinoid (0–40 μM) was incubated with soybean LOX-1 (0.05 units/mL) in 50 mM borax buffer (350 μl, pH 9.0). Data are expressed as a means ± SD value of triplicate assays.

Substrate	kcat (min^{-1})	km (μM)	Kcat/km (min^{-1} μM^{-1})
N-arachidonoyl glycine	1063 ± 49.33	11.60 ± 1.28	92.77 ± 12.83
N-arachidonoyl taurine	1509 ± 139.9	42.29 ± 6.53	36.55 ± 7.63
N-arachidonoyl serine	470.7 ± 13.36	4.46 ± 0.543	107.13 ± 15.47
N-arachidonoyl dopamine	888.5 ± 58.20	18.02 ± 2.74	50.47 ± 9.67
Arachidonoic acid	3301 ± 289.8	51.36 ± 6.36	65.26 ± 11.46

3. Results

3.1. Determination of kinetic values in oxygenation of endocannabinods by soybean LOX-1

When the respective endocannabinoid was incubated with soybean LOX-1, a time-dependent increase in absorbance at 234 nm was observed, indicating the formation of a conjugated diene. Based on this, the concentration-dependent effect of endocannabinoid on LOX-1-catalyzed oxygenation was examined. Fig. 1 showed that the enzyme activity followed a classical Michaelis–Menten kinetics when the concentration of each endocannabinoid was varied. Subsequently, the kinetic value for oxygenation of some endocannabinoids by soybean LOX-1 was determined from non-regression analysis, and compared to that for oxygenation of arachidonoic acid. As displayed in Table 1, most endocannabinoids are found to be favorable substrates for soybean LOX-1 catalyzed oxygenation. In comparison, the most effective substrate was N-arachidonoyl serine (NA-Ser), showing a catalytic efficacy (kcat/km) value of 107.13 min^{-1} μM^{-1}, followed by N-arachidonoyl glycine (NA-Gly) (kcat/km, 92.77 min^{-1} μM^{-1}), possessing a higher value than that of arachidonoic acid (kcat/km, 65.26 min^{-1} μM^{-1}). Meanwhile, N-arachidonoyl dopamine (NA-DA) and N-arachidonoyl

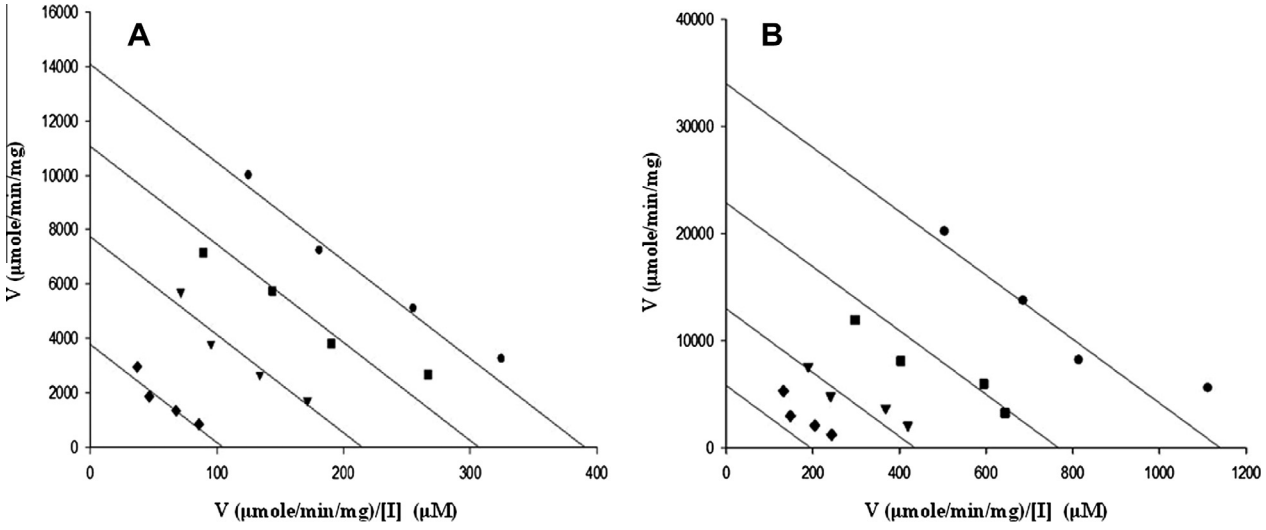


Fig. 2. Inhibitory effect of N-arachidonoyl dopamine (NADA) or N-oleoyl dopamine (NODA) on soybean LOX-1 activity. Soybean LOX-1 (0.05 units/mL) was incubated with linoleic acid (0–40 μM) in the presence of inhibitors in borax buffer (50 mM, pH 9) at 25 °C. (A) NADA (●, 0 μM; ■, 1 μM; ▼, 3 μM and ♦, 10 μM). (B) NODA (●, 0 μM; ■, 3 μM; ▼, 10 μM and ♦, 30 μM). The remaining LOX activity was determined by measuring the A_{234}. Results are expressed by Eadi-Hofee plot using enzyme kinetic analysis in Sigma Plot program.

taurine (NA-Tau) demonstrated a kcat/km value of 50.47 min^{-1} - μM^{-1} and 36.55 min^{-1} μM^{-1}, respectively, which was lower than that of arachidonoic acid. However, oxygenation of N-arachidonoyl serotonin (NA-5HT) by soybean LOX-1 could not be properly determined by UV spectrophotometer (data not shown), since no time-dependent increase in absorbance at 234 nm was observed with NA-5HT. Instead, oxygenation of NA-5HT by soybean LOX-1 was determined by FOX assay but NA-5HT was not an efficient substrate for soybean LOX-1 (data not shown).

3.2. Inhibition of soybean LOX-1 by N-arachidonoyl dopamine or N-oleoyl dopamine

The above results show that arachidonoyl endocannabinoids can be alternative substrates of soybean LOX-1. However, the rate

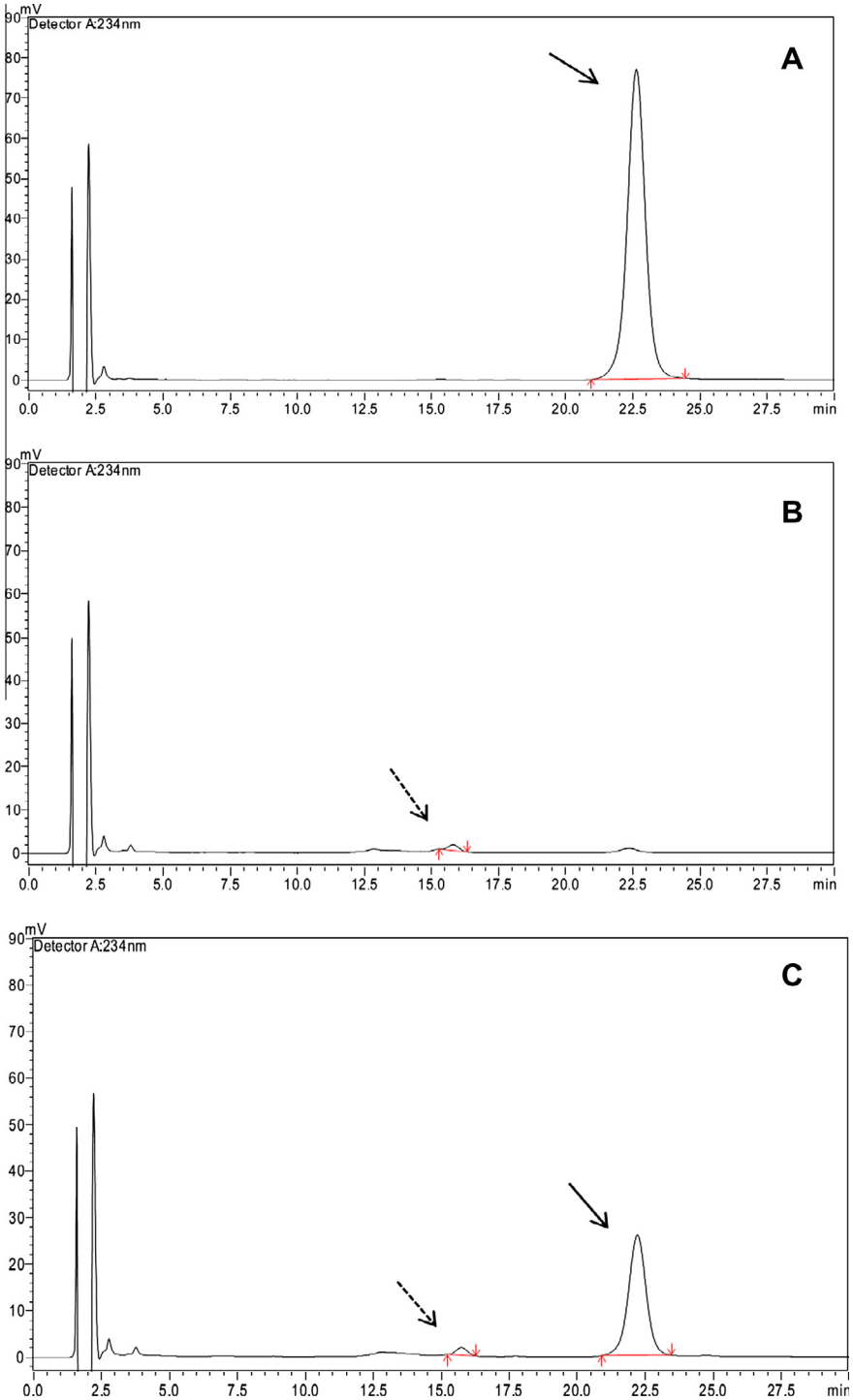


Fig. 3. RP-HPLC chromatogram of products from soybean LOX-1-catalyzed oxygenation of linoleic acid (LA) in presence or absence of N-arachidonoyl dopamine (NADA). Soybean LOX-1 was incubated with LA (50 μM) in the presence or absence of NADA (10 μM) in borax buffer (50 mM, pH 9.0). After 30 min incubation, the mixture was subjected to RP-HPLC analysis (flow rate, 0.4 mL/min, UV detection, 234 nm). (A) Products from incubation of soybean LOX-1 with LA (50 μM) alone (solid arrow); (B) products from incubation of soybean LOX-1 with NADA (10 μM) (dotted arrow) and (C) products from incubation of soybean LOX-1 with LA (50 μM) (solid arrow) in the presence of NADA (10 μM) (dotted arrow).

for oxygenation of endocannabinoids by LOX seemed to differ greatly according to the oxygenation time. From this, it was supposed that oxygenation products of N-arachidonoyl endocannabinoids might affect oxygenation of native substrate by soybean LOX-1. In an attempt to explain this phenomenon, we examined the inhibitory effect of endocannabinoid on soybean LOX-1 activity. First, soybean LOX-1 was incubated with linoleic acid in the presence N-arachidonoyl dopamine (NA-DA) or N-oleoyl dopamine (NO-DA), and the remaining activity of soybean LOX-1 was determined. As shown in Fig. 2, NA-DA or NO-DA was found to inhibit soybean LOX-1 activity strongly. The Eadie–Hofstee plot analysis indicates that NA-DA and NO-DA inhibit Soybean LOX-1, displaying a quasi-noncompetitive type of inhibition (Fig. 2). In contrast, N-arachidonoyl glycine, N-arachidonoyl taurine, N-arachidonoyl serine, N-palmitoyl dopamine or N-arachidonoyl serotonin had no remarkable inhibition up to 30 μM of soybean LOX-1 activity (data not shown). The most potent endocannabinoid in inhibiting soybean LOX-1 activity was NA-DA (Ki, 3.7 μM), followed by NO-DA (Ki, 6.2 μM) and other endocannabinoids (Ki, >100 μM) (data not shown). All these suggest that the presence of phenolic group and unsaturated acyl group may be important for inhibitory action of these compounds. In additional experiment to confirm the inhibitory action of NADA, soybean LOX-1 was incubated with LA in the presence or absence of NADA (10 μM) for 30 min, and the final reaction products was subjected to RP-HPLC analysis. As demonstrated in Fig. 3C, the inclusion of NADA (10 μM) inhibited soybean LOX-1-catalyzed formation of 13-HpODE from LA, compared to control (Fig. 3A). Moreover, the area of the peak corresponding to oxygenation product of NADA was less than 1% of that of LA, when the oxygenation product of NADA and LA were analyzed by HPLC, indicating that under the condition used, the oxygenation of NADA itself could be negligible (Fig. 3 B and C). Furthermore, in a separate experiment, the inhibition of soybean LOX-1 by NA-DA or NO-DA was confirmed by FOX assay. NA-DA or NO-DA was also found to display a strong inhibition of Soybean LOX-1 (data not shown). Meanwhile, it was questioned whether soybean LOX-1 could be inactivated during the pre-incubation of soybean LOX-1 with endocannabinoid, as had been suggested from the inactivation of soybean LOX-1 by unsaturated fatty acids and their peroxide derivatives [22,24,37].

3.3. Inactivation soybean LOX-1 by N-arachidonoyl dopamine and N-arachidonoyl serotonin

Previously, it had been reported that the partially purified soybean LOX-1 was significantly inactivated during incubation with arachidonic acid, but not with linoleic acid. In the present study, the oxygenation of endocannabinoids was also found to be reduced time-dependent during the oxygenation process. To clarify this, each endocannabinoid (30 μM) was pre-incubated with soybean LOX-1 (0.8 units/mL) in 50 mM borax buffer pH 9 (final volume, 300 μL) and after each time interval, the aliquot was taken into borax buffer (50 mM, pH 9.0) containing linoleic acid (30 μM) to determine remaining activity. The remaining activities of soybean LOX-1 after 15 min pre-incubation were shown in Table 2. A time-dependent inactivation was not observed when N-arachidonoyl glycine, N-arachidonoyl taurine, N-arachidonoyl serine, N-oleoyl dopamine, N-palmitoyl dopamine or N-arachidonoyl dopamine was preincubated with soybean LOX-1 (Fig. 4). In contrast, when the soybean LOX-1 was exposed to N-arachidonyl dopamine (NA-DA) or N-arachidonoyl serotonin (NA-5HT), the enzyme was inactivated in a time-dependent manner.

Next, NA-DA of various concentrations was pre-incubated with soybean LOX-1, and an aliquot of the mixture was taken into 350μL of borax buffer (pH 9.0) containing linoleic acid (30 μM). Under the condition used, the concentration of NA-DA (0.125–0.5 μM) in the final assay was not sufficient to affect soybean LOX activity. As shown in Fig. 5, soybean LOX-1 pre-incubated with NA-DA was inactivated gradually in the time-dependent manner, indicative of a typical irreversible inhibition of LOX. When the reciprocals of rate constant of inactivation against the reciprocals of the concentration of NA-DA according to Kitz and Wison [38] plot, a good linear relationship was observed (Fig. 5), reaffirming the irreversible inhibition of soybean LOX-1. Kinetic analyses indicate that the value of K_{in} is 27.6 μM and k_3 is 0.125 min^{-1}. Previously, serotoninamide of arachidonic acid had been reported as an irreversible inhibitor of soybean LOX-1 [39]. Consistent with this previous work, a similar pattern of inactivation of soybean LOX-1 activity was also observed in the prior incubation of soybean LOX-1 with various concentrations of NA-5HT (Fig. 6); the values of K_{in} and k_3 were determined to be 24.5 μM and 0.35 min^{-1},

Table 2
Inactivation of soybean LOX-1 activity after 15 min pre-incubation by endocanabinoids. Each compound (30 μM) was pre-incubated with 30 μl Soybean LOX-1 0.3 mg/ml in 50 mM borax buffer pH 9 (final volume 300 μL). The aliquot (3 μl) was taken into assay mixture containing 30 μM linoleic acid after 15 min pre-incubation. The remaining soybean LOX-1 activity was determined by measuring A_{234}. 100% activity was determined in a control in which soybean LOX-1 was pre-incubated in buffer for the same period of time that soybean LOX-1 was pre-incubated with the several compounds. Data were expressed as means ± SD valued of triplicates experiments.

Compound	% Activity remaining
N-arachidonoyl glycine	80.25 ± 8.92
N-arachidonoyl taurine	76.02 ± 5.46
N-arachidonoyl serine	82.21 ± 0.37
Arachidonoic acid	80.07 ± 1.5
N-oleoyl dopamine	57.02 ± 6.25
N-palmitoyl dopamine	44.63 ± 2.33
N-arachidonoyl dopamine	5.51 ± 1.15
N-arachidonoyl serotonin	0.9 ± 0.12
Dopamine	95.13 ± 5.3
Dopamine + arachidonoic acid	80.3 ± 3.9
Serotonin	93.6 ± 2.2
Arachidonoic acid + serotonin	70.06 ± 4.5

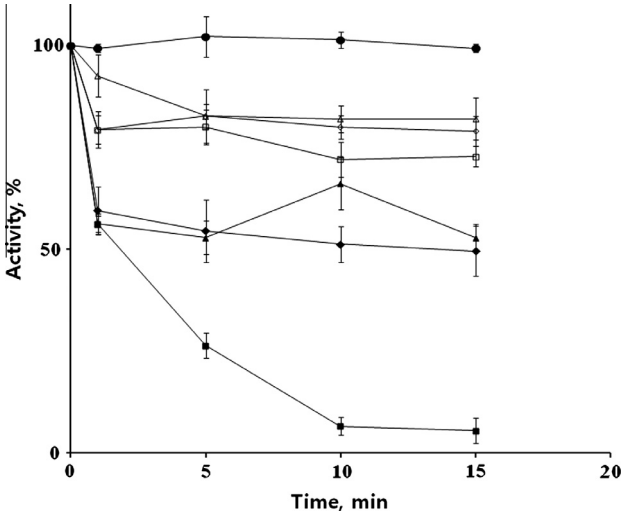


Fig. 4. Time-dependent inactivation of soybean LOX-1 activity during preincubation with each endocannabinoid (25 °C). Each compound (30 μM) was pre-incubated with 30 μl soybean LOX-1 (0.3 mg/ml; 0.8 U/mL) in 50 mM borax buffer pH 9 (final volume 300 μL). The aliquot (3 μl) was taken into the assay mixture containing 30 μM linoleic acid at indicated time pre-incubation. The remaining soybean LOX-1 activity was determined by measuring A_{234}. 100% activity was determined to be an activity of control in which soybean LOX-1 was pre-incubated in buffer for the same period of time that soybean LOX-1 was pre-incubated with each compound. Data were shown as mean ± SD of three parallel experiments. (●), control; (Δ), NA-Ser; (◇), NA-Gly; (□), NA-Tau; (▲), NODA; (♦), NPDA; (■), NADA.

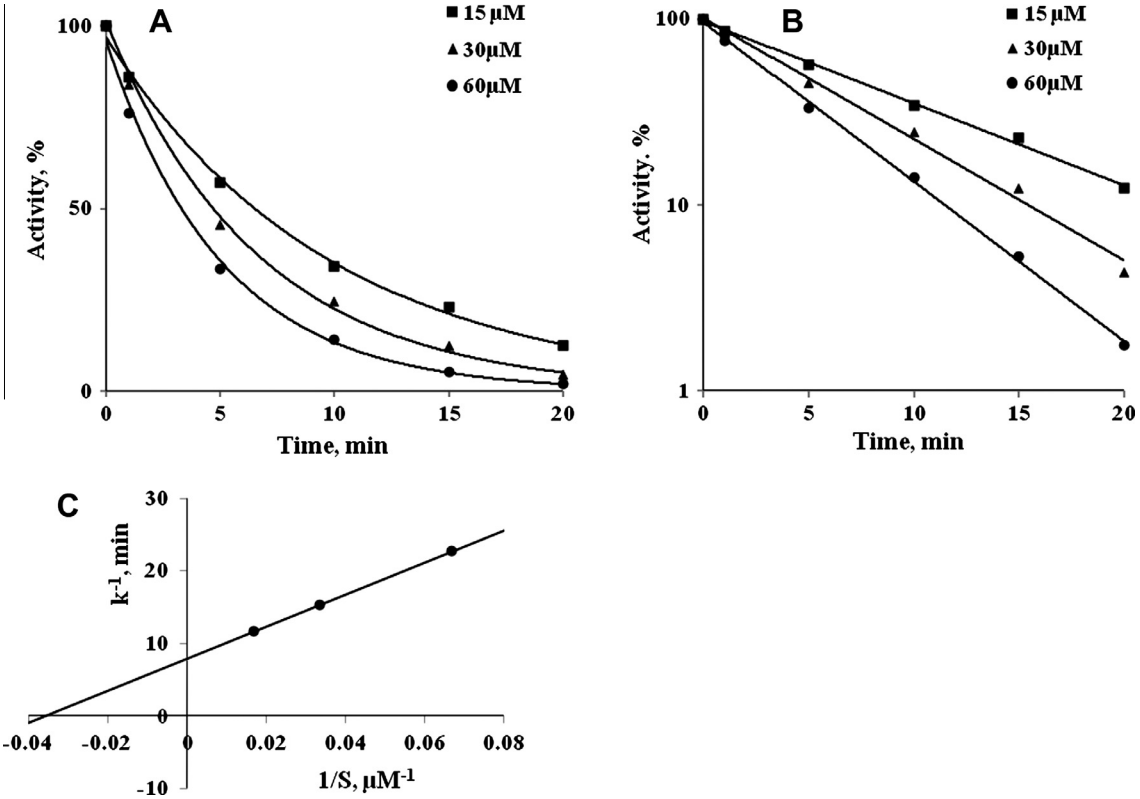


Fig. 5. Inactivation effect of N-arachidonoyl dopamine (NADA) on soybean LOX-1 activity (A) Time-dependent inactivation of soybean-LOX-1 during preincubation (25 °C) with NADA (■, 15 μM; ▲, 30 μM; ●, 60 μM). (B) Pseudo-first order time course of inactivation of soybean LOX-1 by NADA. (C) Reciprocal plot of the observed pseudo-first order rate constant (k) as a function of the concentration (I) of NADA. Incubation was carried out as described in Section 2, and the aliquot (3 μl) was taken into assay mixture containing 30 μM linoleic acid. The remaining soybean LOX-1 activity was determined by measuring A_{234}.

respectively. Taken together, these results suggest that the compounds containing both of adjacent cis, cis-1,4-tetradiene and phenolic group may correspond to an effective inactivator of soybean LOX-1. To further support this assumption, dopamine or serotonin was evaluated for the inactivation of soybean LOX-1 in the presence or absent of arachidonic acid. As exhibited in Table. 2, dopamine or serotonin at 30 μM did not inactivate soybean LOX-1 in the presence or absence of arachidonic acid. Therefore, it is implied that the common structural characteristics of NA-DA or NA-5HT may be a crucial requirement for the inactivation of lipoxygenase. Further, FOX assay was used to determine the amount of hydroperoxy product generated from the incubation of soybean LOX-1 with NA-DA or NA-5HT. Briefly, soybean LOX-1 (0.8 units/mL) was incubated with the respective concentration of NA-DA (15 μM, 30 μM or 60 μM) or NA-5HT (2.5 μM, 5 μM or 10 μM) in borax buffer. The oxygenation reaction was terminated by the addition of FOX reagent mixture at each time period. As exhibited in Fig. 7, the formation of the hydroperoxy product was gradually enhanced up to 1 min, reaching the maximum level.

Since NA-DA and NA-5HT contain a phenolic group, it was supposed that the inactivation by NA-DA and NA-5HT may be due to the presence of phenolic group as had been observed with catechols [27]. The previous study showed that catechols reduced the catalytically active ferric enzyme (E_{ox}) to the catalytically inactive ferrous form (E_{red}). Regeneration of the radical is dependent upon the presence of antioxidant such as ascorbic acid that readily reduces the radical (Scheme 1). To examine the above suggestion, the inactivating capacity of NA-DA or NA-5HT was assessed in the presence of ascorbic acid (100 μM) as antioxidant, 13(S)-hydroperoxy-9,11-octadecadienoic acid (13-HpODE) or 15(S)-hydroperoxy-5,8,11,13-eicosatetraenoic acid (15-HpETE) (5 μM) as oxidants. As exhibited in Fig. 8, the presence of ascorbic acid (100 μM) enhanced the inactivation decrease from 45%, 65% and 81% to 63%, 83% and 92% at time interval periods of 5 min, 10 min and 15 min, respectively. In contrast, 13-HpODE (5 μM) reduced the inactivation degree from 65% and 81% to 57% and 60% at 10 min and 15 min, respectively. This may support our previous study that the hydroperoxy group of 13-HpODE lysophosphatidylcholin may be important for the stimulation of soybean LOX-1 activity [26]. However, 15-HpETE did not express such an effect (data not shown), presumably due to its inhibitory effect on soybean LOX-1 as reported previously [25]. Likewise, a similar result was also found with NA-5HT (data not shown). Taken together, it is suggested that the potency of these compounds in inactivating soybean LOX-1 was improved in the presence of antioxidant and reduced in the presence of oxidant (Scheme 1).

4. Discussion

Lipoxygenases (LOXs) had been known to be regulated by substrates or their hydroperoxy products [40,41]. LOXs had been reported to be inactivated during incubation with polyunsaturated fatty as substrates [22]. Furthermore, LOXs was observed to be inactivated by some hydroperoxy lipoxygenation products [23–25]. Separately, phenolic compounds had been reported to reduce soybean lipoxygenase-1 activity [27–29]. Our present study demonstrates that soybean LOX-1 can be inhibited or inactivated by some endocannabinoids containing phenolic group.

N-arachidonyl-, oleoyl-, or palmitoyl dopamine belonging to endocanabinoids was isolated from bovine brain [42,43]. Especially, N-arachidonoyl dopamine (NA-DA) was found to show various biological activities through CB1 and transient receptor

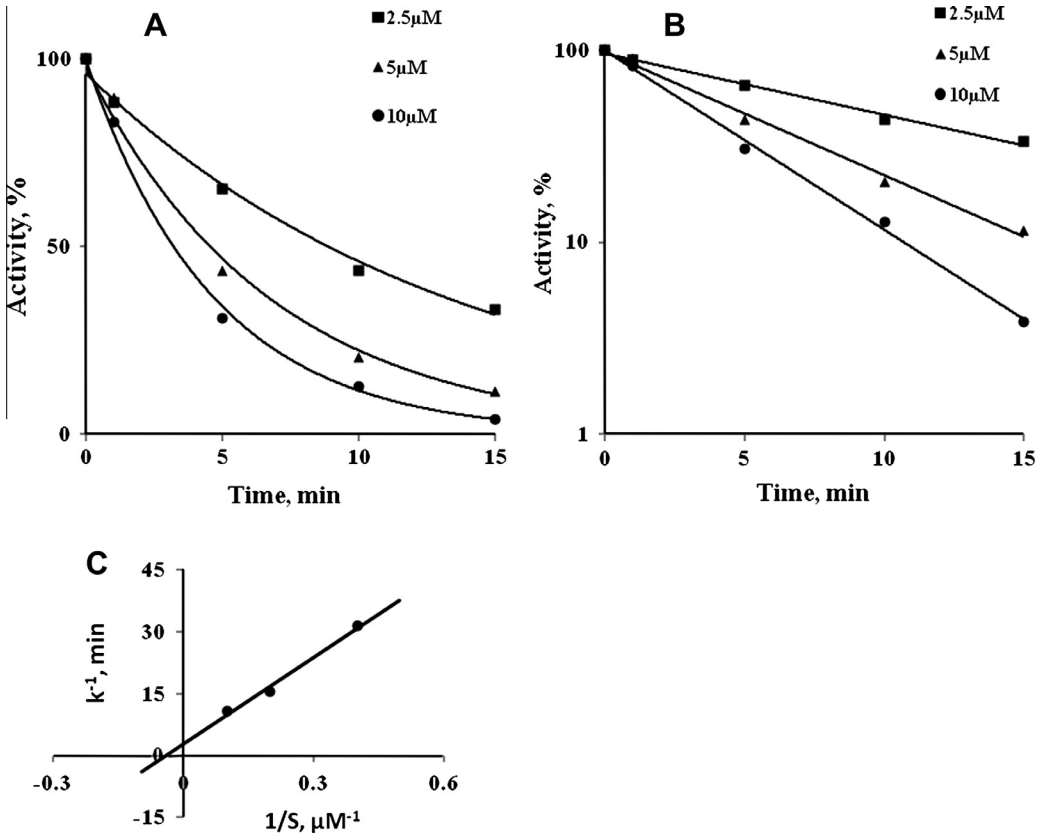


Fig. 6. Inactivation effect of N-arachidonoyl serotonin on soybean LOX-1 activity (A) Time-dependent inactivation of soybean LOX-1 during preincubation (25 °C) with N-arachidonoyl serotonin (■, 2.5 μM; ▲, 5 μM; ●, 10 μM). (B) Pseudo-first order time course of inactivation of soybean LOX-1 by N-arachidonoyl serotonin. (C) Reciprocal plot of the observed pseudo-first order rate constant (k) as a function of the concentration (I) of N-arachidonoyl serotonin. Incubation was carried out as described in Section 2, and the aliquot (3 μl) was taken into assay mixture containing 30 μM linoleic acid. The remaining soybean LOX-1 activity was determined by measuring A_{234}.

potential vanilloid 1 (TRPV1) receptor, and additionally reduce spontaneous motor activity via mechanisms partially independent of transient receptor potential vanilloid 1 or CB1 receptors [44]. Additionally, N-acyl dopamines such as N-oleoyl dopamine (NO-DA), N-linoleoyl dopamine or N-linolenoyl dopamine was reported to be potent inhibitors of 5-lipoxygenase [34]. Further, NA-DA was reported to be a modest inhibitor of fatty acid amide hydrolase (FAAH) [45]. Our study indicates that the NA-DA, but not NO-DA, can be utilized as substrate for soybean LOX-1 (km, 18 μM). Of note, our present study indicates that NA-DA and NO-DA are potent inhibitors of soybean LOX-1, the K_i values of NA-DA and NO-DA in linoleic acid oxygenation were 3.7 μM and 6.2 μM, respectively. However, other endocanabinoids such as N-arachidonoyl glycine, N-arachidonoyl taurine, N-arachidonoyl serine, N-palmitoyl dopamine or N-arachidonoyl serotonin fails to inhibit soybean LOX-1 up to 30 μM. Analysis of the structure–activity relationship indicates that phenolic group and unsaturated fatty acyl group of endocanabinoids are crucial for the inhibitory effect on soybean LOX-1 activity. Previously, oleoyl sulfate has been reported to be a potent inhibitor of soybean LOX-1 that tightly binds to an allosteric site of soybean LOX-1 to cause the non-competitive inhibition [46]. One of mechanism responsible for the inhibition of soybean LOX-1 by NADA or NODA might be related to its binding to an allosteric site of soybean LOX-1. Separately, it had been reported that 2-(3,4-dihidroxyphenyl)ethanol and caffeic acid with two adjacent hydroxyl groups on an aromatic ring inhibited 12-LOX and 5-LOX activities potently, while 2-(4-hydroxyphenyl) ethanol and p-coumaric acid with one hydroxyl group on an aromatic ring had little inhibitory effect on both lipoxygenase activities [47]. These results may agree with previous findings concerning the inhibition of 12-LOX activity by coumarins and 5-LOX activity by cafeic acid methyl derivatives [48]. All these suggest that two adjacent hydroxyl groups on an aromatic ring may be re crucial for potent inhibition of LOX activity.

Meanwhile, soybean LOX-1 had been reported to be inactivated during the incubation with unsaturated fatty acids or their hydroperoxy derivative [22,25,37]. Likewise, in the present study, soybean LOX-1 was also inactivated during the incubation of soybean LOX-1 with phenolic endocanabinoid. Among endocannabinoids examined, NADA expressed the greatest inactivation. Moreover, the time-dependent inactivation of soybean LOX-1 activity was found to be greater with NADA (k_3, 0.125 min^{-1}), compared to NA-5HT (k_3, 0.35 min^{-1}). As far as the mechanistic aspect of such an inactivation is concerned, NA-DA could readily reduce ferric iron of the LOX active form to cause enzymatic inactivation as observed with nordihydroguaiaretic (NDGA) [27]. This might be supported by our finding that ascorbic acid enhanced inactivation on soybean LOX-1 by NA-DA or NA-5HT, while 13(S)-hydroperoxy-9,11-octadecadienoic acid reduced the inactivation degree (Scheme 1). Consistent with this, NADA with higher reducing power was more efficient than NA-5HT with lower reducing power in the inactivation of soybean LOX-1. Thus, the mechanism of inactivation might include redox chemistry between the OH-group of phenolic inactivator and the ferric ion in lipoxygenase. Moreover, N-arachidonoyl endocannabinoid such as NADA or NA-5HT caused an almost comple inactivation, whereas NODA or NPDA showed a partial inactivation. This may suggest that the role of NA-DA or NA-5HT as substrate may be required for the greater inactivation of soybean LOX-1. This suggestion may be consistent with the mechanism proposed for the inactivation of soybean LOX-1 by

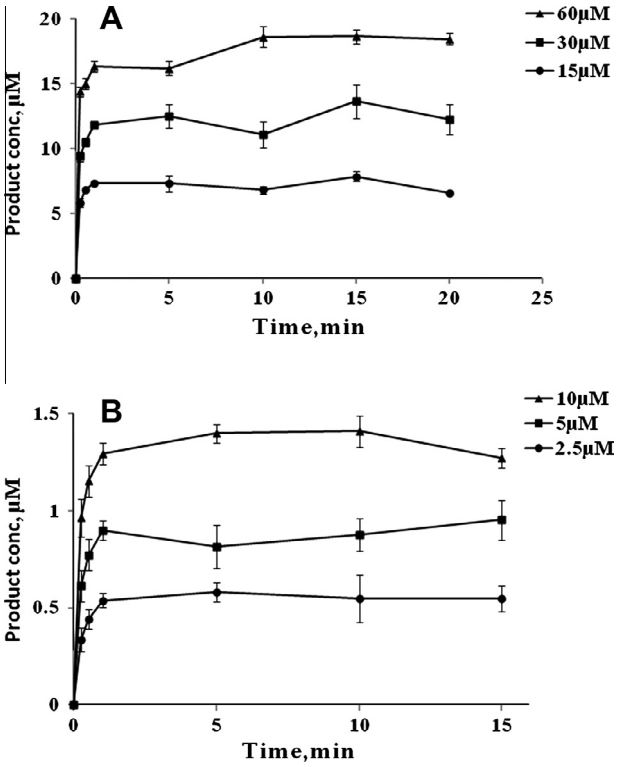


Fig. 7. Effect of substrate concentration on oxygenation of N-arachidonyl dopamine (A) or N-arachidonoyl serotonin (B) by soybean LOX-1. Soybean LOX-1 (0.8 units/mL) was incubated with N-archidonoyl dopamine of various concentrations (●, 15 μM; ■, 30 μM; ▲, 60 μM) or N-arachidonoyl serotonin of various concentrations (●, 2.5 μM; ■, 5 μM; ▲, 10 μM) in 50 mM borax (pH 9.0). The oxygenation reaction was terminated at designed time by the addition of FOX reagent mixture. After 10 min of reaction, A_{550} was measured as described in Section 2. Data expressed as means ± SD of results in three independent experiments.

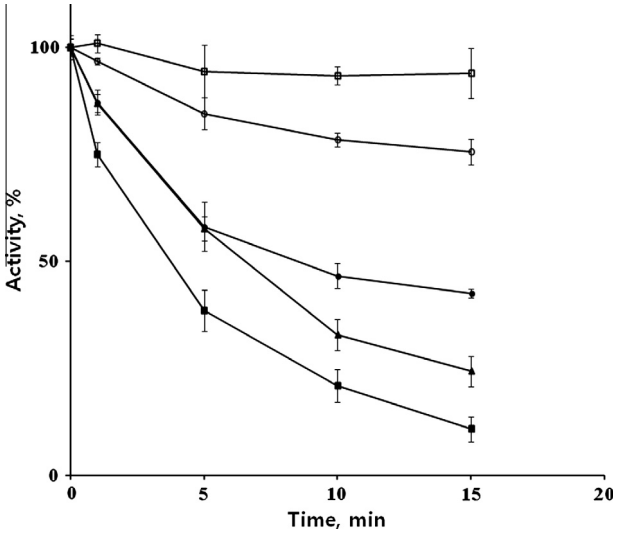
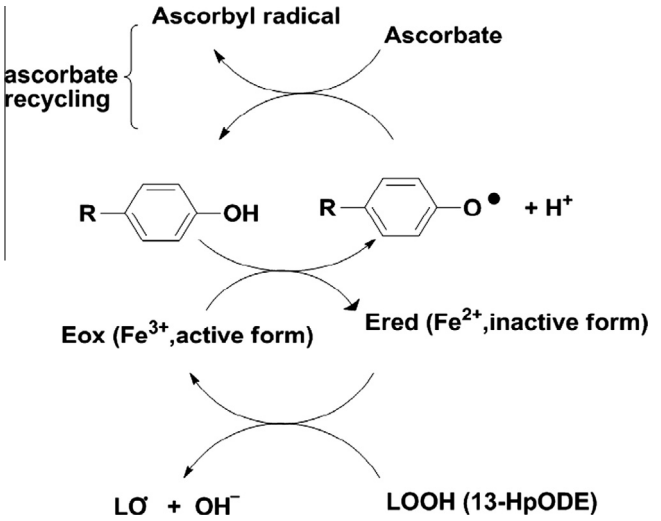


Fig. 8. Effect of 13(S)-hydroperoxy-9,11-octadecadienoic acid (13-HpODE) or ascorbic acid on inactivation of soybean LOX-1 by N-arachidonoyl dopamine (NADA). Soybean LOX-1 (0.8 units/mL) was incubated with 15 μM NADA in absence (▲) or presence of 5 μM 13-HpODE (●) or 100 μM ascorbic acid (■). Separately, the enzyme was pre-incubated with 5 μM 13-HpODE (○) or 100 μM ascorbic acid (□) in 50 mM borax buffer pH 9.0 (final volume 300 μL). The aliquot (3 μl) was taken to determine the remaining activity after indicated time. Data express as means ± SD of results in three independent experiments.



Scheme 1.

hydroperoxy-intermediate [25]. Taken together, both polyunsaturated acyl group and phenolic moiety are crucial for the efficient inactivation of soybean LOX-1.

The aforementioned considerations are based on the observation with soybean LOX-1. Nonetheless, soybean LOX-1 has been used as a suitable model for mammalian 15-LOX activity. Given the sites for oxygenation of polyunsaturated fatty acids, soybean LOX-1 had been reported to be similar to mammalian 15-LOX [1]. Thus, inhibitors of soybean LOX-1 may be expected to be inhibitors of human 15-LOX. A recent study indicated that several enzymatic inhibitors to inactivate both soybean LOX-1 and human 15-LOX may act even more efficiently on the latter than on the former enzyme [49]. Our results suggest that the inhibitory or inactivating effects of N-acyl dopamine or NA-5HT on soybean LOX-1 might be utilized for design of human 15-LOX inhibitor for the prevention and treatment of chronic diseases related to human 15-LOX. Also this study may contribute to further elucidation of in vivo regulation of LOX activity by N-acylated/phenolic endocannabinoids, which need be clarified in future studies using plant or animal cell.

5. Conclusions

Present study demonstrates a structural important of endocannabinoids for the inhibition or inactivation of soybean LOX-1. For the inhibition of soybean LOX-1, the presence of catechol group is required and unsaturation degree of N-acyl group determines the inhibitory potency; N-arachidonoyl dopamine > N-oleoyl dopamine ≫ N-palmitoyl dopamine. Meanwhile, polyunsaturated acyl moiety and phenolic group are requied for the efficient inactivation of soybean LOX-1. The mechanism of inactivation might include redox chemistry between phenolic inactivator and ferric ion in LOX.

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