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Catalytic characterization of heterodimeric linoleate 13*S*-lipoxygenase from black soybean (*Glycine max* (L.) Merr.)

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ABSTRACT

A novel lipoxygenase (BLOX) was purified from black soybean (*Glycine max* (L.) Merr.), and its catalytic properties were characterized. The molecular weight of BLOX was 101 kDa and its unique heterodimeric structure with two different subunits of molecular weight 46 kDa and 55 kDa was elucidated. The optimum pH and temperature of BLOX were pH 9.5 and 40 °C, respectively. BLOX was highly stable at the pH range of 6.0–10.0 and below 40 °C, and was stimulated by adding ferrous ion (Fe^{2+}). In terms of substrate specificity, BLOX showed a substrate preference to linoleic acid that is the main substance to produce hydroperoxides in soybean. When it reacted with linoleic acid, the major product was 13(*S*)-hydroperoxy-9,11-octadecadienoic acid; therefore, it could be classified into the linoleate 13*S*-LOX family (EC 1.13.11.12). Finally, the kinetic parameters (V_{max}, K_m, and k_{cat}) of BLOX were 0.124 mM min^{-1}, 0.636 mM, and 12.28 s^{-1}, respectively, and consequently, the catalytic efficiency (k_{cat}/K_m) was calculated as 1.93×10^4 $M^{-1}{\cdot}s^{-1}$. These catalytic characteristics of BLOX could contribute to understanding the enzymatic rancidification of black soybean, and to further biotechnical approaches to control and mitigate the deterioration.

1. Introduction

Lipoxygenase (LOX, EC 1.13.11.-) is a family of iron-containing enzymes in many plants, animals, fungi, and microalgae [1]. It catalyzes the dioxygenation of polyunsaturated fatty acids (PUFA) containing *cis*,*cis*-1,4-pentadiene units ($-CH=CH-CH_2-CH=CH-$) to produce conjugated unsaturated hydroperoxydiene fatty acid [2]. The roles of LOX in plants and animals are not fully understood; however, it is generally related to the deterioration of plant foods with high lipid contents (*e.g.*, soybean, peanut, oilseeds, and so forth) by the enzymatic rancidification of internal PUFA, that is to say, the production of hydroperoxides. Decomposition products of these hydroperoxides are potentially toxic to human [3]. For example, aldehydes (*e.g.*, hexanal and nonenal) generated from scission of hydroperoxydiene radical can cause an off-flavor in foods. Furthermore, LOX catalyzes the co-oxidation of carotenoids including β-carotene, resulting in the loss of essential nutrients in foods [4]. Furthermore, LOX is also known to play an important role in plant development and response to biotic and abiotic stresses [5].

Black soybean is a black variety of soybean (*Glycine max* (L.) Merr.) that has been widely used as a good source of food proteins and as a medicinal food in Asian countries [6]. Black soybean is very similar to yellow soybean nutritionally; however, its antioxidative activity is generally higher than that of yellow soybean due to genetic variation and different functional polyphenols [7]. The polyphenols in black soybean (particularly its seed coat) have excellent antioxidative activities that inhibit the oxidation of low-density lipoprotein (LDL) in the human body [8]. Nevertheless, the utilization of black soybean as a food ingredient and food itself is often limited because of its beany or grassy flavor, which is caused by enzymatic rancidification of lipids [3,9]. To regulate the deterioration of black soybean and increase the sensory quality, the catalytic characteristics of LOX in black soybean should be sufficiently understood. Numerous studies have investigated LOXs in yellow soybean, uncovering their structures, catalytic

Abbreviations: LOX, lipoxygenase; BLOX, lipoxygenase from black soybean; PUFA, polyunsaturated fatty acid; 13*S*-HpODE, 13(*S*)-hydroperoxy-9,11-octadecadienoic acid

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properties, and reaction mechanism [10–12]. Moreover, several reports have ascertained that the elimination of LOX isozymes in stored soybeans and soy products results in a significant reduction of off-flavor compounds [3]. Unfortunately, however, there is still no reported information about LOX in black soybean and its catalytic characteristics. Soybean cultivars have a lot of genetic variation or protein expression pattern each other; hence, internal LOX of black soybean needs to be uncovered independently to mitigate its enzymatic rancidification biotechnologically.

In the present study, a novel and unique LOX was purified from black soybean and its catalytic properties were investigated for the first time. The sole LOX protein (BLOX) was successfully purified from natural black soybean via sequential chromatographic purification and its structural information was identified using size-exclusion chromatography and electroporation systems, together with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS). The catalytic properties of BLOX, compared with commercial soybean LOX, were determined in terms of optimum conditions for reaction, physicochemical stability, substrate specificity, and enzyme kinetics. The substrate specificity of BLOX was revealed using high-performance liquid chromatography (HPLC) system and ^{13}C-nuclear magnetic resonance (^{13}C-NMR) spectroscopy simultaneously.

2. Materials and methods

2.1. Materials

Fresh black soybean (*G. max* (L.) Merr.) samples were harvested from Yeoncheon-gun (Gyeonggi-do, Republic of Korea) in 2010 and immediately stored in a refrigerator at 4 °C before use. Commercial soybean LOX (≥50,000 units/mg), oleic acid (≥99%), linoleic acid (≥99%), α-linolenic acid (≥99%), γ-linolenic acid (≥99%), docosahexaenoic acid (DHA, ≥98%), and eicosapentaenoic acid (EPA, ≥99%) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). 13(*S*)-Hydroperoxy-9,11-octadecadienoic acid (13*S*-HpODE) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Trifluoroacetic acid (TFA, ≥99.0%) and sinapinic acid (≥99.0%), used as matrix substances for MALDI-TOF/MS, and deuterated chloroform ($CDCl_3$), used as a solvent for the ^{13}C-NMR spectroscopy were purchased from Sigma-Aldrich Co. Coomassie Brilliant Blue R-250 and 30%(w/v) acrylamide/bis solution (37.5:1) were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). All other chemicals were of analytical reagent grade and were used without further purification.

2.2. Purification of lipoxygenase from black soybean

The purification process was carried out as previously described [13] with slight modifications. Natural black soybean (20 g) was ground using a Waring commercial blender and homogenized in 200 mL of 50 mM Tris-HCl buffer (pH 7.0) overnight. The homogenate was centrifuged at 20,000 *g* for 30 min and the supernatant was filtered through a 0.2 μm membrane filter (Advantec MFS Co., Tokyo, Japan). Crude protein extract was fractionated from the filtrate via ammonium sulfate precipitation. The crude protein extract was resuspended with 50 mM Tris-HCl buffer (pH 7.0) and then dialyzed overnight against Tris-HCl buffer (pH 7.0) using a dialysis tube (MWCO 12–14 kDa, Thermo Fisher Scientific, Waltham, MA, USA). The dialyzed fraction was centrifuged at 5000 *g* for 15 min and the supernatant was subjected to further purification. The protein concentration was determined using Bradford method [14] with bovine serum albumin as the standard protein. The chromatographic purification process was conducted using a fast protein liquid chromatography (FPLC) system (GE Healthcare, Uppsala, Sweden) equipped with an ultraviolet (UV) detector (GE Healthcare) to measure the absorbance at 280 nm. The fractions from each following step were analyzed for LOX activity and protein concentration. First, the crude black soybean protein extract was applied to a HiTrap Q HP anion exchange chromatography column (0.7 × 2.5 cm, GE Healthcare) equilibrated with 25 mM Tris-HCl buffer (pH 9.0) and proteins were eluted with a step gradient of sodium chloride (0–1 M NaCl in column buffer) at a flow rate of 1.0 mL/min. Second, the active fractions were concentrated by ultrafiltration (MWCO 10 kDa, MilliporeSigma, Billerica, MA, USA) and subsequently applied to a HiTrap DEAE Sepharose FF anion exchange chromatography column (0.7 × 2.5, GE Healthcare) equilibrated with 25 mM Tris-HCl buffer (pH 8.5). The elution process was conducted as described above. Finally, the active fractions were concentrated as described above and loaded onto a HiPrep 16/60 Sephacryl S-100 HR size exclusion chromatography column (1.6 × 60 cm, GE Healthcare) equilibrated with 25 mM Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl. Proteins were eluted at a flow rate of 0.4 mL/min. The purification process using a HiPrep 16/60 Sephacryl S-100 HR was carried out twice under the same conditions. All purification steps were carried out at 4 °C.

2.3. Lipoxygenase assay

The catalytic activity of LOX was determined using a spectrophotometric method as described previously [15] with slight modifications. The reaction medium (5 mL) was 50 mM Tris-HCl buffer (pH 9.0) containing linoleic acid (100 μM) as a substrate and Tween 20 (100 μM) as an emulsifier. The reaction was initiated by adding LOX solution (0.017 mg/mL) at 25 °C and the absorbance at 234 nm ($\varepsilon = 2.5 \times 10^4\ M^{-1}\,cm^{-1}$) was measured with a UV–vis spectrophotometer (UV-2450, Shimadzu Co., Kyoto, Japan) over reaction time. One unit of LOX activity was defined as the amount of enzyme producing 1 μmol of 13*S*-HpODE per minute under the assay conditions; the calibration curve for 13*S*-HpODE was shown in Fig. S1. The protein concentration was determined as described abov and all assays were run in duplicate. Non-linear regression curve fitting of LOX reaction was performed using SigmaPlot software (ver. 12.5, Systat Software Co., San Jose, CA, USA) and installed enzyme kinetics module to evaluate the exploratory kinetic parameters based on the Michaelis–Menten model. The Michaelis–Menten assumption of the results was subsequently validated using a Hanes–Woolf double-reciprocal plot [16,17]:

$$\frac{[S]}{v} = \frac{1}{V_{max}}[S] + \frac{K_m}{V_{max}}$$

where [*S*] is the concentration of substrate, *v* is the initial velocity of the reaction, VV_{max} is the maximum initial velocity when substrate approaches infinite concentration, and KK_m is the dissociation constant of the enzyme-substrate complex (*i.e.*, the Michaelis constant).

2.4. SDS-PAGE and blue native PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 10%(w/v) polyacrylamide gels based on the buffer system described by Laemmli [18]. Protein samples were diluted in Laemmli buffer and boiled for 5 min before loading them onto a polyacrylamide gel. PRO-STAIN™ protein marker (iNtRON Biotechnology, Inc., Seongnam, Gyeonggi-do, Republic of Korea) was used as a broad range (9–198 kDa) protein standard to estimate the molecular weight of the proteins. The protein samples were separated on a Hoefer SE 250 mini-Gel system (GE Healthcare) using a 0.5 mm thick 10% polyacrylamide gel at a constant current of 15 mA and room temperature. Then, proteins were stained with Coomassie Brilliant Blue R-250 stain [19]. Blue native-PAGE (BN-PAGE) was performed as described previously [20]. The proteins were separated and visualized in the same manner as SDS-PAGE; however, the electric current was 10 mA.

2.5. MALDI-TOF/MS

Proteins were analyzed by MALDI-TOF/MS using an Autoflex II mass spectrometer (Bruker Co., Bremen, Germany) set to the reflectron mode with positive polarity. The matrix employed a solution containing 10 mg of sinapinic acid and 0.1%(v/v) TFA in 1.0 mL acetonitrile/deionized water (1:1, v/v), and desorption/ionization was accomplished using a nitrogen laser (337 nm). Product ions were analyzed using an orthogonal TOF analyzer fitted with a reflector, a microchannel plate detector, and a time-to-digital converter. All processes were conducted according to the manufacturer's instructions. The data were processed using *mMass* software (ver. 5.5.0, www.mmass.org/) [21].

2.6. Product identification by HPLC-UV

The oxygenated products were identified as described previously [22] with slight modifications. The 30 min reaction of LOX with linoleic acid as a substrate was terminated by adding 1 M sodium borohydride ($NaBH_4$), and the products were extracted using ethyl acetate with the same volume as the substrate solution. The supernatant was lyophilized in a Savant SC110 Speed Vac® rotary vacuum concentrator (Savant Instruments, Inc., Farmingdale, NY, USA). Then, the concentrate was subjected to normal-phase HPLC equipped with a μPorasil™ column (10 μm, 3.9 mm × 300 mm, Waters Co., Milford, MA, USA), a low pressure gradient pump (PU-2089 Plus, JASCO Co., Tokyo, Japan), and a UV detector (UV-2075, JASCO Co.). The products of LOX were eluted isocratically with *n*-hexane/2-propanol/acetic acid (98.1:1.9:0.1, v/v/v) solvent at a flow rate of 1.0 mL/min and detected at 234 nm by UV absorption. The injection volume was 20.0 μL, and the column oven was set to 30 °C. The production of 13*S*-HpODE was confirmed using 13*S*-HpODE standard.

2.7. Product identification by ^{13}C-NMR spectroscopy

^{13}C-NMR spectroscopy was performed to validate the production of 13*S*-HpODE from the LOX-catalyzed dioxygenation of linoleic acid. A liquid-state ^{13}C-NMR spectroscopy was conducted using an AVANCE™ 600 MHz NMR spectrometer (Bruker Co.). The LOX reaction products were dissolved in $CDCl_3$ and tetramethylsilane (TMS) was used as an internal standard. The samples were spun at a rate of 20 Hz at 25 °C with a spectral width of 42 kHz. The acquisition time was 0.773 s and the line broadening was 3 Hz. The data were processed using TopSpin™ software (ver. 4.0.6, Bruker Co.) and ^{13}C-NMR prediction was conducted using ChemDraw Professional software (ver. 16.0, PerkinElmer Co., Waltham, MA, USA).

3. Results and discussion

3.1. Identification of purified lipoxygenase from black soybean

The comprehensive results of the purification process of BLOX from natural black soybean are summarized in Table 1. Crude proteins with LOX activity were extracted from 50 mM Tris-HCl (pH 7.0) homogenates of black soybean by ammonium sulfate precipitation at 80% saturation. From the crude extract, BLOX was successfully purified 85-fold with 2.17% yield and 3.00 units/mg of specific activity using FPLC sequential chromatography. Fig. 1 shows chromatographic elution profile in detail. Proteins with LOX activity were initially fractionated using anion exchange chromatography (Fig. 1(a) and (b)), indicating that the putative BLOX protein carried a net negative charge at the pH range of 8.5–9.0. These fractions were applied to size exclusion chromatography and exhibited a sole peak corresponding to purified BLOX after two repetitions (Fig. 1(c) and (d)). Moreover, the results of size exclusion chromatography ascertained the exact molecular weight of BLOX compared with five standard proteins: Carbonic anhydrase (29 kDa), ovalbumin (44 kDa), commercial soybean LOX (96 kDa), alcohol dehydrogenase (150 kDa), and β-amylase (200 kDa). The ratio of elution volume to void volume (V_e/V_o) of BLOX was 1.1876, and the molecular weight was determined to approximately 101 kDa using a calibration curve of standard proteins (Fig. 2a). The molecular weight of BLOX was similar to those of LOXs from yellow soybean and other plants (94–104 kDa) [1].

To provide supportive information for the structure of BLOX, two different electroporation systems were employed. Surprisingly, BLOX migrated as two bands of molecular weight 46 kDa and 55 kDa on an SDS-PAGE gel (Fig. 2b) whereas a single band was observed without any other minor band on a BN-PAGE gel (Fig. 2c), suggesting that BLOX may have a unique heterodimeric structure. The sum of the molecular weights of the two bands on SDS-PAGE was approximately 101 kDa, which is equal to the results of size exclusion chromatography. The purity of the enzyme solution was high enough to be considered as a homogeneous state according to the results of BN-PAGE. Additionally, MALDI-TOF/MS, which is used to analyze biological samples such as peptides and proteins [23,24], was applied to validate the molecular weight of BLOX from the size exclusion chromatography and PAGE analyses. Several molecular ion peaks at *m/z* 7365.3, 7736.0, 8188.5, 9109.8, 9325.7, 12662.2, 19842.9, and 28753.8 expected as random peptides from BLOX, were observed in the MS spectrum (Fig. S2). The sum of these values (~103 kDa) was close to the molecular weight of BLOX (~101 kDa); however, no significant molecular ion peaks at *m/z* 46,000 and 55,000 corresponding to each subunit of BLOX were detected, probably due to the incidental degradation of polypeptides during the ionization process. In other words, MALDI-TOF/MS enabled to crosscheck the total molecular weight of BLOX only.

Although further in-depth studies such as genome sequencing, peptide mapping, gene cloning, and advanced structural analysis should be conducted for uncovering the actual structure of entire BLOX, the results provided corroborative evidence that BLOX could be identified as a heterodimeric enzyme with an approximate molecular weight of 101 kDa composed of 46 kDa and 55 kDa subunits. These findings are surprising because LOXs in yellow soybean, which is conspecific with black soybean (*G. max* (L.) Merr.), have a monomeric structure with two domains [25] and there have been no reports covering a heterodimeric LOX. Several reports suggested that LOX monomers enable to oligomerize with itself (particularly dimerization) to form a stable structure in solution [26–28]; however, this could not account for the peculiar structure of BLOX. Therefore, this appears to be another

Table 1
Purification of BLOX from black soybean (*G. max* (L.) Merr.).

Step	Total volume (mL)	Activity (units/mL)	Protein (mg/mL)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)	Purification fold
50 mM Tris-HCl homogenate	180.00	0.03	0.87	5.53	156.60	0.04	100.00	1
80% $(NH_4)_2SO_4$ precipitation	26.30	0.21	0.79	5.40	20.78	0.26	97.65	7
HiTrap Q HP	20.00	0.25	0.87	5.09	17.40	0.29	92.04	8
HiTrap DEAE Sepharose FF	15.00	0.03	0.09	0.46	1.35	0.34	8.32	10
HiPrep Sephacryl S-100 HR (1st)	0.50	0.26	0.10	0.13	0.05	2.60	2.35	74
HiPrep Sephacryl S-100 HR (2nd)	0.30	0.40	0.13	0.12	0.04	3.00	2.17	85

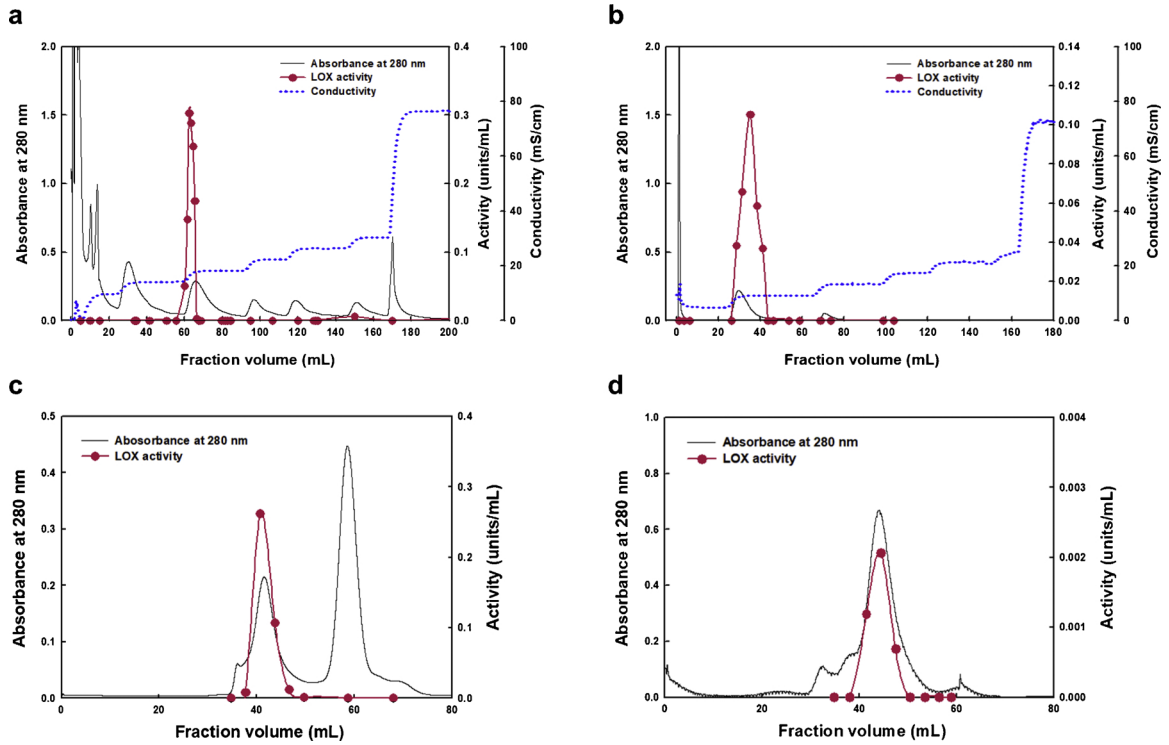

Fig. 1. Sequential chromatography for purification of BLOX from black soybean (*G. max* (L.) Merr.) using an FPLC system. (a) HiTrap Q HP anion exchange chromatography. (b) HiTrap DEAE FF anion exchange chromatography. (c) First HiPrep Sephacryl S-100 HR size exclusion chromatography. (d) Second HiPrep Sephacryl S-100 HR size exclusion chromatography. Proteins with LOX activity were detected by measuring the absorbance at 280 nm and then analyzed for the catalytic activity.

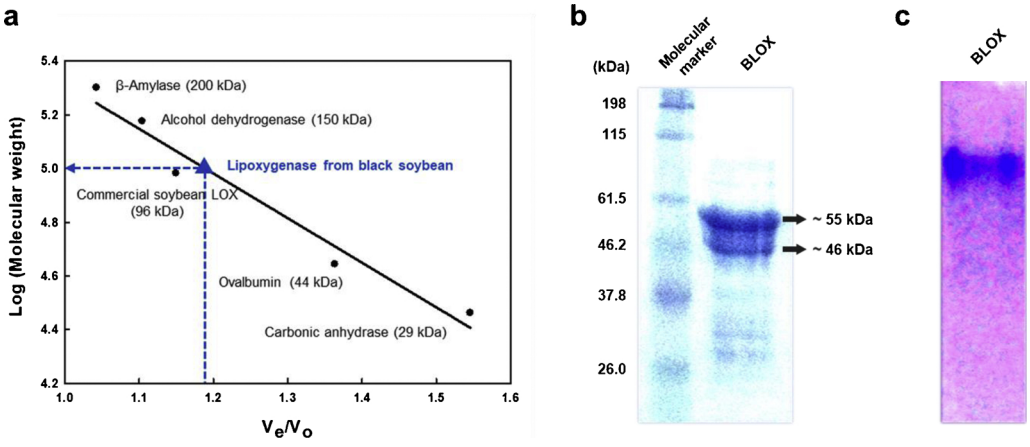

Fig. 2. Structural information of BLOX from black soybean (*G. max* (L.) Merr.). (a) Analytical size exclusion chromatography of BLOX and calibration curve to estimate the molecular weight (kDa). V_e and V_o represent the elution and void volumes of each protein, respectively. (b) SDS-PAGE and (c) BN-PAGE analyses of BLOX. Gels were stained with Coomassie Brilliant Blue R-250 stain. All lanes except the molecular marker lane were loaded with the same BLOX sample.

different case of LOX that is reported for the first time in the present study.

3.2. Optimum reaction conditions for purified lipoxygenase from black soybean

The catalytic activity of enzymes is highly influenced by the physicochemical environment of a reaction system such as pH, temperature, ionic strength, and chemicals [29], which together determine the optimum conditions for a reaction. Considering this aspect, the effects of various physicochemical conditions on BLOX were investigated to determine the optimum conditions for the reaction using linoleic acid as a substrate. First, BLOX worked within a small pH range in the reaction system (Fig. 3a). The catalytic activity of BLOX peaked at pH 9.5 and sharply declined as pH value increased or decreased from the peak value (*i.e.*, a typical bell-shaped curve). Notably, the relative activity shrunk by more than 70% in neutral pH and reached nearly 0% under mild acidic condition (pH 6.0), indicating that the active site of BLOX is more prone to an acidic environment than an alkaline environment. The previously reported LOXs from yellow soybean are in the most active state at pH 6.0–7.0 [15]; however, BLOX was more like those from other sources such as broad bean (*Vicia faba* L., pH 9.0–10.0) [30] and sesame (*Sesamum indicum* L., pH 8.0) [31]. In contrast with pH stability, BLOX showed robustness to reaction temperature at a wide range of 15–55 °C (Fig. 3b). The highest catalytic activity of BLOX was shown at 40 °C (moderately high temperature), exhibiting activity over 50% of the maximum value throughout the conditions. The relative activity at opposite ends of the temperature was 51.51% at 15 °C and 63.97% at 55 °C, respectively. These properties are considered to be derived from the putative intra- or intermolecular bonds (*e.g.*, disulfide bond, hydrogen bond, and so forth.) maintaining the structural rigidity [32] of BLOX. The optimum temperature of BLOX was similar to other

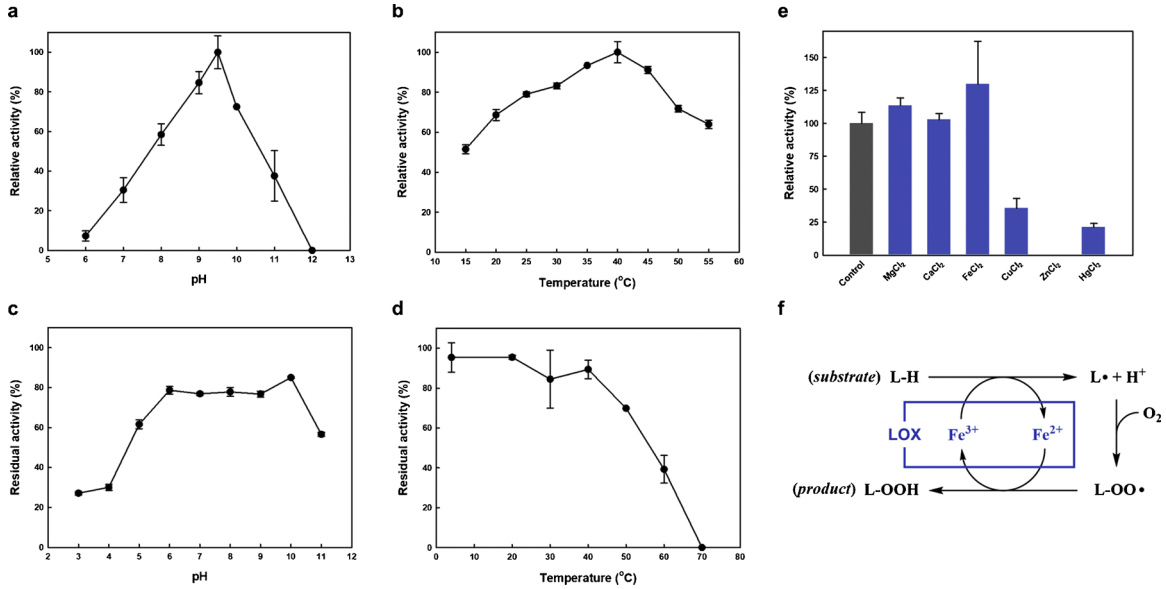

Fig. 3. Effects of physicochemical conditions on the catalytic activity and stability of BLOX from black soybean (*G. max* (L.) Merr.). (a) Effects of pH (6.0–12.0) on the catalytic activity of BLOX. (b) Effects of reaction temperatures (15–55 °C) on the catalytic activity of purified LOX. (c) pH stability of BLOX. The LOX sample was pre-incubated at 4 °C for 24 h under different pH conditions (pH 3.0–11.0). (d) Thermal stability of BLOX. The LOX sample was pre-incubated for 15 min at different temperatures (4–70 °C). (e) Effects of divalent metal ion additives (20 mM) on the catalytic activity of BLOX. (f) Schematic diagram for dioxygenation of unsaturated fatty acid by a non-heme iron-containing LOX.

LOXs (25–45 °C). Therefore, the optimum conditions for BLOX were pH 9.5 and 40 °C.

Next, to investigate the physicochemical stability of BLOX, the residual activity of BLOX was measured after pre-incubation at different pH values (pH 3.0–11.0) or temperatures (4–70 °C) under the optimum reaction conditions (pH 9.5 and 40 °C). For pH stability, BLOX was highly stable at pH 6.0–10.0, retaining over 80% of its original catalytic activity (Fig. 3c). However, the catalytic activity was depleted at higher or especially lower pH, probably due to an irreversible change in the protein structure at extreme pH levels that provoke a disruption of internal ionic bonds [33]. For thermal stability, BLOX showed no loss of catalytic activity at temperatures up to 40 °C (Fig. 3d). When the temperature increased above 40 °C, BLOX dramatically lost its catalytic activity by 56% at 60 °C and it was fully deactivated at 70 °C. The deactivation of BLOX was possibly attributable to irreversible denaturation at high temperature, simultaneously with the dissociation of its heterodimeric structure. These results positively suggest that blanching black soybean at the proper temperature could alleviate the enzymatic rancidification of internal lipids significantly.

Furthermore, LOXs generally have a conserved catalytic domain containing a single atom of non-heme iron that is intimately involved in the reaction [34], which prompted this study to investigate the influence of metal ions on BLOX. The catalytic activity of BLOX was evaluated in the presence of each divalent metal cations (Mg^{2+}, Ca^{2+}, Fe^{2+}, Cu^{2+}, Zn^{2+}, and Hg^{2+}), and consequently, only ferrous ion (Fe^{2+}) performed as an activator of BLOX (129.79% of original activity), as expected (Fig. 3e). Cupric ion (Cu^{2+}), zinc ion (Zn^{2+}), and mercuric ion (Hg^{2+}) strongly inhibited the catalytic activity of BLOX, whereas magnesium ion (Mg^{2+}) and calcium ion (Ca^{2+}) had no effect on the reaction. The proposed mechanism for LOX-catalyzed dioxygenation depends on the capability of inherent iron to participate in the redox reaction (Fig. 3f) via coordination of an octahedral organoiron complex with substrates and metal-chelating ligands (*i.e.*, three histidines and C-terminal isoleucine) within the active site [1,34]. The role of iron in LOX suggests that an abundant supply of the Fe^{2+} ions required for the redox reaction was profitable to enhance the catalytic activity of BLOX. However, heavy metal ions (Cu^{2+}, Zn^{2+}, and Hg^{2+}) with a strong affinity for N-ligands and without catalytic activity on the redox reaction [35] formed a highly stable complex by substituting an internal metal atom and consequentially deactivated BLOX. Finally, Mg^{2+} and Ca^{2+} do not participate in the redox reaction but instead stabilize LOX [36], thereby contributing to maintaining the catalytic activity of BLOX.

3.3. Catalytic properties of purified lipoxygenase from black soybean

In most cases, LOXs are classified according to their substrate specificity based on regioselectivity or stereoselectivity on PUFA substrates; for example, yellow soybean LOX belongs to the linoleate 13*S*-LOX family (EC 1.13.11.12) because it produces 13*S*-HpODE as the major product and 9*S*-HpODE as the minor product from linoleic acid as a substrate [2]. Interestingly, there are no reports on the 13*R*- or 9*R*-LOX in soybean family; hence, this study focused on the regioselectivity of BLOX. First, among six different unsaturated fatty acid substrates (oleic acid, linoleic acid, α-linolenic acid, γ-linolenic acid, DHA, and EPA), BLOX exclusively catalyzed the dioxygenation of linoleic acid (18:2, ω-6) which has only one *cis*,*cis*-1,4-pentadiene unit (Fig. 4a). The catalytic preference of BLOX decreased drastically as the degree of substrate unsaturation increased, although α-linolenic acid (18:3, ω-3) was dioxygenated by BLOX to some degree (51.53% of relative activity to linoleic acid). Oleic acid (18:1, ω-9), which is devoid of the *cis*,*cis*-1,4-pentadiene unit, was not catalyzed by BLOX because LOXs require *cis*,*cis*-1,4-pentadiene unit with at least one olefin in the Z geometry of substrate [37]. Notably, BLOX exhibited a higher substrate affinity for linoleic acid than any other substrate, consistent with the above results. These results suggest that BLOX could catalyze the addition of dioxygen to linoleic acid (major substrate) or α-linolenic acid (minor substrate), similar to other LOXs in plants [12].

Second, BLOX produced 13*S*-HpODE as a major product by regioselective dioxygenation of linoleic acid (Fig. 4b). The elution profiles of normal-phase HPLC analyses for detecting conjugated unsaturated hydroperoxydiene at 234 nm wavelength showed that one dominant peak at 18 – 19 min appeared consistently among the products of BLOX and those of commercial soybean 13*S*-LOX. The same peak was also detected in the chromatogram of 13*S*-HpODE standard but not when linoleic acid standard was injected, indicating that BLOX produced 13*S*-

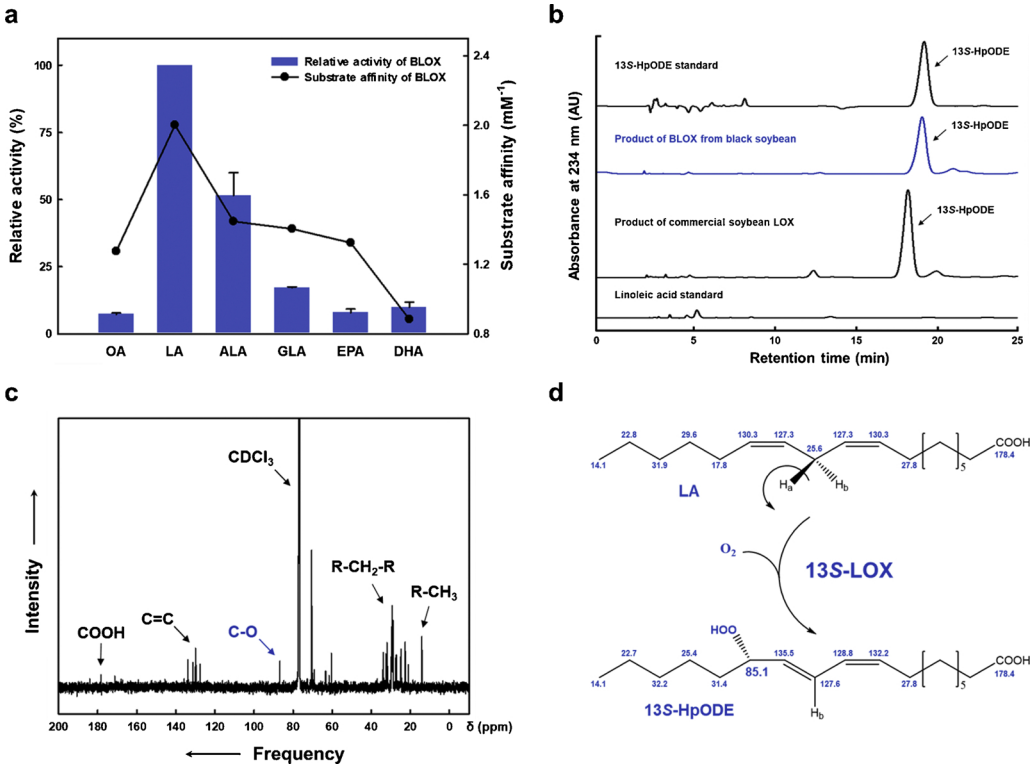

Fig. 4. Substrate specificity of BLOX from black soybean (*G. max* (L.) Merr.). (a) Relative catalytic activity and substrate affinity of BLOX for six different unsaturated fatty acids. Substrate affinity was calculated from the dissociation constant (K_m) of each reaction. (b) HPLC-UV analyses of the products from BLOX-catalyzed dioxygenation of linoleic acid. 13*S*-HpODE and linoleic acid were used as standards for validation. (c) ^{13}C-NMR analyses of the products of BLOX-catalyzed dioxygenation of linoleic acid. (d) Schematic diagram of dioxygenation of linoleic acid by 13*S*-LOX and theoretical ^{13}C-NMR prediction of the chemicals. OA, oleic acid; LA, linoleic acid; ALA, α-linolenic acid; GLA, γ-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; 13*S*-HpODE, 13(*S*)-hydroperoxy-9,11-octadecadienoic acid.

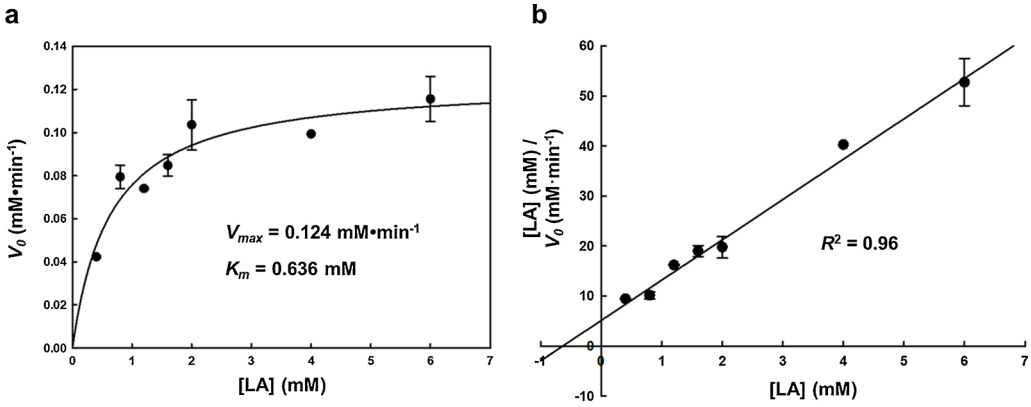

Fig. 5. Enzyme kinetics of the dioxygenation of linoleic acid by BLOX from black soybean (*G. max* (L.) Merr.) at optimum conditions (pH 9.5 and 40 °C). (a) Michaelis–Menten plot of the reaction. (b) Hanes–Woolf plot of the reaction. LA, linoleic acid; V_0, the initial velocity of reaction; V_{max}, the maximum initial velocity of reaction; K_m, the dissociation constant of LOX-LA complex.

HpODE from linoleic acid. Moreover, the elution profiles suggest that BLOX catalyze the dioxygenation of linoleic acid regioselectively because no other possible hydroperoxydienes such as 9-hydroperoxy-11,12-octadecadienoic acid (9*S*-HpODE) were detected. The resolution of HPLC conditions in the present study was enough to separate the two hydroperoxydiene isomers, 13*S*-HpODE and 9*S*-HpODE [22]. Obviously, the stereoselectivity or other specificity of BLOX is still unknown and those need to be analyzed in the future.

In addition, ^{13}C-NMR spectroscopy, which is another clear-cut methodology, was applied to validate the existence of 13*S*-HpODE in the products of BLOX-catalyzed dioxygenation because there could be a remote possibility for peculiar BLOX to generate other hydroperoxydiene similar to 13*S*-HpODE. The ^{13}C-NMR spectra of pure linoleic acid and the BLOX-catalyzed products are shown in Fig. S3 and Fig. 4c, respectively. The similar chemical shifts (δ, ppm) of different carbon atoms were detected in both spectra: primary carbon (R − CH_3, 0–20 ppm), secondary carbon (R − CH_2 − R, 20–40 ppm), olefinic carbon (C = C, 120–140 ppm), and carboxyl carbon (COOH, 175–185 ppm), whereas signals for hydroperoxide carbon (C − O, 50–90 ppm) were only detected in the spectrum of the BLOX-catalyzed products. The theoretical value of the chemical shift for hydroperoxide carbon of 13*S*-HpODE was calculated to be 85.1 ppm according to ^{13}C-NMR prediction in ChemDraw Professional software (Fig. 4d), and a signal corresponding to the theoretical value was also experimentally detected in the products. The strong signals split into a triplet at 77 ppm in both spectra were identified as $CDCl_3$ solvent. The results from HPLC analysis and ^{13}C-NMR spectroscopy demonstrate that 13*S*-HpODE was definitely produced from linoleic acid by BLOX. Therefore, BLOX could be classified into the linoleate 13*S*-LOX family.

Finally, with regard to the enzyme kinetics, the exploratory kinetic parameters of BLOX including V_{max}, K_m, k_{cat}, and k_{cat}/K_m were determined. The kinetics of BLOX-catalyzed dioxygenation of linoleic acid (0.4–6.0 mM) was analyzed under the optimum reaction conditions (pH 9.5 and 40 °C). The values of V_{max} and K_m were determined to be 0.124 mM min^{-1} and 0.636 mM, respectively, according to non-linear regression curve fitting of the data set of initial velocity into the Michaelis–Menten model (Fig. 5a). Then, the validity of the Michaelis–Menten assumption and the calculated kinetic parameters were

statistically examined based on the Hanes–Woolf double-reciprocal plot (Fig. 5b) because the direct linear plot is derived by first assuming that Michaelis–Menten kinetics is applicable. As a result, the kinetics of BLOX-catalyzed dioxygenation of linoleic acid was confirmed to follow the Michaelis–Menten model. Based on these parameters, the turnover number (k_{cat}) and catalytic efficiency (k_{cat}/K_m) of BLOX were calculated as 12.28 s^{-1} and 1.93 × 10^4 $M^{-1}{\cdot}s^{-1}$, respectively. The parameter K_m describes the dissociation constant of the enzyme-substrate complex, and its inverse form implies the substrate-affinity for the enzyme [38]. The affinity of BLOX for linoleic acid was relatively lower than that of LOXs from yellow soybean (0.01–0.03 mM), and consequently, the catalytic efficiency of BLOX was also inferior (1.00–3.00 × 10^7 $M^{-1}{\cdot}s^{-1}$) although the reaction conditions varied among studies [2,39]. Further in-depth comparative analysis of the discrepancy between LOXs from soybean family should be conducted; nevertheless, the catalytic efficiency of BLOX is robust enough to cause enzymatic rancidification intimately. Therefore, BLOX should be practically or biotechnologically controlled using appropriate methods to mitigate enzymatic rancidification of black soybean.

4. Conclusions

In the present study, BLOX was successfully purified from black soybean and identified as a linoleate 13*S*-LOX with a heterodimeric structure. The catalytic activity of BLOX was relatively robust under conditions of alkaline pH and moderately high temperature with sufficient Fe^{2+}. The kinetic parameters (V_{max}, K_m, k_{cat}, and k_{cat}/K_m) of BLOX-catalyzed dioxygenation of linoleic acid at optimum conditions were 0.124 mM min^{-1}, 0.636 mM, 12.28 s^{-1}, and 1.93 × 10^4 $M^{-1}{\cdot}s^{-1}$. Therefore, as well as revealing the nature and catalytic properties of BLOX, the results of the present study provide significant criteria for a biological control of the deterioration of black soybean due to enzymatic rancidification. In addition, one unanswered question from this study is the reason why black soybean has a heterodimeric LOX. For the future, experiments will be focused on addressing the comparative analysis of LOXs in the various cultivars of soybean family to make explicit the findings in this study.

Declaration of Competing Interest

None.

CRediT authorship contribution statement

Jun-Young Park: Data curation, Writing - original draft, Writing - review & editing. **Chae Hyung Kim:** Investigation, Visualization. **Yoonseok Choi:** Writing - review & editing, Validation. **Kyung-Min Park:** Conceptualization, Methodology. **Pahn-Shick Chang:** Conceptualization, Supervision.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.enzmictec.2020.109595.

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