A Thesis for the Degree of Master of Science

Purification and Characterization of Lipoxygenase from Jinuni Bean (*Rhynchosia volubilis*)

쥐눈이콩(*Rhynchosia volubilis*)으로부터 유래한 lipoxygenase 의 분리정제 및 특성규명

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Abstract

In this study, for the determination of lipoxygenase (LOX) activity, the possibility of reversed micelles, widely used as an enzyme reactor for lipases, was investigated. Although it is rapid and simple, reversed micelles have some limitations, such as interference by UV-absorbing materials and surfactant. LOX activity in the reversed micelles was determined by reading the absorbance of the lipid hydroperoxidation product (conjugated diene) at 234 nm. Among surfactants and organic media, AOT and isooctane were most effective for the dioxygenation of linoleic acid in reversed micelles. The strong absorbance of AOT in the UV region is a major obstacle for the direct application of the AOT/isooctane reversed micelles to LOX assay. To prevent interference by AOT, an AOT removal step was inserted to the procedure for LOX activity determination in reversed micelles. The LOX activity was dependent on water content, and maximum activity was obtained at an *R*-value of 10.

LOX from Jinuni bean (*Rhynchosia volubilis)* was purified 13.07-fold by 20-60% saturation of ammonium sulfate fractionation, ion-exchange chromatography on DEAE-sepharose and ANX-sepharose and gel permeation chromatography on sephacryl S-100. The purified LOX showed a monomeric structure with a molecular weight of 66 kDa and the pH and

temperature for optimum activity were pH 8.0 and 15℃, respectively. The LOX from Jinuni bean was stable at pH 4.0-9.0 when stored at 4℃ for 1 day and below 50℃ when stored at pH 7.0 for 30 min. Among the substrate specificity tested, α-linoleic acid (18:2) showed the highest relative activity. When LOX from Jinuni bean reacted with α-linoleic acid, the main product was 13(S)-hydroperoxy-9,11-octadecadienoic acid (13(S)-HPOD). Far-UV circular dichroism revealed that the secondary structures of LOX consist of α-helix (26.2%), antiparallel β-sheet (17.6%), parallel β-sheet (9.9%), β-turn (18.5%) and random coil (35.4%). The kinetic parameters of LOX, V_{max}, K_m, k_{cat} and k_{cat}/K_m, values were 22.07 μmol•min^{-1}•mg^{-1}, 11.80 mM, 24.18 s^{-1}, and 2.05 s^{-1}•mM^{-1}, respectively.

Keywords: lipoxygenase; reversed micelles, AOT; Jinuni bean; circular dichroism

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CHAPTER I

Development of the Simple and Sensitive Method for Lipoxygenase Assay in AOT/Isooctane Reversed Micelles

1. Introduction

Lipoxygenases (LOXs) are a family of iron-containing enzymes found in many plants and animals (Brash, 1999; Kuhn & Thiele, 1999). LOXs are also found in microorganisms such as fungi (Perraud & Kermasha, 2000) and microalgae (Beneytout, Andrianarison, Rakotoarisoa, & Tixier, 1989; Nuñez, Savary, Fogila, & Piazza, 2002). These enzymes catalyze the stereo- and regio-specific dioxygenation of polyunsaturated fatty acids (PUFA) to form fatty acid hydroperoxides (Baysal & Emirdöven, 2007) and convert natural *cis* double bonds to the *trans* double bonds of fatty acids (Maas, Brash, & Oates, 1981). The roles of LOXs in plants and animals remain unclear. Recent studies (Brash, 1999; Siedow, 1991) suggest that they may be involved in plant physiology related to growth and development, such as pest resistance, senescence, and responses to wounding. In mammals, LOXs are involved in the metabolism of eicosanoids such as prostaglandins, leukotrienes, and non-classic eicosanoids. Because LOXs catalyze the insertion of oxygen to polyunsaturated fatty acids, LOXs could be negatively

related to the development of off-flavors in many plant and animal food systems (Hsieh, 1994). Also, LOXs may catalyze the co-oxidation of carotenoids, resulting in a loss of essential nutrients (Robinson, Wu, Domoney, & Casey, 1995). LOXs may play an important role in aroma-compound formation, producing hydroperoxides, precursors for the volatile compounds produced by other enzymes (Robinson,Wu, Domoney, & Casey, 1995). Several methods are available for the determination of LOX activity, including colorimetric methods (Koch, Stern, & Ferrari, 1958), dye solution bleaching methods (Toyosaki, 1992), and spectrophotometric methods (Axelrod, Cheesebrough, & Laakso, 1981). Among them, spectrophotometry is the most accepted method, determining the absorbance of a lipid hydroperoxidation product such as a conjugated diene at 234 nm (Axelrod, Cheesebrough, & Laakso, 1981). Because this spectrophotometric method can simply and directly measure the reaction product, it is widely used for quantitative kinetics determinations with purified LOXs. However, although it is rapid and simple, this method has two limitations. First, if other UV-absorbing materials are present in the crude enzyme solution, the spectrometric assay loses sensitivity. Second, turbidity resulting from the low water solubility of fatty acid substrates can disturb absorbance readings. To avoid interference from fatty acid-induced turbidity and UV-absorbing

materials, water-in-oil emulsions or reversed micelles have received attention as an enzyme reactor system. Reversed micelles have been widely used as an enzyme reactor system to determine lipase activity (Carvalho & Cabral, 2000). LOXs, like lipases, have catalytic activity with water-insoluble substrates. Conducting the enzyme reaction in reversed micelles has a number of advantages, such as solubilization of both hydrophilic and hydrophobic substrates and/or products, a low reaction volume, a large hydrophilic/hydrophobic interfacial area, and control over the activity and stability of enzymes (Carvalho & Cabral, 2000; Martinek, Klyachko, Kabanov, Khmelnitsky, & Levashov, 1989; Martinek, Levashov, Klyachko, Khmelnitsky, & Berezin, 1986). Based on previous studies, it appears that AOT/isooctane reversed micelles are the most suitable for the determination of lipase activity (Carvalho & Cabral, 2000). However, this system developed for the lipase assay cannot be used directly for LOX activity determination because of the overlapping UV-absorbing characteristics of AOT. Thus, in this present study, we investigated the possibility of using AOT/isooctane reversed micelles, developed for the determination of lipase activity, for the determination of LOX activity. We also assessed which modification(s) to the AOT/isooctane reversed micellar system would be

needed to develop a rapid, easy, and sensitive LOX-activity determination method.

2. Materials and methods

2.1. Materials

Soybean LOX type I-B (linoleate:oxygen, oxidoreductase, EC 1.13.11.12) with a reported catalytic activity of 50 units/mg solid (one unit is equivalent to the dioxygenation of 0.12 μmoles of linoleic acid per min at pH 9.0 and 25℃) was purchased from Sigma-Aldrich (Milwaukee, WI). HPLC-grade organic solvents (J.T. Baker, Phillipsburg, NJ), isooctane, cyclohexane, *n*-hexane, *n*-heptane, and hexadecane, were dehydrated with molecular sieves (4 Å, Sigma-Aldrich) and filtered through a membrane filter (0.45 μm) prior to use as a reaction medium for the reversed micelles. Linoleic acid (≥98.0%) and bis(2-ethylhexyl)sulfosuccinate sodium salt (AOT) were purchased from Sigma-Aldrich, and the latter was purified according to the method of Tamamushi & Watanabe (1980). All other chemicals were of extra pure grade and were used without further purification.

2.2. Preparation of reversed micelles

A typical procedure for the formation of reversed micellar system was carried out as follows. First, an appropriate amount of 50 mM Tris-HCl buffer (pH 9.0) containing LOX from soybean was added to 100 mM AOT/isooctane to give the desired *R*-value, which refers to molar ratio of water to surfactant. Subsequently, the mixture was shaken vigorously with a vortex mixer for 60 s until it became clear or optically transparent.

2.3. Procedure for LOX assay

After formation of AOT/isooctane reversed micelles, LOX-catalyzed dioxygenation was initiated by adding 25 μmoles of linoleic acid to the reversed micellar solution pre-incubated in a water-bath at 25℃ with magnetic stirring (800 rpm). Each aliquot (200 μL) of reactant was collected at pre-determined intervals and diluted with 1.8 mL of isooctane. An equal volume of 30% ethanol was added to the dilution, and then the mixture was vortex mixed for 60 s. After centrifugation (5,000×g, 10 min), absorbance of the supernatant (isooctane layer) was measured at 234 nm to quantitatively analyze the conjugated linoleic acids produced. The blank was processed through the same procedure with heat-inactivated enzyme, and the calibration curve (regression equation between molar concentration of

conjugated diene and absorbance at 234 nm) had a coefficient of determination above 0.998. For the evaluation of specific activity, quantitative analysis of protein was performed according to the method of Bradford (1976).

2.4. Statistical analysis

All data are given as mean values and standard deviations of triplicate experiments and were reproducible within ± 10%. The initial velocity of LOX was estimated from the fitted nonlinear regression equation between the reaction time and μmoles of conjugated diene produced, calculated using the SigmaPlot software (ver. 10.0; Systat Software, Inc., San Jose, CA)

3. Results and Discussion

3.1. Effects of AOT in reversed micelles on LOX activity determination

A major limitation on the use of AOT/isooctane reversed micelles as a microreactor for LOX is the strong UV absorption of AOT used as a surfactant to prepare the reversed micelles (Fig. 1). Figure 1 shows that AOT alone in isooctane absorbs strongly at 220 nm, in the UV range. The increase in UV absorption was found to be proportional to the amount of AOT. As

mentioned, LOX activity is generally determined spectrophotometrically by the production of conjugated diene, which shows strong absorbance at 234 nm. To overcome the interference caused by AOT absorption in the UV range, Perez-Gilabert et al. (1992) suggested an AOT concentration below 6 mM. However, in our experiment, 5 mM AOT in isooctane still showed significant UV absorption. Thus, to use an AOT/isooctane reversed micellar system to assay LOX, removal of AOT is required. The removal of AOT from AOT/isooctane reversed micelles was carried out by addition of an ethanol-water mix. Figure 2 shows AOT removal from the isooctane layer as a function of the proportion of ethanol in the added ethanol-water mixture. From preliminary experiments for the selection of AOT extraction solvent, we concluded that ethanol was the most suitable solvent for AOT extraction. Ethanol is immiscible with isooctane, and it effectively extracts only AOT, not the conjugated diene, from the isooctane layer. Addition of ethanol-water mixture with up to 30% ethanol (v/v) had no effect on the absorbance of the isooctane layer at 234 nm, but addition of more than 30% (v/v) ethanol–water mixture gradually decreased the absorbance. However, unlike the change in the absorbance of the isooctane layer, if the ethanol proportion in the added ethanol-water mixture exceeded 30%, the absorbance of the ethanol layer increased in proportion to the added ethanol in the ethanol-

water mixture. These results suggest that the ethanol-water mixture containing up to 30% ethanol selectively extracted AOT from the isooctane layer and that a higher proportion of ethanol may extract not only AOT but also conjugated diene from the isooctane layer. However, the addition of ethanol-water mixture with below 30% ethanol caused turbidity in the isooctane layer, which interfered with the absorbance reading at 234 nm. Thus, for further study, the ethanol proportion of the ethanol–water mixture was fixed at 30% for AOT extraction.

The efficiency of the ethanol-water mixture in AOT extraction depended on AOT concentration (Fig. 3). The dependency on AOT concentration was determined by adding 30% ethanol–water mixture to the isooctane layer containing AOT in a range from 2.5 to 15 mM. Addition of 30% ethanol-water mixture effectively eliminated AOT from the isooctane layer, independent of the AOT concentration in the isooctane layer.

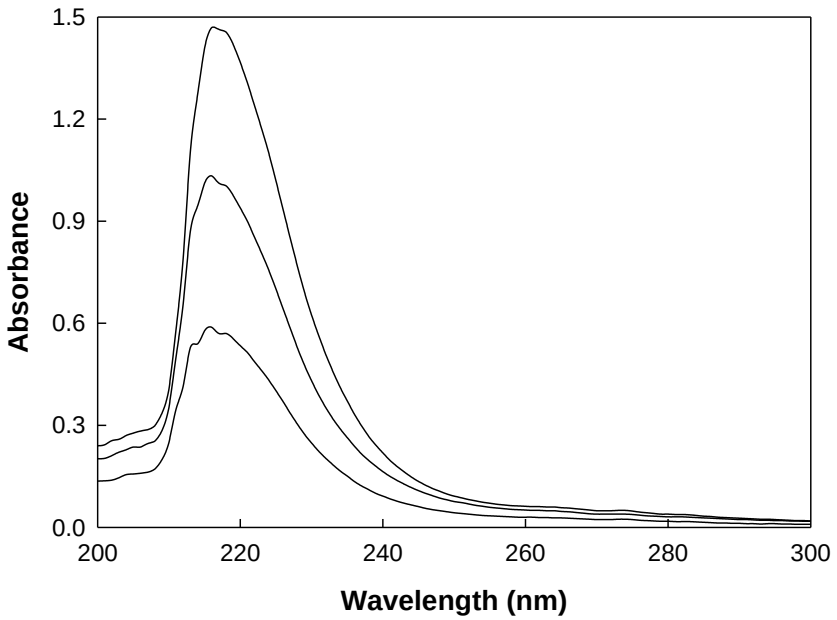

Fig. 1. UV absorption spectra of AOT in isooctane at various concentration.

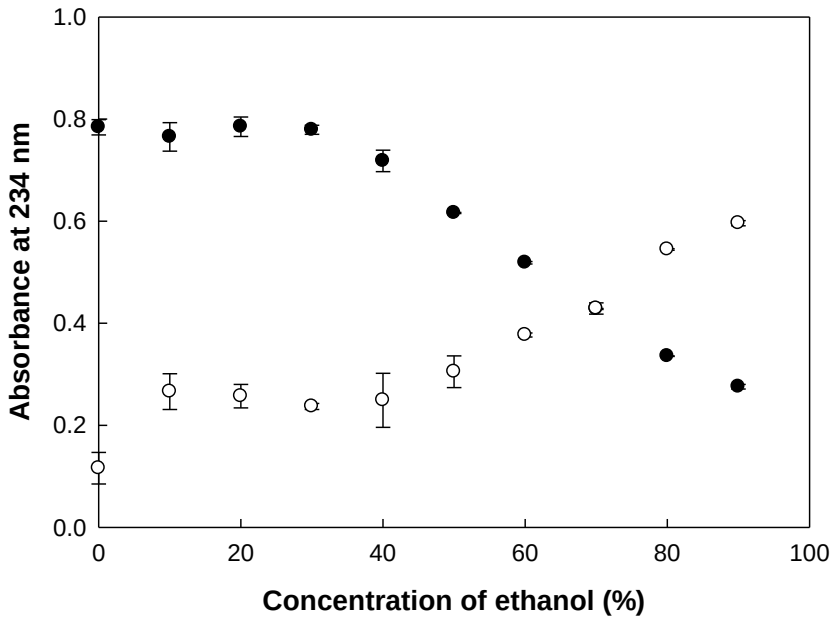

Fig. 2. Effect of the concentration of ethanol added to reaction-terminated reversed micellar system on the content of conjugated diene in isooctane (●) and ethanol layer (○).

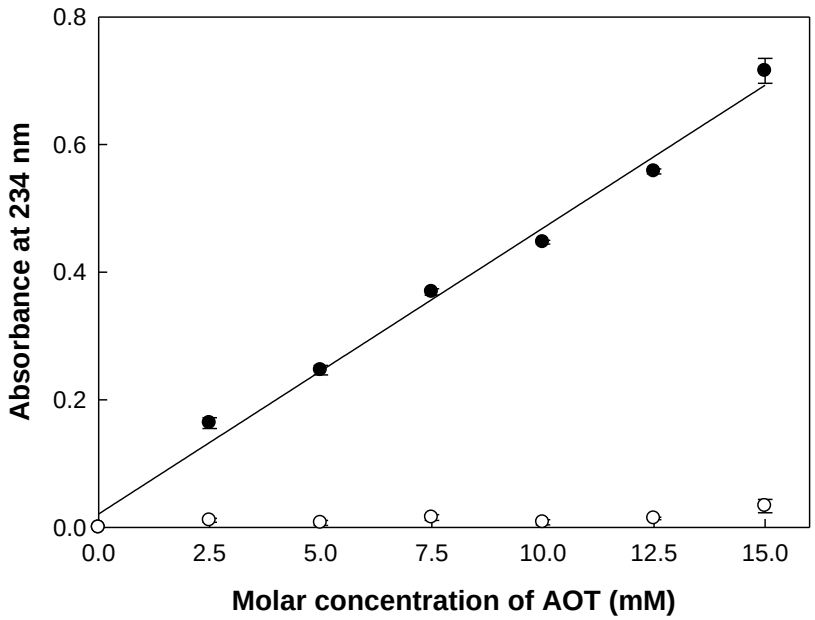

Fig. 3. Elimination of AOT from isooctane layer by addition of 30% ethanol (○) and without treatment (●).

3.2. Selection of organic reaction medium

Five organic solvents, cyclohexane, *n*-heptane, *n*-hexane, isooctane, and hexadecane, were tested because they are frequently used in the determination of lipase activity in reversed micelles (Chang & Rhee, 1990; Han & Rhee, 1986; Kim, Kwon, & Rhee, 1984; Prazeres, Garcia, & Cabral, 1992). Isooctane was the most effective organic solvent tested for the LOX reaction in reversed micelles (Table 1). This result was consistent with previous reports on lipase activity in reversed micelles (Han & Rhee, 1986; Kim, Kwon, & Rhee, 1984). In fact, it has been reported that changes in the biocatalytic activity of lipases and LOXs in organic solvents can be explained by the log*P* value, which is the degree of solvent polarity (Leo, Hansch, & Elkins, 1971). Generally, biocatalysis in organic solvents have been reported to be relatively low in hydrophilic solvents having a log*P* <2, moderate in solvents having a log*P* between 2 and 4, and high in hydrophobic solvents having a log*P* >4. However, recently, Laane and others (1987) have reported that log*P* of the organic solvents may affect the biocatalysis rate when a microemulsion has a very small amount of water only to solubilize and stabilize enzymes. So, following the proposal of Laane and others (1987), relatively low LOX activity should be obtained only in reversed micelles prepared with hexadecane (log*P*=8.8). However, our

results were not consistent with this. The highest LOX activity was obtained with reversed micelles prepared with isooctane (log*P*=4.1). Although cyclohexane (log*P*=3.2), *n*-heptane (log*P*=3.8), and *n*-hexane (log*P*=3.5) have similar log*P* values to isooctane, LOX activities in reversed micelles prepared with them were much lower than those in isooctane reversed micelles. Another possible explanation for this observation is the difference in the molecular structure of the organic media used. The organic solvents we tested were straight-chain alkanes, except cyclohexane and isooctane. Hydrocarbons with short chains can embed in the interfacial membrane formed with AOT molecules; then, hydrocarbons can form an additional layer at the interfacial membrane (Hirai, Kawai-Hirai, Sanada, Iwase, & Mitsuya, 1999). Penetration of the mostly saturated hydrocarbons into the surfactant layer of the reversed micelle impedes the contact and/or interaction between LOX and its substrates, and that could be a reason for the relatively low LOX activity in *n*-hexane and *n*-heptane. The mostly or fully saturated hydrocarbons, with hydrocarbon chains of C9-10, are able to embed in the surfactant layer of the AOT reversed micelle. The stability of the reversed micelles and of enzymes in the water pool could be governed primarily by the packing pattern of surfactant molecules on the interfacial membrane. Although hexadecane has too long a hydrocarbon chain to

penetrate through the AOT interfacial membrane, and cyclohexane does not have the proper structure to penetrate through the AOT interfacial membrane due to its unique ring structure, LOX activities in these media were quite low compared with those in isooctane. This could be due to a more favorable packing pattern of AOT molecules in isooctane than in hexadecane or cyclohexane. The favorable packing pattern of AOT molecules in isooctane could make the reversed micelles and the LOX in them, more stable. In this case, AOT molecules may be packed more appropriately in cyclohexane than in hexadecane. From these results, we concluded that LOX activity was not associated with log*P* values of the organic solvents tested but that the biocatalysis of LOX in reversed micelles had an organic-solvent dependency. In fact, LOX activity was influenced by the structure of the organic medium rather than simply by its polarity. Thus, isooctane was used as the organic reaction medium for the reversed micelles in further studies.

Table 1. Relative activity of the LOX from soybean in reversed micelles formed by AOT in various organic solvents

Organic solvents	Relative activity (%)
Isooctane	100.00
Cyclohexane	38.61±1.94
n-Heptane	19.15±2.48
n-Hexane	18.80±5.50
Hexadecane	16.75±3.25

3.3. Effect of water content in AOT/isooctane reversed micelles

The water content in the reversed micelles is a key factor in biocatalysis using reversed micelles (Avramiotis, Xenakis, & Lianos, 1996; Prazeres, Garcia, & Cabral, 1992), and it may influence the stability of the encapsulated enzyme (Barbaric & Luisi, 1981). Figure 4 shows the effect of the water content in the AOT/isooctane reversed micelles. The water content in the reversed micelle is expressed as the molar ratio of water to AOT, the *R*-value. The specific activity was strongly dependent on the water content, and LOX showed a bell-shaped profile of specific activity, with a maximum at an *R*-value of 10. The cores of the reversed micelles are of nanometer size and are able to solubilize proteins, especially enzymes, into them. The water molecules in the reversed micelles may be free or bound. A previous study showed that two or three molecules of water can strongly bind to thehydrophilic head of an AOT molecule, and approximately molecules of water can be trapped in an AOT molecule (Amararene, Gindre, Le Huérou, Nicot, Urbach, 221 & Waks, 1997). The properties of water molecules that bind to the surfactant polar head at the interface are significantly different from those of bulk water (Faeder & Ladanyi, 2000). At low *R*-values, meaning that size of the reversed micelles is quite small, most of the water molecules may be directly bound to the hydrophilic heads of AOT

surfactants; thus, there may be a lack of water for the hydration of enzymes. The unique internal environment of the reversed micelles with low *R*-values also has effects on the protein folding and unfolding transition and protein–protein interactions. Thus, the low LOX activity at low *R*-values (<5) could be explained by a lack of water for the hydration of the enzyme and/or by incomplete conformational changes into an active form. There are several hypotheses for the reduction of LOX activity with further increases in *R*-value above the *R*-value for maximum LOX activity. One is that the reduction in enzyme activity when the *R*-value exceeds the optimal value could be due to a decrease in the concentrations of enzyme and substrate in the water pool (Chen & Pai, 1991; Han & Rhee, 1986). Electrostatic interactions between the negatively charged AOT and LOX could be another explanation for the low activity in the reversed micelles with high *R*-values. According to the LOX supplier, the pI of LOX used in this study is in the range of 4.5–6.5. Thus, LOX in pH 9 Tris-HCl buffer should be negatively charged. Because of electrostatic repulsion between negatively charged LOX and negatively charged AOT, LOX could be localized to the core of the reversed micelles. The size of the reversed micelles may impact LOX–membrane and/or LOX-solvent interactions. In conclusion, the amount of water molecules in the reversed micelles with an *R*-value of 10 and micellar

size could determine the most appropriate conditions for the highest stability and activity of LOX. The bell-shaped profile of LOX activity, depending on the *R*-value, is similar to those with lipase (Han & Rhee, 1986; Han, Walde, & Luisi, 1990; Prazeres, Garcia, & Cabral, 1992).

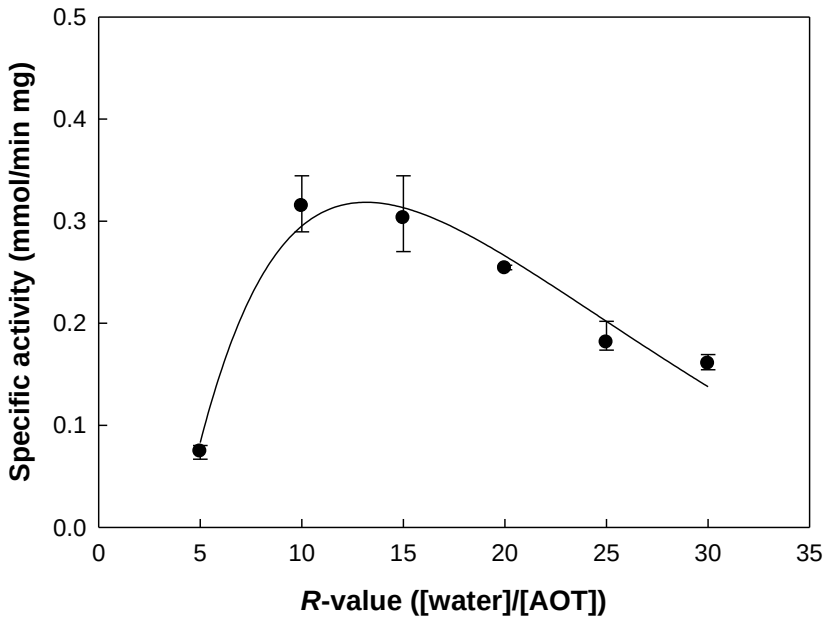

Fig. 4. Effect of *R*-value ([Water]/[AOT]) on specific activity of soybean LOX in reversed micellar system.

3.4. Verification of LOX assay in reversed micellar system

To assess the LOX assay developed here, experiments monitoring changes in initial velocity according to enzyme concentration were performed using commercial LOX I-B from soybean. Figure 5 shows that the accumulation of conjugated diene produced by LOX-catalyzed dioxygenation at different enzyme concentration and a fixed substrate concentration (5 μmol/mL reactor). The range of enzyme concentrations was determined from preliminary experiments in which the enzyme concentrations did not reach a saturated level. The fitted equations for a hyperbolic curve at different enzyme concentrations are given in Figure 5, which shows non-linear regression between the reaction time and the amount of conjugated diene produced. Initial velocities calculated from the fitted equation of the LOX-catalyzed reaction with 18, 36, 54, and 72 μg /mL were determined as 0.046, 0.114, 0.197, and 0.268 μmol/min, respectively. These values of initial velocities were significantly proportional to the enzyme concentration within the range evaluated. These results agreed with the pattern of fatty acid liberation as a result of lipase-catalyzed hydrolysis in reversed micelles reported by Han & Rhee (1986). It was also observed that absorbance at 234 nm of the blank test rarely fluctuated regardless of enzyme concentration, indicating that there was no interference caused by the increase in enzyme

concentration. This was likely because enzyme that participated in the reaction did not remain in the isooctane phase to be analyzed by UV spectrophotometry. By the same token, it is also reliable evidence that UV-absorbing materials present in the crude enzyme extract did not affect the quantitative analysis of conjugated diene because of their water-soluble properties. From these results, it was determined that the LOX assay in the reversed micellar system could be useful as a novel method for screening LOX activity in crude enzyme extracts from various sources in the food industry.

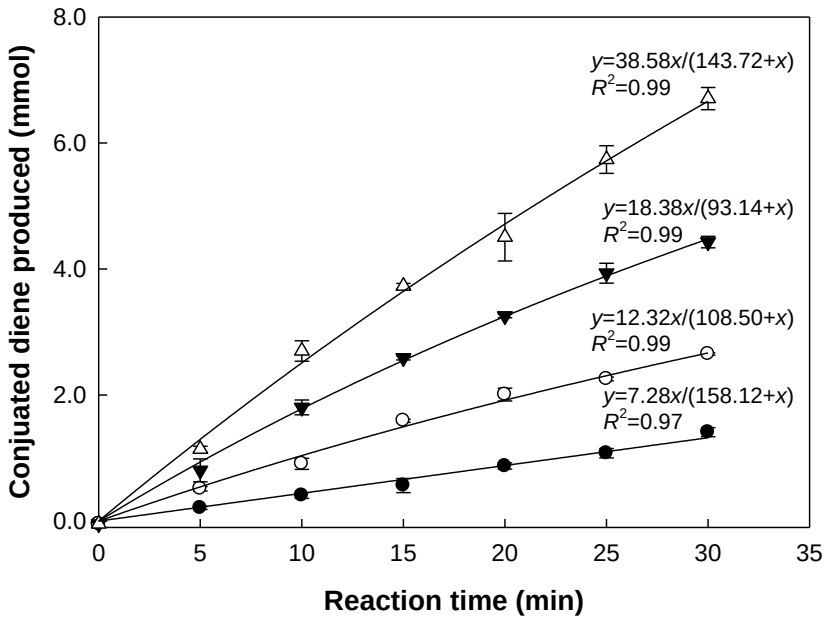

Fig. 5. Formation of conjugated dienes in reversed micellar system, showining the changes in initial velocity of the LOX-catalyzed dioxygenation according to enzyme concentation (●, 18 μg/mL; ○, 36 μg/mL; ▼, 54 μg/mL; △, 72 μg/mL).

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CHAPTER Ⅱ

Purification and Characterization of Lipoxygenase from Jinuni Bean (*Rhynchosia volubilis*)

1. Introduction

Lipoxygenases (LOXs) are present in a wide range of animals and plants, but they are particularly abundant in grain legume seeds (beans and peas) and potato tubers (Casey R., 1998). LOXs catalyze the regionselective and stereoselective dioxygenation of 1,4-pentadiene *cis*-polyunsaturated fatty acids (PUFA) into their corresponding hydroperoxy derivatives (Gardner, H. W., 1991). In plants, linolenic acid and linoleic acid are the most common substrates for LOXs. In the case of linoleic acid, this leads to two possible products, the 9- and 13-hydroperoxy fatty acids (Siedow, 1991).

LOXs not only have food-related applications in bread making (Casey R., 1997) and aroma production (Whitehead IM., 1995) but also have negative implications for color, off-flavor, and antioxidant status of foods (Casey R., & Domoney C., 1996). The details of the catalytic cycle of LOXs have been proposed based on X-ray crystallography, sitedirected mutagenesis, electron paramagnetic resonance studies, and spectroscopic measurements (Chasteen, N. D., 1993; Segraves, E. N., 2006). A novel scheme is proposed for the

mechanism of the LOX-catalyzed dioxygenation of PUFA in which two different pathways are suggested for the anaerobic and aerobic oxidations (Fig. 1). In this present study, the AOT/isooctane reversed micellar system has been applied to the Jinuni beans LOX purification and characterization. This system was developed for the rapid, easy, and sensitive LOX activity assay and modified for AOT removal after enzyme reaction because of the overlapping UV-absorbing characteristics.

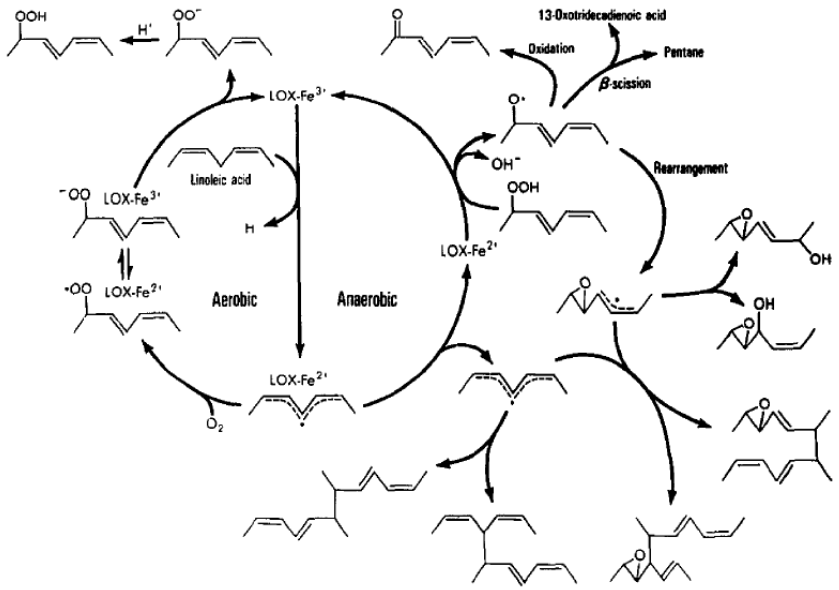


Fig. 1. Pathway of LOX-catalyzed oxidation (Gardner H. W., 1988).

2. Materials and Methods

2-1. Plant and chemical materials

Jinuni beans (*Rhynchosia volubilis*) were obtained from the local market of Gyeonggi-do, Korea and harvested in 2010. The beans were stored at 4^{o}C until used for experiments. Trizma® base (≥99.9%), N,N,N′,N′-tetramethylethylenediamine (TEMED), 2-mercaptoethanol (≥99%), sodium dodecyl sulfate (SDS), glycerol (≥99%) and glycine (≥99%) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Ethyl alcohol (>99.5%), isooctane (>98%) and hydrochloric acid (35~37%) were obtained from Daejung Chemicals & Metals Co. (Shihueng, Gyeonggi-do, Korea). Triton X-100, ammonium sulfate (99.5%), and sodium chloride (99.5%) were purchased from Junsei Chemical Co., Ltd. (Tokyo, Japan). Bio-Rad protein assay kit, *N,N'*-methylenebis-acrylamide (30% acrylamide/bis 37.5:1), ammonium persulfate (APS), and bromophenol blue were obtained from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Methanol (100%) and acetic acid (100%) were purchased from J.T. Backer Co., (Phillipsburg, NJ, USA). 13(S)-Hydroperoxy-9,11-octadecadienoic acid (13(S)-HPOD) was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

2-2. Preparation of crude enzyme

Five hundred grams of fresh jinuni beans were homogenized at 4℃ for 3 hours with 1 L of 0.5 M Tris-HCl buffer (pH 7.0) and 0.1% Triton X-100. The supernatant was filtered through a 0.45 μm filter paper (Advantec MFS, Inc., Tokyo, Japan) after the mixture was centrifuged at 20,000 × g for 30 min. Then ammonium sulfate was added to the supernatant to 20% saturation and the mixture was stirred for 1 hour at 4℃. The solution was centrifuged at 20,000 × g for 30 min at 4 ℃ and the precipitate was discarded. Further addition of ammonium sulfate to 60% saturation was performed. The mixture was centrifuged again as stated earlier and the supernatant was discarded. The precipitate was dissolved in 0.5 M Tris-HCl buffer (pH 7.0) and dialyzed against the same buffer to remove ammonium sulfate using dialysis membrane (MWCO 12,000-14000, Fisherbrand® , Fisher Scientific, Pittsburgh, PA, USA). The previous solution was filtered through a 0.20 μm filter paper for chromatography. All extraction procedures were performed at 4℃.

2-3. Assay of LOX activity in reversed micellar system

Assay of LOX activity was performed according to the reversed micellar system method. Typical procedure for the formation of reversed micelles was carried out as follows. First, an appropriate amount of 50 mM Tris-HCl buffer (pH 7.0) containing extract from Jinuni beans was added to 100 mM AOT/isooctane to give the *R*-value 10, which refers to molar ratio of water to surfactant. Subsequently, the mixture was shaken vigorously with a vortex mixer for 1 min until it became clear or optically transparent. After formation of AOT/isooctane reversed micelles, LOX-catalyzed dioxygenation was initiated by adding 50 μmoles of linoleic acid to the reversed micelles solution pre-incubated in a water-bath at 15℃ with magnetic stirring (800 rpm). Each aliquot (100 μL) of reactant was collected at pre-determined intervals and diluted with 1.9 mL of isooctane. An equal volume of 30% ethanol was added to the dilution, and then the mixture was vortex mixed for 1 min. After centrifugation (5,000×*g*, 5 min), absorbance of the supernatant (isooctane layer) was measured at 234 nm to quantitatively analyze the conjugated linoleic acids produced.

2-4. Purification of Jinuni bean LOX

The purification procedure was performed according to the flow scheme shown in Figure 2. First, the crude enzyme solution was loaded on a DEAE sepharose F.F. column (0.7 × 2.5 cm) equilibrated with 50 mM Tris-HCl buffer (pH 7.0) using a flow rate of 1 mL/min and eluted by a five step gradient (0.5, 0.10, 0.15, 0.2, 1.0 M NaCl). The LOX active fractions were collected and concentrated using ultrafiltration (molecular mass cutoff of 10 kDa, Millipore, Billerica, MA, USA). Second, the active fractions obtained after step using DEAE sepharose F.F. column were applied to an ANX sepharose F.F. column (0.7 × 2.5 cm) equilibrated with 50 mM Tri-HCl buffer (pH 8.0) using a flow rate of 1 mL/min and eluted by four step gradient (0.1, 0.2, 0.3, 1.0 M NaCl). The fractions indicated LOX activity were collected and concentrated. Finally, the active fractions obtained after step using ANX sepharose F.F. column were loaded on a sephacryl S-100 (1.6 × 60 cm) column equilibrated with 0.15 M NaCl in 50 mM Tri-HCl buffer (pH 7.0) using flow rate at 0.4 mL/min. The active fractions using a sephacryl S-100 column were carried out **once again** at same flow rate. All steps were performed at 4℃ and performed using a fast protein liquid chromatography system (GE Healthcare, Uppsala, Sweden) with UV detector at 280 nm.

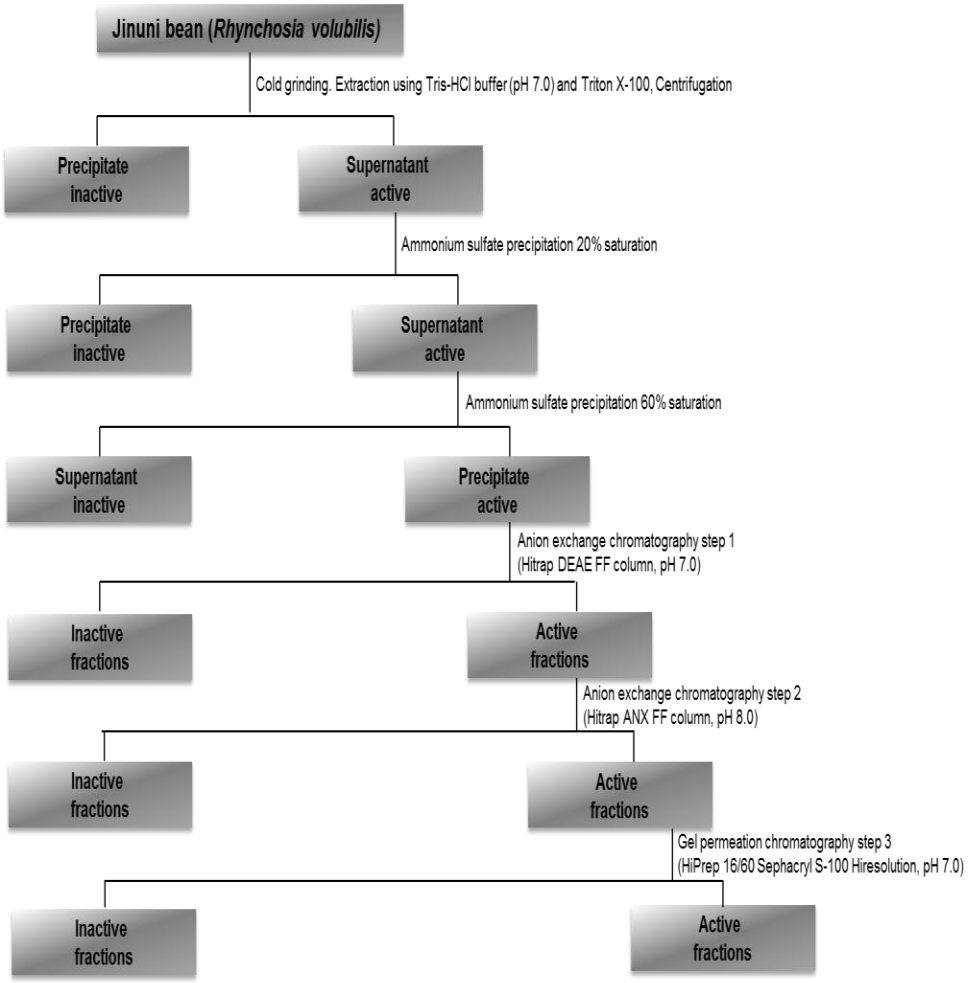


Fig. 2. Scheme of the purification procedure of LOX from Jinuni bean.

2-5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the Laemmli's system (Laemmli, 1970). The buffer system for resolving gel was 0.5 M Tris-HCl (pH 6.8) and stacking gel was 1.5 M Tris-HCl (pH 8.8). The composition of 12% concentrated resolving gel stock solution was 4 mL acrylamide (30%), 2.5 mL resolving gel buffer, 3.3 mL deionized distilled water (D.D.W), 0.1 mL sodium dodecyl sulfate (SDS, 10%), 0.75 mL ammonium sulfate solution (APS, 10%), and 5 μL N,N,N′,N′-Tetramethylethylenediamine (TEMED). Resolving gel stock solution was loaded onto 0.5 mm thick plate. After polymerization of resolving gel, 3% stacking gel was poured. The composition of 3% stacking gel stock solution was 0.66 mL acrylamide (30%), 1 mL stacking gel buffer, 2.27 mL D.D.W, 40μL SDS (10%), 20 μL APS (10%) and 4 μL TEMED. Protein from Jinuni bean was mixed in a 1:1 ratio with sample buffer containing 2 mL stacking buffer, 1.6 mL glycerol, 3.2 mL SDS (10%), 0.8 mL 2-mercaptoethanol and 0.4 mL bromophenol blue (1%) and boiled for 2 min at 95^{o}C before loaded. Protein sample was separated on Hoefer SE 250 mini-gel system (GE Healthcare) at a room temperature at a constant current of 20 mA for 1 h. The proteins were stained

with Coomassie Brilliant Blue R-250 (Cleveland, Fischer, Kirschner, & Laemmli, 1977).

2-6. Identification of LOX product

The products from Jinuni bean LOX were produced in 50 mL of reaction mixture including 50 mM Tris-HCl (pH 8.0), 500 μmol linoleic acid, and purified LOX from Jinuni bean at 15℃ for 3 h. An equal volume of 30% ethanol was added to the reactant and vortex mixed for 1 min. After centrifugation at 5,000×g for 10 min, the resulting compounds were separated with an isooctane layer and 30% ethanol layer. The supernatant was evaporated by rotary vacuum concentrator at 4℃ (Speed Vac® SC110, Savant Instrument Inc., NY, USA). Normal phase-high pressure liquid chromatographic (NP-HPLC) analysis were performed on a μPorasil™ silica column (3.9 × 300 mm, 10 μm, Waters, Milford, MA, USA) equipped with a pump (PU-2089 plus, JASCO, Inc., Tokyo, Japan) and a UV detector (UV-2075, JASCO). The hydroperoxy derivatives were eluted with the solvent system of *n*-hexane/2-propanol/acetic acid (98:1.9:0.1, v/v/v) at a flow rate of 1.0 mL/min and detected at 234 nm (Kuo J.M., 1997). The LOX-catalyzed product, 13-hydroperoxy-9,11-octadecadienoic acid (13-HPODE), was confirmed in comparison to standard.

2-7. Amino acid analysis

The LOX from Jinuni bean was hydrolyzed by 6 N HCl at 130 ℃ for 24 h. The amino acid composition of LOX from Jinuni bean was analyzed using a high-performance liquid chromatograph (HPLC) instrument (Agilent 1200LC, Agilent Technologies, Inc., CA, USA) equipped with a C18 column (5 um, 4.6 ×150 mm), fluorescence detector (EX=340 nm, Em=450 nm) and a UV detector (338 nm). The column temperature was set at 40℃. A calibration chromatogram was established for 16 known amino acids (aspartate, glutamate, serine, histidine, glycine, threonine, arginine, alanine, tyrosine, valine, methionine, phenylalanine, isoleucine, leucine, lysine, and proline). The mobile phase A and B were 20 mM sodium phosphate monobasic (pH 7.8) buffer and water/acetonitrile/methanol (10:45:45 v/v/v) at 1.5 mL/min flow rate for 30 min.

2-8. Circular Dichroism (CD)

Circular dichroism (CD) is being increasingly recognized as a valuable technique for examining the structure of proteins in solution (Kelly S. M., 2005). CD spectrometer (Chirascan™-plus, Applied Photophysics, Ltd., Leatherhead, Surrey, UK) was performed on homogeneous samples at a protein concentration of 0.5 mg/mL in 50 mM sodium phosphate (pH 7.0) in the Far-UV regions (190-260 nm), path length (0.5 mm) and bandwidth (1 nm). The composition of secondary structure in Jinuni bean LOX was determined by CDNN secondary structure analysis software.

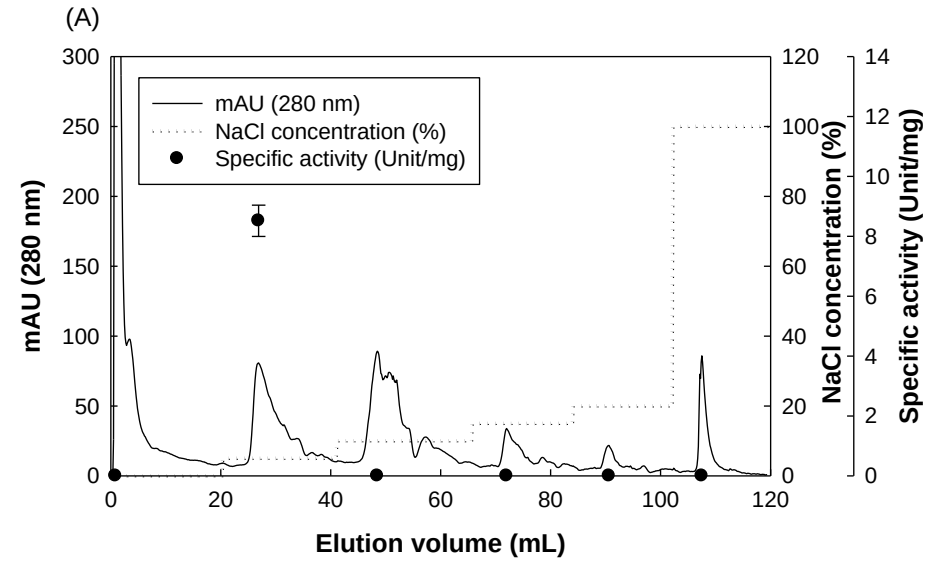
3. Results and Discussion

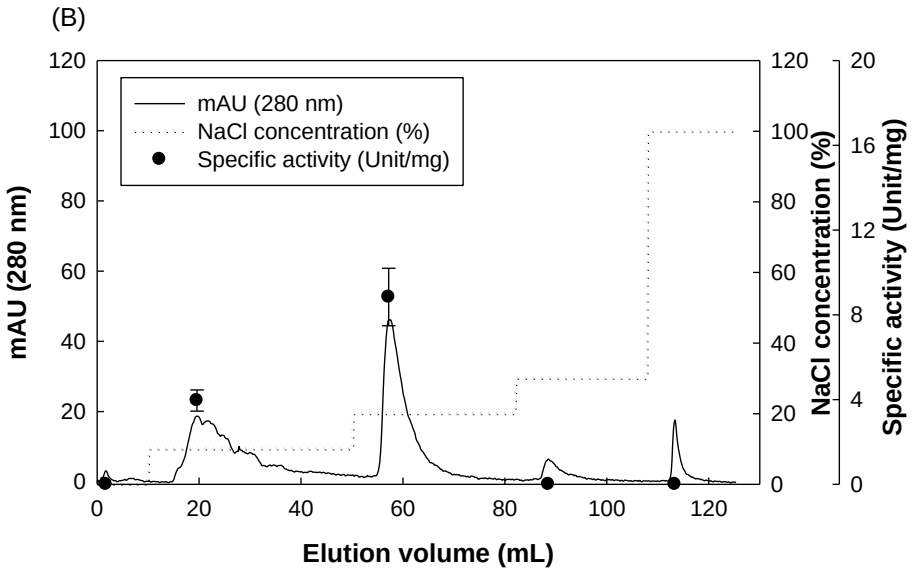
3-1. Purification of Jinuni bean LOX

Table 1 summarizes the purification process of Jinuni bean LOX. The LOX from Jinuni bean purified 13.07-fold; ion-exchange chromatography on DEAE-sepharose F. F. column was performed (Fig. 3A), resulting in a 7.04-fold purification with 27.18% recovery. Second step, ion-exchange chromatography on ANX-sepharose F. F. column was performed (Fig. 3B). The recovery of LOX activity in this fraction was 3.59% with 7.33-fold purification. Final purification step was performed by gel permeation chromatography on sephacryl S-100 column (Fig. 3C-D) with a final 13.07-fold purification with 3.47% recovery and specific activity of 15.82 units/mg. Thus, the LOX active fractions from the first gel permeation using sephacryl S-100 column were collected (elution volume; 40-50 mL), concentrated, and loaded again onto the sephacryl S-100 column for a 0.4 mL/min flow rate separation. As a result, the one peak was confirmed and the purity of Jinuni bean LOX was identified by SDS-PAGE (Fig. 4). The purified LOX showed only one band with molecular weight of 65 kDa.

Table 1. Purification summary of LOX from Jinuni bean

Stage	Total volume (mL)	Total activity (Units)	Total protein (mg)	Specific activity (Units/mg)	Yield (%)	Purification fold
Crude extract	660	7702.20	6349.20	1.21	100.00	1.00
20-60% $(NH_4)_2SO_4$	290	4381.90	3123.30	1.40	56.89	1.16
Ion-exchange chromatography on DEAE Sepharose	74	2093.46	245.68	8.52	27.18	7.04
Ion-exchange chromatography on ANX Sepharose	30	276.60	31.20	8.87	3.59	7.33
Gel permeation chromatography on sephacryl S-100	12	267.60	16.92	15.82	3.47	13.07


(A)
mAU (280 nm)
NaCl concentration (%)
Specific activity (Unit/mg)
mAU (280 nm)
NaCl concentration (%)
Specific activity (Unit/mg)
Elution volume (mL)


(B)
mAU (280 nm)
NaCl concentration (%)
Specific activity (Unit/mg)
mAU (280 nm)
NaCl concentration (%)
Specific activity (Unit/mg)
Elution volume (mL)

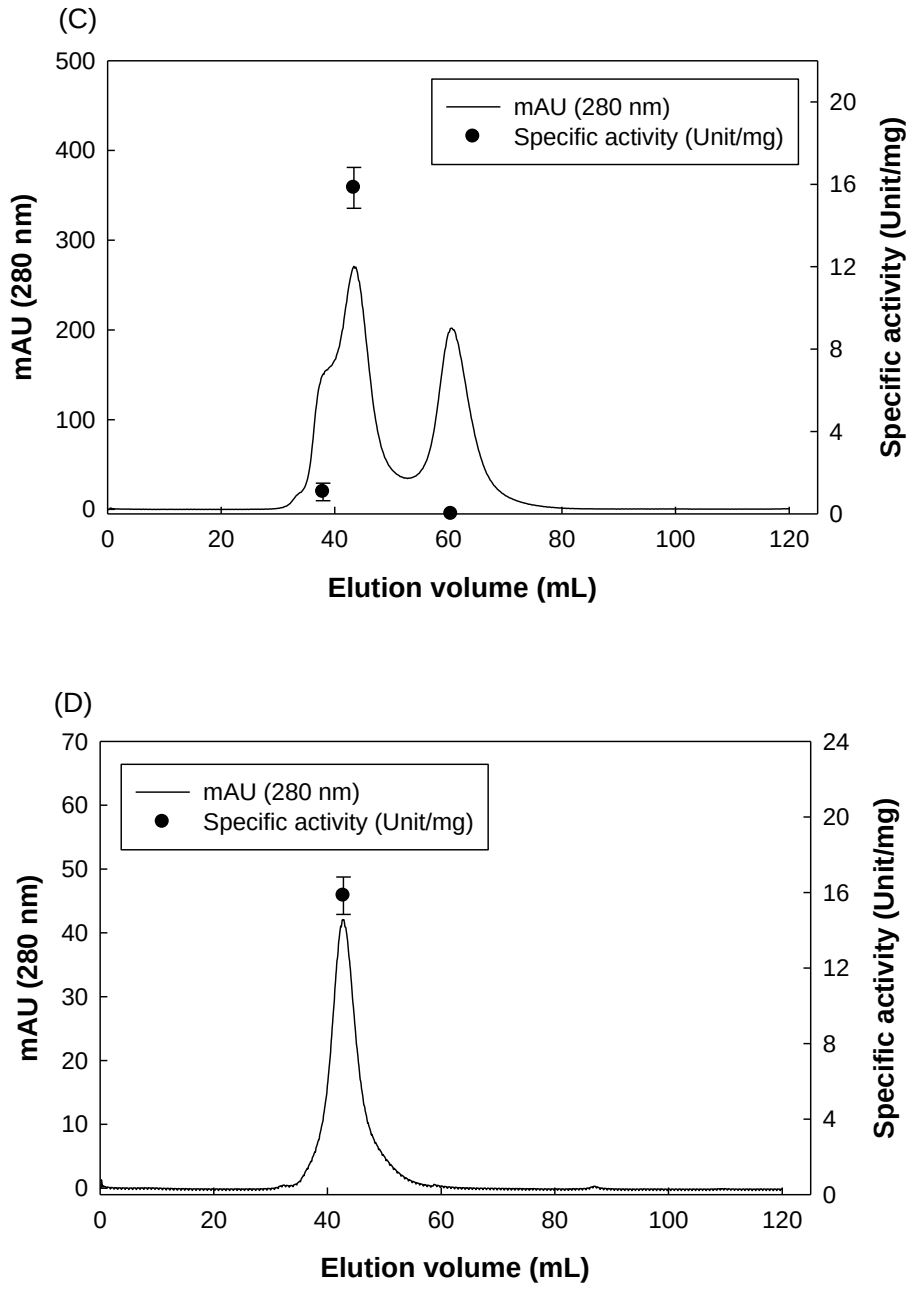


Fig. 3. Elution profile of Jinuni bean LOX on DEAE sepharose F.F. (A), ANX sepharose F.F. (B) and sephacryl S-100 column. (C-D).

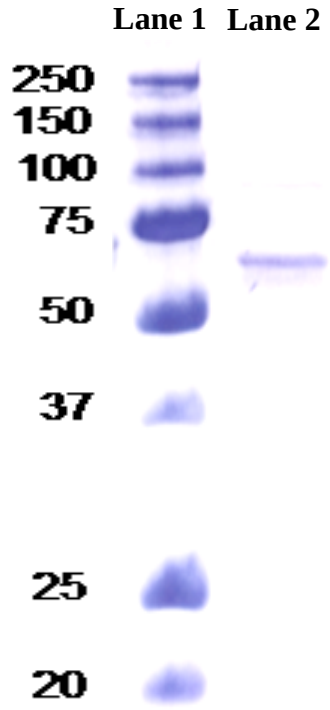


Fig. 4. SDS-PAGE of the purified LOX from Jinuni bean. Lane 1, Marker; lane 2, purified LOX from Jinuni bean.

3-2. Determination of molecular weight

To estimate the molecular weight of LOX from Jinuni bean, gel permeation chromatography on sephacryl S-100 was carried out. 0.2 mL of the purified enzyme (1.41 mg/mL) was loaded on a sephacryl S-100 column by a FPLC system, at a flow rate of 0.4 mL/min. The column was previously equilibrated with 50 mM Tris-HCl/0.15 mM NaCl (pH 7.0) and calibrated with the following standards (Fig. 5): bovine serum albumin (66 kDa), ovalbumin (44 kDa), carbonic anhydrase (29 kDa) and ribonuclease A (13.7 kDa). It could be found out that LOX purified from Jinuni bean has a molecular weight of 65,729 Da.

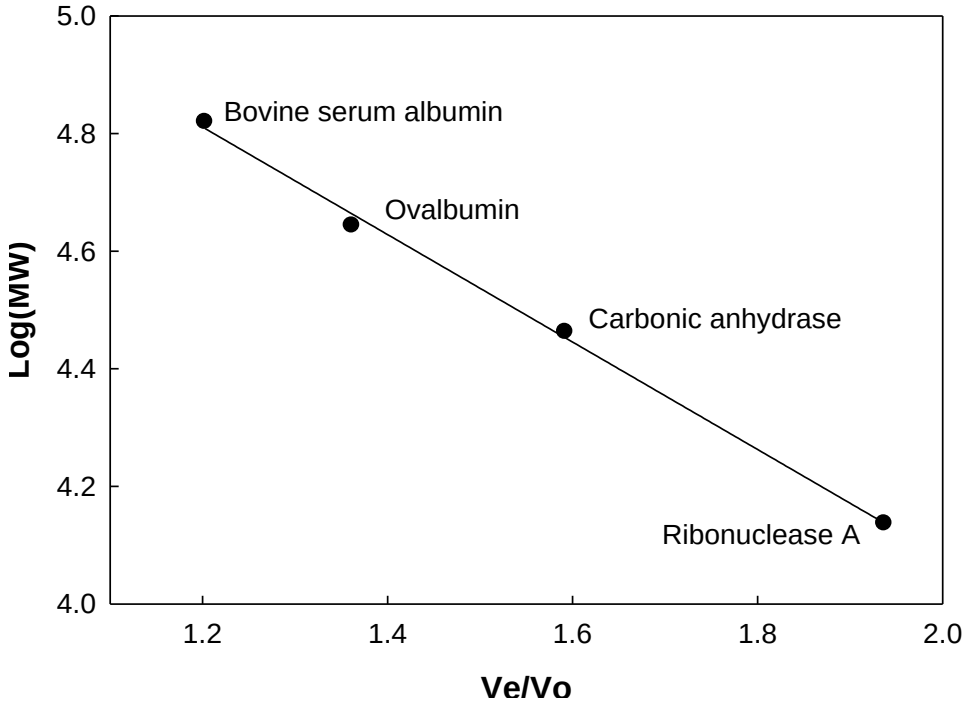


Fig. 5. Determination of molecular weight of LOX from Jinuni bean by gel permeation chromatography on sephacryl S-100, Ve and Vo mean the elution volume of each protein and the void volume.

3-3. Effect of pH and temperature on LOX activity

3-3-1. Optimum pH and stability

The optimal pH of purified LOX from Jinuni bean was measured at ranging from 3.0 to 11.0 with linoleic acid as a substrate. The Britton-Robinson buffer system for determining pH profile consists of a mixture of 50 mM boric acid (H_3BO_3), phosphoric acid (H_3PO_4) and acetic acid (CH_3COOH). The optimal pH was 8.0 (Fig. 6). At pH 11.0, 21.52% relative activity was observed and at pH 3.0, only 5.11% relative activity.

The pH stability was performed by pre-incubating Jinuni bean LOX at different pH values for 1 day and assayed for LOX activity. The stability was shown in Figure 7. The Jinuni bean LOX was stable over a pH range from 4.0 to 9.0.

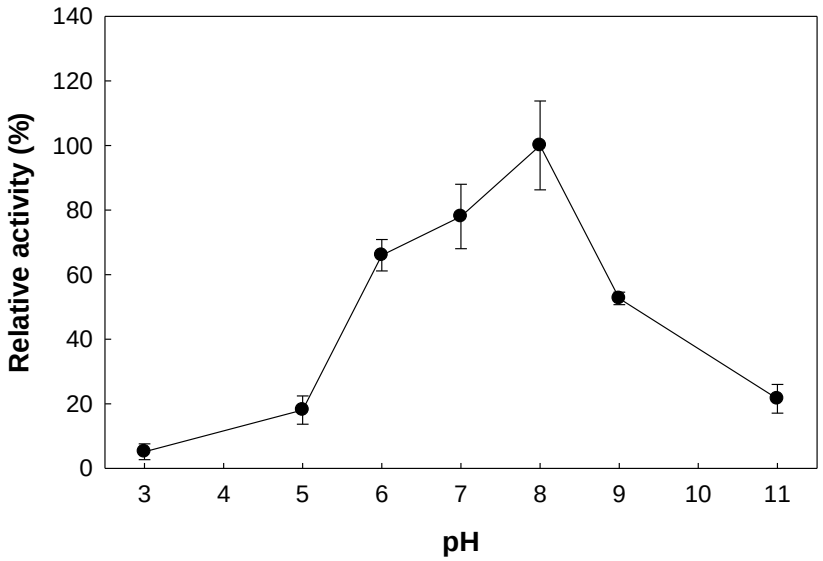


Fig. 6. Effect of pH on the LOX activity purified from Jinuni bean.

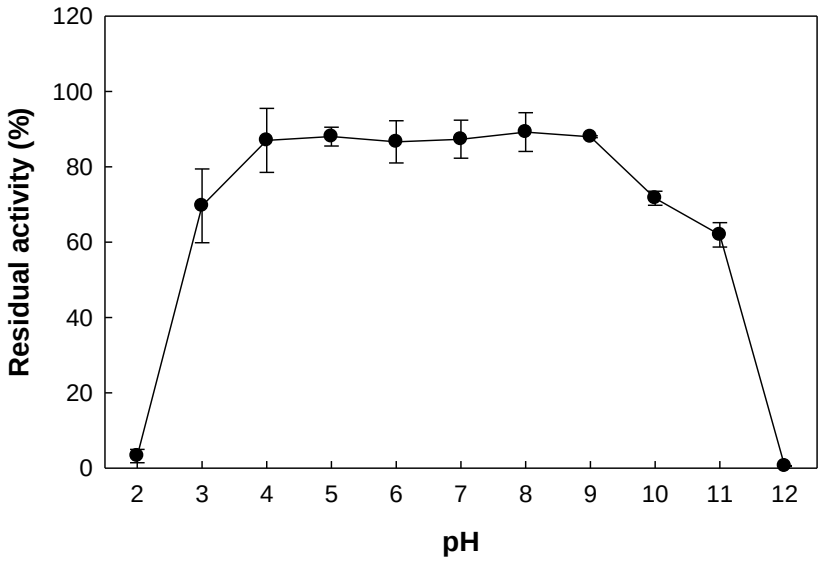


Fig. 7. Effect of pH on the stability of the purified LOX from Jinuni bean.

3-3-2. Optimum temperature and stability

The optimal temperature of purified LOX from Jinuni bean was measured at ranging from 5.0 to 45.0℃ with linoleic acid as a substrate. The optimal temperature was 15.0℃ (Fig. 8). At 5^{o}C, 74.65% relative activity was observed and at 45℃, only 3.32% relative activity. The optimal temperature of LOX from banana leaf (Giant Cavendishii, AAA) was 40℃ (Kuo J.M., 2006), avocado (Persea americana Mill, cv. Hass) was 40℃ (Daniel A. J.V., 2010) and wheat germ was 45℃ (Xu B., 2012). The optimal temperature of Jinuni bean LOX was relatively low when compared with other LOX. The temperature stability was performed by pre-incubating Jinuni bean LOX at different temperature values for 30 min and assayed for LOX activity. The stability was shown in Figure 9. The Jinuni bean LOX was stable below 50.0℃. The residual activity after pre-incubation at temperature over 60℃ rapidly decreased.

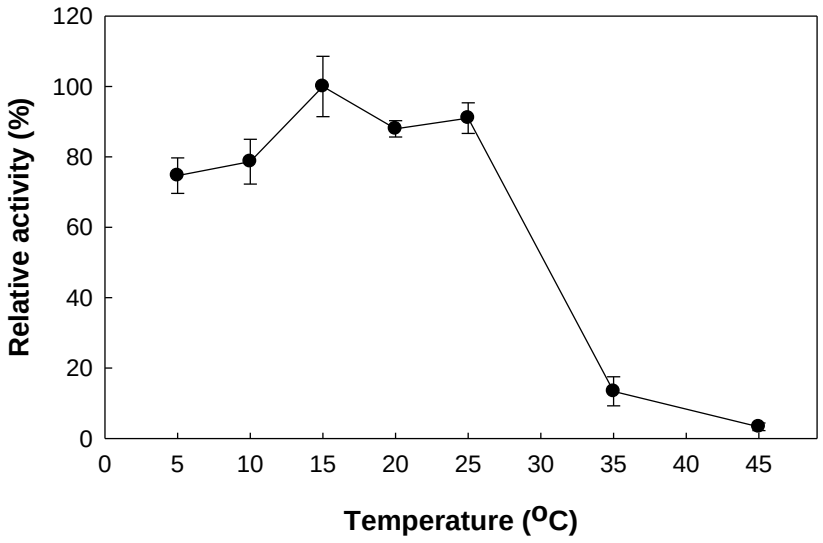


Fig. 8. Effect of temperature on the LOX activity purified from Jinuni bean.

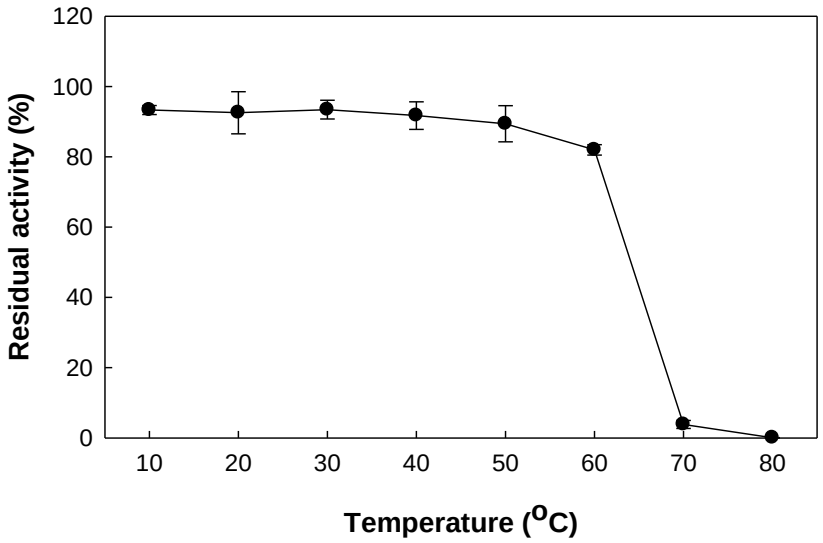


Fig. 9. Effect of temperature on the stability of the purified LOX from Jinuni bean.

3-4. Substrate and positional specificity

Since LOX catalyzes hydroperoxidation of polyunsaturated fatty acids (PUFA) possessing a *cis*,*cis*-1,4-pentadiene unit, fatty acids with chain of 18 carbons and single, double and triple bonds can serve as substrates of LOX. The relative LOX activity of these fatty acids was assayed at optimum pH and temperature conditions (Fig. 10). The purified LOX from Jinuni bean showed the highest relative activity toward linoleic acid (18:2) and linolenic acid (18:3) was 92.91%. One the other hand, oleic acid (18:1) as substrate of LOX showed almost zero activity (1.30%).

The NP-HPLC analysis was performed to characterize the positional specificity of product produced by LOX from Jinuni bean (Fig. 14). The NP-HPLC elution profile containing only *n*-hexane solvent showed one peak at 2.88 min (Figure 11A) and the retention time of standard 13-HPOD was 16.69 min (Figure 11B). The NP-HPLC elution profile of the product produced using linoleic acid as substrate by LOX from Jinuni bean showed major peak at 16.58 min (Fig. 11C). The positional specificity of LOX from Jinuni bean was showed at 13-LOX produced 13(S)-Hydroperoxy-9,11-octadecadienoic acid.

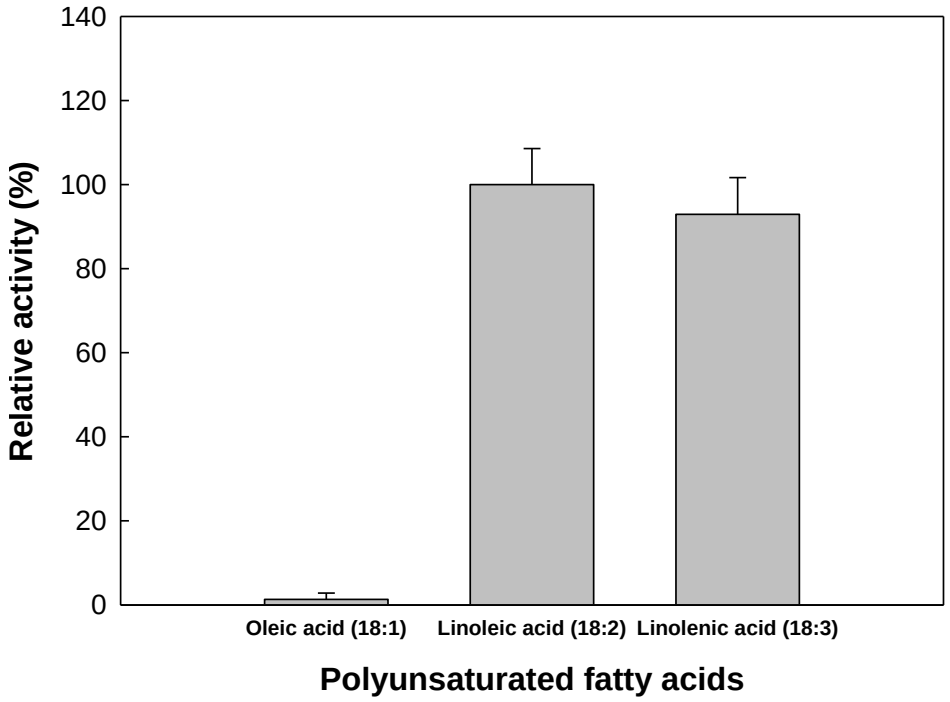


Fig. 10. Substrate specificity from polyunsaturated fatty acid reacted with purified LOX from Jinuni bean.

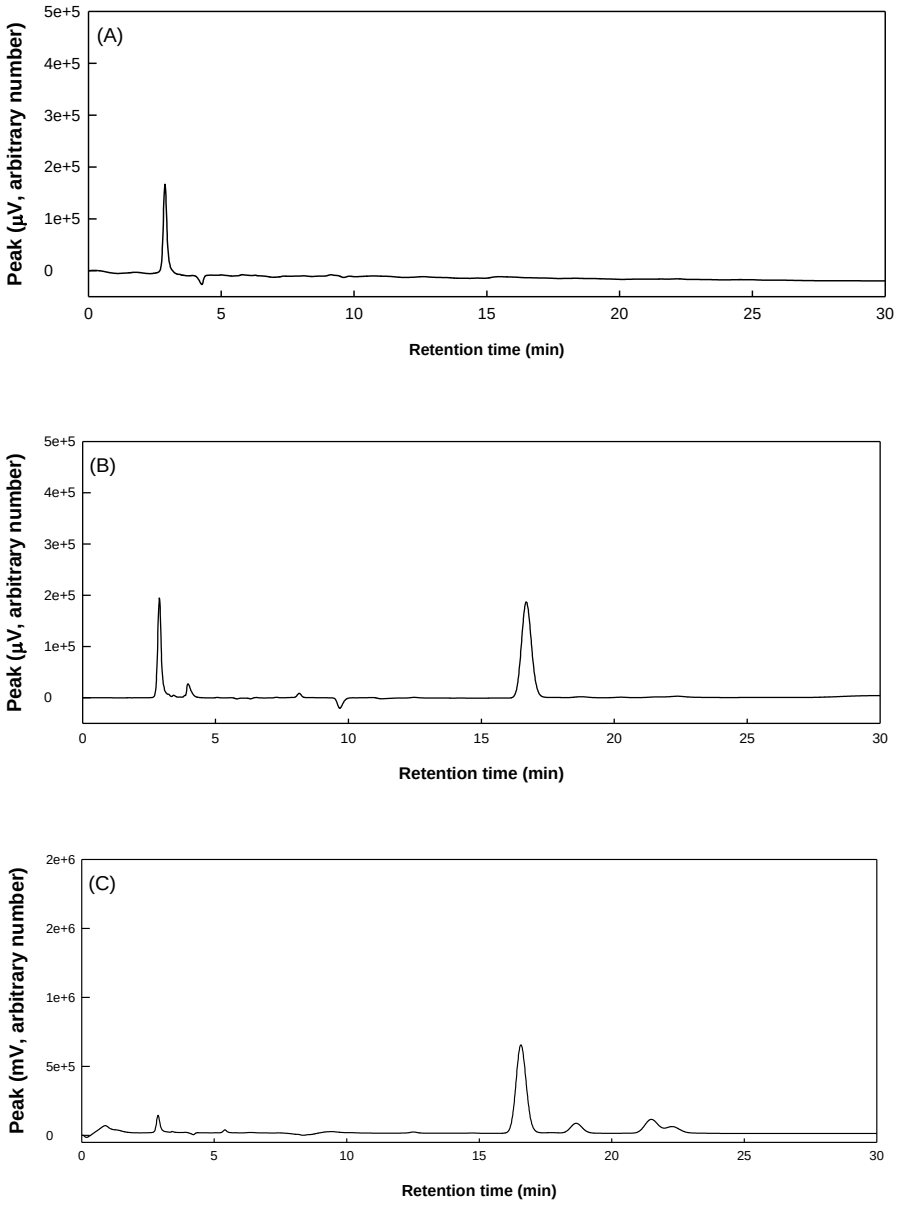


Fig. 11. NP-HPLC chromatogram (A) *n*-hexane, (B) standard of 13-HPODE, (C) α-linolenic acid treated with purified LOX of Jinuni bean.

3-5. Amino acid analysis

The amino acid composition of LOX from Jinuni bean is shown in Table 2, which reveals that, a total of 16 amino acids were recorded in the Jinuni bean LOX. The number of residues of each amino acid was calculated on the basis of a monomer molecular weight of 66 kDa and the cysteine and tryptophan residue content were not detectable by the used methods. The most abundant essential amino acid in the Jinuni bean LOX was glycine (Gly), recorded at 13.18%. The total number of amino acids is thus 546.

Table 2. Amino acid composition of LOX from Jinuni bean

Amino acid	Integer	Amino acid	Integer
Aspartate (Asp)	62	Valine (Val)	30
Glutamate (Glu)	54	Methionine (Met)	6
Serine (Ser)	53	Phenylalanine (Phe)	16
Histidine (His)	11	Isoleucine (Ile)	23
Glycine (Gly)	72	Leucine (Leu)	52
Threonine (Thr)	33	Lysine (Lys)	6
Arginine (Arg)	19	Proline (Pro)	30
Alanine (Ala)	63	Cysteine[a] (Cys)	nd
Tyrosine (Tyr)	14	Tryptophan[a] (Trp)	nd

[a]Cysteine and tryptophan residue content was not detectable by the used methods.

3-6. Secondary structure contents

Far-UV CD spectra of LOX from Jinuni bean recorded between 190 and 260 nm (Fig. 12). The far-UV CD of a protein generally reflects its secondary structure content. α-Helix proteins have negative bands at 222 and 208 nm and a positive band at 193 nm and proteins with β-sheet have a negative band at 218 nm and a positive band at 195 nm, whereas disordered proteins such as β-turn and random coil have negative bands at 189 nm and 198 nm and positive bands at 210 nm and 212 nm, respectively (Greenfield N.J., 2007). The secondary structures of LOX from Jinuni bean consist of α-helix (26.2%), antiparallel β-sheet (17.6%), parallel β-sheet (9.9%), β-turn (18.5%) and random coil (35.4%) (Table 3).

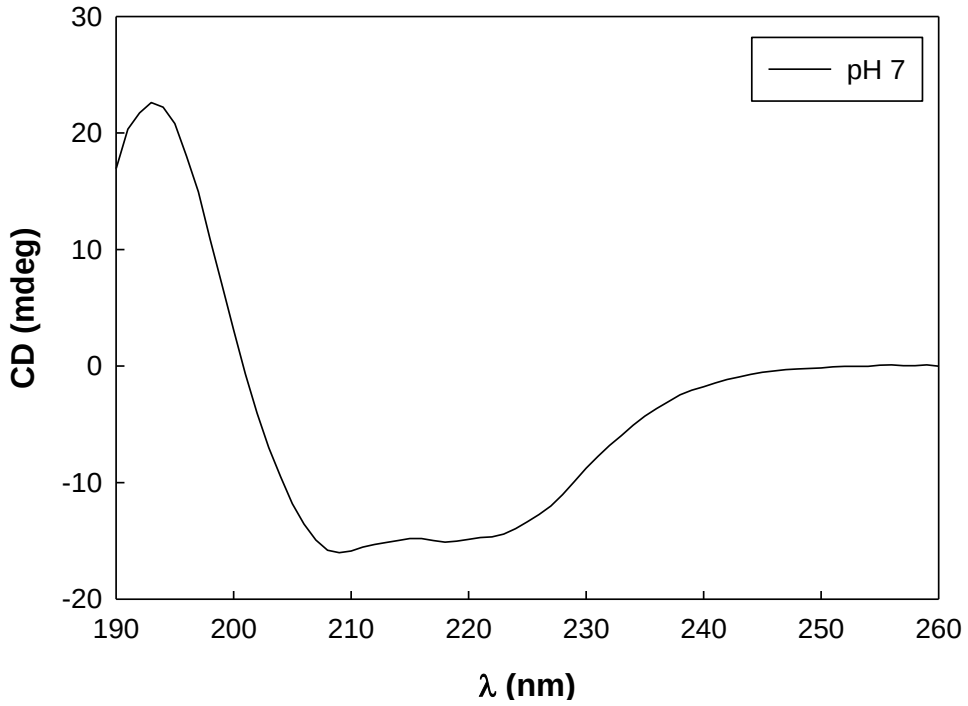


Fig. 12. Far-UV CD spectrum of LOX from Jinuni bean at pH 7.0.

Table 3. The secondary structure contents of LOX from Jinuni bean

Secondary structure	Contents (%)
α- Helix	26.2
Antiparallel β-sheet	17.6
Parallel β-sheet	9.9
Beta-turn	18.5
Random coil	35.4

3.7. Kinetics of purified LOX from Jinuni bean

The V_{max}, K_m, k_{cat} and k_{cat}/K_m values were determined from Hanes-Woolf plot under optimum conditions of LOX activity (Fig. 13). The V_{max}, K_m, k_{cat} and k_{cat}/K_m values are 22.07 μmol•min^{-1}•mg^{-1}, 11.80 mM, 24.18 s^{-1} and 2.05 s^{-1}•mM^{-1}, respectively. The K_m value of LOX from Pea Seeds (Reynolds P. A, 1982) was 0.20 mM at pH 9.0 and from Banana Leaf (Kuo J.M, 2006) was 0.15 mM. The K_m value signified substrate affinity of Jinuni bean LOX was relatively high when compared with other LOX.

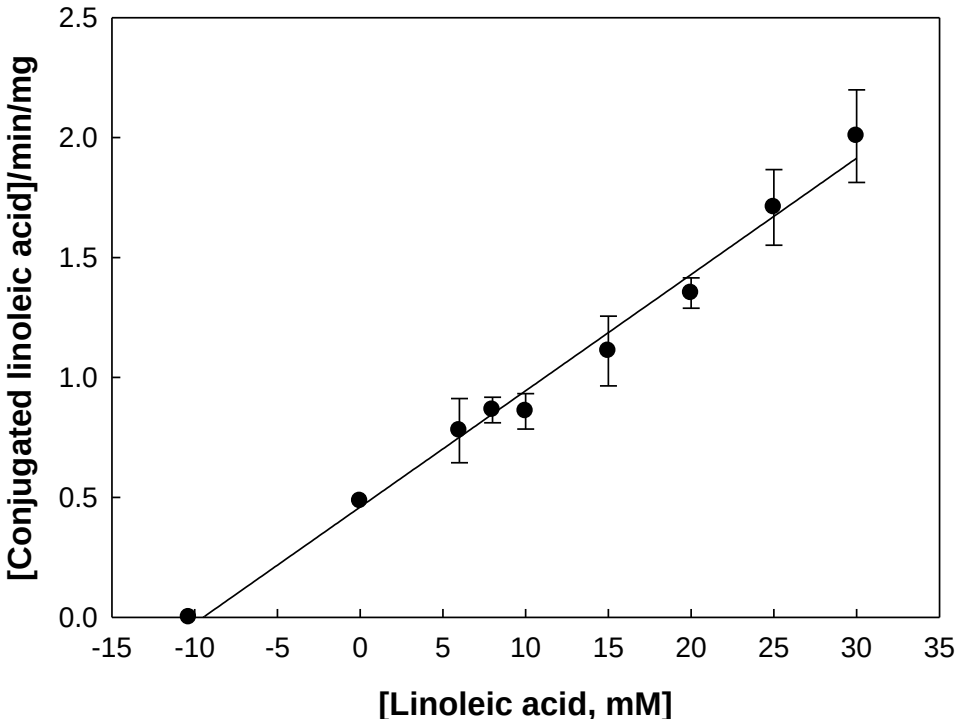


Fig. 13. Hanes-Woolf plot of the initial rate of 13-HPOD formation catalyzed by Jinuni bean LOX at optimum conditions according linoleic acid concentration.

4. Conclusions

This study was performed for purification and characterization of Jinuni bean LOX using modified AOT/isooctane reversed micellar system. This method was used to characterize LOX from Jinuni bean in terms of optimum pH and temperature, pH and thermal stability and kinetic parameters. The LOX from Jinuni bean differ from previously characterized other plants in many respects. The molecular weight of Jinuni bean LOX (66 kDa) was relatively small when compared with other LOX (e.g. almond LOX; 98 kDa, Giovanni M., 2001, banana leaf LOX; 85 kDa, Kuo J.M., 2006, and durum wheat semolina; 95 kDa, Roberto B, 1999). The optimal temperature of Jinuni bean LOX was relatively low when compared with other LOX. The purified LOX from Jinuni bean showed that the pH and temperature for optimum activity were pH 8.0 and 15℃, respectively. The LOX from Jinuni bean was stable at pH 4.0-9.0 and below 50℃. Among the substrate specificity tested, linoleic acid (18:2) showed the highest relative activity and the kinetic parameters of LOX, V_{max}, K_m, k_{cat} and $k_{cat}/K_{m,}$ values were 22.07 μmol•min^{-1}•mg^{-1}, 11.80 mM, 24.18 s^{-1}, and 2.05 s^{-1}•mM^{-1}, respectively. These results suggest that reaction of Jinuni bean LOX will be able to control and further studies on the inhibitors will contribute to solving the problem of LOX activity regulation in food industry.

5. References

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국문초록

식물체 내에서 지질산화 반응에 관여하는 대표적인 효소인 lipoxygenases(LOXs)는 식물체의 성장 및 외부 스트레스에 대한 방어물질을 생성하지만, 식품공학적 측면에서는 색을 변질 시키고 이미·이취 유발 및 관련 영양성분을 산화시킴으로써 유통 및 저장기간을 단축시키는 부정적인 요인으로 작용하는 것으로 알려져 있다. 곡류, 서류, 콩류, 견과류, 과실류, 채소류 및 버섯류 등에 존재하는 LOXs 의 특성 조사를 통하여 상대적으로 활성이 높고, 다량의 생리활성 성분을 함유하고 있는 쥐눈이콩(서목태)을 선정하여 부정적인 요인을 제어하기 위한 기초 연구로써, LOXs 를 분리•정제하여 그 특성을 규명하고자 하였다. 또한, 본 연구에서는 LOX 의 활성측정은 역미셀계 내에서의 새로운 분석법을 도입함으로써, 빠르고 간편하게 효소의 활성을 측정할 수 있도록 하였다. 쥐눈이콩으로부터 LOX 을 황산 암모늄 분획(20-60%), DEAE-Sepharose 와 ANX-sepharose ion exchange chromatography, sephacryl S-100 gel permeation chromatography 을 이용하여 정제한 결과 3.47%의 수율 및 13.07 의 정제도를 나타냈다. 정제된 LOX 의 분자량은 SDS-PAGE 및 gel permeation chromatography 를 통하여 약 66 KDa 으로 판명되었다. Linoleic acid 를 기질로 사용하였을 때, 최적의 반응조건은 각각 pH 8.0, 온도 15℃로 확인되었으며, pH 4.0-9.0 및 50℃이하에서 효소의 안정성이 높은 것으로 나타났다. 쥐눈이콩 유래 정제된 LOX 는 13 번 위치에 hydroperoxy 유도체를 생성하는 13-LOX 로 판명되었으며, 정제된 LOX 단백질의 2 차 구조는 α-helix (26.2%), antiparallel β-sheet (17.6%), parallel β-sheet (9.9%), β-turn (18.5%) 그리고 random coil (35.4%)의 구조 등을 이루고 있는 것을 circular dichroism (CD)를 통해 확인하였다. 효소의 동력학적 상수를

분석한 결과 V_{max}, K_m, k_{cat}, k_{cat}/K_m 값은 각각 22.07 μmol•min^{-1}•mg^{-1}, 11.80 mM, 24.18 s^{-1}, 2.05 s^{-1}•mM^{-1} 으로 나타났다. 이러한 결과를 바탕으로 쥐눈이콩 유래 LOX 의 활성을 저해 할 수 있는 저해제 탐색을 통하여 식품가공적 측면에서의 부정적인 요인을 제어할 수 있을 것으로 사료된다.