

## Isolation and Characterization of Lipoxygenase from *Fusarium oxysporum*

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### Abstract

*Lipoxygenase was isolated from cultured fungus Fusarium oxysporum and partially purified by ammonium sulphate precipitation, dialysis and ion-exchange chromatography on DEAE – cellulose column. The enzyme was partially purified 3.9 fold with 26% recovery of enzyme activity. The isoelectric point of the enzyme was pH 5.0±1.19 and its K<sub>m</sub> was 1.7 x 10<sup>-4</sup>M, while the V<sub>max</sub> was 0.12 µmole/min. The optimum pH was 8.0 and the optimum temperature was 35°C. The enzyme was most stable at 50°C.*

**Keywords:** *Fusarium oxysporum*, lipoxygenase, linoleic acid

### Introduction

Lipoxygenase (1.99.2.1) is an enzyme that, in the presence of oxygen, catalyses the oxidation of unsaturated fatty acids having cis, cis 1, 4-pentadiene structures (such as linoleic, linolenic, and arachidonic acids) to produce cis, trans – conjugated diene hydroperoxides as primary products (Dolev *et al.*, 1967; Hsu *et al.*, 1998; Diel and Star, 2000). Lipoxygenase initiates free radical chain reaction in lipids (Tappel *et al.*, 1953; Stevens *et al.*, 1970; Srivastava *et al.*, 2003). Its primary products, cis, trans-conjugated diene hydroperoxides and the secondary products such as aldehydes, ketones, alcohols and short hydrocarbons are the causes of either desirable flavours like fresh-vegetable flavours and scents of flowers or undesirable off-flavours that occur in stored and processed foods (Sessa, 1979; Frankel, 1984; Sicdow, 2000).

*Fusarium oxysporum*, on the other hand, is a mould found in the soil, decaying foods, decayed plants and animals (Prescott and Harley, 1999). It is characterized by its multicellular sickle – shaped conidia that are borne on short conidiophores. Its body is filamentous and branched with spiral microconidia. The macroconidia are multiseptate and usually crescent – shaped (Pelczar *et al.*, 1995).

Lipoxygenase was initially found in soybean and later in beans, wheat, radish, white lupine, alfalfa, potatoes, in a variety of legumes and in algae (Beneytout *et al.*, 1989). The enzyme had been used to bleach carotene (Tookey *et al.*, 1958; Rosa, 2003) yellow wheat flour to white one (Furia, 1975) and browned yam tubers to their original white or yellow colour (Anokwulu, 2001). There is the need therefore to find most economic ways of getting lipoxygenase for biotechnology. The present work investigates the presence of lipoxygenase in *F. oxysporum*; characterizes the enzyme and finds at what concentration it exists in the microorganism.

### Materials and Methods

**Collection of soil samples:** Soil samples used in the isolation of the fungi were collected from an agricultural farm in Nsukka. The soil samples were sieved with a 2 mm mesh to remove large particles.

**Isolation of *Fusarium oxysporum*:** *F. oxysporum* was isolated from a sandy loam soil. Soil samples (10.0g each) were placed in 250 ml Erlenmeyer flasks containing 90 ml of distilled water and shaken for about 2 min and allowed to settle. Soil suspension (1.0 ml) was inoculated in 9 ml of sterile basal medium of the following composition: NaNO<sub>3</sub>, 0.5%; MgSO<sub>4</sub>.7H<sub>2</sub>O, 0.05%; KH<sub>2</sub>PO<sub>4</sub>, 0.5%; (pH 6.0); soybean oil, 5%. Cultivation was carried out with shaking on a mechanical shaker at 27°C for one week. An aliquot (0.1 ml) of the culture was spread on sabourand dextrose agar (SDA) plates and incubated at room temperature for one week. Fungi that grew in the plates were subcultured and purified on SDA plates.

**Identification of *Fusarium oxysporum*:** The isolate selected for lipoxygenase study, namely, *F. oxysporum*, was characterized and identified based on the schemes of Smith (1971). All experiments were carried out in triplicate.

**Extraction of crude lipoxygenase:** The isolated *F. oxysporum* was precultured without shaking in 100 ml of the basal medium at 27°C for a week. The cells were collected with cheese – cloth, washed with 50 mM phosphate buffer pH 7.0 and ground in a mortar with washed sand and the phosphate buffer. The homogenate was filtered through cheese – cloth and centrifuged at 2000 rpm for 30 min at 5°C. The supernatant which was the crude enzyme was stored at – 20°C.

**Purification of crude enzyme:** Crystallized ammonium sulphate was added slowly to the crude enzyme with stirring to 30% saturation. The precipitate was dissolved in 50 mM phosphate buffer pH 7.0 and dialysed for 18 h against 200 ml

**Table 1: Purification profile of *Fusarium oxysporum* lipoxygenase**

Purification step	Total protein (mg/ml)	Total Activity (unit/ml)	Specific activity (units/mg protein)	Yield (%)	Purification (fold)
Crude lipoxygenase extract	11.66	$3.6 \times 10^{-2}$	$3.1 \times 10^{-3}$	100	1.00
30% Ammonium sulphate precipitation	9.84	$5.9 \times 10^{-2}$	$4.8 \times 10^{-3}$	68	1.2
Dialysis	5.65	$7.1 \times 10^{-2}$	$6.4 \times 10^{-3}$	47	2.5
Ion-exchange chromatography on DEAE - cellulose	2.78	$9.5 \times 10^{-2}$	$10.2 \times 10^{-3}$	26	3.9

of the same buffer with 3 changes. The dialysed proteins were applied to DEAE - cellulose column (2.5 x 40 cm) previously equilibrated with the same buffer.

The proteins were eluted with 100 ml of 50 mM phosphate buffer pH 7.0 at a flow rate of 0.5 ml/min and at room temperature then with a linear salt gradient from 0.1 to 0.9 M NaCl in 100 ml of the phosphate buffer at the same flow rate. Those fractions that showed enzyme activity were collected and stored at 0°C for further studies.

**Determination of protein content and enzyme activity:** The methods used to determine protein content and enzyme activity were as described by Anokwulu (2003). The formula for the determination of protein content was; Protein (mg/ml) =  $1.45 \text{ O.D.}_{280} - 0.76 \cdot \text{O.D.}_{260}$ . Unit of enzyme (unit/ml) = Specific activity X protein content. One enzyme unit was defined as a change in absorbance of 0.01 in 1 min. The a 1 cm / 1% value was used to determine the extinction coefficient.

**Isoelectric focusing:** The method for isoelectric focusing was according to O'Farrell (1972) and the use of ampholites at a pH range of 3 to 10 was as described by Asano *et al.* (1987).

**Effect of substrate concentration:** The preparation of substrate solution was as described by Anokwulu (2003). Linoleic acid (0.5 ml) and 0.5 ml of Tween 20 were dissolved in 2 ml of distilled water. A few drops of 2M NaOH were added to the mixture until the turbidity was cleared. Distilled water was added to make up the volume to 25 ml. Lipoxygenase solution (0.1 ml) was added to each test tube containing different substrate solutions of 1 to 10 ml. The absorbance of each mixture of the enzyme and substrate solutions was read at 234 nm.

**Effect of enzyme concentration:** Lipoxygenase solution (0 to 10 ml) was added to different test tubes containing constant volume (2.9 ml) of substrate solution. Their absorption spectra were read at 234 nm.

**Effect of pH:** The enzyme solution (1.0 ml) was added to 1 ml of 0.1 M sodium acetate for pH 3.0 to 5.0, 1 ml of 0.05 M potassium phosphate for pH 6.0 and 7.0, 1 ml of 0.05 M tris - hydrochloric acid for pH 8.0 and 9.0, and 1 ml of 0.05 M of glycine sodium hydroxide for pH 10.0. After the incubation of the enzyme in its various buffers for 10 min at

25°C, 1 ml of each of the above mixtures was added to 2 ml of the substrate solution and the absorbance was read at 234 nm.

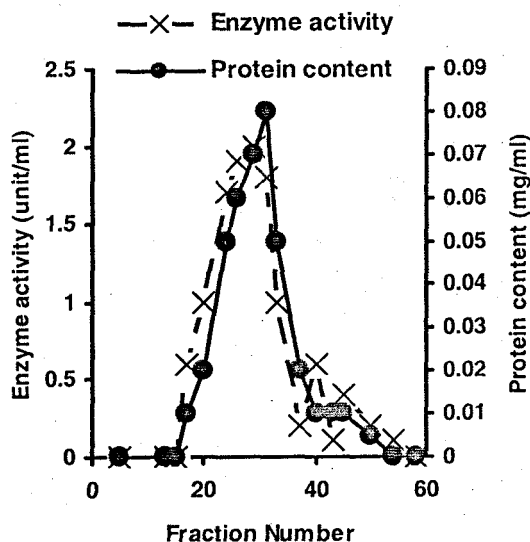
**Effect of temperature:** The method for determining the effect of temperature on the enzyme's activity was as described by Beneytout *et al.* (1989). The

enzyme solution was heated in water bath for 5 min at different temperatures (0 to 80°C), cooled in ice and lipoxygenase activity determined.

**Stability of the enzyme:** The method of Al-Obaidy and Siddiqi (1981) was used in the study of the stability of *F. oxysporum* lipoxygenase. The enzyme solution was heated at fixed temperature for various lengths of time (0 to 30 sec), cooled in ice and lipoxygenase activity found.

## Results and Discussion

Table 1 is the purification profile of *F.oxysporum*. There was 26% recovery of enzyme activity in the purification and a purification fold of 3.9.



**Fig. 1: Elution of *Fusarium oxysporum* lipoxygenase on DEAE-cellulose ion exchange column**

The total enzyme activity and protein content were  $9.5 \times 10^{-2}$  unit/ml and 2.78 mg/ml respectively; these are very low when compared to 41.65 unit/ml for total enzyme activity and 28.38 mg/ml for total protein obtained in the purification of soybean

lipoxygenase by Shibba *et al.* (2001) since soybean is often considered as standard for the source of lipoxygenase (Al-Obaidy and Siddiqi, 1981).

As Fig. 1 shows, most of the enzyme and other proteins were eluted at the first peak before salt gradient was used. The salt gradient succeeded in eluting the enzyme and other proteins that were tightly bound to the column (shown at the second and third peaks). The extinction coefficient of the product *cis-trans* conjugated diene hydroperoxide from linoleic acid at the absorption spectrum of 234 nm was  $2.6 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ . The pI (isoelectric point) was  $\text{pH } 5.0 \pm 1.19$  which is lower than pH 5.4 obtained by Tappel *et al.* (1953) from soybean lipoxygenase.

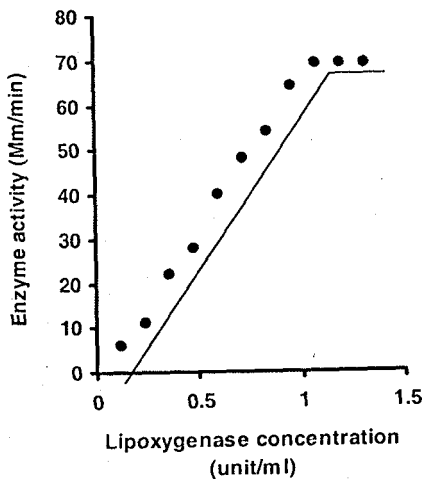


Fig. 2: Effect of the enzyme's concentration on the enzyme activity

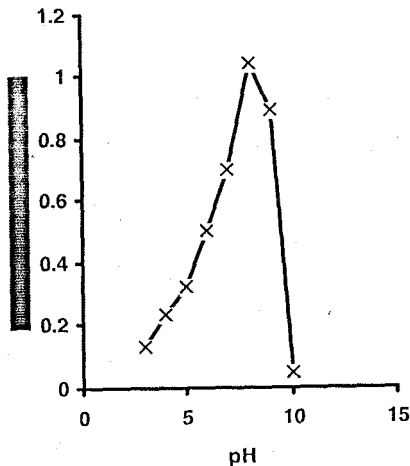


Fig. 3: Effect of pH on the enzyme's catalysed linoleic acid oxidation

Using Lineweaver Burk Plot, the  $K_m$  and  $V_{max}$  of the enzyme's activity on the oxidation of linoleic acid were calculated to be  $1.7 \times 10^{-4} \text{ M}$  and 0.12

$\mu\text{mole/min}$  respectively. The plot of enzyme concentration on the oxidation of linoleic acid was initially first - order kinetics (Fig. 2). This was followed by zero - order kinetics probably caused by the depletion of the substrate.

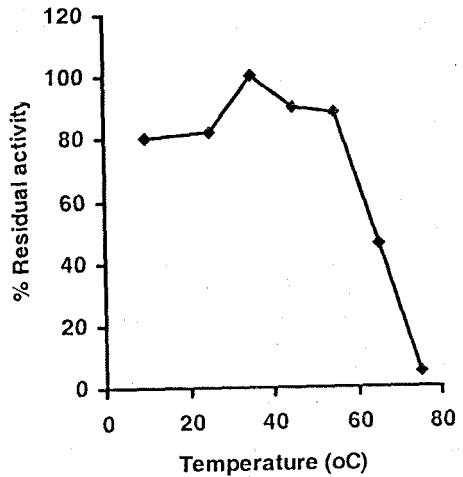


Fig. 4: Effect of temperature on the enzyme's activity

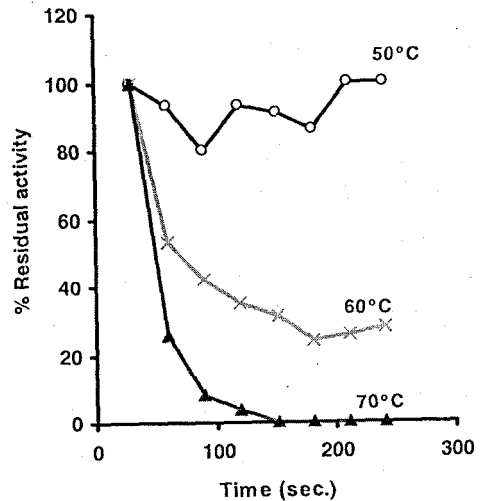


Fig. 5: Temperature stability of *Fusarium oxysporum* lipoxygenase at different temperatures.

The maximum pH of *F. oxysporum* lipoxygenase was 8.0 (Fig. 3). According to Boyer *et al.* (1963), lipoxygenase is in most active state at pH 6.5 to 7.0. The optimum pH of lipoxygenase from *F. oxysporum* was therefore higher than the normal optimum pH for lipoxygenase. However, its pH is lower than pH 8.8 obtained as maximum pH for the enzyme from green algae *Oscillatoria* Sp. (Beneytout *et al.*, 1989).

The optimum temperature of *F. oxysporum* lipoxxygenase was 35°C (Fig. 4). There was a sharp decline in enzyme activity from 65 to 75°C. Fig. 5 shows that the enzyme was most stable at the temperature of 50°C. Al-Obaidy and Siddiqi (1981) obtained 55°C as the most stable temperature for the activity of broad bean lipoxxygenase.

From the results, lipoxxygenase is present in *F. oxysporum* though in small amount ( $9.5 \times 10^{-2}$  unit/ml) when compared to soybean lipoxxygenase (41.65 unit/ml). Nevertheless, the source of *F. oxysporum* lipoxxygenase is inexpensive since the organism is from soil while soybean was bought.

## References

- Al-Obaidy, H.M. and Siddiqi, A.M. (1981). Properties of broad bean lipoxxygenase. *J. Food Sci.* 46: 622 – 626.
- Anokwulu, M.N. (2001). Bleaching of polyphenols in various yam species with rubber (*Hevea brasiliensis*) seed lipoxxygenase. *Nigerian Journal of Biochemistry and Molecular Biology* 16:112-118.
- Anokwulu, M.N. (2003). Purification and some properties of rubber seed lipoxxygenase. *J. of Bio. Res. & Biotech.* 1: 21-31.
- Asano, Y., Nakazawa, A. and Endo, K. (1987). Novel phenylalanine dehydrogenases from *Sporosarcina ureae* and *Bacillus sphaericus*. Purification and characterization. *Amer. Soc. Biol. Chem.* 262: 10346-10354.
- Beneytout, J., Andrianarison, R., Rakotoarisoa, Z. and Tixier, M. (1989). Properties of a lipoxxygenase in green algae (*Oscillatoria* Sp.). *Plant Physiol.* 91:367-372.
- Boyer, P.D., Lardy, H. and Myrback, K. (1963). *The enzymes*. Academic Press, New York, pp 275-285.
- Diel, E. and Star, H. (2000). Purification and characterization of lipoxxygenase from soybean. *Plants* 178:321-328.
- Dolev, A., Rohwedder, W.K. and Dutton, H.J. (1967). Mechanism of lipoxidase reaction. 1. Specificity of hydroperoxidation of linoleic acid. *Lipids* 2: 28-31.
- Frankel, E.N. (1984). Volatile lipid oxidation products. *Prog. Lipid Res.* 22:1-6.
- Furia, T.E. (1975). *Handbook of food additives*. 2<sup>nd</sup> Edition. Academic Press, New York, pp 46-54.
- Hsu, A., Shen, S., Wu, E. and Foglia, T.A. (1998). Characterization of soybean lipoxxygenase immobilized in cross-linked phyllosilicates. *Biotechnol. Appl. Biochem.* 28:55-59.
- O'Farrel, P.H. (1975). High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250: 4007-4021.
- Pelczar, M.J., Reid, R.D. and Chan, E.C.S. (1995). *Microbiology* 8<sup>th</sup> Edition. McGraw-Hill Inc., New York, pp 312-331.
- Prescott, L.M. and Harley, J. (1999). *Microbiology*. 4<sup>th</sup> Edition. The McGraw-Hill Inc., New York, pp 522-538.
- Rosa, S.R. (2003). Improving food processing. <http://us.f807.mail.yahoo.com/ym/showletter?MsgId=826-132047-10105-1033-4312-...>
- Sessa, D.J. (1979). Biochemical aspects of lipid-derived flavours in legumes. *J. Agr. Food Chem.* 27:234-238.
- Shibba, K., Negishi, Y., Okada, K. and Nagao, S. (2001). Purification and characterization of lipoxxygenase from soybean. *Agr. Bio. Chem.* 164:1029 - 1034.
- Sicdow, J. N. (2000). Plant lipoxxygenases, structure and function. *Plant Molecular Biol.* 46:145-188.
- Smith, G. (1971). *An introduction to industrial mycology*. 6<sup>th</sup> Edition. Edward Arnold (Pub.) Ltd., London, pp 102 –107.
- Srivastava, S., Melkani, G.C., Singh, S., Gupta, V.D. and Gupta, V.P. (2003). Hydroperoxidation of unsaturated fatty acids by lipoxxygenase. <http://alfa.ist.utl.pt/~fidel/creac/sec41b.html>
- Stevens, F.C., Brown, D.M. and Smith, E.L. (1970). Some properties of soybean lipoxxygenase. *Achives of Biochem. And Biophy.* 136:413-421.
- Tappel, A. L., Boyer, P. D. and Lundberg, W.O. (1953). The reaction mechanism of soybean lipoxidase. *J. Biol. Chem.* 199:267-281.
- Tooke, H. L., Wilson, R. G., Lobmar, R. L. and Dutton, H. J. (1958). Coupled oxidation of carotene and linoleate catalyzed by lipoxidase. *J. Biol. Chem.* 230:65-72.