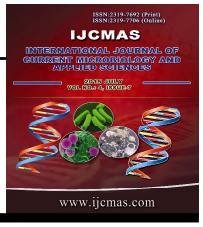
Int.J.Curr.Microbiol.App.Sci (2015) 4(8): 621-628

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 4 Number 8 (2015) pp. 621-628**
http://www.ijcmas.com



Original Research Article

Enzymatic Activity Levels of Lipoxygenase (Lox) in Germinating Green Gram Seeds (V*igna radiate*)

Trupthi Deshpande, A. Sai Padma*, M.K. Sukumaran and D. Rajani

Department of Biochemistry, Bhavan's Vivekananda College, Sainikpuri, Secunderabad, India
**Corresponding author*

Keywords

Lipoxygenase (Lox), Green Gram Seeds, *Vigna radiate*, SDS-PAGE, Arachidonic acid (AA)

A B S T R A C T

Present study describes the enzymatic activity of lipoxygenase (LOX) in germinating green gram seeds. The enzyme activity gradually increased from day 2 of germination and was optimum on day 4 and declined thereafter. On incubation of green gram LOX with arachidonic acid (AA), the LOX product formed was identified as 15-hydroperoxyeicosatetraenoic acid (15-HPETE) based on TLC technique. Maximum absorbance for the LOX product by spectrophotometric analysis was found at 230 nm, a characteristic wavelength for HPETEs. The product concentration was high on 4th day of germination, which is also reflected by highest enzyme activity and protein content on that day. Based on the formation of 15-HPETE on incubation with AA, green gram LOX was confirmed as 15-LOX. Immunoblot analysis revealed that there was no sequence homology between green gram and animal 15-LOX. The relative molecular weight of the LOX enzyme as determined by SDS-PAGE analysis was 51,000 Daltons.

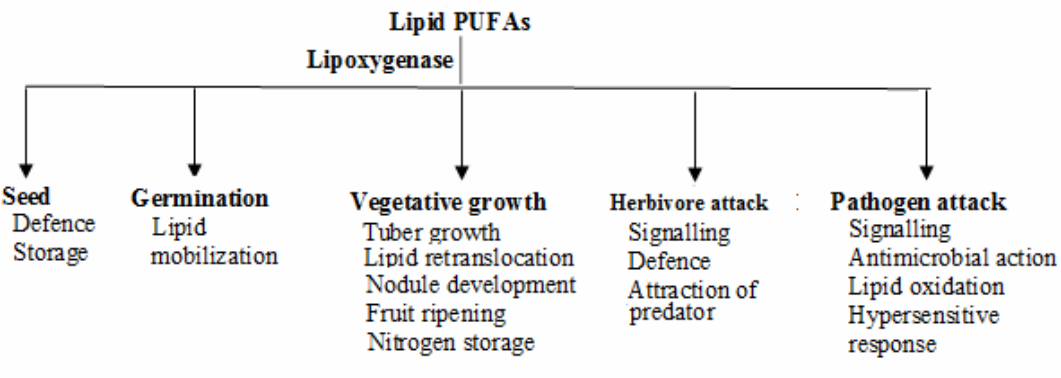
Introduction

Lipoxygenases

Lipoxygenases (LOXs; EC 1.13.11.12) are non-heme iron containing dioxygenases that are widely distributed in plants and animals. LOX initiates the synthesis of a group of acyclic or cyclic compounds collectively called 'oxylipins', which are products of fatty acid oxidation with diverse functions in the plant cells (Helena Porta and Mario Rocha-Sosa, 2002).

The products of the LOX pathway in normal vegetative growth performs various functions like signaling in wounding and pathogen attack, tendril coiling, attractors to enemies of herbivores, antimicrobial, antifungal and induction of cell death (Siedow, 1991; Kolomiets *et al.*, 2001). LOX has also been associated with some processes in a number of developmental stages; one of the functions is mobilization of storage lipids during germination (Feussner *et al.*, 2001). Lipoxygenases also play a vital role in other developmental processes like fruit ripening and senescence.

LOXs have active roles in several processes during plant life (Helena Porta and Mario Rocha-Sosa, 2002)



Lipoxygenases catalysis

Lipoxygenases catalyze the addition of molecular oxygen to polyunsaturated fatty acids containing a (cis, cis)-1, 4-pentadiene system to yield an unsaturated fatty acid hydroperoxides. Oxygen can be added to either of the pentadiene system with high stereo specificity, and in the case of linolenic and α-linolenic acids, result in the formation of 9(S) - or 13(S)-hydroperoxy derivatives or both depending on the specific iso-form of the enzyme (Siedow, 1991).

In vitro, most LOXs prefer free fatty acids, though it has been shown that sterified fatty acids are also the substrates for LOX *in vivo*, suggesting that membrane lipids could be the substrates for oxylipins biosynthesis.

The hydroperoxy fatty acid products of the LOX reaction are substrates of at least seven different enzyme families. Signaling compounds such as jasmonates (triggers gene activation during wound responses in plants), antimicrobial and antifungal compounds such as leaf aldehydes or divinyl ethers, and a plant specific blend of volatiles including leaf alcohols are among the numerous products.

Role of lipoxygenase in seed germination

LOX are normally present in the seeds of plants. During germination, the levels of LOX enzyme were found to increase which suggests that the enzyme may play a physiological role during maturation process (Devi P. Uma Maheswari *et al.*, 2005). Maximal accumulation of LOX protein and the corresponding mRNAs lasts from a few hours to few days after germination and the mRNAs synthesized during germination could also be found in the mature plants. Their levels were increased by the application of abscisic acid and jasmonic acid, or by stresses such as wounding, pathogen infection, or water deficit (Park *et al.*, 1994).

Presence of lipoxygenase contributes to favour emergence speed of soybean seedlings in germinating soybean seeds although lipoxygenases were found not to be involved in lipid mobilization (Wang *et al.*, 1999). The lack of substantial oxygenation of polyunsaturated fatty acids during the germination process supports the idea that the soybean seed LOX might have been recruited to function as storage proteins despite their intact but obsolete enzymatic capacity.

The presence of LOX in the germinative process was also detected in pea seeds (*Pisum sativum* L), in which there is a shift in LOX activity from radical to shoot that accompanies the transition from seed lipoxygenases to vegetative lipoxygenases (Mo and Koster, 2006).

Keeping in view of the above findings, the present study was aimed to analyze and compare the levels of lipoxygenase enzyme activity on different days after the initiation of Green gram seed germination.

Materials and Methods

Seed germination

The green gram seeds were surface sterilized with 0.1% mercuric chloride for few minutes and washed with autoclaved water. The above treatment was repeated twice and the seeds were allowed to germinate under constant light and ambient temperature for a maximum of 8 days.

Preparation of crude extract

100g of non-germinating and germinating green gram seeds (day 0, 2, 4, 6 and 8 of germination) were homogenized in 100mM potassium phosphate buffer pH 6.3 containing 2mM ascorbic acid, 2mM EDTA, and 5mM sodium metabisulphite. The resulting homogenate was passed through two layers of cheesecloth and the extract was subjected to centrifugation at 10,000 rpm for 20 min at 4°C. The clear supernatant thus obtained was used as crude enzyme for estimation of lipoxygenase enzyme activity.

Ammonium sulphate fractionation

The above crude enzyme extract was subjected to a 45% ammonium sulphate fractionation. The ammonium sulphate fractionated enzyme extract was centrifuged at 10,000 rpm for 15 min at 4^0C. After centrifugation, the supernatant was discarded and the pellet was resuspended in 50mM phosphate buffer, pH 6.3 and the contents were dialyzed against the same buffer. After dialysis, the enzyme extract was immediately used for further analysis or stored frozen until use. Protein concentrations were measured by Lowry method (Lowry *et al.,* 1951).

Spectrophotometric enzyme assay

Enzyme activity was measured spectrophotometrically basically following the procedure as described by Ben Aziz *et al* (Ben Aziz *et al.,* 1970). The reaction mixture contained ~40µg of green gram LOX in 100mM phosphate, pH 6.3 buffer at room temperature. The reaction was initiated by addition of 250µM AA and followed for 1 min. The enzyme activity was measured at 235 nm in a UV-Vis spectrophotometer and enzyme activity is defined as µmoles of hydroperoxide formed per min/mL of enzyme.

$$\text{Enzyme Activity} = \frac{\text{Volume of reaction mixture} \times \text{absorbance difference}}{\varepsilon \times \text{volume of enzyme}}$$

Where ε = 27,500.

SDS-PAGE analysis

SDS-PAGE was essentially carried out as described by Laemmli (1970). 100µg of LOX (45% Ammonium sulphate fractionated) obtained on days 0,2,4,6 and 8 days of germination was precipitated with 100% TCA at 40C for 1-2 h. The pellet was washed twice with 100% ethanol and dissolved in 30 µL of SDS sample buffer. The sample was boiled for 5 min and

electrophoreses was carried out at 100V along with standard protein markers. After completion, the gel was subjected to Coomassie Brilliant Blue R 250 staining.

Western blotting

Western blot analysis was carried out for the 100% TCA precipitated LOX as described by Towbin *et al.* (1979). Proteins from the gel were electro-blotted onto nitrocellulose membrane and treated with rabbit polyclonal 15-LOX specific antibodies and developed with goat anti-rabbit IgG-HRP conjugate and TMB/H_2O_2 system.

Extraction of LOX products

The active enzyme was incubated with 250 µM AA in 100mM potassium phosphate buffer, pH 6.3 for 2 min in 100 ml reactions. The reaction was terminated by acidifying the reaction mixture with 6 N HCl. The products were extracted with hexane**:** ether in a 1**:**1 ratio and the organic phase was passed through anhydrous granular sodium sulphate. The sample was evaporated under nitrogen gas and the residue was dissolved in methanol and analyzed further.

Separation and identification of LOX products of AA

The LOX products (0, 2, 4, 6 and 8 days of germination) were separated on TLC with the mobile phase of hexane **:** ether **:** acetic acid in the ratio 40**:**60**:**1 and the separated products were detected by exposing the plates to iodine vapours. LOX products were compared with the standard HPETEs that were treated similarly.

Determining of absorption maxima for LOX Products

The extracted compounds were subjected for wavelength scan between 200 and 300 nm in a UV-Vis spectrophotometer using methanol as blank.

Results and Discussion

Enzyme assay

LOX activity was found to increase gradually over a period of 60 sec (Figure 1). The activity increased with the onset of germination followed by a marked increase on 4th day and decreased during subsequent stages of germination. The enzyme activity was found to be 5709µmoles/min/ml on day 4 (Table 1 & Figure 2). Products were not formed on heat inactivation of enzyme (data not shown).

SDS-PAGE analysis of LOX

The LOX enzyme in 45% ammonium sulphate fractionated extract was analyzed using SDS-PAGE. A band corresponding to molecular weight 51,000 Daltons was observed as the major band. Similar to the increase in the LOX activity after the onset of germination, the protein content (band corresponding to 51,000 Daltons) increased with the onset of germination followed by a marked increase on 4th day and decreased during subsequent stages of germination. The high protein content could reflect synthesis of high amounts of LOX during germination as observed in spectrophotometric analysis. The molecular weight of the LOX corresponded to 51,000 Daltons (Figure 3).

Immunoblot of LOX

It was observed that there was no cross reactivity of green gram LOX with animal 15-LOX antibodies (figure not shown since there was no band developed against control animal 15-LOX).

Table.1 Spectrophotometric analysis of LOX activity on different days of germination

Day of germination	0	2	4	6	8
Difference in absorbance from 1 min to 0 min.	0.16	0.53	1.57	0.90	0.42
Enzyme activity (µmoles/min/ml)	581.8	1927.2	5709	3272	1527

Figure.1 Assay of green gram lipoxygenase on day 4 of germination

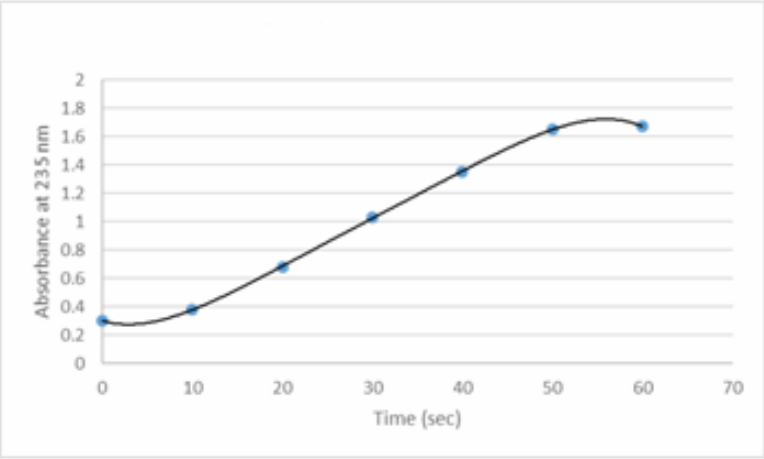


Figure.2 Spectrophotometric analysis of LOX activity on different days of germination

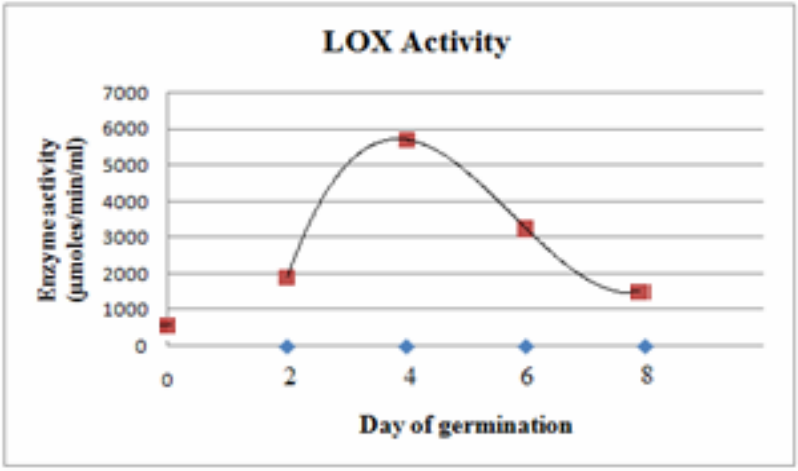
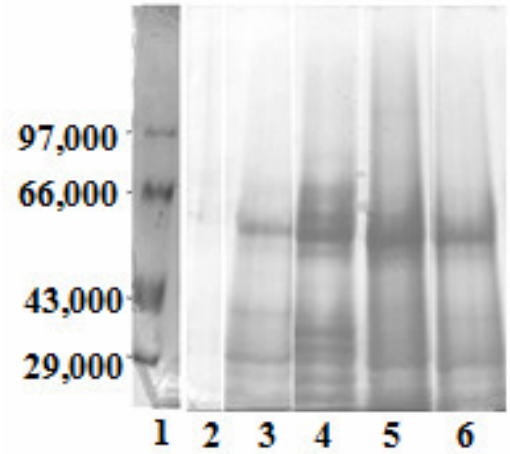


Figure.3 SDS-PAGE Analysis of Green Gram LOX on 0, 2, 4, 6, 8 days of germination



Lane 1, standard protein molecular marker, Lane 2 – 6: LOX enzyme on 0, 2, 4, 6, 8 days of germination.

Figure.4 Spectral analysis of LOX products

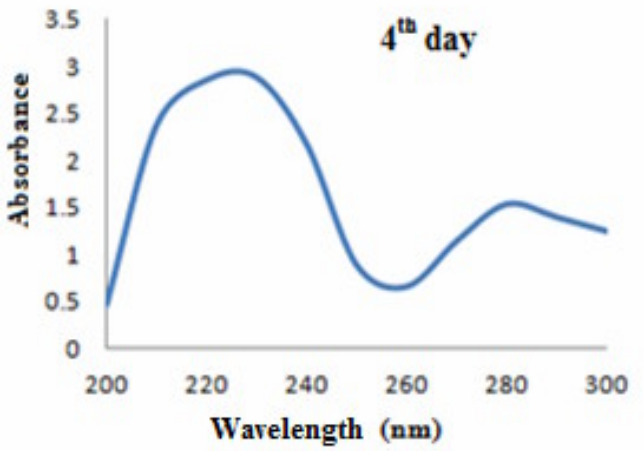
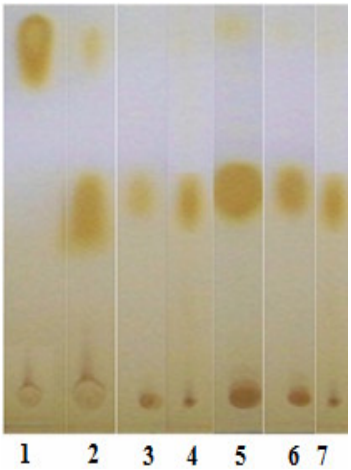


Figure.5 TLC analysis LOX products on different days of germination



Lane 1, Arachidonic acid; Lane 2, standard 15-HPETE, Lane3-7, LOX products at different days of germination: lane 3 –day 0, lane 4-day 2, lane 5-day 4, lane 6-day 6, lane 7-day 8

This clearly suggested that the sequence homology between plant LOX and animal LOX might be entirely different even though they produce the same type of products i.e., 15-HPETEs. This observation is in accordance with earlier reports (Fleming *et al.,* 1989; Funk *et al.,* 1990; Sigal *et al.,* 1998 and Yoshimoto *et al.,* 1990).

Spectral analysis of LOX products

The 45% ammonium sulphate fractionated protein obtained on different days of germination was analyzed for LOX activity spectrophotometrically using AA as substrate. The results of spectrophotometer analysis are shown in figure 4. The optimum absorbance in all the samples examined was obtained at 230nm, which confirms the presence of HPETE products.

TLC analysis of LOX products

The LOX enzyme from different days of germination gave a single product on TLC which co-migrated with the standard 15-HPETE (Lane 2, Figure 5). This result coincides with higher LOX activity and protein content on day 4 after initiation of seed germination (Lane 5, Figure 5). This result has also confirmed that green gram lipoxygenase is of type 15-LOX, which is in support to the earlier reports (Kiran *et al.,* 1992).

In the present study on the comparison of levels of lipoxygenase enzyme activity and its product analysis from germinating Green gram seeds, the following conclusions can be drawn.

The enzyme activity was found to be increased from day 2 of germination and was optimum on 4th day. The enzyme activity was reported as 5709 µmoles/min/ml on day 4, which showed maximum activity. The approximate molecular weight of the LOX protein as determined by SDS-PAGE analysis was 51,000 Daltons. The Immunoblot analysis showed no structural similarities among green gram and animal 15-LOX. The optimum spectrophotometric absorbance for the LOX products of different days of germination was found to be 230nm, confirming the products are HPETEs. The products concentration was high on 4th day of germination, which in turn reflects highest enzyme activity and protein content on that day. TLC analysis of LOX products has confirmed that the LOX enzyme of green gram is of type 15-LOX.

\

Acknowledgment

Authors are thankful to Management and Prof. Y. Ashok, Principal of Bhavan's Vivekananda College for providing necessary facilities and constant encouragement.

References

Ben Aziz, S., Grossman, I.A., Budowoki, B. 1970. Linoleate oxidation induced by lipoxygenase and heme proteins: A direct spectrophotometric assay. *Anal. Biochem.,* 34: 88–100.

Devi P. Uma Maheswari, Lakshmi K. Vijaya, Reddy K. Janardhan, Reddanna, P. 2005. Differential expression of upoxygenase in gree gram (*Vigna radiata* L.). *Legume Res.,* 28: 87–93.

Feussner, I., Ku¨hn, H., Wasternack, C. 2001. Lipoxygenase-dependent degradation of storage lipids. *Trends Plant Sci.,* 6: 268–273.

Fleming, J., Thiele, B.J., Chester, J., Oprey, J., Janetzki, S., Aitken, A., Anton, I.A., Rapoport, S.M., Harisson, P.R. 1989. The complete sequence of the rabbit erythroid cell-specific 15-lipoxygenase

mRNA: comparison of the predicted amino acid sequence of the erythrocyte lipoxygenase with other lipoxygenases. *Gene*, 79: 181–188.

Funk, C.D., Furci, L., Fitzgerald, G.A. 1990. Molecular cloning, primary structure, and expression of the human platelet/erythroleukemia cell 12-lipoxygenase, *Proc. Natl. Acad. Sci. U.S.A.*, 87: 5638–5642.

Helena Porta, Mario Rocha-Sosa, 2002. Plant lipoxygenases: Physiological and molecular features. *Plant Physiol.*, 130: 15–21.

Kiran, K.Y.V., Sailesh, S., Prasad, M., Reddanna, P. 1992. Identification and product profiles of some plant lipoxygenases. *Biochem. Arch.*, 8: 17-22.

Kolomiets, M.V., Hannapel, D.J., Chen, H., Tymeson, M., Gladon, R.J. 2001. Lipoxygenase is involved in the control of potato tuber development. *Plant Cell*, 13: 613–626.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227: 680–685.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randal, R.L. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, 193: 265–275.

Mo, B., Koster, K.L. 2006. Changes in lipoxygenase isoforms during germination and early seedling growth of *Pisum sativum L. Seed Sci. Res.*, 16: 97–106.

Park, T.K., Holland, M.A., Laskey, J.G., Polacco, J.C. 1994. Germination-associated lipoxygenase transcripts persist in maturing soybean plants and are induced by jasmonate. *Plant Sci.*, 96: 109–117.

Siedow, J.N. 1991. Plant lipoxygenase: structure and function. *Ann. Rev. Plant Physiol. Plant Mol. Biol.*, 42: 145–188.

Sigal, E., Craik, C.S., Highland, E., Grunberger, D., Costello, L., Dioxon, R.A.F., Nadel, J.A. 1988. Molecular cloning and primary structure of human 15-lipoxygenase, *Biochem. Biophys. Res. Commun.*, 157: 457–464.

Towbin, H., Staehelin, T., Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. (USA)*, 76: 4350–4354.

Wang, C., Croft, K.P.C., Jarlfors, U., Hildebrand, D.F. 1999. Subcellular localization studies indicate that lipoxygenases 1 to 6 are not involved in lipid mobilization during soybean germination. *Plant Physiol.*, 120: 227–236.

Yoshimoto, T., Suzuki, H., Yamamoto, S., Takai, T., Yokoyama, C., Tanabe, T. 1990. Cloning and sequence analysis of the cDNA for arachidonate 12-lipoxygenase of porcine leukocytes, *Proc. Natl. Acad. Sci. U.S.A.*, 87: 2142–2146.