Introduction

Lipoxygenase (LOX) (EC 1.13.11.12) is widely distributed and studied enzyme in higher plants, mammals and fish (Hsieh R.J 1994), and also reported in microorganisms, including bacteria (Iny et al,1993), fungi (Bisakowski B et al,1997; Su C et al,1998) and algae (Kuo J et al,1996,1997). Lipoxygenases (linoleate: oxygen oxidoreductase, LOX, are iron containing dioxygenase, which catalyze the addition of oxygen to polyunsaturated fatty acids to form hydroperoxides (HPOD) of fatty acids (Hildbrand, D.F, et al., 1989; Rasahl S, Feussner I et al, 2006). The product of lox – HPOD - is a precursor for formation of number of volatile aroma giving aldehydes and other bioactive compounds involved in plant development and response to biotic and abiotic stresses. Soybean lipoxygenase has the highest activity of the various lipoxygenases and is the most economical source of lipoxygenase, Lipoxygenase has been isolated from many other plant sources (Axelrod et al, 1974), LOXes are used in food-related applications such as in bread making, aroma production, etc. Presence of LOX marks negative implications on food products like color, off flavour and antioxidant status (Baysal and Demirdoven, 2007). Loxs have a single polypeptide chain with a molecular mass of 94–104 kDa.

Many fungal lipoxygenases have been studied like **Fusarium oxysporum**, **F. proliferatum**, **Aspergillus niger**, **Penicillium caseicolum**, **Geotrichum candidum**, **Mortierella**; oomycetes **Lagenidium giganteum**, **Saprolegnia parasitica**, **Acylia ambisexualis**; mushroom **Morchella esculenta**, **Pleurotus ostreatus**; ascomycetes **Saccharomyces vini**, **S. cerevisiae**, **Gaeumannomyces graminis**; thermophilic actinomycete **Thermoactinomyces vulgaris** etc (Fillipovich et al, 2000; Kuribayashi et al, 2002; Perez-Gilabert et al, 2005).

LOX pathway produces varieties of important molecules in fungi. In Oomycete, **Saprolegnia parasitica** lipoxygenase convert arachidonic acid into 15S-hydroxy eicosatetraenoic acid, which is isomerized in to epoxy alcohol by the enzyme epoxy alcohol synthase (Oliw et al, 2002). The characteristic flavour of mushroom **Psalliota bispora** is due to the 1-octen 3-ol and 10–oxo- trans-8-decenoic acid, which is reduced by oxygenation of 10-hydroxy linolenic acid. (Oliw et al, 2002).
Lasiodiplodia theobromae produces one of such important metabolite by lipoygenase pathway, jasmonic acid, which has various physiological functions in plants such as seed development, germination, vegetative growth, signalling in wounding, stress response, senescence and cell signalling. Considering the importance of LOX enzyme in producing JA in this fungus, the aim of the present work was to study this enzyme by purification and characterization.
Materials and Methods

1) Growth of *Lasiodiplodia theobromae*

Growth of *L. theobromae* was done on Basal salt media. 8 day old fungal mycelia was selected for the purification of lipoxygenase. Mycelia were crushed in liquid nitrogen and stored at -20°C.

2) Preparation of crude enzyme extract

Dried mycelia were taken and crushed in 10mM potassium phosphate buffer (pH: 7.4) containing 0.5mM PMSF. Homogenized mycelia were centrifuged at 7000 rpm for 20 min. Supernatant were collected whereas pellets were discarded. Resulting extract was considered as a crude enzymatic extract.

3) Ammonium sulphate precipitation

Crude enzyme extract was precipitated at (0-55) % saturation of ammonium sulphate. Precipitation was kept for overnight at 40°C and then centrifuged at 5000rpm for 15 mins. Pellets were collected and supernatant was discarded. Pellets were dissolved in minimum amount of 10mM potassium phosphate buffer. This fraction was lyophilized and concentrated.

4) Purification of lipoxygenase

Lipoxygenase was purified by size exclusion chromatography using sephadex G100 (Pharmacia). Column bed volume was calculated by formula $V_t = \pi r^2h$. Column void volume measurement was done by blue dextran (2,00,000 kD). Column calibration was done by running two standards - BSA (66,000 D) and RNase (13,700D). 16.5 mg of APS precipitated protein was loaded on column and ran with a potassium phosphate buffer (pH: 7.4) at a flow rate of 30ml/hr. 12 fractions of 4ml each were eluted. Protein content of each fraction was measured by Lowry et al. 1951. Lipoxygenase activity of all fractions was assayed by method of Surry et al 1964. All fractions containing protein were also subjected to LOX activity staining. Fractions showing positive LOX activity were selected and pooled for further purification. Pooled fractions were lyophilized and subjected to ion exchange chromatography. DEAE–cellulose (Hi media) was used as an anion exchanger after activation as per
manufacturer’s protocol. Lyophilized sample of protein was loaded on ion exchange column. Proteins were first eluted with a potassium phosphate buffer (pH7.4) and then separately with NaCl gradients (0.2M, 0.4M 0.6M, 0.8M and 1M NaCl) prepared in potassium phosphate buffer (pH7.4). 25 fractions of 2 ml each were collected at a flow rate of 60ml/hr. All fractions were lyophilized and concentrated to 0.5 ml. Protein content of fractions was measured by Lowry et al. Lipoxygenase activities of all fractions were measured by Surry (1964) method. Fractions containing LOX activity were subjected to activity staining. Native PAGE and SDS-PAGE were also performed on 8% separating gel according to the method of Davis & Laemmli respectively (Davis et al, 1964 & Laemmli et al, 1967) and stained with silver nitrate. Activity staining gel was performed on 8% separating gel containing 10% starch and ran at 12mA for 2 hours. After running, the gel was incubated for 1hr at 24°C in 0.1M potassium phosphate buffer (pH 7), containing 25mM substrate. After 1 hr gel was washed with deionised water for three times and incubated with 5% KI in 15% glacial acetic acid till white band appeared (Chen, 1986).

5) Electrophoresis
Native PAGE and SDS-PAGE were performed according to the method of Davis & Laemmli respectively. Native PAGE and SDS-PAGE were performed on 8% separating gel and stained with silver nitrate. Activity staining gel was performed on 8% separating gel containing 10% starch and run the gel 12mA for 2 hours. Gel was incubated for 1hr in 0.1M pottasium phosphate buffer having pH7 containing 25mM substrate at 24°C. After 1 hr gel was washed with deionized water for three times and incubated with 5% KI in 15% glacial acetic acid till white band appeared (Chen, 1986).

6) Protein estimation
Protein content was measured by (Lowry et al, 1951) using BSA as a standard.

7) LOX assay
LOX activity was measured UV Spectrophotometrically at 234 nm as an appearance of conjugated diene. Linoleic acid was used as a substrate for LOX assay. Same protocol was used for LOX activity as described in chapter 1.
8) Enzyme characterization
Stability of pH of two isoenzymes of LOX was studied by incubating enzyme at different pH from 4 to 9 for 5 min and activity was measured as described in chapter 1. For pH 4 & 5 – 10mM, Acetate buffer was used, for pH 6 & 7 - 10mM Potassium phosphate buffer was used, and for pH 8 & 9 - 10mM Tris buffer was used. Optimum temperature of LOX isoenzymes was found out by incubating the enzyme at different temperature i.e 30°C, 40°C, 50°C, 60°C, 70°C for 5 min and LOX activity was measured as described in chapter 1.

9) Enzyme Kinetics
Lipoxygenase kinetics was done using different linoleic acid concentration -15mM, 20mM, 25mM, 30mM, 35mM. Lipoxygenase activity was performed as described in chapter 1.

10) LOX reaction product identification and characterization
To identify LOX product one unit of purified enzyme was added to the reaction tube containing 2ml of 10mM potassium phosphate buffer of pH 6 and 1ml 25mM linoleic acid as a substrate. The mixture was incubated at 40°C for 1 hr. Immediately after 1 hrs reaction was stopped by addition of 1M HCL. Product was extracted thrice-using equal volume of chloroform: methanol (2:1). The product was concentrated by evaporating and then dissolved in HPLC grade methanol.
High performance liquid chromatography (HPLC) was used to identify which product is formed. HPOD were separated on normal phase Silica column and eluant solvent was n-Haxane: iso propanol: acetic acid (98.7:1.2:0.1) at a flow rate of 1ml/min. HPLC was used to analyse reaction product (9&13HPOD) was Agilent1100 equip with a fixed wave length UV detector. Detection was performed at 234nm.
Liquid chromatography-Mass spectrophotometry(LC-MS) was used to conformed which product is formed. Binary solvent system was used Methanol:Water (50:50) and flow rate was 1ml/min.LC-MS was use to analyse is of Shimadzu,Nexera highest pressure (UHPLC).Triple Quadrupole Lans CMS-8030.
11) **Effect of purified enzyme LOX1 and LOX2 on dough rheology**

Bleach wheat flour was used to see the effect of purified enzyme. Bleached wheat flour (10gm) and 7gm MQ water was mixed in a blender with or without 10 units of LOX for 5 min and the dough obtained was placed on Modular Compact Rheometer (Anton Paar, Graz, Austria). To study effect of addition of LOX, 0.01 mM Linolenic acid (LA) was added. Dynamic properties of dough, storage and loss modules were measured at different frequency of pressure and elasticity strains.
Results

1) Day wise Lipoxygenase activity with comparison to DMW

It was already known that Lipoxygenase is the first enzyme of the pathway that is responsible for JA production in *L. theobromae*. To find out the time of highest LOX activity in this fungus, day-wise LOX activity in the mycelial crude extract was assayed by UV Spectrophotometric method using linoleic acid as a substrate at pH 6 and 40°C temperature. LOX activity was found higher in the beginning of the growth (initial lag phase) and then reduced in the log and late log phase (Figure 1). The LOX activity was once again increased at the start of the stationary phase. The highest activity was found to be on 8th day old fungal mycelia extract, which happens to be stationary phase (Figure 1).

Figure 1: Day wise Dry mycelia weight (DMW) with lipoxygenase activity from *L.theobromae*.
2) Purification of lipoxigenase

To characterize the LOX of *L. theobromae*, it was purified from the eighth day old mycelial extract by APS precipitation, gel permeation chromatography and ion exchange chromatography. Addition of 0-55% of APS to 8-day-old mycelial extract precipitated the proteins, which gave positive LOX activity. The precipitated proteins were further purified by gel permeation chromatography, in which three fractions with positive LOX activity were eluted. Protein profile in crude extract, ammonium sulphate precipitation, and in size exclusion chromatography was done on SDS-PAGE (Figure 2) The fractions giving LOX activity were pooled and loaded in to DEAE-cellulose ion exchange chromatography column. Using NaCl gradient (0 to 1M) as a mobile phase, 27 fractions were eluted; out of which, only one fraction showing positive lox activity was separated in 0.4 M NaCl mobile phase gradient. A summary of the purification is given in table 1. An overall 68-fold purification was achieved with a yield of 0.018%. The specific activity of pure LOX reached 1851.81 Units/mg−1 when linoleic acid was used as a substrate. The homogeneity of sample obtained at each stage of purified enzyme was analyzed by SDS-PAGE. The single fraction obtained in ion exchange chromatography showed two prominent bands in SDS PAGE. To confirm, whether it is an impurity or both of these peptides are LOX, it was subjected to activity staining, which showed the presence of two bands (Figure 4). The presence of LOX isozyme was repeatedly confirmed by activity staining. The molecular weight of two LOXes were analyzed on the SDS gel using alphadigidoc densitometry software and found to be 93kD and 45kD (Figure 4).
Table 1: Purification of LOX.

<table>
<thead>
<tr>
<th>Purification steps</th>
<th>Total protein (mg)</th>
<th>LOX activity (units)</th>
<th>Specific activity (units/mg)</th>
<th>Recovery (%)</th>
<th>Purification (fold)</th>
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<tbody>
<tr>
<td>Crude enzyme</td>
<td>3.88</td>
<td>10560</td>
<td>27.22</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Ammonium sulphate (0-55)%</td>
<td>48</td>
<td>1800</td>
<td>37</td>
<td>12.4</td>
<td>1.38</td>
</tr>
<tr>
<td>SephadexG100</td>
<td>9.02</td>
<td>890</td>
<td>88.73</td>
<td>2.32</td>
<td>3.25</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>0.072</td>
<td>133.33</td>
<td>1851.81</td>
<td>0.018</td>
<td>68.03</td>
</tr>
<tr>
<td>SephadexG100 LOX1 (93 kD)</td>
<td>0.009</td>
<td>141.6</td>
<td>15733.33</td>
<td>0.002</td>
<td>578</td>
</tr>
<tr>
<td>SephadexG100 LOX2 (45 kD)</td>
<td>0.039</td>
<td>216</td>
<td>5538</td>
<td>0.010</td>
<td>203.45</td>
</tr>
</tbody>
</table>

Figure 2: SDS-PAGE GEL. Lane 1: crude extract, Lane 2: Broad range molecular weight marker, Lane 3: Ammonium sulphate fraction, Lane 4, 5, 6: Size exclusion fractions of 2, 3, 4.
Figure 3: SDS-PAGE of purified enzyme. Lane 1: 45kD LOX, Lane 2: 93kD LOX, Lane 3: Molecular weight marker.

Figure 4: LOX Activity staining of ion exchange Fraction
3) Effect of pH and temperature on enzyme activity

To see the effect of different pH on purified LOXes, LOX activity was done and it showed their maximum activity at pH 6.0 (Figure 5). Optimum temperature of LOX activity was found to be different for LOX1 (93kD) and LOX2 (45kD). Optimum temperature of LOX1 was found to be 50°C and of LOX2 was 40°C (Figure 6).

![Figure 5: Effect of pH on purified LOX1 and LOX2.](image)

![Figure 6: Effect of temperature on purified LOX1 and LOX2.](image)
4) Kinetic analysis of purified LOXes

Both the purified LOXes were subjected to kinetic analysis using linoleic acid as a substrate. The Km value of LOX1 was found to be 100mM and of LOX2 was found to be 59mM (Figure 7, 8). The Vmax value of LOX1 was found to be 25000μM/min/mg of protein and of LOX2 was found to be 16666μM/min/mg of protein. The Kcat value was found to be 13×10⁴ sec⁻¹ for LOX1 (93kD) and 99×10⁹ sec⁻¹ for Lox2 (45kD). The catalytic efficiency of LOX1 was 1300 and of LOX2 was 1.67×10⁹.

Figure 7: L.B Plot of Purified LOX1
Figure 8: L.B Plot of Purified LOX2

<table>
<thead>
<tr>
<th></th>
<th>Km</th>
<th>Vmax</th>
<th>Kcat</th>
<th>Catalytic efficiency</th>
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<tr>
<td>LOX1</td>
<td>100mM</td>
<td>25000</td>
<td>1.3×10⁴</td>
<td>1300</td>
</tr>
<tr>
<td>LOX2</td>
<td>59mM</td>
<td>16666</td>
<td>9.9×10⁹</td>
<td>1.67×10⁹</td>
</tr>
</tbody>
</table>

Table 2: Enzyme Kinetics

5) Characterization of Hydro peroxides products of LOXes

HPLC of products of purified 93kD & 45kD LOX showed two major peaks at 2.8 min and 3.5 min as detected by UV detector of HPLC (Figure 9, 11). Peak obtained at 2.8 min could be of 13-HPOD, whereas peak obtained at 3.5 min could be of 9- HPOD. Number of researchers have separated LOX products using HPLC and obtained retention time of 13- HPOD and 9- HPOD similar to our results using similar column...
and mobile phase (Kato TS. et al, 1992). Although both LOXes produced similar products, LOX1 produced more of 9- HPOD (retention time 3.5 min) and LOX 2 produced more of 13- HPOD (retention time 2.9 min).

LC-MS analysis was performed to confirm the products of both the isoenzymes of LOX. LOX1 products showed maximum peak at 312.00, 171.90, 144.2 m/z ratio where as LC-MS analysis of LOX2 products showed maximum peaks at 312.00, 296.30, 267.00, 185.40, 145.20 m/z ratio. The peak at 312 could be due to 9/13-HPOD, the peak at 296 could be due to removal of –OH group from HPOD (312 – 17 = 295), the peak at 171 and 144/145 could be of the two fragments of 9/13 HPOD (Figure 10, 12).

![HPLC chromatogram of LOX1 reaction product.](image)

Figure 9: HPLC chromatogram of LOX1 reaction product.
Figure 11: MS chromatogram of LOX1 reaction product

Figure 10: HPLC chromatogram of LOX2 reaction product
6) Effect of addition of LOX 1 and LOX 2 on elasticity and viscosity of dough

Dough rheology measurements, viscosity (G”, storage module) and elasticity (G’, loss module) were studied to, know what is the effect of LOX1 and LOX2 on Viscous-Elastic nature of bleached wheat flour. At low strain, the elasticity (G’) was observed greater than viscosity (G”) in presence of LOX 1 as well as LOX 2. Addition of LOX 1 reduced G’ as well as G”, however addition of LOX 2 increased G’ and G” values, giving a properties of dough as per different food products’ requirements (Figure 11,12). Addition of LA reduced the G’ and G” of dough in presence of either LOX 1 or LOX2.
Figure 13: Effect of LOX1 on Storage ($G'$) and Loss ($G''$) Module of dough.

Figure 14: Effect of LOX2 on Storage ($G'$) and Loss ($G''$) Module of dough.
Discussion

Lipoxygenase has an importance in food and medicinal industry; in food industries it is used in bread making, as a bleaching agent, and also involved in to improve dough rheology. (Whitehead et al, 1995; Baysal and Demirdöven, 2007, Cumbee et al, 1997; Borellib et al, 1999). *L. theobromae* produces large amount of JA (Robert et al, 1992; Jerneren et al, 2012) so it was considered as a good source of lipoxygenase enzyme. Considering the importance of LOX in food and medicine industry and its study in *L. theobromae* might also reveal metabolic details, LOX was purified and studied from this fungus. Maximum lipoxygenase activity was to be found on 8th day as it shows maximum growth on that day in *L. theobromae*.

LOX was purified by 0 - 55 % Ammonium sulphate precipitation, Sephadex G 100 size exclusion and DEAE-cellulose ion exchange chromatography. When LOX was purified from *L. theobromae*, through ion exchange and it showed two proteins with LOX activity, one of 93kD (LOX1) and another of 45kD (LOX2). LOX1, whose molecular weight was found to be 93kD, was similar to the soybean LOX (94kD), which was reported by Max et al. in 1986 as well as by Brash in 1999 (Zhang et al, 2012) and to the pea seed LOX which was reported by Szymanowska et al., 2009, (Zhang et al, 2012). LOX2, which has molecular weight around 45kD, it shows similarity to the 47kD LOX reported in *Pseudomonas aeruginosa* and in *Anabaena sp.* (Zhang et al, 2012). This is the first report showing presence of LOX isoenzymes in *L. theobromae*. LOX1 of 93kD appeared to be constitutive and major enzyme in *L. theobromae* and its km value was found to be higher than the LOX2 (45kD). The turn over number of LOX1 enzyme was found to be $13 \times 10^4$ sec$^{-1}$ and of LOX2 was $99 \times 10^6$ sec$^{-1}$, which is higher than soybean LOX (232 S$^{-1}$). Higher catalytic efficiency of LOX isolated from *L. theobromae* vouch for its industrial application.

To understand the activities of the two LOX isoenzymes in *L. theobromae*, products of both the LOXes were subjected to LC-MS and MS-MS analysis. Product analysis of LOX1 and LOX2 showed two major peaks of 13-HPOD and 9 –HPOD. Peaks conformation was done by their mass as well as by their fragments which was produced during MS Spectra. It was derived that both the LOXes produced 9 & 13 HPOD, however, LOX1 produced more of 9- HPOD (retention time 3.5 min) and LOX2 produced more of 13- HPOD (retention time 2.9 min) (Figure 8,9). Soyabean
seed lipoxygenase also produced 13-HPOD and 9–HPOD (Fukushige et al., 2005). *F. proliferantum* was also produce 9 and 13 HPOD in different proportion. Similar results were obtained in fungus like *Desert Truffle* and *Thermomyces lanuginosus*, *Gaumannomyces graminis* lipoxygenase (Su & Oliw 1998), also in the olive LOX. It shows that 13 hydroperoxide is the major product which are responsible for the synthesis of jasmonates and other volatile compounds. LC-MS data also prove that 13 HPOD formed which m/z 312 which completely match to the standard value.

Apparent Km and Vmax value of purified LOX 1 is 100mM, 25000 µmol/min/mg and for LOX 2 is 59mM, 16666µmol/min/mg. Kcat value of 13×10⁴ sec⁻¹ for LOX 1 and 99×10⁹ sec⁻¹ for LOX 2. Iny et al. (1993b) reported that the LOX of *vulgaris* showed optimum LOX activity at pH 6.0. *F. proliferantum* has optimum pH 6.0, Km, and Vmax 5.15 X10⁻⁵ M & 1.61, respectively. Slightly higher Km values of 2.0 x 1, 2.6 X 10 and 2.8 X 10 M were reported for pea seed LOX (Reynolds and Klein, 1982a; Chen and Whitaker, 1986). The highest Km of 1 mM was reported by Iny et al. (1993b) for the enzymatic extract from *T. vulgaris*.

Altunkaya A et al., (2011) reported two LOX isoforms having Km and Vmax 0.33 mM and 0.24µ mol/min for LOX1 and for LOX 2 Km and Vmax is 0.98 mM and Vmax0.24 µmol/min at pH= 6.0 from Freshly Cut Lettuce(*L. sativa*). High km was reported for human 5-LOX having 63.1 mM (Soberman et al. 1985; Denis et al. 1991) all the reported results shows that lower the Km and Vmax value which ever reported. Catalytic efficiency of enzyme Kcat for LOX1 is 13×10⁴ sec⁻¹ and LOX2 is 99×10⁹ sec⁻¹. Which is higher then soybean LOX 232 S⁻¹. Higher Km, Vmax and Kcat shows higher lox activity and turnover number which shows more catalytic activity and higher the production of hydroperoxide.

Optimum pH for LOX 1 and LOX 2 is 6. Altunkaya A et al., (2011) in freshly cut Lettuce reported that pH of LOX 1 is 6 which is same and LOX2 is 7 while for both LOX temperature is 40°C. Our result of LOX1 & 2 has optimum activity at 50°C and 40°C respectively. Ana-rLOX, *Pseudomonas aeruginosa* LOX, and *Thermomyces lanuginosus* LOX has optimum pH is 6. Optimum temperature for banana leaf LOX is 40°C, *Thermomyces lanuginosus* LOX is 55°C, which was quite nearer to LOX 1.
Study of effect of LOX isoenzymes isolated from *L. theobromae* on bleached dough rheology showed different kind of effects by LOX1 and LOX2. Addition of LOX1 reduced the $G'$ and $G''$ of dough whereas addition of LOX2 increased $G'$ and $G''$ values. Addition of LOX2 maintained higher elasticity ($G'$) than by addition of LOX1, which showed their usefulness in different types of bakery products. Knowledge of rheological properties of bread dough is important to understand mechanical properties of the dough and control finished products (Mirsaeedghaz et al, 2008; Barajas, 2012).