

INTRODUCTION

Legumes are important sources of protein for populations in developing countries (Kadam and Salunke, 1985). The seeds are consumed after cooking, sprouting or fermentation. The important legumes include soyabean (*Glycine max*), navy bean (*Phaseolus vulgaris*), moth bean (*Vigna aconitifolia*), broad bean (*Vicia faba*), green gram (*Phaseolus aureus*), black gram (*Phaseolus mungo*), chickpea (*Cicer arietinum*), horsegram (*Dolichos biflorus*) and peas (*Pisum sativum*).

Legume seeds comprise the embryo (including cotyledons), the endosperm and the testa or seed coat. The testa is the only protective barrier between the embryo and the external environment.

The seed, principally, is a storehouse of carbohydrates, fat and proteins (Bewley and Black, 1985). Legume seeds, in general, contain 20 –25% protein, 1 – 2% fat and 40 – 50% carbohydrates. Soyabean, winged bean and lupine seeds are an exception with higher contents of protein and fat (Kadam and Salunke, 1985). In addition to the major constituents, several antinutritional factors like protease inhibitors, phenolic compounds, lectins, α - amylase inhibitors and oligosaccharides are also present in the seeds.

Horsegram

Horsegram is one of the underutilized legumes of the tropics. It has a significantly higher proportion of non-protein nitrogen than other legumes (Kadam and Salunkhe, 1985). Horsegram (*D. biflorus*) is extensively cultivated in the dry regions of South India in the states of Andhra Pradesh, Karnataka and Tamil

Nadu. It is a slender, trailing or erect, annual herb with downy stems and branches (Fig. 1A). The crop, besides having good adaptability to adverse climate conditions, is drought resistant. The leaves are trifoliolate and the flowers are pale yellow in color. Seeds (5 – 7) are present in pods, which are linear and flattened (Fig. 1B and 1C).

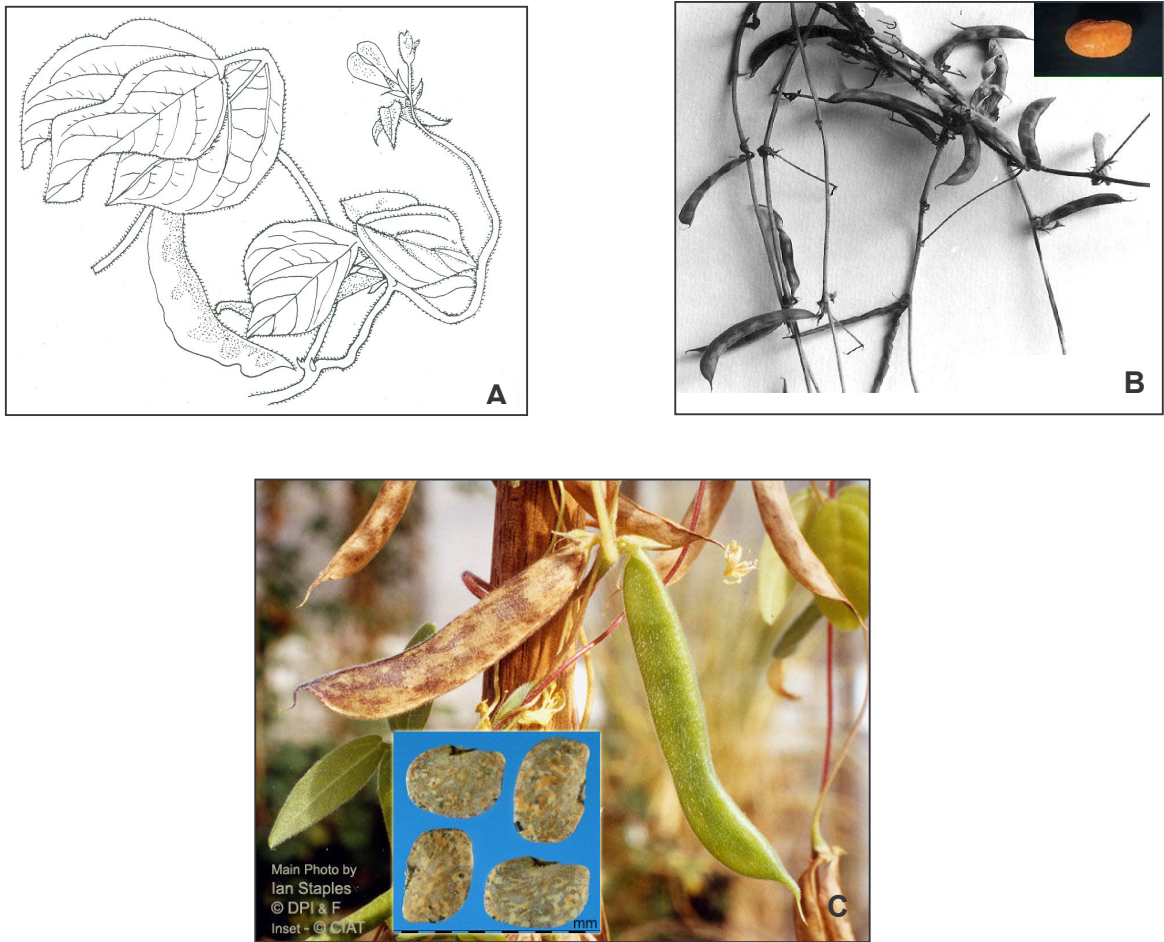


Figure 1. A. Horsegram plant showing a pod and flower. B. Pods containing horsegram seeds and a single seed (*Inset*). C. Photograph of horsegram pods – both immature and mature showing the seeds in the *inset*.

Horsegram seeds have been identified as a potential food source for the tropics by the National Academy of Sciences, USA, 1979.

Chemical composition of horsegram seeds

Horsegram seed comprises 57% carbohydrate, 22% protein and 2.5% fat (Sudha *et al*, 1995, Mahadevappa and Raina, 1978). It is an excellent source of iron, calcium and molybdenum. The bioavailability of iron is limited by the presence of antinutritional factors like phytates, tannins and oxalic acid (Sudha *et al*, 1995).

The lipid content of horsegram seeds is ~2.2 – 2.5%. The saturated fatty acid content is ~28.8%, while the unsaturated fatty acids form ~71.2% (Kadam and Salunkhe, 1985). Neutral lipids, glycolipids and phospholipids constitute 20, 30 and 30% respectively, of the total lipids. Palmitic and linoleic acids are the principal fatty acids in horsegram. The unsaturated fatty acids include 18: 1 (13.0%), 18: 2 (44.6%) and 18: 3 (13.7%). The sterols, sterol esters and phospholipids in horsegram have been shown to defend against infective duodenal ulcer due to *Helicobacter pylori* infection (Jayaraj *et al*, 2000).

The presence of several antinutritional factors like trypsin inhibitors, lectins, phytic acid, polyphenols and oligosaccharides in horsegram seeds is known. Dehulling, cooking, sprouting, roasting and germination reduce these factors and improve the digestibility of the legume. Antinutritional factors present in horsegram are given in Table 1. The haemagglutinin content of horsegram is high (2.6 HU / g seeds x 10³). The haemagglutinins of horsegram have a molecular weight in the range of 113, 000 - 109, 000 Da. Germination of horsegram for 72 h is found to decrease haemagglutinin activity from 2.6 to 0.6 HU/ g seeds x 10³. Horsegram

seeds are resistant to infestation. This may be in part due to the high content of trypsin inhibitors and haemagglutinin activities compared to other legumes.

Table 1. Antinutritional factors present in horsegram seeds

Antinutritional factor	Content
Polyphenols (%)	1.6
Phytic acid (%)	0.66
Oligosaccharides (raffinose, stachyose & verbacose) (%)	5.8
Haemagglutinin (HU/ g seeds x 10 ³)	2.6
Trypsin inhibitor (U/ g flour)	94.0

Kadam and Salunkhe, 1985

Plant defense against pests: The exemplary horsegram

Horsegram is known for its resistance to attack by pests and insects. This feature of horsegram has prompted investigation to understand the plant defense mechanisms against pests with a view to develop resistance in other plants. Plant defenses (Fig. 2) against insect herbivores include physical barriers (eg, a thick cuticle). However, the most important deterrents are secondary metabolic compounds such as terpenes, phenolics, tannins, alkaloids, enzyme inhibitors,

hemagglutinins and non-protein amino acids that are localized in the plant in a vacuole or in the cell wall. They are not toxic to the cell that synthesizes them (Kessler and Baldwin, 2002, Ferry *et al*, 2004).

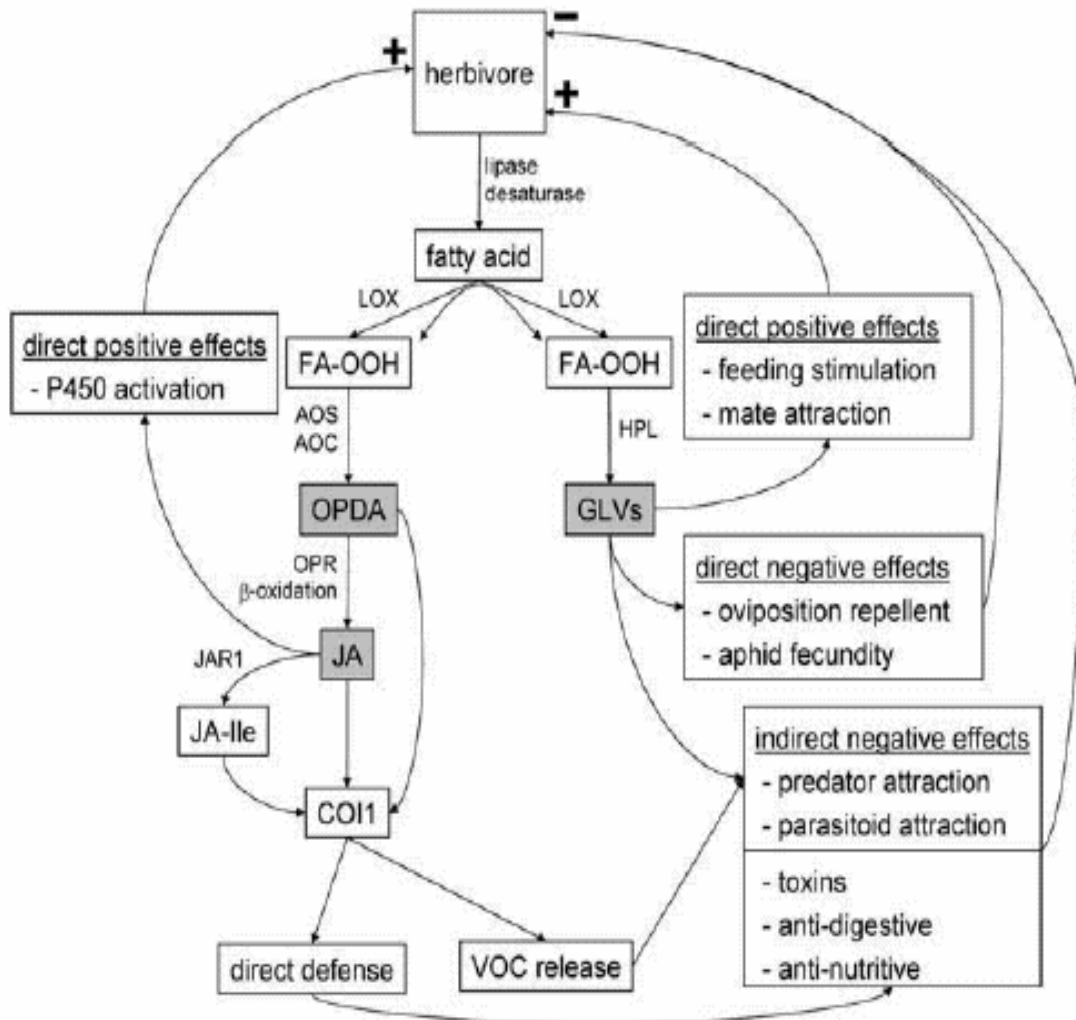


Figure 2. Plant response to stress – direct and indirect defense mechanisms (Adapted from Ferry *et al*, 2004).

These compounds act in several ways:

- They may be directly poisonous eg. Pyrethroids
- Digestion inhibitors eg. Enzyme inhibitors
- Irritants or increase unpalatability of the plant eg. Saponins, tannins

When plants are attacked by bacterial or fungal pathogens, lipases are activated to release the unsaturated fatty acids and trigger the synthesis of a range of oxylipins with diverse roles. Some of these have direct antimicrobial or anti-insect functions, while others, especially the jasmonates and their precursors (the oxo-phytodienoic acids), are potent regulators of defense mechanisms; as an example, by stimulating proteinases or promoting the accumulation of antimicrobial plant oxylipins (Fig. 2).

The role of lectins and LOXS in plant defense is elaborated further.

Role of lectins in plant defense

A defensive role is suggested for lectins (haemagglutinins). Lectins are proteins having affinity for specific carbohydrate moieties. They bind to glycoproteins - in the peritrophic matrix lining the insect midgut - disrupt digestive processes and nutrient assimilation (Ranjekar *et al.*, 2003). Legume lectins may also function as defense proteins. One recent finding suggests that the ability of some legume lectins to protect the plant against predators is not related to carbohydrate binding activities. Studies have addressed the possibility that lectin gene expression can be triggered in vegetative tissues by defense related stimuli (Etzler, 1992). Some possible natural functions (Cummings, 1999) of plant lectins are:

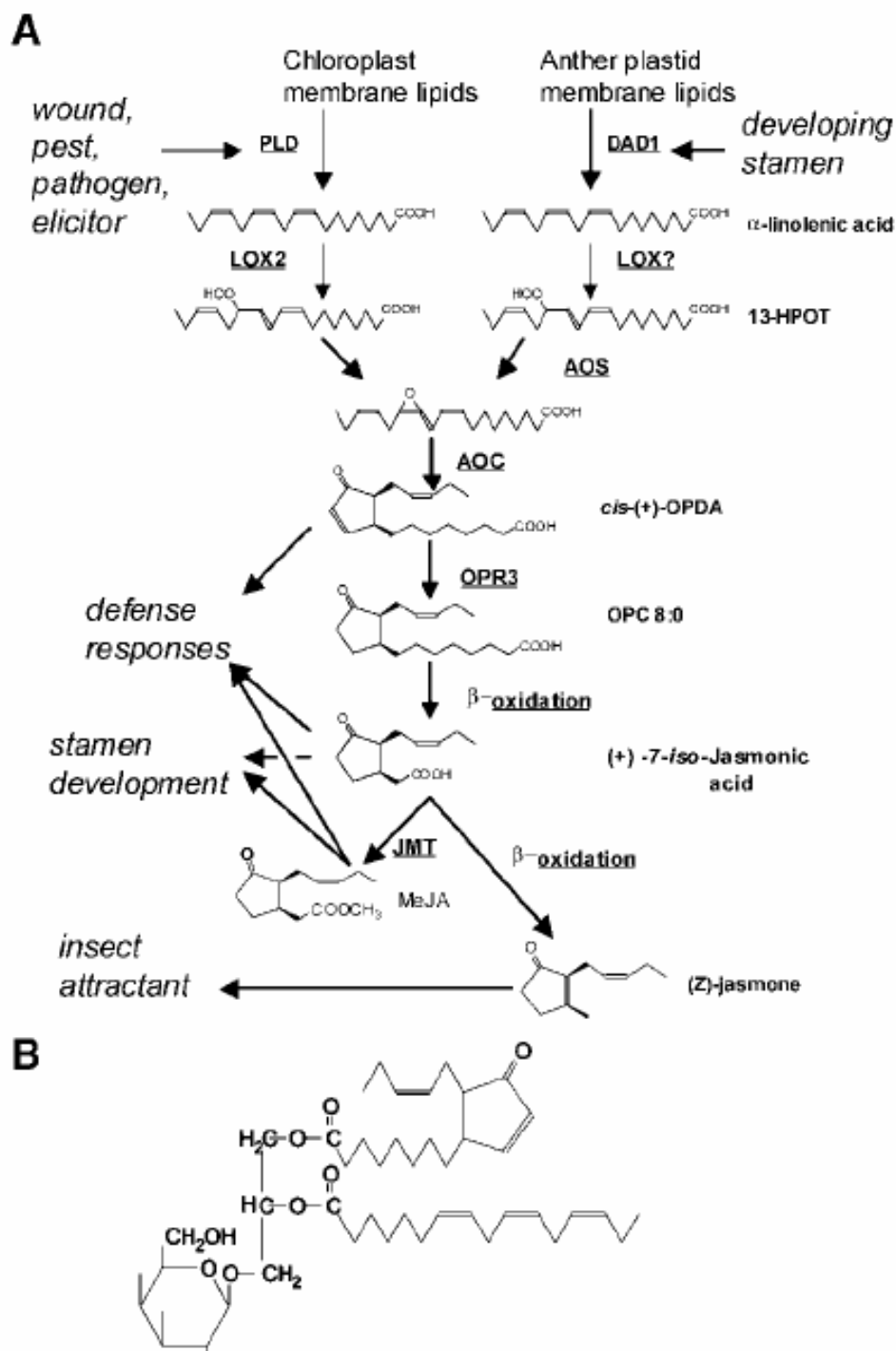


Figure 3. Biosynthesis of jasmonic acid (Model). **A.** Pathways inputs and outputs are in italics. Enzyme names in abbreviations are underlined. Abbreviations for intermediates are in bold. **B.** Structure of sn1-O-(12-oxophytodienoyl)-sn2-O-(hexadecatrienyl)-monogalactosyl diglyceride, a chloroplast oxylipin containing esterified OPDA. (Adapted from Wasternack et al, 1998).

1. Seed storage
2. Aid in maintaining seed dormancy
3. Defense against predators
4. Symbiosis in legumes
5. Transport of carbohydrates
6. Mitogenic stimulation of embryonic plant cells
7. Elongation of cell walls
8. Recognition of pollen

Role of lipoxygenases in plant defense

Lipoxygenases are one of the key enzymes in plant defense. They act on unsaturated fatty acids, producing hydroperoxides, the precursors of oxylipins leading to an indirect plant defense mechanism. The reactions take place in the peroxisome and chloroplasts (Wasternack *et al*, 1998).

Lipoxygenase pathway (Feussner and Wasternack, 2002)

Majority of hydroperoxides arise from LOXs action in plants (9- and 13-hydroperoxy derivatives of PUFAs). These are subsequently metabolized through a number of secondary reactions. Four major metabolic routes for the metabolism have been characterized (Fig. 3 and 4):

(1) *The peroxygenase pathway*: The epoxy and hydroxy derivatives of linoleic acid resulting from the peroxygenase pathway are toxic to fungal pathogens (Blee, 1998).

(2) *The allene oxide synthase (AOS) pathway*: AOS mediated pathway results in unstable allene oxides. These undergo nonenzymatic hydrolysis leading to the formation of α - and γ -ketols. They may also be enzymatically metabolized to oxo-phytodienoic acid (OPDA) by an allene oxide cyclase). This OPDA is known to accumulate upon wounding or other stresses (Hamberg, 1988).

OPDA exhibits signaling properties distinct from jasmonic acid (JA). JA and related compounds are essential signals in the wound response of leaves, leading to synthesis of proteinase inhibitors. Jasmonate derivatives, namely, the free acid, methyl ester and conjugates with amino acids, have distinct biological effects. Methyl jasmonate, in particular, is important in initiating defensive strategies. They are believed to interact with receptors in the cell to activate signaling pathways both intra- and inter-cellularly that modulate the expression of a number of genes, and hence the synthesis of many key proteins (Fig. 4). Volatile jasmonate metabolites, such as *cis*-jasmone, may regulate the behavior of some insects by deterring herbivorous species or attracting their predators (Hamberg and Gardner, 1992).

(3) *The hydroperoxide lyase (HPL) pathway*: Hydroperoxide lyase catalyzes the oxidative cleavage of the hydrocarbon backbone of fatty acid hydroperoxides, which leads to the formation of short chain aldehydes (C₆-or C₉-) and the corresponding C₁₂- or C₉- fatty acids (Matsui, 1998). These fatty acids have potent antimicrobial effects and reduce the fecundity of insect pests. These fatty acids lead to the production of traumatin, which is implicated in defense pathway.

(4) *The DES pathway*: It leads to the formation of divinyl ethers such as colneleic acid and colnelic acid (Grechkin, 1998), which are produced quickly in leaves of

potato plants infected by fungi or viruses, and they are believed to have a defensive role.

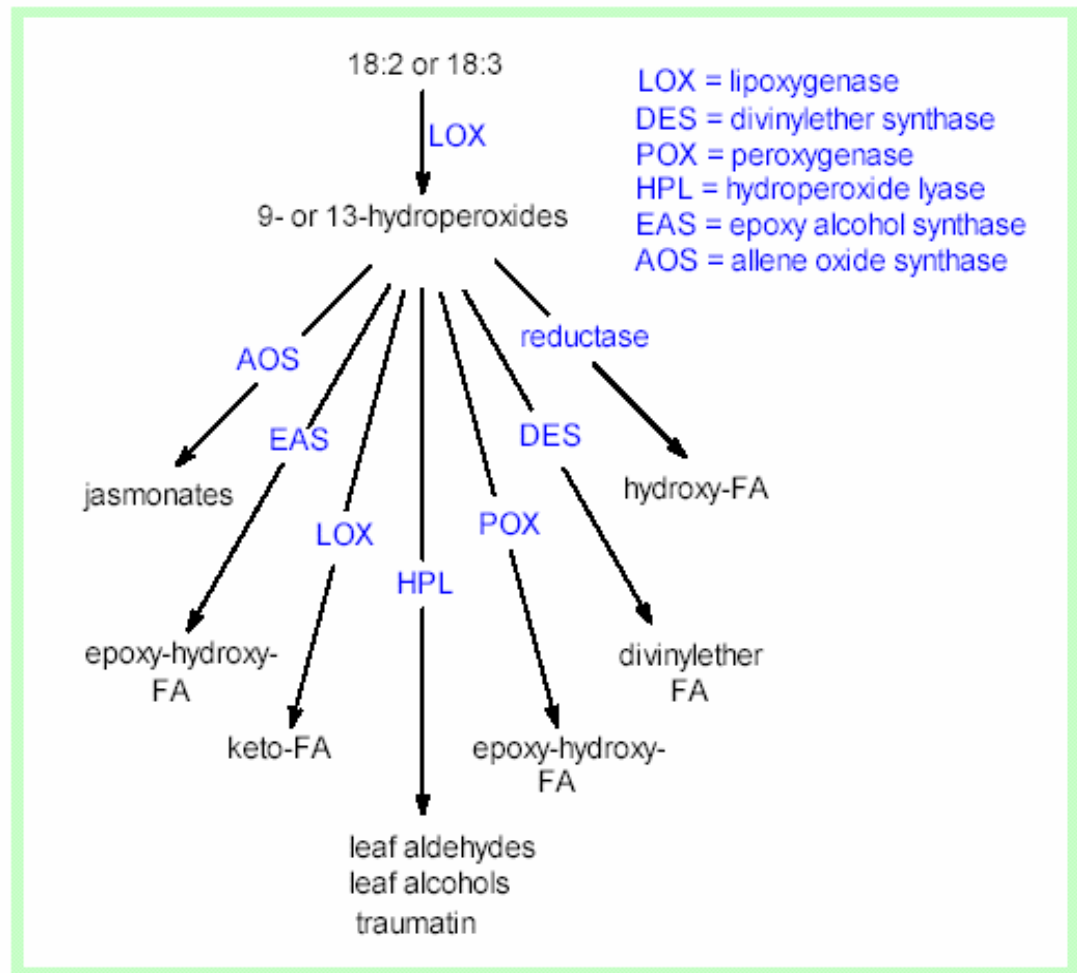


Figure 4. Enzyme mediated pathway for the production of oxylipins in plants (Adapted from Feussner and Wasternack, 2002).

In addition to the four major pathways, there are other hydroperoxide reactions, which are not well characterized. These include:

(a) *The LOX catalyzed hydroperoxidase reaction (ketodiene forming pathway):*

Under certain conditions such as low oxygen pressure, LOXs are capable of catalyzing the hemolytic cleavage of O-O bond forming alkoxy radicals, which may rearrange to ketodienes. Their physiological role has to be elucidated (Kuhn *et al*, 1991a).

(b) *The EAS pathway:* Products of epoxy alcohol synthase (EAS) reactions may be regiochemically identical to the peroxidase (POX) reaction but they differ with respect to stereochemistry. The EAS generated oxylipins may be involved in defense mechanism (Hamberg, 1999).

(c) *The reductase pathway:* In this POX independent reduction, hydroperoxy fatty acids are reduced to their corresponding hydroxyl derivatives. Their role again is not fully understood (Feussner and Wasternack, 2002).

Many of the products of the lipoxygenase pathway are implicated in plant defense. Such compounds are highly reactive, and they are quickly metabolized by various enzymes into a series of oxylipins (Fig. 4) with distinct biological activities.

Thus, the emerging picture of plant defense is a highly complex one, and many aspects need clarification. The functional differences between species that are yet to be understood, and the relationship with other defense mechanisms including those based on ethylene and salicylic acid are among them. One of the models proposed illustrates the cross talk between jasmonic acid, ethylene and salicylic acid pathways (Fig. 5).

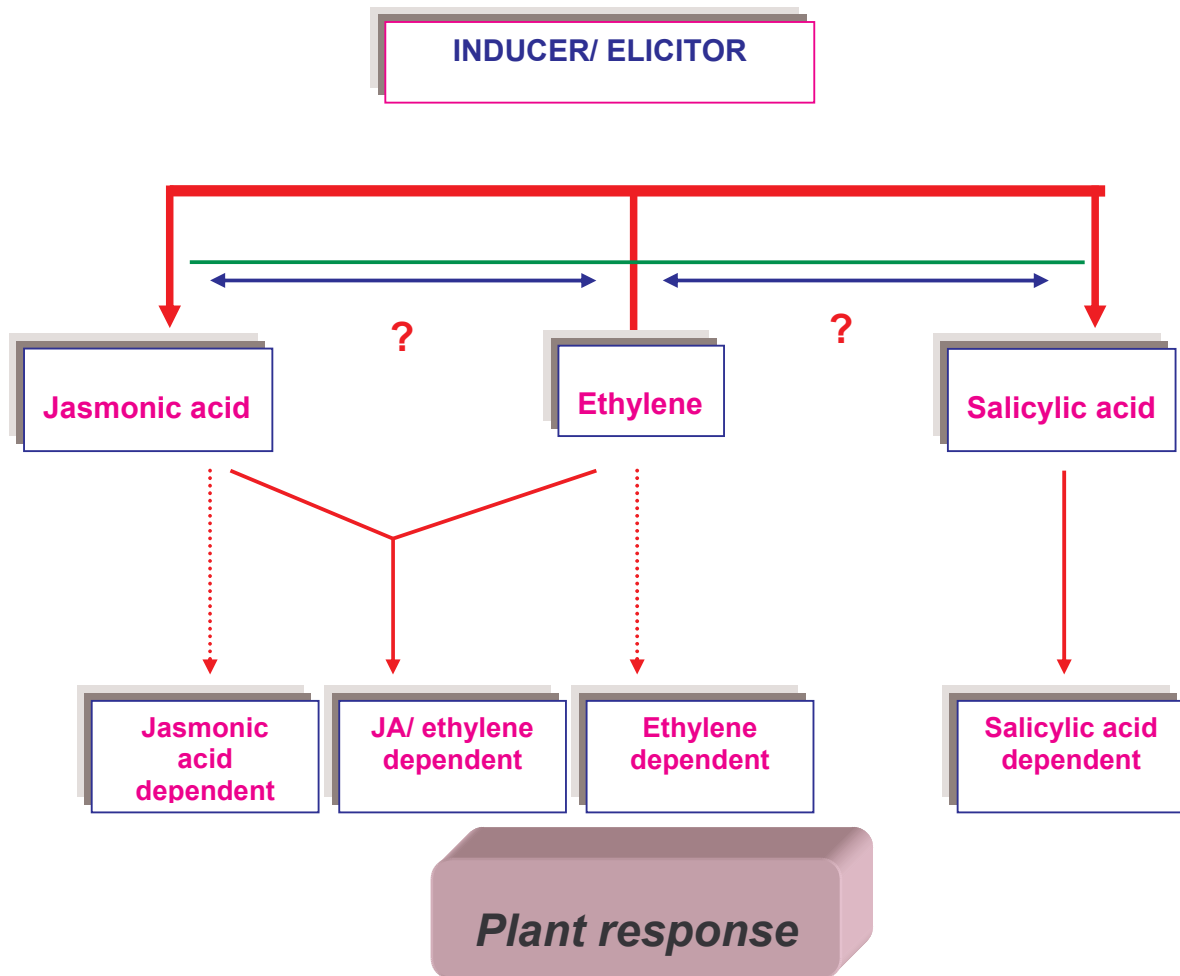


Figure 5. Hypothetical model illustrating the cross-talk between salicylic acid signalling and jasmonic acid/ ethylene pathways for regulating plant response to stress factors. (Adapted from Kunkel and Brooks, 2002).

LECTINS

Lectins are a structurally diverse class of proteins that reversibly bind carbohydrates. However, they do not exhibit enzymatic activity towards these carbohydrates. They are ubiquitous in nature and are found widely in plants, fungi, bacteria, viruses, invertebrates and a few vertebrates (Lis and Sharon, 1998). Their ability to agglutinate cells results from their ability to bind specifically to saccharides on the surface of cells/ membranes and act as bridges between cells. This property of lectins has provided a new tool for cell biologists to investigate the cell surface architecture. The abundance of lectins in the seeds of legumes, their solubilities and wide range of carbohydrate specificities have made the legume lectins useful tools in a variety of analytical, preparative, biomedical and other biotechnological applications (Hamelryck *et al.*, 1998). Lectins are particularly abundant in the seeds of legumes, where they are localized in the protein bodies and account for approximately 10% of the soluble protein of the seed extracts. The seeds of a number of legumes have been found to contain more than one lectin, which differ from one another in carbohydrate specificity and are encoded by related genes from different loci. Lectins are also present in other legume tissues, including stems, leaves, bark, roots, nodules, pods and flowers (Etzler, 1998). Although the genes encoding most of the vegetative tissue lectins described to date are closely related to the seed lectin genes, no such relationship has been reported in a recent study of a root lectin gene from *Dolichos biflorus* thereby raising the possibility of the existence of multiple classes of lectins exist in the same plant (Etzler, 1992).

Carbohydrate specificity of lectins

Legume lectins are known to bind mono-and oligosaccharides. This framework consists of:

- (a) A conserved monosaccharide binding site in which four residues confer affinity, (b) a variable loop that confers monosaccharide specificity and (c) a number of sub sites that harbor additional sugar residues or hydrophobic groups (Hamelryck *et al*, 1998).

Legume lectins can be subdivided into five groups according to the specificity of the monosaccharide binding site: (i) Fuc specific, (ii) GlcNAc/GlcNAc (β 1-4) GlcNAc specific, (iii) Glc/Man specific, (iv) Gal/GalNAc specific, and (v) those that do not bind any simple monosaccharide (eg. *Dolichos biflorus* lectin, which binds GalNAc but not Gal) (Hamelryck *et al*, 1998).

Structure of lectins

The complete amino acid sequences of a large number of legume lectins have been determined, either directly by protein sequencing or indirectly from their complementary or genomic DNAs. At present, about 14 crystal structures are available. In addition, two plant defense proteins that are truncated - hence non-sugar binding - from kidney bean have been crystallized (Hamelryck *et al*, 1999). These structures are enabling information on the three dimensional structures of related lectins with known primary structures and carbohydrate specificities through molecular modeling. Despite their diversity in carbohydrate specificities, the legume seed lectins have shown a remarkable conservation in primary, secondary and tertiary structure. Comparisons of these sequences and structures have established that the differences in carbohydrate specificity appear to be primarily due to differences in amino acid residues residing in loops adjacent to the carbohydrate binding site. The association constants of the individual carbohydrate binding sites of the legume lectins are quite low in the range of 10^4 - 10^5 M⁻¹ but increases with the oligomerization of the subunits *in vitro* and

presumably *in vivo*. Multivalent binding is of great advantage in biological systems in which the overall interactions between components must not only be strong, but also allow the individual point detachments, e.g., in cellular adhesion and motility (Etzler, 1998, Hamelryck, *et al.*, 1999).

Early studies with fluorescent probes established that a number of the legume seed lectins have both low affinity and high affinity hydrophobic binding sites. The high affinity hydrophobic binding sites bind adenine and adenine derivatives with a value two order of magnitude higher than the affinities of the individual carbohydrate binding sites. No significant differences have been observed in the affinity binding sites of any of the legume lectins examined. The conservation of the specificity of this adenine-binding site, despite variations in carbohydrate specificity and tissue distribution of these legume lectins, suggests that this site may serve an important purpose in the roles of these lectins in the plant. To date, no interactions have been found between the adenine binding sites and the carbohydrate binding sites (Etzler, 1998).

Physiological role of lectins

Several hypotheses have been proposed on the role of legume lectins. However, no role has been firmly established for any legume lectin (Etzler, 1998). A single legume plant contains a variety of legume lectins, some of which are located in different plant tissues. The ability of lectins to bind specific carbohydrates enables them to bind glycoproteins also, suggesting a role for lectins in oligosaccharide signaling events that occur in plant development, defense and other interactions of plants with their environment. Lectins are known for their haemagglutination properties. This function is attributed to their ability to bind carbohydrates (Etzler, 1998). The carbohydrate-binding site may play a

regulatory role or have transducing functions. The preservation of this activity during evolution suggests an essential role of these proteins. The presence of adenine binding sites in legume lectins suggests these proteins may have a primary role related to this hormone-binding site (Hamelryck *et al*, 1999). It is of interest that two animal proteins have structures similar to legume lectins and are involved in glycoprotein trafficking events in the secretory system (Feider and Siemens, 1994 and 1995). Despite their lack of knowledge about their function *in vivo*, legume lectins are widely used as a model system for protein-carbohydrate interactions.

In contrast to the above studies, which invoke lectins in a positive role in plant defense systems, there is a suggestion that lectins may help in the invasion of plants by serving as receptors for attachment of pathogen (Romantschuk, 1992).

***Dolichos biflorus* seed lectin (DBSL)**

Seed lectin from the leguminous plant, *Dolichos biflorus*, has a unique specificity among the members of the legume lectin family because of its high preference for GalNAc over Galactose. The *Dolichos biflorus* seed lectin (DBSL) is one of at least four blood A + H substance – binding lectins present in this plant. It is a tetrameric glycoprotein with a molecular mass of 110 kDa. The protein is composed of two dimers – each comprising two unidentical subunits, designated subunit I and subunit II (Fig. 6A) (Carter and Etzler, 1975a).

A lectin isolated from the roots of *Dolichos biflorus* is reported to have phosphatase activity (Etzler, 1998) in addition to its carbohydrate binding activity.

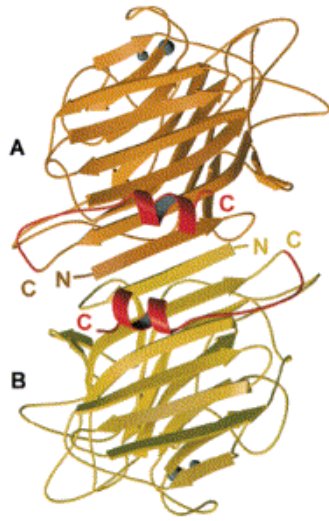
The deduced sequence of this lectin shows no similarity with other reported legume lectins suggesting that this belongs to a new class of lectin. Studies with this lectin showed that there is interaction between carbohydrate binding site and enzyme activity.

Structure of DBSL (Hamelryck *et al*, 1999)

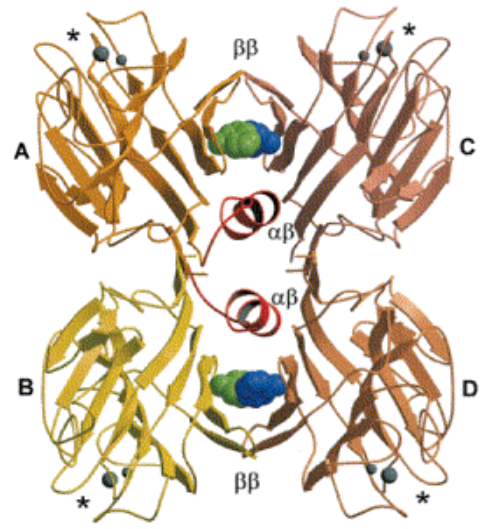
The overall structure of DBSL in complex with adenine is shown in Fig. 6. This tetramer type consists of two canonical legume lectin dimers that pack against each other in a parallel fashion. A first interface between two canonical dimers is present at the outer ends of the tetramer. This β, β - interface consists of two strands that pack together by zipper like intercalation of their side chains. This mode of association creates a large channel running through the center of the tetramer. Two α -helices are sandwiched between the β - sheets of two pairs of facing monomers (monomer pairs AC and BD in Fig. 6b) forming the second interface i.e. $\alpha\beta$ interface. This is an important stabilization factor for the tetramer. Subunit II is post translationally formed from subunit I (253 amino acid residues) by the removal of 12 amino acid residues from its C-terminus.

The unique architecture of DBSL explains why two subunits (subunit II) are truncated and two subunits (subunit I) remain intact. The two subunits (subunit I) that have their C-terminal region buried in the central cavity remains intact while the other two are proteolytically cleaved resulting in the formation of a heterodimer.

(a)



(b)



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The large, hydrophobic cavity. side chains of residues Ser 246 and Arg 250 of both helices protrude into a large, water accessible cavity situated in the center of the tetramer. On the opposite side of α helices, the side chains of residues Leu244 and Leu248 form the bottom of cavity, side chains of residues Ser 246 and Arg 250 of

both helices protrude into a large, water accessible cavity situated in the center of the tetramer.

Adenine binding site in DBSL.

Two identical adenine binding cavities which are predominantly hydrophobic are found at opposite ends of the tetramer (Fig 6). The top of this cavity is formed by the β , β -interface (Ile189), while the bottom consists of the side chains that protrude from the sandwiched α helices (Leu244 and Leu248; Fig 7). The side chains of the two C-terminal residues (subunit I) also add to the hydrophobic character of the cavity. The walls are formed by the protruding side-chains from the back sheets of a pair of facing monomers. All the side chains that form the cavity are aliphatic (Leu, val and Ile) or contain a hydroxyl group. The two cavities each have internal pseudo 2-fold symmetry and are related to each other by a non-crystallographic 2-fold axis. The adenine molecules are bound by hydrogen bonds and hydrophobic interactions with side chains from β -strands. In addition, two residues belonging to the sandwiched β -helix are directly below the adenine rings. All nitrogen atoms of the adenine molecules are involved in hydrogen bonds, except N-9 (Fig. 7).

Weak density in the neighborhood of the N-9 atom indicates that it is probably involved in a water bridge with the main chain oxygen atom of Glu136. DBSL binds two adenine molecules per tetramer despite the presence of four

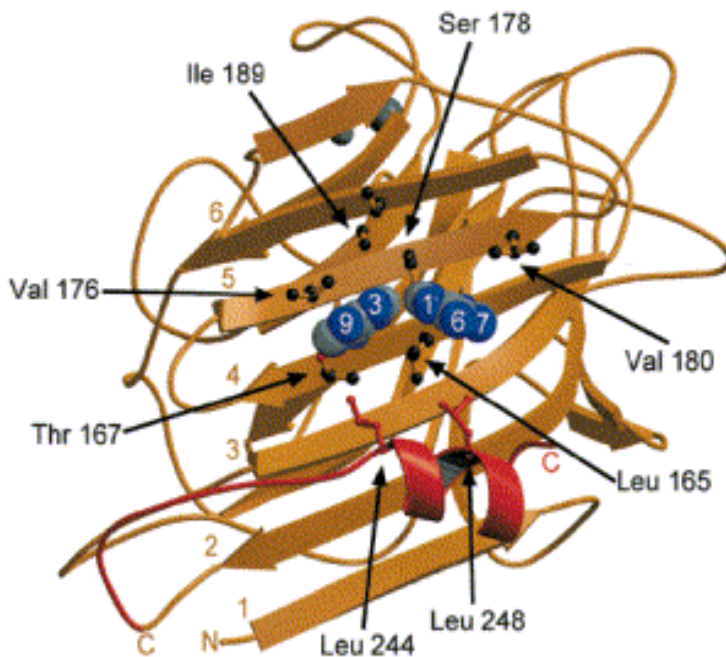


Figure 7. A view on a pair of neighboring adenine binding sites. The adenine molecules are shown as space filling models. The six β -strands that form the back sheet are numbered. The side chains of residues involved in adenine binding and the sandwiched α helix is shown as ball and stick representations. (*Adapted from Hamelryck et al, 1999*).

adenine binding sites, probably, because of the unavailability of hydrogen bond donors. Thus, DBSL has a novel and unique quaternary structure, involving a

characteristic α helix sandwiched between two monomers. It has no counterparts among other legume lectins of known structure (Lis and Sharon, 1998).

Role of metal ions in lectin structure and activity

DBSL specifically binds N-acetylgalactosamine (GalNAc). The carbohydrate binding and haemagglutination activity is dependent on metal ions. Ca^{2+} is essential for carbohydrate binding activity Fig. 8A. Native lectin contains Ca^{2+} , Mg^{2+} , Mn^{2+} , Zn^{2+} and Cu^{2+} . The established presence of Ca^{2+} in high concentrations (3-5 mol/mol lectin) has been shown to be essential for haemagglutination activity (Lis and Sharon, 1986). Both the monosaccharide binding site and the neighboring metal binding site are highly conserved. In the monosaccharide binding site, three of the four conserved residues (the Gly-Asn-Asp triad) hydrogen bond to the sugar (GalNAc) while a fourth aromatic residue stacks against the hydrophobic part of the sugar. These four residues are held in the correct position by two metal ions (a Ca^{2+} ion and a Mn^{2+} ion), either directly or via water molecules (Fig. 8A). Most importantly, the metal ions stabilize an unusual *cis*- peptide bond between an Ala and Asp, thereby positioning the Asp side chain in the correct position for sugar binding. Ca^{2+} is bound to two Asp residues, one Asn residue and two water molecules. Mn^{2+} is bound to two Asp residues, one Glu, one His and two water molecules. (Fig. 8B). Hydrophobic interactions also play a major role for sugar binding (Hamelryck *et al*, 1998).

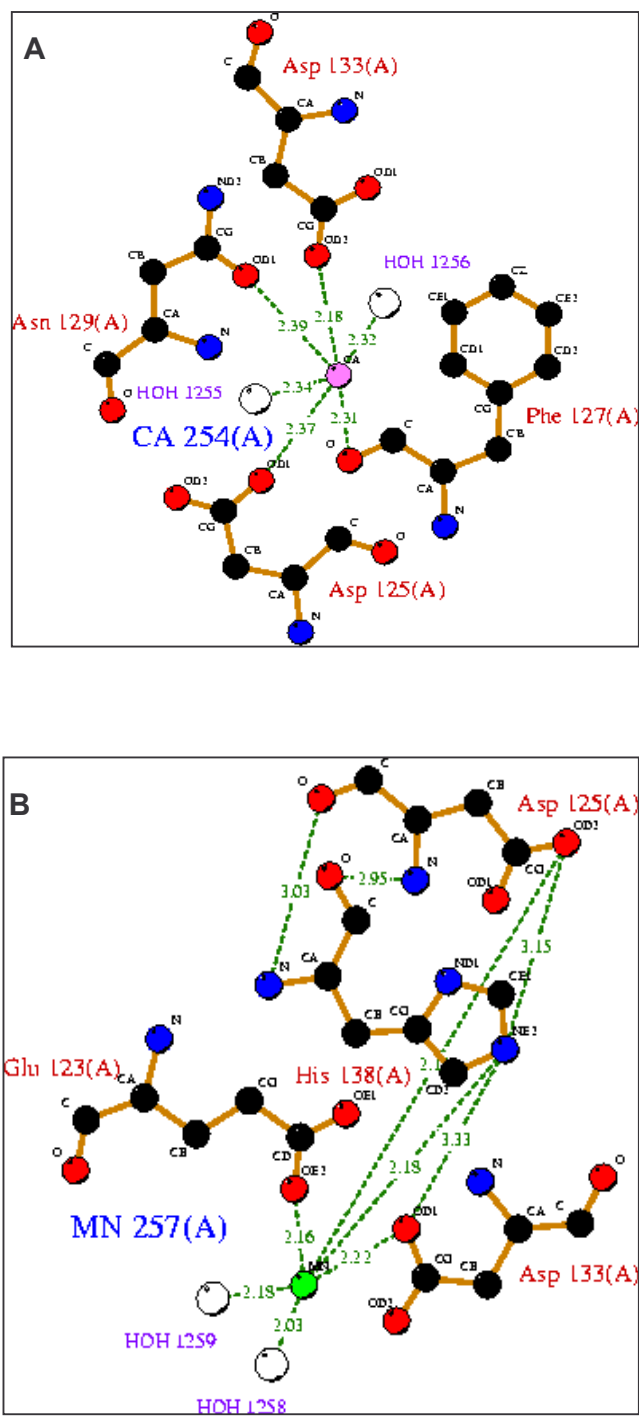


Figure 8. A. Calcium binding site of DBSL **B.** Manganese binding site of DBSL

(PDB No. 1BJQ, Hamelryck et al, 1999)

Lipoxygenases

Lipoxygenases (LOX) (linoleate: oxygen oxidoreductase, 1.13.11.12) are a family of non-heme, non-sulfur iron monomeric dioxygenases, which catalyze the conversion of polyunsaturated fatty acids into conjugated hydroperoxides. They induce lipoperoxidation of membranes and the synthesis of signaling molecules or structure/ metabolic changes in the cell (Maccarone *et al*, 2001). Their substrates contain a (1Z, 4Z) – pentadiene system. LOXs from mammals, plants and microorganisms differ from each other in their substrates as well as products formed as a result of catalysis.

Occurrence

LOXs are widely expressed in animal and plant cells. Plant and animal LOXs have evolved separately as shown by the phylogenetic tree, each forming different subgroups (Brash, 1999, Feussner and Wasternack, 2002). Lipoxygenases are reported from a few fungal sources. LOX from the fungus, *G. graminis*, an important wheat pathogen, forms the only characterized enzyme (LOX) containing manganese at the catalytic center (Su and Oliw, 1998). LOXs have been reported from algae and molds (Kuo *et al.*, 1997, Brash, 1999) and bacteria (Porta and Socha, 2001).

Nomenaclature. Different LOX enzymes can add a hydroperoxy group at carbons 5, 8, 12 or 15 when arachidonic acid (C20: 4) is the substrate. Hence, these isozymes are designated as 5-, 8-, 12- or 15-lipoxygenases (Maccarone *et al.*, 2001). Since, arachidonic acid is a minor polyunsaturated fatty acid (PUFA) in the plant kingdom, plant LOXs are classified based on their positional specificity for linoleic acid (LA) oxygenation. LA is oxygenated at either the C-9 or C-13 of the

hydrocarbon backbone of the fatty acid resulting in the formation of either 9-hydroperoxy or 13-hydroperoxy derivatives of LA (Feussner and Wasternack, 2002). Thus, soya LOX-1 is a 13-LOX while potato LOX is a 9-LOX based on this classification.

Biological role of LOXs

5- and 15-LOXs are present in both animals and plants, while, 8- and 12-LOXs are present only in animals (Yamamoto *et al.*, 1997). Animal LOXs are involved in the arachidonic acid cascade for the biosynthesis of lipid hormones – leukotrienes, lipoxins and / or hydroxy fatty acids (Fig. 9). The lipoxygenase pathway forms hydroperoxyeicosatetraenoic acids (HPETEs) and dihydroxyeicosatetraenoic acid (DEA) and subsequently converts these to (1) hydroxyeicosatetraenoic acids (HETEs) by peroxidases, (2) leukotrienes (e.g. LTC₄) by lipoxygenase, hydrolase and glutathione S-transferase (GST), and (3) lipoxins by lipoxygenases. Leukotrienes and lipoxins have potent biological functions like leukotactic and myotropic effects (Samuelsson *et al.*, 1987). They also constitute mediators of anaphylactic and inflammatory disorder (Samuelsson *et al.*, 1987). Leukotriene antagonists and leukotrienes synthesis inhibitors are currently being tested in clinical trials for their antagonist properties, which are promising. The ability of 15-LOX to oxidize LDL is suggestive of an important asthmatic and/ or anti-inflammatory potencies (Samuelsson *et al.*, 1987). However, certain LOX subtypes (reticulocyte-type 15(S)-LOXs, leukocyte-type 12(S)-LOXs) may play a biological role outside the arachidonic acid cascade. Lipoxygenases are known to play a role in cell death (Maccarrone *et al.*, 2001). The products formed as a result of LOX action in animals is shown schematically in Fig. 10. The biological role of most lipoxygenases is not clear.

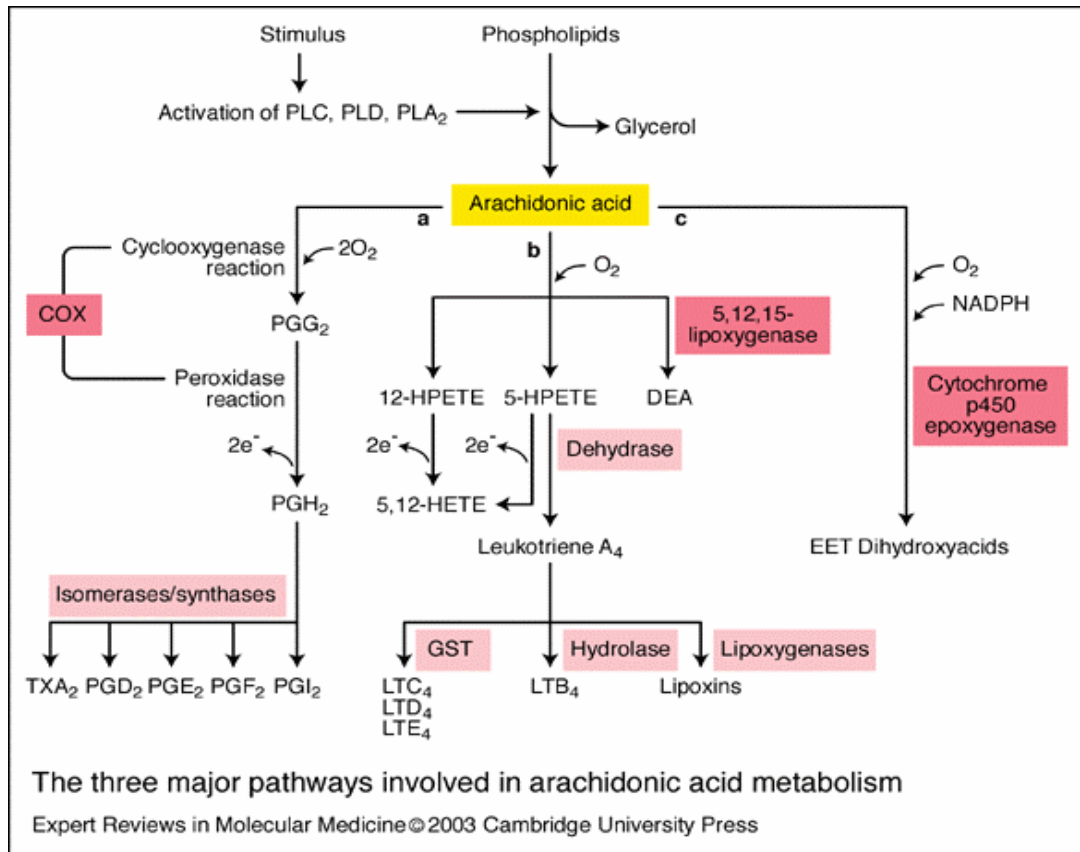


Figure 9. Arachidonic acid metabolism.

Ref. <http://www-ermm.cbcu.can.ac.uk/03006288a.pdf>

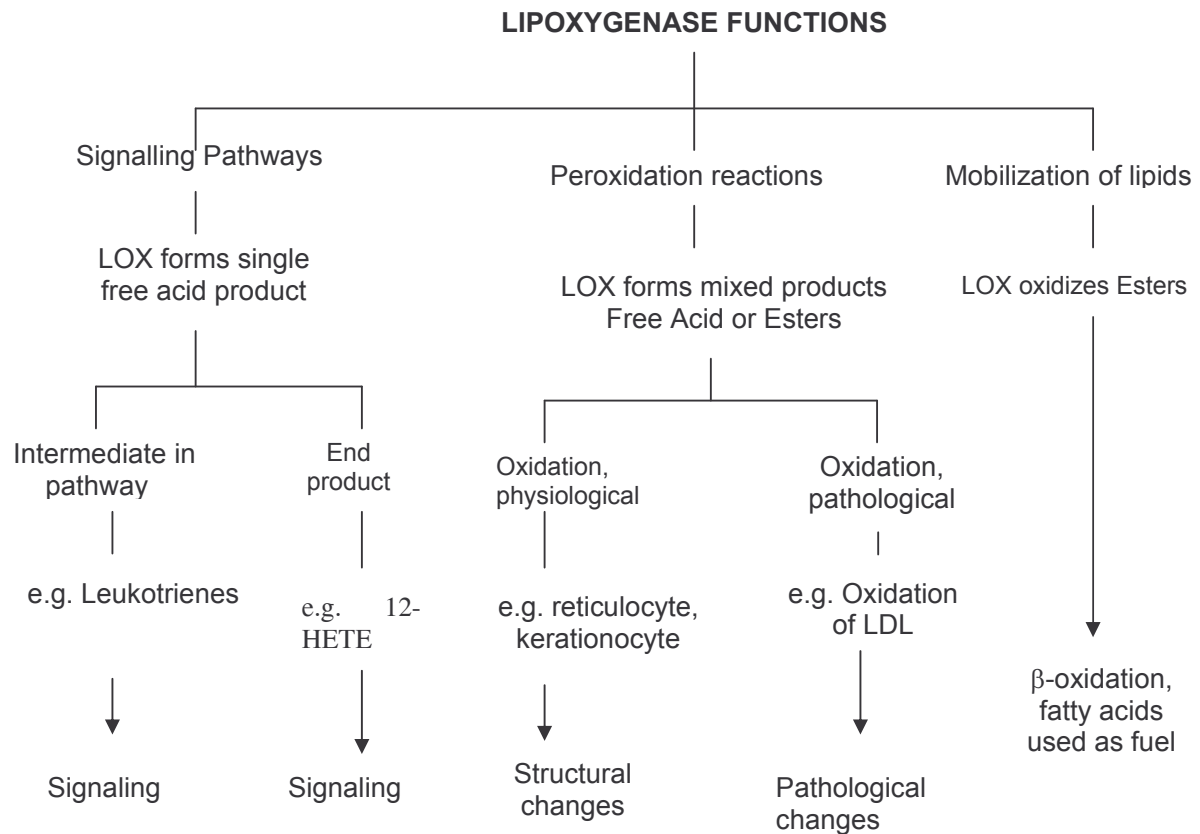


Figure 10. Biological role of mammalian lipoxygenases

Some of the biological properties of the products of LOX reactions in plants are given in Table 2. The products of LOX pathway are involved in plant defense, plant growth and development, senescence, organogenesis and maintenance of homeostasis (Siedow, 1991, Gardner, 1991, Grechkin, 1998). However, the physiological aspects of the plant pathway are poorly understood. Fatty acid derivatives and other oxylipins derived from LOX pathway is involved in plant defense as described in detail earlier (Fig. 4).

Table 2

Recently it has been shown that storage lipids are mobilized by lipid body lipoxygenase through their oxidation and hydrolysis (Feussner and Kuhn, 1995).

Catalytic mechanism of lipoxygenases

Lipoxygenases contain non-heme iron, which is covalently bound to the protein, as the co-factor. Iron exists in one of the two oxidation states; Fe^{2+} (inactive enzyme) and Fe^{3+} (active form). In most of the mechanisms proposed, iron oscillates between the Fe^{2+} and Fe^{3+} forms during catalysis (Fig.11). Lipoxygenases contain Fe^{2+} in resting state. Assays initiated with ferrous enzyme (Fe^{2+}) show significant lag period in their reaction rates. Two hypotheses exist to explain this (Nelson and Seitz, 1994).

- (1) Product activation model: This model suggests that the hydroperoxide produced is required to activate the enzyme by oxidizing the ferrous ion to ferric ion. In this hypothesis, only the ferric enzyme is active.
- (2) Substrate inhibition model: In this model, both ferric form and ferrous form are active and are inhibited by the substrate binding to a non-catalytic site. Here the lag period reflects the displacement of substrate in the regulatory sites by the products as they accumulate.

Two schemes have been proposed to explain the mechanism of catalysis by lipoxygenase. Both these schemes begin with the active Fe^{3+} form of lipoxygenase.

In the first scheme proposed (Nelson *et al.*, 1990), the 1, 4-diene moiety of the substrate is oxidized by Fe^{3+} and a proton as abstracted by a base (water molecule) to form a pentadienyl radical (free-radical intermediate) and free Fe^{2+} enzyme. The radical reacts with molecular oxygen to produce a peroxy radical,

which abstracts an electron from the metal, regenerating Fe^{3+} enzyme and producing a peroxidate anion. The peroxidate receives the proton from the base (water) yielding a hydroperoxide product.

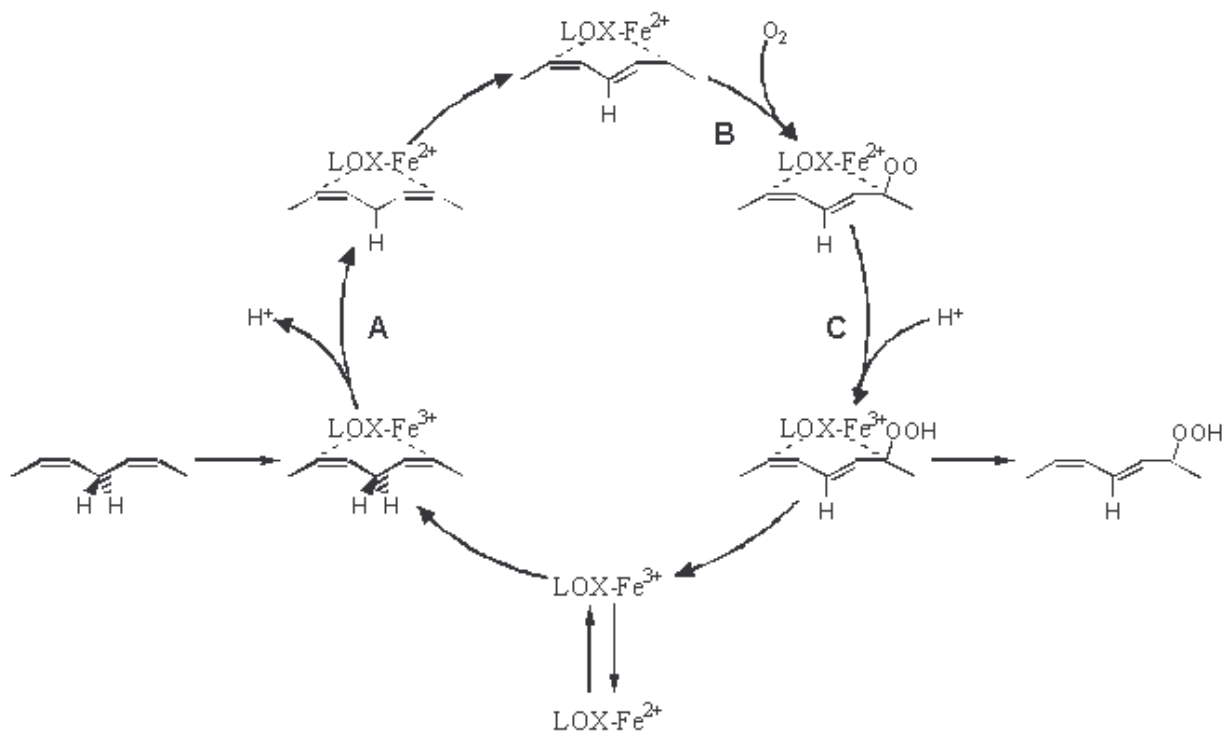


Figure. 11 . Catalytic mechanism of Lipoyxygenase enzyme.

Ref. www.dkfz.de/spec/lox-db/show-literature.

In the second mechanism proposed by Corey and Nagata (1987), the Fe^{3+} assists in the deprotonation by making a direct bond with resulting radical (organoiron intermediate). The path continues with the insertion of dioxygen into

the Fe-C bond followed by cleavage of the Fe-O bond. This mechanism offers an explanation for the stereo and regiospecificity of the enzyme. The other steps proposed are similar to the first scheme.

From a chemical point, transition metals such as manganese can also fulfill the requirement of the metal ligand in lipoxygenases. A manganese dependent lipoxygenase lacking iron has been reported (Su and Oliw, 1998) from the fungus, *G. graminis*, a pathogen of wheat. The manganese lipoxygenase reaction, using linoleic acid as substrate, results in the formation of two hydroperoxides - 11(*S*)-HPOD and 13(*R*)-HPOD (Cristea *et al*, 2005). Furthermore, the enzyme catalyzes not only dioxygenation of the substrate (fatty acid) but also a slower isomerization of the 11-hydroperoxide into the thermodynamically more stable 13-hydroperoxide (Fig. 12). Isotope studies using O-18 as well as deuterated linoleic acids indicate that the conversion of 11(*S*)-HPOD to 13(*R*)-HPOD takes place via a carbon-centered linoleyl radical intermediate. This mechanism is reminiscent of the one earlier proposed to explain the slow chemical rearrangement of 9-hydroperoxide to 13-hydroperoxide, which takes place at elevated temperatures.

Regiospecificity of Lipoxygenases

Lipoxygenases are known to catalyze at least three types of reactions (a) dioxygenation of lipid substrates. (b) Secondary conversion of the hydroperoxy lipids and (c) formation of epoxy leukotrienes (Feussner and wasternack, 2002). However, under physiological conditions, the first reaction is more prevalent in plants. Positional specificity of the products (formed by lipoxygenase reaction) is determined by two independent properties of site of oxygen insertion and via rearrangement of the intermediate fatty acid radical.

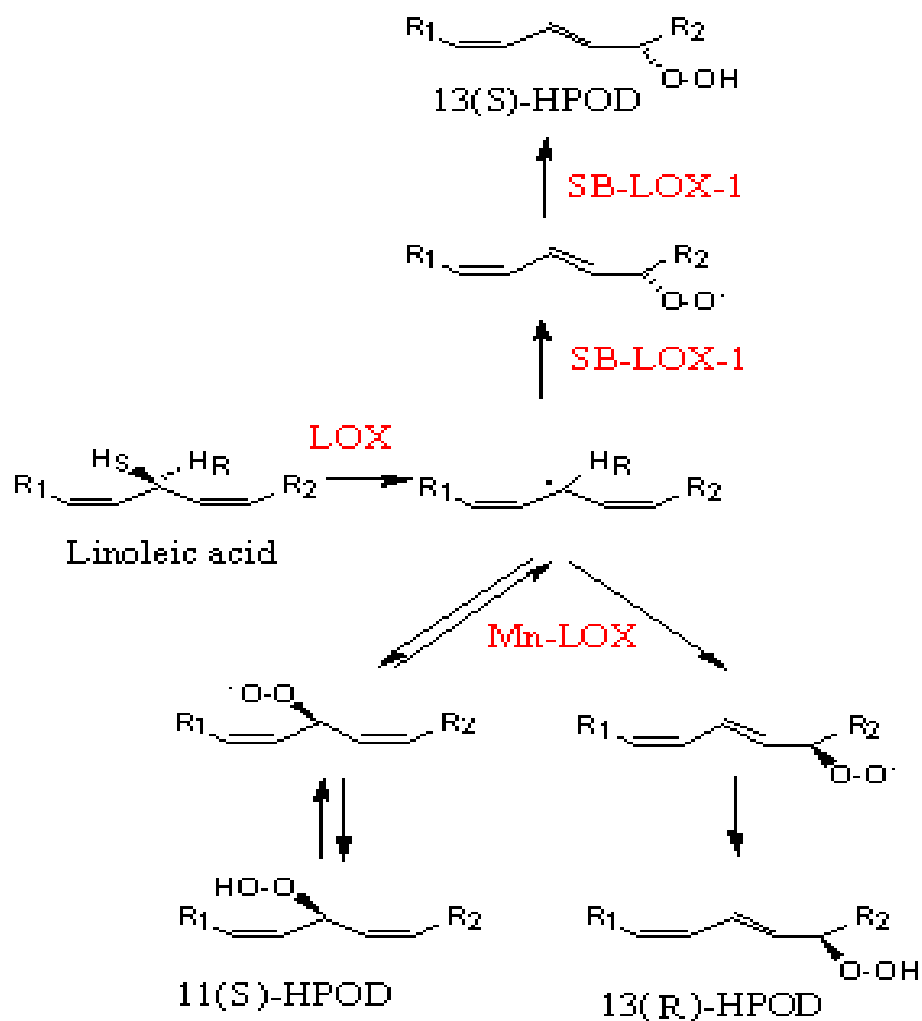


Figure 12. Comparison of reaction mechanism of soya lipoxygenase with manganese lipoxygenase (Ref. Su et al, 2000). The soya LOX-1 converts Linoleic acid to 13(S) HPOD, while Mn-LOX converts Linoleic acid to 11(S) and 13(R) HPOD.

Consequently when hydrogen is abstracted at C-11, molecular oxygen can be introduced at position [+2] or [-2] leading to dioxygen insertion at C-13 or C-9. The two different regioisomers of hydroxy PUFAs may be determined by two independent properties of catalysis (Hamberg and Samuelsson, 1967): (a) selectivity in the initial hydrogen removal and (b) selectivity in the site of oxygen insertion through arrangement of the of the intermediate fatty acid radical.

Two models have been proposed regarding the binding of substrate and its stereo specificity (Fig. 13) (Feussner and Wasternack 2002).

- (1) Space related theory: This theory has been established based on the data obtained from mammalian LOXs. The substrate (fatty acid) penetrates into the active site generally with its methyl end first. Then the depth of the substrate pocket determines the site of hydrogen abstraction, which in turn leads to the positional specificity of molecular oxygen insertion. However in plant LOXs only one double allylic methylene group of the substrate is accessible lending less credence to this hypothesis.
- (2) Orientation dependent theory: According to this, the regiospecificities of different isozymes is determined by the substrate orientation at the active site (either through carboxyl end or methyl end). Ruddat *et al* (2004) reported that both product and substrate bind “carboxylate end first” in the active site in soya LOX. Trp500 and Arg707 play a major role in substrate binding. At the lower end of the substrate binding pocket, a space filling His or Phe residue is present. One of these residues is strictly conserved in all plant 13-LOXs. In contrast, for all

plant 9-LOXs, a valine residue was identified at this highly conserved position was identified.

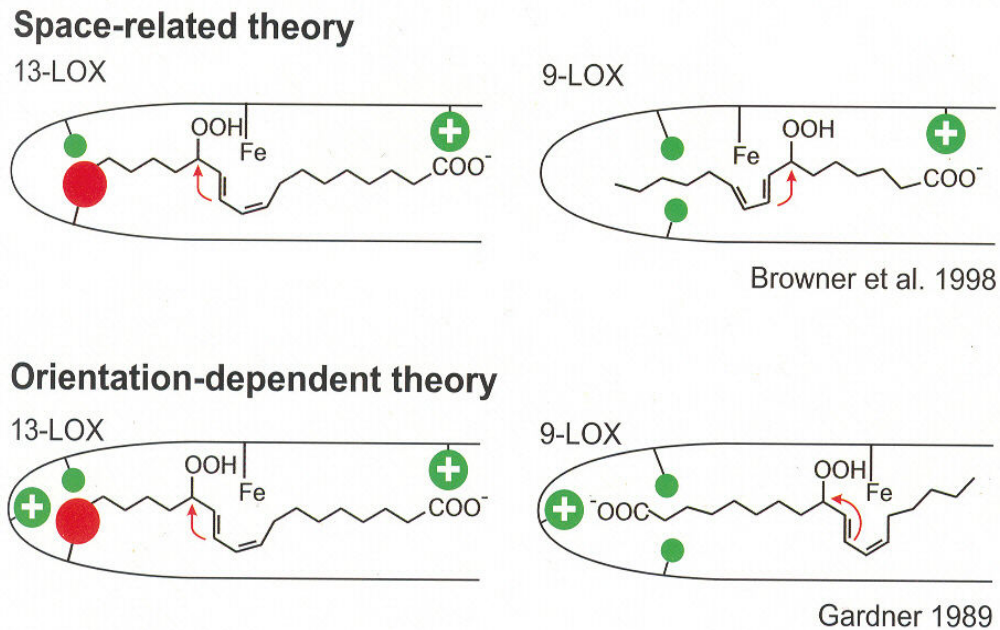


Figure 13: Models detailing the underlying reaction mechanism of regiospecificities

of LOX reaction (Adapted from Feussner and Wasternack, 2002).

Manganese lipoxygenase from *Gaeumannomyces graminis* has the unique ability to oxygenate the fatty acid carbon between the *cis* double bonds (C-11 of linoleic acid) (Su and Oliw, 1998). This has given rise to several new questions, which are yet to be answered. In summary, the space within the active site and the orientation of the substrate are important determinants for the positional specificity of LOXs.

Secondary reactions of LOX activity

Apart from the production of fatty acid hydroperoxides (primary products), lipoxygenases from plants catalyze the formation of secondary products – carbonyl compounds (ketodienoic fatty acids) under both anaerobic and aerobic conditions. Some lipoxygenases require anaerobic conditions while others are capable of producing these products under aerobic conditions. Pistorius (1974) reported the formation of ketodienes under aerobic conditions, in the soya LOX-3 catalyzed reaction. Soya LOX-1, with an alkaline pH optimum, produced carbonyl compounds under anaerobic conditions. The conjugated oxodiene chromophore has an absorption maximum at 285 nm. The aerobic formation of carbonyl compounds by lipoxygenases from other plant sources has also been reported (Kuhn *et al.*, 1991b, Wu *et al.*, 1995).

Co-oxidation reactions of LOX

Lipoxygenases can be used to bleach carotenoids in cereal flours by a cooxidation reaction, which requires the presence of a polyunsaturated fatty acid. The cooxidation reaction starts with the abstraction of a hydrogen atom from a carotenoid, resulting in the formation of a resonance stabilized radical which can combine with oxygen to produce carbonyl compounds (Klein *et al.*, 1985).

It is well known that a wide range of substances are cooxidized during the LOX catalyzed oxidation of linoleic acid observed only in plants (Axelrod 1974). This is a coupled oxidation reaction and requires both the substrate and product. Bleaching of carotene is commonly used as a measure of cooxidation potential. Lipoxygenases from different sources differ largely in their bleaching ability. Soya

LOX-2 and 3 are more effective in bleaching carotene compared to LOX-1 (Ramadoss *et al*, 1978).

Acquisition and substrate specificity of LOXs

The substrate for LOX should contain a *cis cis* 1, 4 pentadiene moiety, with an activated methylene group between the two double bonds. Majority of LOXs prefer free fatty acids as substrates. However, two 13-LOXs - soya LOX-1 and cucumber lipoxygenase - oxygenate the PUFA moieties esterified to phospholipids in biomembranes (Kuhn and Theile, 1999). Among mammalian LOXs rabbit reticulocyte is known to act on biomembranes (Kuhn *et al*, 2005). This suggests a role for LOXs in membrane permeabilization. For some LOXs from other sources, activity with neutral lipids (such as triglycerides) has been established suggesting their involvement in triglyceride catabolism (Gardner, 1991).

A clue to the mechanism of substrate acquisition by LOX comes from the conserved β -barrel domain (located at the C-terminus) of mammalian lipases (Gillmor *et al*, 1997). Like lipoxygenases, lipases (known to act on fatty acid substrates) are cytosolic. However, they need to access substrate in a hydrophobic environment. The β - barrel of lipase helps in the acquisition of substrate through protein - protein receptor interactions as well as hydrophobic interactions with lipoprotein particles (Brash, 1999). In lipoxygenases, the β - barrel is not essential for catalysis and is implicated in membrane interactions and substrate acquisition (Coffa *et al.*, 2005). However, there is no direct evidence of a role for the β - barrel (present in the N-terminal) of LOX in acquiring or binding the substrate.

A novel potato lipoxygenase has been shown to require hydrophobic surface loop for substrate binding (Ruddat, *et al.*, 2004). Knapp *et al* (2003) suggest that substrate oxygen enters through a specific protein channel through diffusion.

Chirality of products formed by LOXs

Most plant and animal lipoxygenases produces products of 'S' stereochemistry. Nonetheless, lipoxygenases producing mirror image 'R' configuration products are also wide spread, being found among aquatic invertebrates, plants and recently in humans (Brash *et al*, 1996, Coffa *et al.*, 2005). A key determinant in the chirality of products produced is a single active residue that is conserved. The conserved residue is an Ala in *S*-lipoxygenases and a Gly in *R*- lipoxygenases. These residues determine the position of the oxygen being inserted, which in turn determines the configuration of the hydroperoxide formed (Coffa *et al.*, 2005).

Acidic lipoxygenases

Most plant, animal and microbial lipoxygenases show broader pH activity profiles in the range 5.5 – 9.0. Potato lipoxygenases is active in the pH range of 4.5 – 7.0 with an optimum activity at pH 5.5 (Shimizu *et al*, 1990). It produces 9-hydroperoxides predominantly, which in turn are utilized for the production of traumatic acid (involved plant defense mechanism). Rice LOX is the only lipoxygenase, which is reported to be active only in acidic pH (Shibata and Axelrod, 1995). The enzyme introduces oxygen exclusively into the C-13 position of the linolenic acid. Maize, pepper, olive and tomato lipoxygenases are also

active in acidic pH range. However, they have broader pH profiles (Gardner, 1991).

Thermal stability of lipoxygenases

Lipoxygenase and lipoxygenase pathway enzymes produce some useful compounds for industrial applications due to implications in diseases like asthma, arthritis and related allergies (Chikere *et al*, 2001). The hydroperoxide products have been recognized as versatile reaction intermediates in the production of different fine chemicals (Gardner, 1996). Lipoxygenase and hydroperoxide lyase (HPL) produce volatile aldehydes like (3Z) hexanal, which give a distinctive odor akin to freshly mowed grass. LOX, combined with HPL, has the potential of providing the flavor industry with fresh odors associated with unprocessed fruits and vegetables. Also there is a possibility of these aldehydes being used as antifungal and antimicrobial agents. Certain compounds such as epoxy hydroxyl fatty acids formed by LOX pathway are used in plant defense against pathogen invasion (Gardner, 1996).

Though the industrial applications of lipoxygenase are large, the potential application of this enzyme as a versatile biocatalyst is limited by its poor stability towards reaction conditions. Rapid inactivation of the enzyme in purified and crude form is a major problem affecting turnover as well as storage stability (Chikere *et al*, 2001). Attempts to stabilize the enzyme through different ways have not been successful. Enzymes, active and stable at high temperatures, possess a great technological potential (Illanes, 1999).

Generally plant lipoxygenases are more stable compared to animal lipoxygenases (Shibata and Axelrod, 1995). However, thermal stability in plant

lipoxygenases varies depending on the source. Soya LOX-1 has a half-life of 25 min ($t_{1/2}$) at 69°C, the midpoint of thermal inactivation (T_m) temperature. Soya LOX-2 is also reported to have a similar $t_{1/2}$ (Christopher *et al*, 1972.) Pea LOX (*Pisum sativum*) is inactivated completely after 4 min at 65°C (Chen and Whitaker, 1986) while, cowpea lipoxygenases are found to be unstable beyond 60°C (Den and Mendoza, 1982). Similarly, potato tuber lipoxygenases (acidic LOXs) are not stable beyond 65°C (Park *et al*, 1988). Most LOXs from other plant sources are rapidly inactivated by exposure to temperatures beyond 70°C. Among plant lipoxygenases, soya LOX-1 is reported to be the most stable lipoxygenase (Shibata and Axelrod, 1995). Manganese dependent LOX from *G. graminis* has higher thermal stability than soya LOX-1. The midpoint of thermal inactivation temperature (T_m) for activity is reported to be 70°C (Su and Oliw, 1998).

Structure of lipoxygenases: Soya LOX-1

Plant lipoxygenases have been investigated since the early 1930s and much of the knowledge gained has contributed to the advance of mammalian lipoxygenase research (Shibata and Axelrod, 1995). Soya LOX-1 has been intensively studied for the past 25 years and is a model for this group of proteins. Soya LOX-1 contains one atom of non heme iron per molecule of the enzyme. EPR studies with resting (Fe^{2+}) and active form (Fe^{3+}) of enzyme revealed that iron is essential for catalytic activity and is in a spherically coordinate structure. The iron containing active site (Fig. 14A) is in the center of the catalytic domain. Four conserved His residues and the carboxyl group of the conserved Ile (at the C – terminal) is involved in iron binding and has been identified as the putative iron ligands (Shibata and Axelrod, 1995).

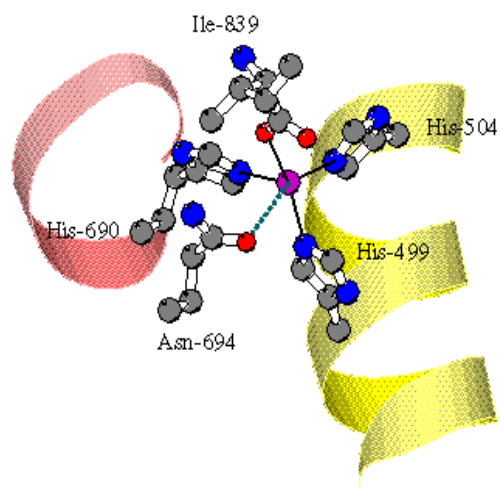


Figure 14 A. Active site of Soya LOX-1. Residues involved in iron binding are shown. Iron is shown in pink.

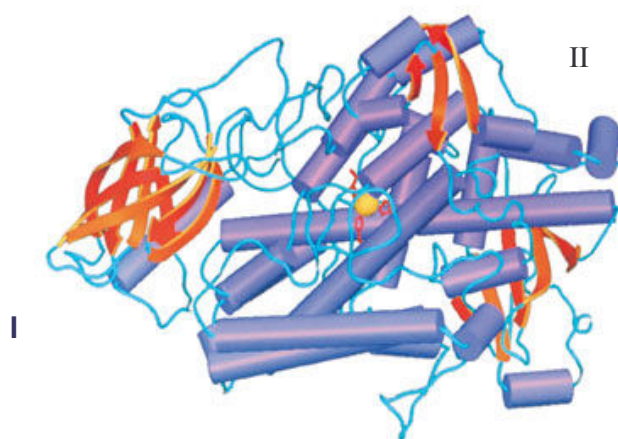


Fig 14 B. Schematic diagram of the three dimensional structure of soya LOX-1. The two domains - small N-terminal domain I (146 residues) and the large C-terminal domain II (693 residues) are shown. The iron containing active site is located in domain II. (*PDB No. 1yge, Minor et al, 1996*)

The primary sequence and three dimensional structure of LOX -1 have been determined, showing that it is an ellipsoid of 90 Å by 65 Å by 60Å, with 839 amino acid residues and a molecular mass of 93,840 Da (Fig. 14B) (Boyington *et al.*, 1993). LOX – 1 consists of two domains: a β -barrel domain at the N-terminal (146 residues) and a helical bundle at the C–terminal (693 residues). The small N-terminal domain comprises an eight-stranded β -barrel while the C -terminal domain contains the active site. In soya LOX-1, the C-terminal domain (catalytic domain) consists of 18-22 helices and two β -antiparallel sheets at the opposite end from the N-terminal β -barrel (Boyington *et al*, 1993). The two long central helices cross at the active site. Both helices include internal stretches of helix that provide three histidine ligands to the active site iron (Fig. 14A). Cavities I and II in the major domain extend from the surface to the active site. Cavity I (funnel shaped) may play role in dioxygen channel while cavity II (long and narrow) is, presumably, the surface to the active site. Cavity I (funnel shaped) may play role in dioxygen channel while cavity II (long and narrow) is, presumably, the substrate-binding pocket. Cavity I presents an ideal path for the access of molecular oxygen to iron whereas cavity II can accommodate arachidonic or even slightly larger fatty acids (Shibata and Axelrod., 1995).

The N-terminal domain in LOX -1 is distinct from the C-terminal domain with only a few contacts. However, limited proteolysis experiments indicated that the two domains are tightly associated and that domain interaction plays a role in reversible unfolding of LOX-1 through ionic interactions (Sudharshan and Rao, 1997).

Structure of mammalian lipoxygenases: rabbit 15-LOX

Mammalian lipoxygenases have smaller molecular mass (75 – 80 kDa) as compared to plants (94 – 104 kDa). Their overall structure is very similar and the similarity in the catalytic domain is very high - almost superimposable (Coffa *et al*, 2005). The overall shape of Rabbit 15-LOX resembles an elliptic cylinder (Fig. 15). Rabbit 15-LOX, comprises of two domains- one small N-terminal domain (110 amino acids, 8 β -barrels) and the C-terminal catalytic domain (Kuhn *et al.*, 2005).



Figure 15. Structure of rabbit reticulocyte 15-LOX

(PDB No. 1-LOX, Gillmor *et al* 1997)

The large catalytic domain consists of 18 helices, which are interrupted by a small β -sheet sub-domain (Fig. 15). A random coil, without defined structure, links both domains. The center of the C-terminal domain consists of 2 long helices. It contains four of the five protein ligands - His361, His366, His541 and His545 that coordinate non-heme catalytic iron. The fifth protein ligand is the carboxylate of the C-terminal residue Ile663 while water may be the sixth ligand required for enzyme catalysis. These five ligands coordinate the iron with excellent octahedral geometry. The substrate binding cavity is boot shaped and is directly accessible from the surface of the protein (Kuhn *et al*, 2005). Arg403, Gly407 and Leu597 line its entrance and the side chains of Phe353, Ile418 and Ile593 define the bottom of the substrate binding cavity. Arg403 might interact with the carboxylic group of fatty acid substrate, while other residues have been identified as sequence determinants for the positional specificity of the enzyme.

Microbial LOX: Manganese dependent lipoxygenase from *G.graminis*

All plant and mammalian lipoxygenases are believed to contain non - heme iron as prosthetic group. *G. graminis* is so far reported to produce the only LOX with manganese at its catalytic center (Su *et al.*, 2000). Its protein sequence has 26% similarity with mammalian lipoxygenases and 40% similarity with plant LOXs (Cristea *et al.*, 2005). Manganese is tightly bound to the apoenzyme in a 1:1 stoichiometry. Site directed mutagenesis suggested that three histidine residues and C-terminal valine were essential for lipoxygenase activity and metal binding (Fig. 16). The metal also likely binds water. The catalytic mechanism of Mn- LO is similar to iron lipoxygenases (Cristea *et al*, 2005).

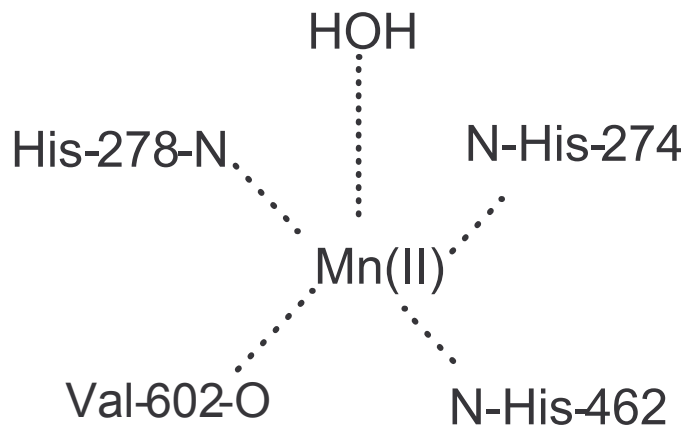


Figure 16. Proposed metal ligands of manganese in manganese lipoyxygenase (Adapted from Cristea *et al.*, 2005)

Dual function proteins

The number of proteins present in any cell is limited; yet, the functions performed by them are many. Many molecules of the protein are present in the cell. The same protein can be assigned for different tasks in different parts of the cell. Proteins have large surfaces that can provide multiple active sites of various catalytic potentials. There are many proteins, which are reported to have multiple functions associated with them (Jeffery, 1999). These proteins are often oligomeric containing catalytic sites at the interface of its subunits (Kirschner *et al*, 1976). One such protein is lectin from *Vicia faba* seeds having α - galactosidase activity (Dey *et al*, 1982). The multiple functions of such moonlighting proteins add another dimension to cellular complexity and benefit cells in several ways. These moonlighting proteins provide one way of coordinating cellular activities. This makes sense in the best interest of cellular economy.

AIM AND SCOPE OF PRESENT INVESTIGATION

Legume seeds play an important role as protein sources in the vegetarian diet of developing countries. Most seeds contain secondary compounds like protease inhibitors, phytates, polyphenols, lectins and products of lipoxygenase pathway, which play an important role in plant defense. These compounds are toxic and have a negative impact on consumer. Soybean, winged bean, chickpea and horsegram are some of the legumes that have become a part of the daily diet.

Horsegram (*Dolichos biflorus*) is a leguminous pulse crop native to South East Asian subcontinent and tropical Africa. The U.S National academy of Sciences has identified this legume as a potential food source of the future as it is found to be good source of proteins and calcium. Horsegram contains antinutritional factors like trypsin inhibitors and lectin. It contains 20 – 25% dry weight of proteins and 2.5% dry weight of lipids. The unsaturated fatty acids constitute 71.2% of total lipids. Horsegram lipids are known to be protective against duodenal ulcers *in vivo*. The major unsaturated fatty acid is linoleic acid, which can be oxidized by lipoxygenase to its hydroperoxides.

Lipid peroxidation is a phenomenon that commonly occurs in all biological systems, in general, and in environmentally/ developmentally regulated processes of plants, in particular. Lipoxygenase (linoleate oxygen oxidoreductase, EC 1.13.11.12 LOX) catalyzes the region / stereospecific dioxygenation of polyunsaturated fatty acids containing cis, cis 1, 4 – pentadiene system. Linoleic acid is oxygenated either at carbon atom 9 or at C-13 of the hydrocarbon backbone of the fatty acid, leading to two groups of compounds, the (9S)-hydroperoxy and the (13S)- hydroperoxy derivatives of polyunsaturated fatty

acids. Lipoxygenases contain ferrous iron as its cofactor, which is oxidized into the ferric state. The ferric form of LOXs is catalytically active. Mammalian and plant LOXs so far studied, catalyze production of hydroperoxides that have the “S” absolute configuration. Manganese LOX, purified from the fungus *Gäeumannomyces graminis* is reported to contain 0.5 – 1.0 atom of manganese per molecule. In contrast, manganese LOX catalyze the formation of “R” hydroperoxides.

Horsegram lectin has unique specificity among the legume lectin family. It has a high content of lectins – proteins having affinity for specific carbohydrate moieties – are particularly abundant in the seeds of legumes. The lectins bind to carbohydrates and are used as a model system for studying protein – carbohydrate interactions. The different legume lectins show a remarkable range of sugar specificity, despite the high sequential and structural similarity of their subunits.

The number of proteins present in any cell is limited; yet, the functions performed by them are many. Proteins, being large, their surfaces can provide multiple active sites of various catalytic potentials. Thus, there are many proteins, which are reported to have multiple functions associated with them. The multiple functions of such moonlighting proteins add another dimension to cellular complexity and benefit cells in several ways. The moonlighting proteins provide one way of coordinating cellular activities.

Comparative structural studies of lipoxygenases and their interaction would help in understanding the role of these enzymes and their products. With the objective of

understanding the structure- function relationship and stability of lipoxygenases from horsegram, the following studies were undertaken.

1. Identification, isolation, purification and characterization of lipoxygenases from horsegram; identifying the cofactor participating in lipoxygenase activity.
2. Enzymatic and molecular properties; inhibition of lipoxygenases with inhibitors; mechanism and nature of inhibition.
3. Thermal stability of lipoxygenases and the role of protein- protein interactions in structural stability.

Lipoxygenases from legumes will be studied to understand the relation between their structure, function and stability. Lipoxygenases from horsegram and their role in the indomitable pest resistance of this legume will also be the focus of this investigation.

MATERIALS AND METHODS

Materials. Horsegram seeds (*Dolichos biflorus*), Nagamangala Pac 9 Strain, were procured from University of Agricultural Sciences, Bangalore, India. Horsegram seeds were dehulled and ground to fine powder and defatted thoroughly by extraction with hexane. Hexane was removed by decantation. The flour was air dried and stored in an airtight container at 4°C.

Linoleic acid (>99%) and linolenic acid (>99%) were obtained from Nuchek Prep Inc. (Elysian Mn). Arachidonic acid, DEAE-Sephacel, Sephacryl S-200, Con A Sepharose, ω - amino hexyl agarose, Tween-20, protein molecular weight markers, protease inhibitor cocktail, urea, nor NDGA, 4-nitrocatechol, ANS, NBT, BCIP, BSTFA, *Dolichos biflorus* lectin, nitrocellulose membrane, β -carotene, adenine, kinetin, trypsin, ponceau stain, eicosatetraynoic acid, N-acetyl galactosamine, methyl α -D- mannopyranoside, phosphatidyl choline (PC), deoxycholic acid (DOC), periodic acid (PA) Freund's complete adjuvant, Freund's incomplete adjuvant, goat anti-rabbit IgG conjugate, diazald and CAPS were from Sigma Chemical Co., St. Louis. Solvents used were of HPLC grade. All other chemicals were of analytical grade.

HPLC Columns

Sorbax- Sil column (4.6 mm x 250 mm, 5 μ m), Shim Pak C₁₈ (10 μ , 4.6 mm x 250 mm) column was from Shimadzu, Japan, TSK G-2000 SW_{XL} (7.8 mm x 300 mm, 5 μ m) column from Supelco, Bakerbond chiral phase column; J. T. Baker Res. Products, USA, 250 x 4 mm, 5 μ m).

GC Column. 30 m DB-1, film, 0.25 μ m; diameter 0.25 mm J&W Scientific, USA.

Enzyme assays

Lipoxygenase assay. LOX activity was monitored by UV analysis, according to the method of Shimizu *et al* (1990), by following the increase in absorbance at 234 nm due to the formation of conjugated diene fatty acid hydroperoxide. One unit of enzyme activity was defined as the amount of enzyme required to form 1 μ mole of product/min at 25°C under the assay conditions. The standard assay mixture consisted of 150 μ M linoleic acid in 0.1 M sodium acetate buffer (pH 5.0) containing Tween-20 (2 nM) for HGLOXI. For HGLOXII the standard assay mixture comprised of 250 μ M linoleic acid in 0.1 M sodium acetate buffer (pH 5.0) containing Tween-20 (0.1 μ M). Fresh stock solutions (10 mM) of the desired fatty acid in absolute ethanol were prepared for enzyme assay. The reaction was initiated with the addition of enzyme to substrate in a total volume of 3 ml at 25°C and monitored for 3 min. To the reference cuvette, buffer with alcohol was added instead of enzyme. The rate was estimated from the linear version of the curve. Kinetics constants were determined at 25°C in triplicate for 18:2n-6, 18:3n-3 and 20:4n-6. Protein concentrations were determined by the method of Lowry *et al.* (1951), using BSA as standard.

Lipoxygenase assay of horsegram extract was carried out with following substrates: (i) sodium linoleate, (ii) ammonium linoleate and (iii) PC micelles with linoleic acid. The standard assay mixture comprised of 100 μ M of substrate in 0.1 M sodium acetate buffer (pH 5.0). Fresh stock solutions (10 mM) of the fatty acid were prepared for enzyme assay.

Sodium linoleate was prepared according to Axelrod *et al* (1981). Linoleic acid (70 mg) and 70 mg of Tween -20 were dissolved in 4 ml of oxygen free water.

The resulting suspension was clarified by adding sufficient amount of 0.5 M NaOH. Final volume was made up to 25 ml with water. This stock solution was divided into 1 ml portions in small vials and flushed with nitrogen gas before use.

Ammonium linoleate was prepared according to Mulliez *et al* (1987). Linoleic acid (70 mg) and 70 mg of Tween -20 were dissolved in 4 ml of oxygen free water. The resulting suspension was clarified by adding sufficient amount of 0.5 M ammonium hydroxide. Final volume was made up to 25 ml with water. This stock solution was divided into 1 ml portions in small vials and flushed with nitrogen gas before use.

PC micelles were prepared according to Began *et al* (1999) using a mixture of phosphatidyl choline and deoxy cholate. After solubilization of the PC and DOC in chloroform / methanol (2:1), the solvent was evaporated and dried in the presence of nitrogen gas. The resulting thin film was solubilized in 50 mM Tris HCl (pH 7.4) and then sonicated for 5 min using a bath type sonicator. Fatty acid insertion was carried out by adding fatty acid solubilized in ethyl alcohol (30 μ M) to PC micelles (30 μ M) with 0.1 M sodium acetate buffer (pH 5.0).

Haemagglutination assay. Haemagglutination assay was carried out by serial dilution of the lectin in phosphate buffered saline (0.01 M sodium phosphate, 0.15 M sodium chloride, pH 7.0) using 2% trypsinised A⁺ human blood as reported earlier (Sivakumar and Rao, 1990). Human blood was collected by hand vein puncture into Alsevier's solution containing 2.05% glucose, 0.89% sodium citrate, 0.42% sodium chloride and 0.05% citric acid. It was centrifuged at 3000 rpm for 10 min at 4°C. The sedimented erythrocyte pellet was washed thrice with 0.9% saline and the pellet was made to 4% suspension with saline. Processed

erythrocytes were treated with 0.1% trypsin and incubated at 37°C for 1 h. Erythrocytes were centrifuged at 3000 rpm for 10 min at 4°C and then made up to their original volume. 200 µl of the protein sample serially diluted with 200 µl of saline and 200 µl of the trypsin treated erythrocytes were added separately in a plexi plate and incubated at 37°C for 1 h. Haemagglutination was visually observed (1 HU is defined as the amount of protein required to cause visible agglutination using human A⁺ erythrocytes. The specific activity is mentioned as units/mg protein).

Cooxidation of β-carotene. Bleaching of the pigment, β-carotene, by LOX was followed spectrophotometrically at 460 nm (Sanz *et al*, 1994). For analysis of the pigment cooxidation power of LOX, pigment substrate solution was prepared as follows: β-carotene (1 mg) was dissolved in 1ml of chloroform to which 36 µl of Triton X -100 was added. From this stock solution, an aliquot of 0.1 ml was taken. The solvent was evaporated under nitrogen flow and the residue was dissolved in 1 ml of 50 mM sodium phosphate buffer (pH 6.0). To this solution, 2.5 ml of linoleic acid solution (10 mM), prepared according to Axelrod *et al*, (1981) was added and 16.5 ml of sodium phosphate buffer (50 mM, pH 6.0). The cooxidation reaction was carried out by incubating 2 ml of the substrate solution with different amounts of LOX for 10 min at 20°C.

PROTEIN PURIFICATION

Purification of all proteins. HGLOXI, HGLOXII, *D.biflorus* lectin from horsegram seeds, lipoxygenase from soybean seeds, pea and potato were carried out at 4°C.

(A) Purification of HGLOXI

Extraction. Defatted horsegram flour (2.5 g) was extracted with 0.1 M sodium phosphate buffer (pH 6.0) (12.5 ml) containing protease inhibitor cocktail and 0.01% sodium azide for 2 h with gentle stirring. The slurry was centrifuged for 30 min, at 6000 rpm to recover the clear supernatant.

Ammonium sulfate fractionation. The above supernatant was subjected to 0 -70% ammonium sulfate saturation and the pellet was recovered by centrifuging at 6000 rpm for 30 minutes. The pellet was dissolved in 25 ml of 20 mM phosphate buffer (pH 6.0) and dialyzed against the same buffer containing 5% glycerol and 0.01% sodium azide (Buffer A) (1000 ml x 6) before loading onto a Sephacryl S-200 column.

Sephacryl S-200 column. The dialyzed protein was fractionated on a Sephacryl S-200 column (2.7 x 120 cm, 700 ml) with buffer A at a flow rate of 20 ml/ h. Activity was recovered in 3 peaks with highest specific activity (94 U/ mg) in the 2nd peak (35% of total activity loaded). This peak was pooled, dialyzed against buffer A containing 0.05 M NaCl.

DEAE Sephacel Column. The dialyzed fraction was loaded onto a DEAE Sephacel column (2.1x 7 cm, 25 ml) equilibrated with buffer A containing 0.05 M NaCl. The column was eluted with a linear gradient of 0.05 M – 0.3 M NaCl in buffer A at a flow rate of 10 ml/ h. The active fractions were pooled, flushed with nitrogen and stored at -20°C, after checking for the homogeneity of the protein.

(B) Purification of HGLOXII from *D.biflorus*

Extraction, ammonium sulfate saturation and gel filtration chromatography was carried out as described above. The third peak with lipoxygenase activity obtained from Sephacryl S-200 column was pooled, concentrated with 70% ammonium sulfate saturation. The pellet obtained was dialyzed against 5 mM sodium phosphate buffer (pH 6.0) for twelve hours with six changes. The dialyzed fraction was loaded onto ω - amino hexyl linolyl agarose column (10 ml) pre equilibrated with 5 mM sodium phosphate buffer (pH 6.0). The column was eluted with a linear sodium phosphate gradient of 5 – 20 mM. The active fractions were pooled, checked for homogeneity and stored at -20° C after flushing with nitrogen.

Preparation of ω - linoleyl amino hexyl agarose. ω - linoleyl amino hexyl agarose (10 ml) was prepared according Grossman *et al* (1972). Linoleic acid was coupled to agarose derivative in 1:2 ratio. Linoleic acid was dissolved in 4 ml of dimethyl formamide, which was added to 10 ml of ω -amino hexyl agarose. The pH was adjusted to 5.0 to which 1 milli mole of carbodimide was added. The reaction mixture was stirred at 25°C for 4 h under nitrogen and washed extensively with sodium phosphate buffer (5 mM, pH 6.0) until no absorption at 280 nm and 234 nm was detected.

(C) Purification of lectin from *D. biflorus*

The lectin from horsegram seeds was purified as reported earlier (Kocourek *et al*, 1977) with slight modifications.

Extraction. Defatted finely ground seeds were extracted for 6 h at 4°C by stirring in 0.1 M sodium phosphate buffer, pH 6.0 instead of saline. The suspension was centrifuged for 30 min, at 6000 rpm and the clear supernatant was recovered.

Ammonium sulfate saturation. The supernatant was saturated to 0 - 40% by ammonium sulfate. After standing overnight at 4°C the precipitate formed was separated by centrifugation and discarded. The supernatant was brought to 60% saturation with ammonium sulfate and stored at 4°C for 24 hr. The precipitated active fraction, separated by centrifugation, after 24 h was dissolved in 50 mM sodium phosphate buffer, pH 7.5, containing 0.02% sodium azide (Buffer B). The dissolved precipitate was dialyzed against buffer B.

DEAE Cellulose column. The active protein fraction was applied onto a DEAE Cellulose column (2.8 X 8 cm, 50 ml) equilibrated with buffer B. The elution was carried out by 70 mM phosphate buffer, pH 7.5 (buffer C). 5 ml / tube fractions were collected at a flow rate of 25 ml/hr. Fractions showing haemagglutination activity were pooled and checked for LOX activity before ammonium sulfate precipitation (0 – 70%). The pellet obtained was dialyzed against buffer B and centrifuged before loading onto a Sephadex G-100 column.

Sephadex G-100. The clear supernatant was applied to a Sephadex G-100 column (0.9 X 130 cm, 90 ml) equilibrated with buffer B. The elution was performed with buffer C at a flow rate of 10 ml / h. the fractions were collected at 15 min time interval and that containing haemagglutination activity were pooled and dialyzed against buffer B.

DEAE Cellulose Column. The dialyzed sample was loaded onto a DEAE Cellulose column (2.1x 7 cm, 25 ml) preequilibrated with buffer A. Following elution with buffer B (5 ml fractions, 20 ml/h flow rate) the fractions containing haemagglutination activity were pooled and checked for homogeneity by SDS Page and the protein was stored at -20°C after flushing with nitrogen.

(D) Purification of soy lipoxygenase I from soybean seeds

Purification was done according to Sudarshan and Rao (1997). The defatted soya bean meal (25 g) was extracted with 300 ml of ice cold 0.2 M sodium acetate buffer, pH 4.5 for 1 hour. The suspension was centrifuged at 6000 rpm for 30 min and pH of the supernatant solution was adjusted to 6.8. The pellet formed was discarded and the supernatant was brought to 0 - 30% ammonium sulfate saturation. The resultant pellet was removed by centrifugation. The clarified supernatant was brought to 60% ammonium sulfate saturation. The protein fraction that precipitates at this stage was dissolved in 20 mM sodium phosphate buffer, pH 6.8. This solution was dialyzed against the same buffer and chromatographed on DEAE- Sephadex A-50 (2.8 X 8 cm, 50 ml) which was preequilibrated with 20 mM sodium phosphate buffer, pH 6.8. The column was developed with a linear gradient formed from 20 mM to 170 mM of phosphate buffer pH 6.8 and enzyme was eluted with a linear gradient of 170 mM to 240 mM phosphate buffer pH 6.8. The fraction containing maximum LOX activity was pooled and the solution was brought to 0 - 70% ammonium sulfate saturation. The precipitate formed was centrifuged and dialyzed in 20 mM phosphate buffer pH 6.8. The dialyzed protein was loaded on a Sephadex G100 column (0.9 X 130 cm, 90 ml), which was pre-equilibrated with 20 mM phosphate buffer, pH 6.8. The enzyme was eluted with the same buffer. The protein fractions with maximum activity were pooled and concentrated using an Amicon ultrafiltration cell with a 30kD cut-off membrane.

(E) Purification of potato lipoxygenase

The purification was done according to Shimizu *et al*, (1990). After peeling, potatoes were cut into small slices and homogenized in a Warring blender with freshly prepared 0.1 M acetate buffer (pH 4.5) containing 2 mM ascorbic acid and 2 mM sodium meta bisulphate. The homogenate is filtered and the supernatant was subjected to 25 – 40% ammonium sulfate saturation. The precipitate formed was dialyzed against sodium phosphate buffer, pH 6.8, 50 mM and was stored at 4°C.

(F) Purification of pea lipoxygenase I

The purification of pea LOX-1 was done according to Reynolds and Klein (1982) with slight modifications. Acetone defatted ground pea seeds were extracted with 10 volumes of 50 mM sodium phosphate buffer, pH 6.8, for 5 h and centrifuged. The supernatant obtained was brought to 0 - 25% saturation with ammonium sulfate. The precipitate obtained after centrifugation was discarded. The supernatant was brought to 60% saturation with ammonium sulfate, centrifuged to recover the pellet. The precipitate obtained was dissolved in 50 mM sodium phosphate buffer, pH 6.8 and dialyzed overnight against the same buffer. The dialyzed sample was applied to a Sephadex G-200 column (0.9 X 130 cm, 90 ml) equilibrated with 50 mM sodium phosphate buffer, pH 6.8. Fractions of 5 ml was eluted with the same buffer, at a flow rate of 20 ml/h. Fractions with LOX activity were pooled and concentrated by ultrafiltration using a membrane with low protein binding (PLGC, NMWL 30,000, Millipore). The concentrated solution was dialyzed overnight against 50 mM sodium phosphate buffer, pH 6.8 containing 50 mM NaCl.

The dialysate was applied to a DEAE-Sephadex A-50 column (2.1x 7 cm, 25 ml) and eluted with a linear salt gradient increasing from 0.05 – 0.4M NaCl in 50 mM sodium phosphate buffer, pH 6.8. Fractions of 3 ml were collected at a flow rate of 20 ml/h. and LOX active fractions were pooled. Pooled fractions with LOX activity were dialyzed against deionised distilled water before loading onto a preparative gel electro focusing. The enzyme solution was mixed with 5 ml of ampholine solution (40%, w/v), pH 5 – 7 and diluted to 100 ml with deionised water. 4 g of Ultradex was added slowly to the ampholine –enzyme solution. The gel bed was dried until a loss of 35% water occurred. Electrofocussing was performed at 10 mA for 14 h, with a constant power of 8 W. The separated zones were collected and eluted with 50 mM phosphate buffer, pH 6.8. Each fraction was assayed for LOX activity.

Gel electrophoretic analysis for homogeneity, activity and glycosylation

Native – PAGE - Native-PAGE was carried out using 12% gels without SDS or β -mercaptoethanol as described earlier by Laemmli, (1970). The molarity of the electrophoretic buffer used was 50 mM Tris and 284 mM glycine. The dialyzed protein samples were centrifuged prior to loading the gel. The gels were run for 6 hr at 10 mA constant current at 25°C. The bands were visualized by staining with coomassie brilliant blue R-250.

Activity staining. Activity staining was done to detect LOX activity in polyacrylamide gels under native conditions. The gels were run as described above at 4°C and incubated in sodium acetate buffer (0.1 M, pH 5.0) for 15 min at 4°C. The gel was transferred to 100 ml sodium phosphate buffer (0.1 M, pH 6.0)

containing 10% alcohol and linoleic acid (50 mg). The gel was incubated for 6 h at 4°C. After incubation, the gels were removed, rinsed thoroughly with water and placed containing acidic potassium iodide (5 ml saturated aqueous KI per 100 ml of 15% acetic acid). The brown activity bands appeared within 15-20 min indicating LOX activity (Guss *et al*, 1967).

Glycoprotein staining. The purified protein was subjected to polyacrylamide gel electrophoresis under native conditions. The molarity of the electrophoretic buffer used was 50 mM Tris and 284 mM glycine, pH 8.3. The gel was immersed in 12.5% trichloroacetic acid (25 – 50 ml/ gel) for 30min followed by rinsing with distilled water. The gel was immersed in 1% periodic acid in 3% acetic acid for 50 min and washed with distilled water (6 x 10 min) till the gel was iodate- free. The gel was stained with fuchsin – sulfite in dark and washed with freshly prepared 0.5% metabisulfite to develop the pink bands indicating the presence of glycosylation (Zacharius *et al*, 1969).

Gel electrophoretic analysis for nature of the subunits

SDS –PAGE. SDS-PAGE was carried out using 12% gels (Laemmli, 1970). The dialyzed sample was heated in a boiling water bath for 90s prior to loading on the gels. The gels were run at a constant current of 10 mA for 3 h at 25°C. Staining was by coomassie brilliant blue R-250 for the gels. Phosphorylase-97,000, Bovine serum albumin- 68,000, ovalbumin – 43,000, carbonic anhydrase – 29,000, soybean trypsin inhibitor -20,000, lysozyme -14,000 and insulin – 6,000 were used as molecular mass markers

Urea PAGE. The nature of the subunits of the purified protein was also determined by urea gel electrophoresis as described by Goldenberg (1997).

Briefly, 9 % slab gels were prepared containing 8 M urea. Electrophoretic buffer used was 50 mM Tris acetate pH 8.0. Protein sample was prepared in the same buffer containing 8 M urea and was electrophoresed at 25°C for 3 h at 9 mA constant current. Pre electrophoresis was performed for 1 h. Protein was visualized by staining the gel with coomassie brilliant blue.

Western Blotting (Semi Dry blot) of proteins. Preparation of PVDF membrane: The PVDF membrane cut to the required size was soaked in methanol for 1-2 min before use. The membranes were immersed in transfer buffer (10 mM CAPS, pH 11.0) containing 10% (v/v) methanol and equilibrated for 10 min (Matsudaira 1987).

Blotting: Following electrophoresis, the gel was immediately rinsed 2-3 times in water to remove the buffer ions and equilibrated in CAPS buffer (10 mM, pH 11.0 containing 10% v/v HPLC grade methanol) for 10min. Semi-dry electro blotting was carried out using a semi dry blotting apparatus (Towbin *et al.*, 1979). The transfer was carried out for 120 min using a current of 0.8 mA / cm² of the filter paper.

Determination of homogeneity of purified protein by RP HPLC. Homogeneity was also ascertained by reversed phase HPLC, using a Shim Pak C₁₈ (10μ, 4.6 mm x 250 mm) column. The column was preequilibrated with solvent A (0.1% TFA in water). Protein (20 μg) was injected at a flow rate of 1 ml/ min. The column was washed with solvent A for 5 min and brought to 50% acetonitrile (containing 0.1%TFA) in 10 min. The bound protein was eluted by a linear gradient of acetonitrile (50-75%) over a period of 45 min. Detection was monitored at 280 nm using Waters® 2996 photodiode array detector.

Determination of homogeneity and molecular weight by size exclusion chromatography. The molecular mass of purified LOX's from horsegram seeds was determined by gel filtration on HPLC using a TSK G-2000 SW_{XL} (7.8 mm x 300 mm, 5 μ m) column, equilibrated in sodium phosphate buffer (20 mM, pH 6.0) containing 0.3 M NaCl. Elution was carried out at a flow rate of 0.2 ml/min for 45 min; protein was detected at 280 nm. The column was calibrated using standard proteins (alcohol dehydrogenase -150 kDa, Soya LOX1- 94 kDa, BSA-68 kDa, Carbonic anhydrase-29 kDa, lysozyme - 14 kDa).

Mass spectrometry. The subunits of LOX were analyzed by MALDI-TOF after gel filtration of HGLOX on HPLC (as described above). The peak fraction was collected and analyzed. Matrix Assisted Laser Desorption Ionization Mass Spectroscopy (MALDI/MS) analyses were performed on a Bruker Daltonics Ultra flex MALDI TOF/TOF system (Bruker- Daltonic, Bremen, Germany) in the reflection positive ion mode, equipped with a nitrogen laser of 337 nm. The samples were prepared by mixing equal volumes of sample (prepared in H₂O/TFA (100:0.1)) and saturated matrix prepared separately in CH₃CN/H₂O/TFA (80:20:0.1) (α -cyano-4- hydroxycinnamic acid (Sigma Aldrich Chemie GmbH). The amount of protein loaded on the probe slide was ~ 10 pmol. The samples were then dried at 25°C under atmospheric pressure. Data was collected between 0 and 100 kDa.

pH optimum for LOX activity. LOX activity was tested in 0.5 pH unit intervals from pH 2.5 – 9. Sodium acetate buffer (0.1 M, 2.5 – 5.5), phosphate buffer (0.1 M, pH 6.0 – 8.0), borate buffer (0.1 M, pH 8.5 – 9.0) were used. The assay was carried out as described above.

pH stability of LOX activity. Stability of the horse gram extract was studied at different pH by incubating the extract for different time intervals, in different buffers (sodium acetate buffer (0.1 M, pH 2.5 – 5.5), phosphate buffer (0.1 M, pH 6.0 – 8.0), borate buffer (0.1 M, pH 8.5 – 9.0) and assaying the residual activity at optimum pH.

Substrate affinity for LOX activity. Substrate affinity of the purified protein towards different fatty acids was carried out at the optimum pH of 4.5 sodium acetate buffer (0.1 M) at 25°C. The protein was incubated with different concentrations (10 - 300 μ M) of linoleic acid, linolenic acid and arachidonic acid and assay was performed as given above. The K_m and V_{max} were estimated from a double reciprocal plot of substrate vs reaction rate.

Inhibition studies

Inhibition of LOX activity. LOX activity was carried out in presence of varying concentration of (i) 4-nitrocatechol (0 – 600 μ M), (ii) ETYA (0 – 200 μ M) and (iii) NDGA (0 – 500 μ M) (iv) adenine (0-500 μ M), (v) kinetin (0-500 μ M) and (vi) ANS (0-100 μ M). LOX activity was studied after preincubation of the protein for 5 min in the presence of the test inhibitor at 25°C. Stock solutions of NDGA, ETYA, adenine, kinetin and ANS were prepared in absolute ethanol while 4-Nitrocatechol was prepared in 50 mM phosphate buffer, pH 6.0. LOX assay was performed as described above.

Detection of LOX and lectin activity loci. To confirm the distinctness of the LOX locus of the protein from its sugar binding (lectin) locus, N-acetyl galactosamine

(500 μM) - in phosphate buffered saline- was added to the protein and the assay for LOX was carried out using appropriate blanks. Similarly, haemagglutination activity was assayed in the presence of the LOX inhibitors NDGA (500 μM) and ETYA (300 μM).

Lectin inhibition studies. The protein was incubated with N-acetyl galactosamine (0 – 1 mM) for 5 min at 25°C. The lectin assay was carried out as described above.

Product analyses

Isolation, identification and characterization of lipoxygenase products. The products of the reaction of LOX with linoleic acid were isolated as described earlier (Feussner and Kuhn, 1995), and identified by straight phase HPLC. For product analysis, the protein was incubated with 150 μM linoleic acid in 0.1 M sodium acetate buffer (pH 5.0), for 15 min. The reactions were stopped by the addition of sodium borohydride, which reduces the hydroperoxy fatty acids (products of LOX reaction) to their corresponding hydroxy compounds. The mixture was acidified to pH 3.0 with glacial acetic acid. The lipophilic products were extracted twice with equal volume of ethyl acetate. The lipids that remained following evaporation of the organic solvent with nitrogen were treated with diazomethane for 15 min. Diazomethane was evaporated using nitrogen flushing. The residue was redissolved in methanol, filtered and injected.

The products were identified by SP-HPLC using a Waters[®] Chromatograph equipped with Sorbax- Sil column (4.6 mm x 250 mm, 5 μm). Positional isomers of the linoleic acid oxygenation products were separated and detected at 234 nm using isocratic elution with the solvent system (n-Hexane: isopropyl alcohol: acetic

acid, 100: 2: 0.1). The products of potato LOX and soy LOX using alcohol-solubilized fatty acids at pH 5.5 and 9.0 were used as standards.

Chiral analyses of 13HOD. Enantiomer composition of the hydroxyl fatty acid was analyzed by chiral phase HPLC on a Bakerbond chiral phase column (dinitrobenzoyl phenylglycine coupled ionically over aminopropyl residues on silica gel as chiral phase; J. T. Baker Res. Products, USA, 250 x 4 mm, 5 μ m). The column was eluted with 0.5% isopropanol in n-hexane (Kuhn *et al*, 1987) at a flow rate of 0.8 ml/min. The linoleic acid oxygenation products thus obtained were methylated with etheric diazomethane at 25°C and subjected to SP-HPLC for separation of the positional isomers. The fractions containing the different positional isomers were collected separately and the acetic acid in the eluant was washed away. Aliquots of the isomers were concentrated, methylated with diazomethane, and then subjected to chiral phase HPLC. Products of pea seed lipoxygenase 1 (13(S) -23%, 13(R) – 16% of products produced) (Kuhn *et al*, 1987, Reynolds *and* Klein, 1982) and soya LOX 1 (predominantly 13(S) – 98%) were used as the standards (Kuhn *et al*, 1987). Molar extinction coefficient of 29,000 was used for determining the concentration of the hydroxyl fatty acid derivatives.

GC- MS analyses. GC-MS analysis was performed on a GC17A QP5000 Mass Spectrometer (Shimadzu, Japan). A capillary GC (Shimadzu, Japan) with a non-polar column (30 m DB-1, J&W Scientific, USA; film, 0.25 μ m; diameter 0.25 mm) was used. The GC was programmed from 120°C to 280°C with temperature increase of 10°C per min (Hamberg and Gardner, 1992). Methyl esters of the products were hydrogenated using platinum oxide in ethanol for 2 min with a general flow of hydrogen. For GC-MS analysis, the hydroxy-fatty acids were

converted to the corresponding trimethylsilyl ethers by reaction with BSTFA (15 min at 60°C).

Amino acid composition. The peak fraction of purified LOX was analyzed directly for total amino acids. The enzyme was blotted onto polyvinylidene difluoride membrane after SDS-PAGE. Electrophoretic transfer (mini Trans-Blot) was in 10 mM 3-[cyclohexylamine]-1-propane sulfonic acid (pH 11.0) with 10% methanol (v/v) (100 V, 4 h at 25°C). The membranes were stained for proteins with Ponceau stain. The excised protein band was subjected to total amino acid analysis. Amino acid analyses were performed according to the method of Bidlingmeyer *et al* (1984) using a Waters Associate Pico-tag amino acid analysis system. Samples (10 µg of protein) were placed in a tube (6 x 50mm, Pyrex™) and placed in a special vacuum vial. The vial was then attached to a Waters Associate Pico-tag workstation system manifold, and the samples were dried under vacuum to 50-60 mtorr. After drying, the vacuum was released and 200 µl of HCl (6 N) containing phenol 1 % (v/v) was pipetted in to the bottom of the vacuum vial. The vial was reattached to the manifold and evacuated and sealed under vacuum. Samples were hydrolyzed in the workstation at 110°C for 24 h, using a PICO-TAG™ workstation. Standard free amino acid as a mixture (Pierce H) containing up to 25 nmol of each amino acid were placed in the tubes and dried under vacuum. Free amino acids and hydrolyzed samples were dried under vacuum after redrying solution (10-20 µl) containing ethanol: water: triethanolamine (TEA) (2: 2:1) to each tube. When the vacuum reached 50-60 mtorr, the samples were ready for derivatization. The amino acids were derivatized with phenylisothiocyanate. Amino acid analysis was performed by precolumn derivatization. The phenyl thiocarbonyl amino acids were analyzed by reversed-phase HPLC using a

Waters Associates HPLC and an application-specific PICO-TAG™ amino acid analysis column (15 cm x 3.9 mm) with a binary gradient system. Temperature was controlled to $38 \pm 1^\circ\text{C}$ with a column heater. The solvents used were (A) aqueous buffer of 0.14 M sodium acetate containing 0.5 ml of triethylamine and titrated to pH 6.4 with glacial acetic acid and 6% acetonitrile and (B) 60% acetonitrile in water. A gradient was run from 0% solvent A to 46% solvent B in 10 min at 1 ml/min. The phenyl thiocarbamyl amino acids were detected at 254 nm.

Amino acid sequence of the protein. The peak fraction of purified protein and fractionated subunits were analyzed directly for N-terminal amino acid sequence on an Applied Biosystems Procise® 4.0 instrument. The protein/subunit was blotted onto a polyvinylidene difluoride membrane after SDS-PAGE. Electrophoretic transfer (mini Trans-Blot) was in 10 mM CAPS (pH 11.0) with 10% methanol (v/v) (100 V, 4 h at 25°C). The membranes were stained with Ponceau stain to identify the protein/ subunits. The excised band was subjected to N- terminal sequencing. Automated gas phase sequencing was carried out on the protein sequenator PSQ-1 (Shimadzu). This sequenator carries out edman degradation by supplying gaseous reagents for the coupling and cleavage reactions. The cut PVDF membrane was applied directly on the glass fibre disc. The coupling reaction is carried out with phenyl isothiocyanate in presence of gaseous trimethylamine. Excess reagents and byproducts were washed with *n*-heptane and ethyl acetate. The cleavage reaction was carried out with gaseous TFA to form an anilino thiazolinone derivative. Both the coupling and cleavage reactions were performed in a temperature controlled reaction chamber. The free ATZ-aminoacid was extracted from the conversion flask by *n*-butyl hydrochloride. The ATZ-aminoacid was converted to a more stable PTH-aminoacid by reaction with 25% TFA. The

PTH –aminoacid was dissolved in acetonitrile and automatically injected to HPLC. The PTH-aminoacids were separated by RP-HPLC. The PTH aminoacid in each cycle identified, quantified and recovery percentage calculated using an online CR4A system. The results were displayed and recorded.

Enzymatic cleavage of HGLOXI by α -TPCK trypsin. HGLOXI was cleaved at R-X and K-X bond by digesting with TPCK-trypsin at 1:100 enzyme to protein ratio in 0.1 M ammonium hydrogen carbonate, pH 8.2 containing 2 M urea for 2 h at 37°C with continuous stirring. The trypsin was added from a stock of 10 mg/ml and the reaction was terminated by reducing the pH of the reaction to 2.0 and by boiling the samples for 5-10 min. The sample was concentrated to dryness and redissolved in 0.5 ml of 0.1% TFA (Leavis et al, 1978). Internal peptide sequencing was carried out after tryptic digestion. The large peptide fragments obtained by limited digestion with TPCK-treated trypsin were separated by RP-HPLC using a Shim Pak C₁₈ (10 μ m, 4.6 mm x 250 mm) column with solvent A (0.1% TFA in water) and solvent B (70% acetonitrile containing 0.05% TFA). Tryptic digest was injected at a flow rate of 0.8 ml/ min. The column was washed with solvent A for 5 min and the bound protein was eluted by a linear gradient of acetonitrile (0-70%) over a period of 90 min. Detection was monitored at 280 nm using Waters® 2996 photodiode array detector.

Sequence alignment. The N-terminal sequence of 30 amino acids and two internal peptide sequences obtained by tryptic digestion were aligned with the Dolichos biflorus lectin PDB 1LU1 using Clustal W version 1.82.

Prosthetic group. The manganese and iron content was estimated by atomic absorption spectroscopy of the lyophilized protein. A Thermo Jarrel Ash atomic

absorption spectrometer equipped with hollow cathode lamps and an ultraviolet-sensitive photomultiplier was used. Absorption was measured at the 2795.6Å – the analytical line for Mn and 2486.2 Å –the analytical line for Fe.1 mg / ml solutions of protein were used. The protein was dialyzed extensively with triple distilled water prior all estimations. Appropriate blanks were used.

Carbohydrate analysis. The carbohydrate content of HGLOXI was estimated by the Phenol- sulfuric acid method (Dubois *et al*, 1956). Glucose (1 mg/ml) was used as standard solution. The assay was monitored at 490 nm.

Spectroscopic measurements

Absorption spectra. Light absorption spectra of LOX and its products were recorded at 25°C on a Shimadzu UV-1601 double beam Spectrophotometer in the range 220 – 600 nm with a 10 mm pathlength cell. Samples were in 20 mM phosphate buffer, pH 6.0.

Fluorescence measurements. Fluorescence measurements were carried out using a Shimadzu RF 5000 spectrofluorimeter at 25°C. Protein concentrations of 50 µg/ ml in 20 mM sodium phosphate buffer (pH 6.0) were used. The sample was excited at 280 nm (excitation maxima) and the emission was recorded in the range 300 – 400 nm using slit widths of 5 nm for both excitation and emission.

CD measurements. Circular dichroism measurements were performed on a Jasco J-810 automatic recording spectropolarimeter at 25°C. Far UV CD spectra (260 – 190 nm) were recorded from 200 – 260 nm using a 1 mm path length cell while near UV CD spectra (320 – 240 nm) was recorded using a 10 mm path length cell. Protein concentrations of 0.42 mg/ ml and 1-1.5 mg/ ml in 0.02 M sodium

phosphate buffer (pH 6.0) were used for far UV and near UV CD spectra respectively. An average of 3 scans was obtained for each spectrum at a scan speed of 10 nm/ min. The mean residue ellipticity $[\theta]_{MRW}$ was calculated using a value of 115 as calculated from amino acid data.

Thermal inactivation studies

Transition temperature measurements. The loss of enzyme activity as a function of temperature was followed in 20 mM phosphate buffer (pH 6.0). The enzyme samples were incubated for 30 min at different temperatures ranging from 4 to 95°C. After cooling to 4°C, the residual activity was measured at 25°C by transferring an aliquot to the assay mixture. The midpoint of thermal inactivation, T_m , at which the activity was diminished by 50%, was calculated from the plot of percent residual activity versus temperature. Activity of the unincubated enzyme was taken as 100%.

Kinetics of thermal inactivation of LOX. The thermal stability of LOX activity was studied at different temperatures in the range 78 – 95°C. Enzyme in 20 mM sodium phosphate (pH 6.0) was incubated in a water bath at the test temperature and aliquots were withdrawn at appropriate time intervals. The enzyme was immediately cooled to 4°C in an ice bath and the residual activity was measured at 25°C, as described above. Activity of the enzyme at 4°C was taken as 100%. From a semilogarithmic plot of residual activity versus time, the inactivation rate constant k_f was calculated. The temperature dependence of k_f was analyzed from the Arrhenius plot to obtain the inactivation constants. Activation parameters were then calculated as described by Moore. Activation enthalpies ($^*\Delta H$) were calculated according to Eq. 5

$$\Delta H^* = E_a - RT \quad (5)$$

where R = the gas constant and T is the absolute temperature. Activation free energy values (ΔG^*) were calculated according to Eq.6.

$$\Delta G^* = -RT \ln [k_r h / K t] \quad (6)$$

where k_r = the rate constant, h = Plank's constant, and K = Boltzmann constant. Activation entropy values ($^* \Delta S$) were then calculated according to Eq. 7.

$$\Delta S^* = \Delta H^* - T \Delta S^* \quad (7)$$

Thermal unfolding measurements: Circular Dichroism studies. Thermal transition curves were obtained from the data collected at 217 nm by CD measurements, in the temperature range of 25-85°C using a Peltier attachment (PMH 354WI), at a heating rate of 1°C/ min. Scans were made in the range of 200 – 600 nm at three different temperatures (25°C, 65°C, 85°C). Samples were degassed to prevent formation of air bubbles at high temperatures.

Fractionation of subunits. Fractionation of subunits was carried out by ion exchange chromatography on a DEAE-cellulose column (2.1 x 7 cm) equilibrated with 8.0 M urea in 0.04 M Tris HCl buffer (pH 7.3) at 25°C. Sample (15 mg) was dialyzed against 10.0 M urea in 0.04 M Tris HCl (pH 7.3) and applied to the column (Carter and Etzler, 1975a). The column was washed with one bed volume of 8.0 M urea in 0.04 M Tris HCl (pH 7.3) followed by elution with 250 ml linear gradient of 0-0.075 M NaCl in 0.04 M Tris HCl (pH 7.3) containing 8.0 M urea at a flow rate of 10 ml / h. The subunits, recovered in two separate peaks, were pooled and extensively dialyzed against 0.04 M Tris HCl (pH 7.3) containing 0.02%

sodium azide at 4°C. LOX and haemagglutination activity of the separated subunits was checked.

Fractionation of HGLOXI on Concanavalin A Sepharose. Isoforms of HGLOXI was separated on a concanavalin A Sepharose column (10 ml) according to Carter and Etzler (1975b). The column was equilibrated with 10 mM sodium phosphate buffer containing 0.5 M NaCl and 0.02% sodium azide (Buffer A) at 4°C. 5 mg of HGLOXI was dialyzed against buffer A for 6 hrs and loaded onto the column. 0.6 mg (~12%) of the applied HGLOXI did not bind to the column. The bound sample was eluted with a linear gradient of 0 – 0.3 M methyl α -D-mannopyranoside in buffer A. Three major fractions were eluted and were checked for LOX activity.

Production of polyclonal antibodies against soy lipoxygenase1. Albino rabbits were immunized with 300 μ g of soy lipoxygenase 1 dissolved in 0.5 ml of sodium phosphate buffer 20 mM, pH 6.8, mixed with equal volumes of Freund's complete adjuvant as the first injection (Freund, 1947). Booster doses of 150 μ g of soya LOX-1 with Freund's incomplete adjuvant were administered weekly after resting the animals for 21 days. After each booster dose, blood was collected from the ear vein of the animal, allowed to clot at 25°C and centrifuged to recover the serum. Cross reactivity with *Dolichos biflorus* purified lectin, purified lectin with LOX activity, soy LOX1, horsegram extract were evaluated by ELISA.

Analysis of Cross reactivity by ELISA

SDS-PAGE was carried out using 12% gels. The dialyzed protein was heated in a boiling water bath for 90s prior to loading on the gels. Electrophoresis

was carried out for 6 h at 10 mA. Following electrophoresis, the protein was blotted onto nitrocellulose membrane as described earlier.

Immunodetection. Following blotting, the nitrocellulose membrane was washed several times in immunoblot buffer (5 % skimmed milk powder in phosphate buffered saline, pH 7.0). The membrane was incubated overnight in immunoblot buffer containing antibodies raised against soya LOXI (1: 500 dilution). After repeated washes in the immunoblot buffer, the membrane was incubated with the secondary antibody alkaline phosphatase conjugated goat anti-rabbit immunoglobulins for 2 h at 25°C. After several washes in immunoblot buffer and finally in substrate buffer (100 mM Tris, 0.5 M NaCl, 5 mM MgCl₂, pH 9.5), the alkaline phosphatase activity was detected with a mixture of BCIP and NBT in substrate buffer.

Dot blot analysis of LOX's from horse gram seeds

About 100 µg of the purified protein was immobilized on a nitrocellulose membrane by repeated application employing a current of hot dry air to accelerate the drying until the required protein was immobilized. Immuno detection was done as described above.

RESULTS AND DISCUSSION

SECTION 1: IDENTIFICATION AND CHARACTERIZATION OF A THERMOSTABLE LOX ACTIVITY FROM HORSEGRAM SEEDS

In this section, the identification of a thermostable LOX activity from defatted horsegram is described. This is the richest source of LOX activity reported so far in plants. Activity is highly thermostable, retaining at least 75% activity after incubation for 30 minutes at 90°C. Both 13- and 9- hydroperoxides were produced with linoleic acid as substrate.

Identification of LOX activity of horsegram seeds. Horsegram seeds were powdered and defatted with hexane. Horsegram flour (44 mesh), having a protein content of 22% was extracted with sodium phosphate buffer (pH 6.0) and centrifuged before checking for LOX activity. Activity of the extract with (alcohol solubilized) linoleic acid, sodium linoleate, ammonium linoleate and fatty acid solubilized PC micelles was calculated and given in Table. 3.

Table 3. Lipoygenase activity of horsegram seed extract with different forms of linoleic acid.

Substrate	Activity U/mg
Alcohol solubilized linoleic acid	1800
Sodium linoleate	1500
Ammonium linoleate	1412
Linoleic acid solubilized PC micelles	1200

Alcohol solubilized linoleic acid was a better substrate when compared to others and hence was chosen as substrate to check LOX activity. The LOX activity of horsegram flour was 1800 U/ g of flour. Specific activity of LOX activity was found to be 37 U/ mg protein

Recovery of LOX activity from horsegram flour. The extract with LOX activity was brought to 70% ammonium sulfate saturation by the addition of powdered ammonium sulphate. The contents were centrifuged after 12 h. The precipitate was dissolved in 20 mM phosphate buffer (pH 6.0) and dialyzed before assaying for LOX activity. 75% of the activity was recovered with a specific activity of 31 U/ mg protein. LOX activity was also verified by activity staining as visualized by brown bands (Fig.17).

Characterization of LOX activity from horsegram

pH optimum of LOX activity. Optimum pH for LOX activity was determined by assaying the activity in the pH range 2.0 – 9.0. The optimum LOX activity was found in the pH range of 4.0 - 5.0 (Fig.18). The enzyme was found to be inactive at pH below 2.5 and above 6.5.

pH stability of LOX activity. The stability of LOX activity was studied at different pH levels (2 – 9) and constant ionic strength (20 mM) for 24 h. LOX activity - found to be stable in basic pH (6 – 8) - was highly unstable in acidic range (Fig.18). The LOX activity, which was stable at pH 6.0 could be completely recovered by ammonium sulfate precipitation. The precipitate can retain most of the activity for at least one month at -20°C.



Figure 17: Activity staining of lipoxxygenase activity from horse gram seeds. Protein extract from horse gram seeds was loaded on 12% gels as described earlier (Materials and methods). Gels were incubated in sodium acetate buffer (0.1 M, pH 5.0) for 15 min at 4°C and transferred to sodium phosphate buffer (0.1 M, pH 6.0) containing linoleic acid and incubated for 6 h at 4°C and stained with acidic KI solution. Brown bands developed after 15-20 minutes. Lane 1- Horse gram extract, 300 µg/ 50 µl. Lane 2 – Dialyzed Ammonium sulfate precipitate-300 µg/ 50 µl

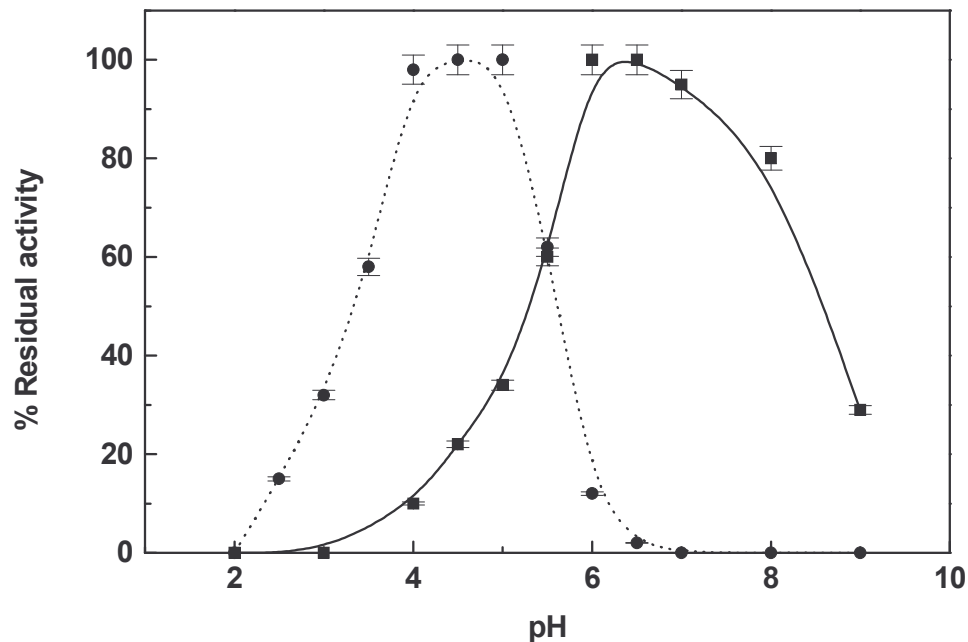


Figure 18: Effect of pH on the activity and stability of LOX activity from horse gram seed extract. pH optimum was determined in the pH range of 2 - 9 by assaying enzyme activity at 25°C with substrate linoleic acid. 0.1 M acetate (pH 2 – 5.5), phosphate (pH 6 – 7.5) and borate buffers (8 – 9) were prepared and used for assaying the enzyme. Reaction mixture included 150 μ M substrate in 1 ml of test buffer containing 2 nM Tween 20 and protein extract from horsegram seeds. All measurements are the average of 3 estimations. For stability measurements the extract was dialyzed against buffer at test pH. The same buffers were used as for measuring pH optimum for LOX activity without Tween 20. The dialyzed extract was further incubated for 24 h at the test pH at 25°C and was assayed for lipoxygenase activity at optimum pH (pH 5) for activity. pH optimum of LOX activity. (dotted line). Stability of LOX activity as a function of pH (solid line).

Identification of reaction products

Absorption spectra of products. Products of linoleic acid oxidation by LOX activity of horsegram seeds were analyzed spectroscopically. The products of LOX activity on linoleic acid were isolated from the reaction mixture as described under materials and methods. The absorption spectra of the methanolic solutions of the products were taken after sodium borohydride reduction. The reduced products had absorption maxima at 234 nm (Fig. 19), characteristic of conjugated diene.

Identification of the positional specificity of the reaction products. The positional specificity of the reaction products was analyzed by straight phase HPLC. It is known that soya LOX-1 exclusively produces 13- hydroperoxides, while potato LOX produces 9- hydroperoxides exclusively. The HPLC analysis of LOX activity from horsegram seeds gave two peaks corresponding to the 13- and 9- hydroperoxides in the ratio of 60: 40 (Fig. 20 A). Soya LOX-1 and potato LOX were used as standards (Fig. 20 B).

Thermal stability of LOX activity from horsegram seeds. The extract of horsegram with LOX activity as well as ammonium sulfate precipitate was highly thermostable retaining 75% of its activity when incubated at 90°C for 50 min. The enzyme had a half- life of 100 minutes (50% of the original activity was retained) when incubated at 90°C in 20 mM sodium phosphate buffer (pH 6.0) and a half life of 125 minutes when incubated at 85°C in 20 mM sodium phosphate buffer, (pH 6.0) (Fig 21).

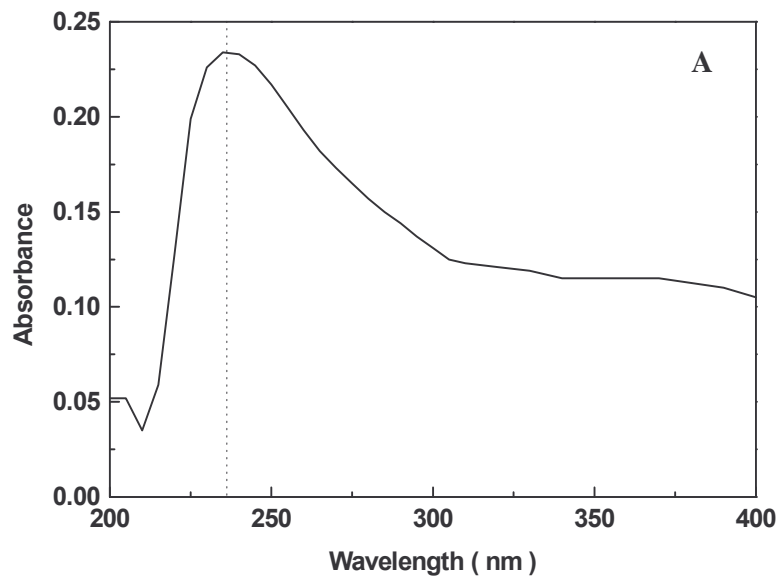


Figure 19: Absorption spectra of products of LOX activity with linoleic acid as substrate. 150 μ M linoleic acid was used as substrate in absolute ethanol. The reaction was carried out in 0.1M acetate buffer, pH 5.0 containing 2 nM Tween 20. The reaction was allowed to proceed at ambient temperature for 15 min, reduced using sodium borohydride (see methods). The products were extracted with ethyl acetate and the spectrum recorded. The products of LOX activity have an absorption maximum at 234 nm characteristic of conjugated diene.

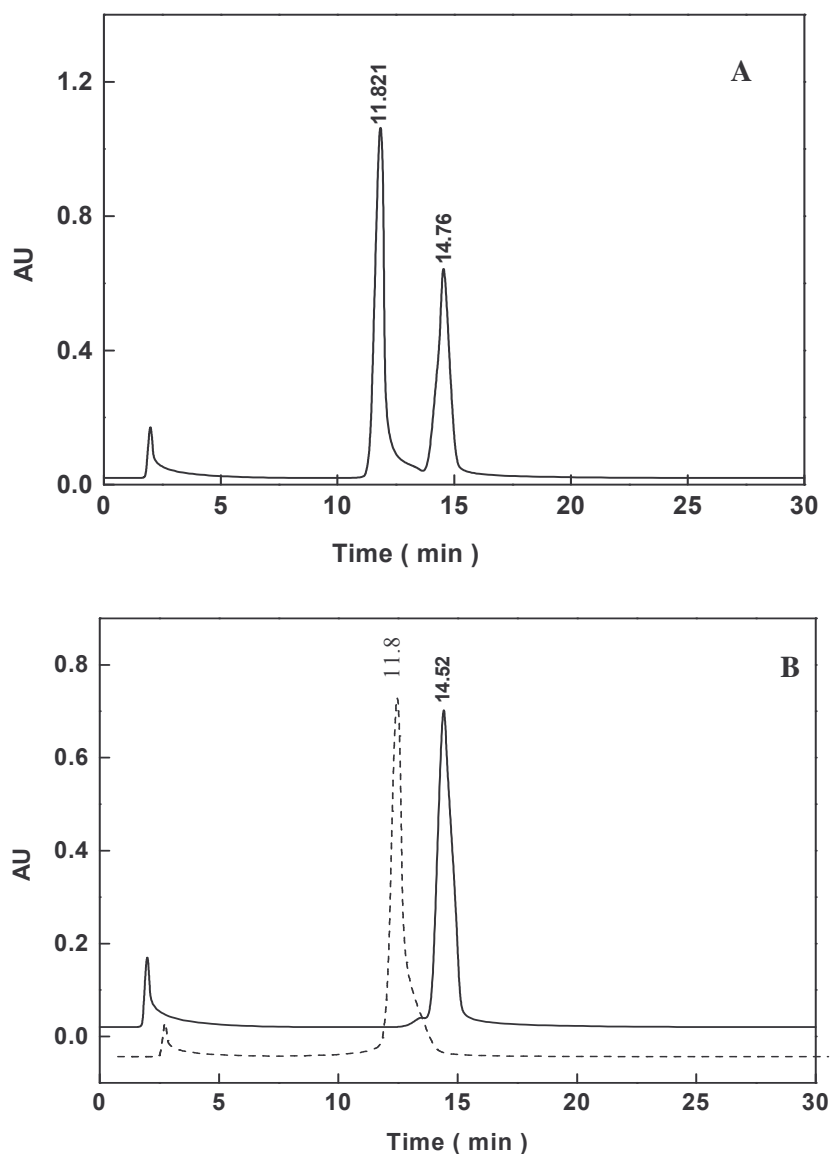


Figure 20. SP HPLC analyses of products of LOX activity from horse gram seeds with linoleic acid as substrate. A. Dialyzed ammonium sulfate precipitate was added to 0.1 M acetate buffer pH 5.0 containing 2 nM Tween 20 and 150 μ M substrate. The reaction was carried out for 15 min. The products formed were reduced with NaBH_4 and was extracted with ethyl acetate. Products were analyzed by SP-HPLC using a Sorbax-sil column (4.6 mm x 250 mm, 5 μ particle size). The products were separated by isocratic elution with solvent system n-Hexane/isopropyl alcohol /acetic acid (100:2:0.1) and a flow rate of 1 ml / min. The main products could be identified as 9- and 13-hydroperoxides of linoleic acid in the ratio of 40:60. **B.** Soya LOX-1 producing exclusively 13 – HPOD and potato LOX producing 9 – HPOD were used as standards.

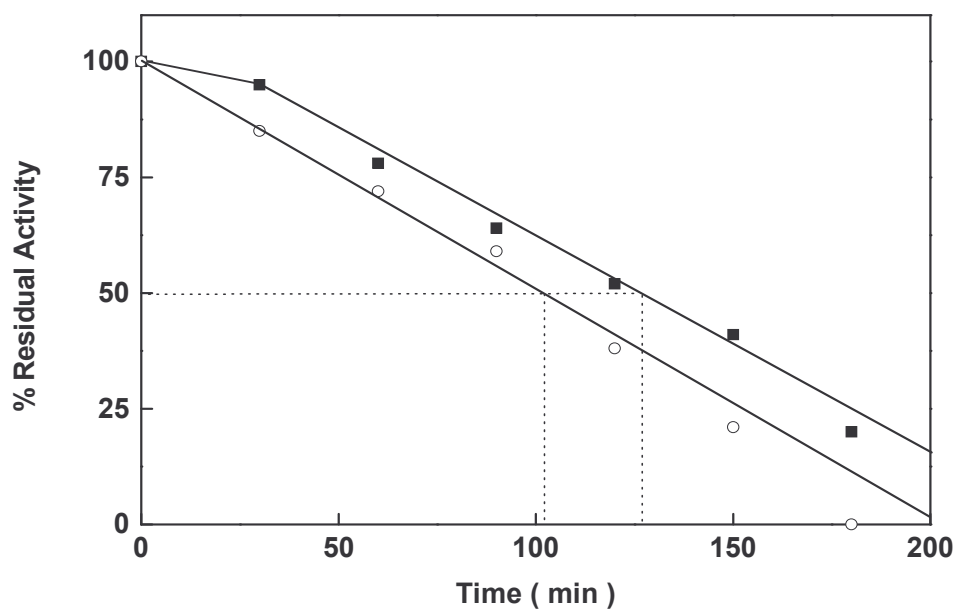


Figure 21: Thermal stability of LOX activity from horsegram flour. Protein was extract from horsegram, precipitated by ammonium sulfate was used (see materials and methods). The precipitate was dialyzed against 20 mM sodium phosphate buffer, pH 6.0. The thermal stability was measured by incubating the dialyzed ammonium sulfate extract at the test temperature (85°C and 90°C). Aliquots were drawn at different time intervals, cooled to 4°C and activity was assayed at 25°C under optimum conditions as given under materials and methods. Residual activity was determined assuming activity of unincubated enzyme as 100%.

— ■ — 85°C, — ○ — 90°C.

Structural similarity with other plant lipoxygenases. Sequence homology among various plant LOXs range from 40 – 70%. Soya LOX-1 is considered as the model lipoxygenase because of ease of purification, stability, and resemblance to animal lipoxygenases (Shibata and Axelrod, 1995). Soya LOX-1 was purified to homogeneity (Fig 22A) and antibodies were raised against it in albino rabbit. Rabbit antisera developed against soya LOX-1 was checked for cross reactivity with horsegram dialyzed extract with LOX activity. There was no cross reaction as seen from the Fig.22B, indicating no structural similarity with soya LOX-1.

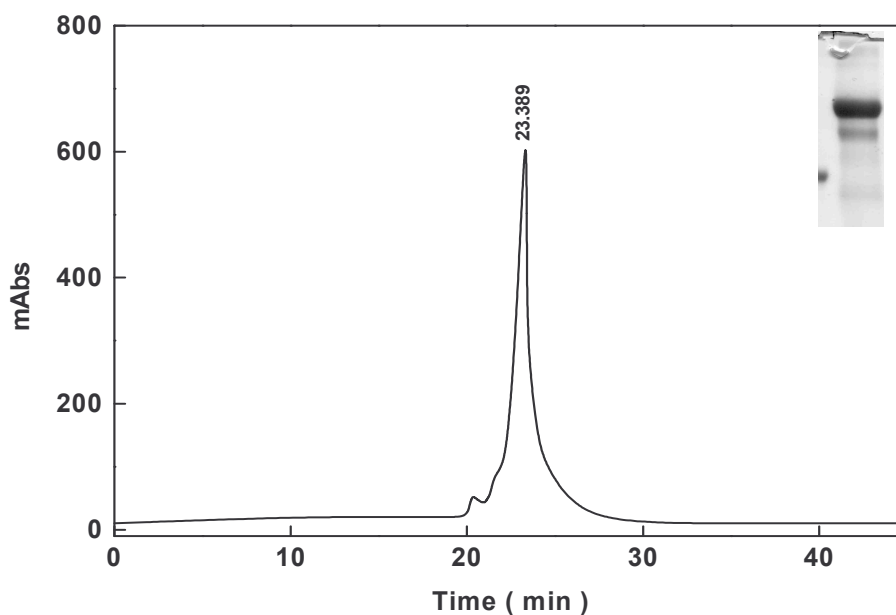


Figure 22 A: *Homogeneity of soya LOX-1 used for raising antibodies in rabbit.*

Soya LOX-1 was purified as reported earlier (Sudharshan and Rao, 1997). Homogeneity of the preparation was ascertained by size exclusion HPLC and SDS PAGE (*Inset*). Size exclusion HPLC was carried out on a TSK G 2000 SW_{XL} gel column (7.8 mm x 300 mm, 5 μ m) at a flow rate of 0.2 ml / min. Elution was isocratic using 20 mM phosphate buffer, pH 6.0 with 0.3 M NaCl. Detection was at 280 nm using a photo diode array detector. 100 μ g of purified soya LOX-1 was injected and the peak fraction collected, concentrated and injected into rabbits for raising antibodies as given under materials and methods. The serum was collected and tested for cross reactivity with horsegram extract having LOX activity. *Inset*: SDS PAGE was performed in 12% separating and 3% stacking gels. The gel was loaded with 50 μ g of soya LOX-1 and run for 3 h at 10 mA and stained with coomassie brilliant blue R-250.



Figure 22 B: *Dot blot analysis of interaction of Soya LOX antibodies with soya LOX and Horse gram extract.* Soya LOX-1 and horse gram extract having LOX activity was blotted on a nitrocellulose membrane. The membrane was washed in immunoblot buffer and was incubated with immunoblot buffer containing antibodies against Soya LOX-1. The membrane was treated with secondary antibody and incubated in substrate buffer. The alkaline phosphatase activity was detected with a mixture of BCIP and NBT in substrate buffer showing cross reactivity. Lane 1. Horse gram extract – 300 μ g, Lane 2. Soya LOX-1- 50 μ g. Soya LOX-1 showed cross reactivity, while horsegram extract did not show any cross reactivity.

DISCUSSION

The occurrence of lipoxygenase in plants is ubiquitous. Legumes, in particular, are rich sources of these enzymes. (Axelrod, 1974, Galliard and Chan, 1980).

Table 4: Lipoxygenases from different plant sources and their activities

Sl. No.	Source	Activity (U/ g)
1	Soybean (<i>Glycine max</i>)	350
2	Winged bean (<i>Psopocarpus tetragonolobus</i>)	150
3	Field bean (<i>Dolichos lablab</i>)	180
4	Mothbean (<i>Phaseolus aronitifolius</i>)	225
5	Pea (<i>Pisum sativum</i>)	250
6	Bengal gram (<i>Cicer arietinum</i>)	250
7	Green gram (<i>Phaseolus aureus</i>)	200
8	Black gram (<i>Phaseolus mungo</i>)	250
9	Potato (<i>Solanum tuberosum</i>)	160
10	Horsegram extract (<i>Dolichos biflorus</i>)*	1800

Sl.No 1-9. Borathakur, 1987. * LOX activity from the present study.

Chang and McCurdy (1985) compared lipoxygenase activities in different legumes and found that soybean, cowpea and lentil possess high level of lipoxygenase

activity. So far, the richest reported source of LOX is soybean, which has 350 U/g of LOX activity and at least 3 isoenzymes (Table 4) (Chang and McCurdy, 1985).

Horsegram is a leguminous pulse crop native to South East Asian sub continent and tropical Africa. Like other leguminous seeds, horsegram seeds are also a good source of protein with an estimated content of 22% in the dehulled horsegram seeds (Begum *et al.*, 1977). It is known to be resistant to infestation from pests. The seeds are known to contain high activity of trypsin inhibitor (Ramasarma and Rao, 1991) and lectin (Etzler, 1998), which is known to play a major role in plant defense.

LOX activity in plants is reported to be fungitoxic and antimicrobial and hence plays an important role in plant defense (Siedow, 1991). The lipoxygenase activity from horsegram is 1800 U/ g of seeds (Table 3), which makes it as the richest source of lipoxygenase activity so far known in plants or animals or fungal sources.

Lipoxygenase activities from different sources are generally active in neutral and alkaline pH (Shibata and Axelrod, 1995). The pH optima for most lipoxygenases are in the range of 5.5 – 9.0. Very few lipoxygenases - potato, maize and tomato are active in acidic range but have broader pH profiles. It should be noted that the LOX activity from horsegram seeds is active in only acidic pH (3.0 - 6.0) with optimum activity from 4.0 – 5.0 in contrast to most plant lipoxygenases, which show broader active pH - activity profiles. Only rice lipoxygenase is known to act in narrow acidic pH range (Shibata and Axelrod, 1995).

The primary product of oxygenation of linoleic acid can be either 9 – or 13 – hydroperoxides in the case of plant and animal lipoxygenases (Gardner, 1991) and 11- in the case of fungal lipoxygenase (Su and Oliw, 1998). The positional specificity for the oxygenation of linoleic acid varies with lipoxygenases depending on the reaction conditions like pH, temperature and oxygen level (Gardner, 1991). Lipoxygenases, other than rice LOX, active in acidic pH generates 9-hydroperoxides preferentially. The exclusive formation of 9-hydroperoxide has been shown for the enzymes from potato tuber, maize (Gardner, 1991) and corn (Gardner and Weiselder, 1970). Though rice LOX is active in acidic pH, it produces 13-HPOD, predominantly (Shibata and Axelrod, 1995). In plants, the products of LOX are potent bioregulators, playing important roles in signaling cascades, plant growth and development, senescence, organogenesis, maintenance of homeostasis (Siedow, 1991; Gardner, 1991). LOX activity from horsegram seeds on linoleic acid as substrate, releases both 13 – and 9 – hydroperoxides as the products in nearly equal quantities of 3: 2. These products are highly reactive and could be the starting materials for the preparation of several pharmacologically important biomolecules like lipoxins and leukotrienes (Gardner, 1991).

Soya LOX-1 has been studied intensively over the past 25 years (Shibata and Axelrod, 1995). LOXs in general are not very thermostable. Soya LOX, the most stable enzyme among lipoxygenases loses half of its activity in 25 mins at 69°C (Christopher *et al*, 1972). Most lipoxygenases reported earlier are known to have their activity beyond 70°C. The present study reports a source with LOX activity that is highly thermostable retaining more than 70% activity after incubation for 50 min at 90°C. To see whether any protein from horsegram extract

had similarity in structure with soya LOX-1, antibodies raised against soya LOX-1 were subjected to cross reactivity with horsegram extract. The absence of cross reaction observed points to the absence of a protein with structure similar to soya LOX-1 in horsegram. This immunological distinction implies the structural differences between protein having lipoxygenase activity from horsegram and soya LOX-1. Comparison of amino acid sequences of different plant lipoxygenases has revealed significant conservation of some regions indicating a possible evolutionary relationship (Shibata and Axelrod, 1995). The presence of such regions needs further probing. In the light of these observations, horsegram forms a new source of rich thermostable LOX activity has been identified.

SECTION 2: PURIFICATION AND CHARACTERIZATION OF A DUAL FUNCTION PROTEIN WITH LIPOXYGENASE AND HAEMAGGLUTINATION ACTIVITY (HGLOXI).

In this section, the purification of a dual function protein with LOX and lectin activity is described. The LOX activity associated with this protein is characterized for its (i) enzymatic properties such as K_m , V_{max} , inhibition and products of reaction (ii) molecular properties such as amino acid composition, amino acid sequence and mass. Identification of LOX activity with lectin comes from (i) MALDI-TOF, (ii) N-terminal sequence, (iii) Partial sequencing of the tryptic fragments of the protein, (iv) amino acid composition and (v) the presence of manganese ion. This is the first novel report of a lectin having LOX activity which may probably help explain the unusual pest resistance associated with this legume.

Purification of a protein with LOX activity. A protein having LOX activity was purified from defatted horsegram flour. The flour, on extraction with 0.1 M sodium phosphate buffer (pH 6.0) had an activity of 4500 U and 120 mg protein. The specific activity was 37 U / mg. Activity was recovered to 75% by ammonium sulfate precipitation (0 – 70%). The precipitate after dialysis against 20 mM sodium phosphate buffer (pH 6.0) was resolved on a Sephacryl S-200 column (Fig.23). The activity eluted in three peaks. The SDS PAGE pattern of the three peaks with LOX activity is shown in Fig 24. The first peak had 1080 units with a specific activity of 51 U /mg and a yield of 24%.

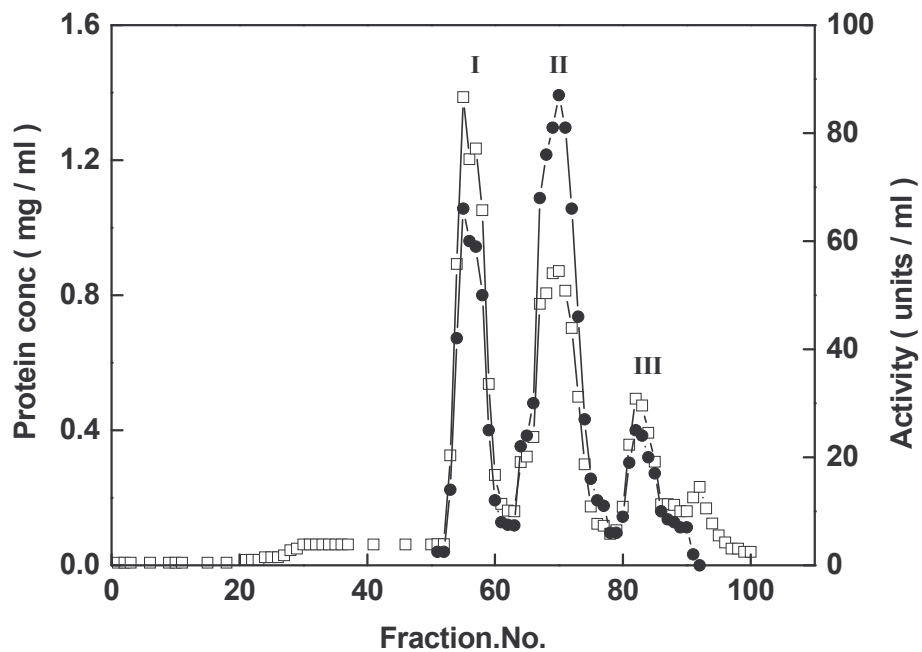


Figure 23: Elution profile of HGLOXI on a Sephacryl S-200 column. Protein extracted from defatted horsegram flour was precipitated with ammonium sulfate (70% saturation), redissolved in 20 mM phosphate buffer and dialyzed against phosphate buffer containing 5% glycerol and 0.02% sodium azide. The dialysate was loaded on a Sephacryl S- 200 column (2.7 x 120 cm, 700 ml). The elution buffer used was 20 mM sodium phosphate buffer (pH 6.0) containing 5% glycerol and 0.02% sodium azide. Flow rate, 20 ml/hr, fraction size, 5ml / tube.

— ● — Activity, — □ — mg/ml.

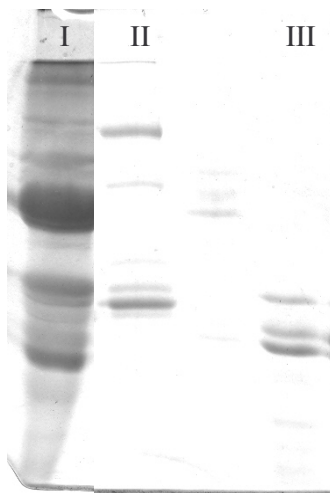


Figure. 24. SDS PAGE pattern of the protein of the three peaks obtained from Sephacryl S-200 column. The three peaks obtained from Sephacryl S-200 (Fig.13) was dialyzed and analyzed on SDS PAGE. SDS PAGE was performed in 12% separating and 3% stacking gels according to Laemmli (1970). The gel was run for 3 hr at 10 mA and stained by coomassie brilliant blue R-250. Lane I – Peak I - 1 mg / ml, Lane II – Peak II – 1 mg / ml, Lane III – Peak III – 1 mg / ml.

The protein content (as visualized by the SDS PAGE) is very high. An attempt was made to purify LOX activity from this peak. The enzyme purified was a heme protein and accounted for 10% of the total LOX activity of the first peak. The second peak had 35% of LOX activity (1560 U) with a good specific activity of 97 U/mg. A purification of 2.6 was achieved in this step. The active fractions were pooled and loaded on DEAE Sephacel column (Fig.25). Activity was recovered in two peaks with major activity (1155 U) in the second peak. The specific activity was 154 U/mg. A 4 fold purification was obtained in this step. The LOX activity in the protein was detected by activity staining (Fig. 26). An overall yield of 25% was achieved. The summary of the details of the purification are summarized in Table 5. The protein was designated as HGLOXI.

Homogeneity of protein with LOX activity. The homogeneity of HGLOXI was ascertained by native PAGE analysis (Fig. 27A), which showed a single band. Urea PAGE analysis (Fig. 27B) under non reducing conditions revealed two closely moving bands suggesting that HGLOXI is composed of two subunits which were linked by non-covalent interactions. Polyacrylamide gel electrophoresis, in presence of SDS under non reducing conditions (Fig. 27C) was carried out. The gel pattern analyzed. Two closely moving bands were detected indicating the presence of 2 subunits (Fig. 27C). The homogeneity of the HGLOXI preparation was further ascertained by reverse phase HPLC on a C₁₈ column. The chromatographic pattern revealed a single peak with a retention time of 37.4 min (Fig 28) and was ~ 95% homogeneity (peak area analysis).

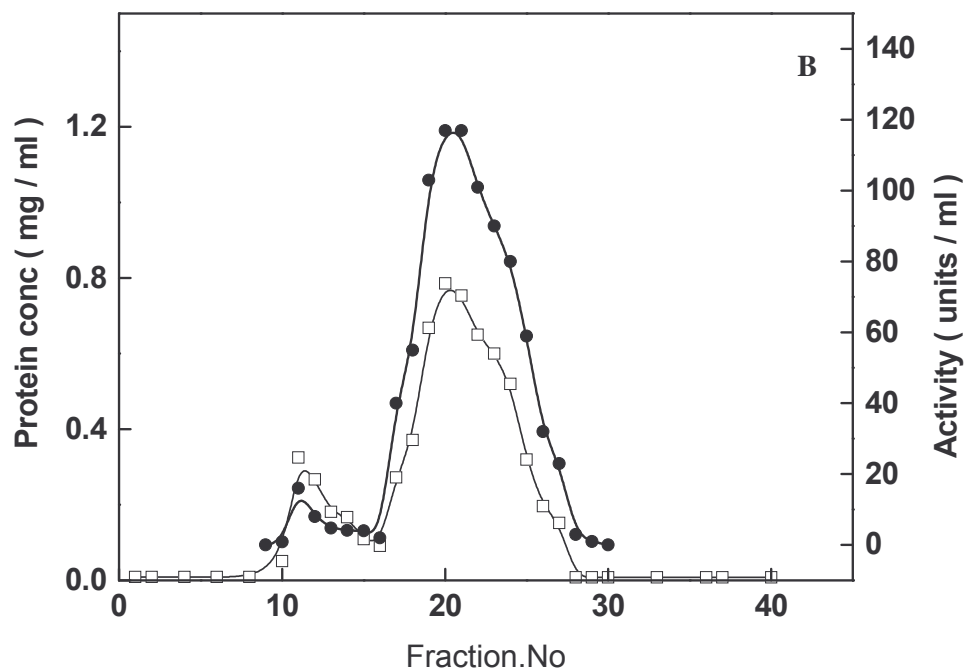


Figure 25: Elution profile of HGLOXI on a DEAE Sephacel column. 2nd peak from gel filtration on Sephacryl S 200 column was pooled and dialyzed against 20 mM sodium phosphate buffer (pH 6.0) containing 5% glycerol, 0.05 M NaCl and 0.02% sodium azide. This was loaded onto a DEAE Sephacel Column (2.1x 7 cm, 25 ml). Elution was carried out with 0.05 M – 0.3 M NaCl gradient in same buffer. Flow rate, 10 ml / hr, fraction size-3 ml / tube. — ● — Activity, — □ — mg/ml.

Table 5: Purification of HGLOX I from horsegram

	Protein (mg)	LOX Activity (Units)	Specific activity (units mg⁻¹ protein)	Fold purification	Yield (%)
Crude extract	120	4500	37.5	1	100
Ammonium Sulfate (0-70%)	114	3384	30	0.8	75
Sephacryl S-200					
Fraction I	21	1080	51	1.36	24
Fraction II	16	1560	97	2.6	35
Fraction III	8	432	54	1.44	9.6
DEAE- Sephacel	7.5	1155	154	4.1	25

These are the results of a typical purification starting from 2.5g of defatted flour. These values were reproduced in two separate purifications.



Figure 26: Activity staining of HGLOXI. Activity staining was performed with polyacrylamide gels under native conditions (protein concentration 300 μg), as described in materials and methods and incubated at 4°C in sodium acetate buffer (0.1 M, pH 5.0) for 15 min. The gel was transferred to sodium phosphate buffer (0.1 M, pH 6.0) containing linoleic acid and incubated for 6 h at 4°C and stained with acidic KI solution. Brown bands developed after 15-20 minutes indicating LOX activity.

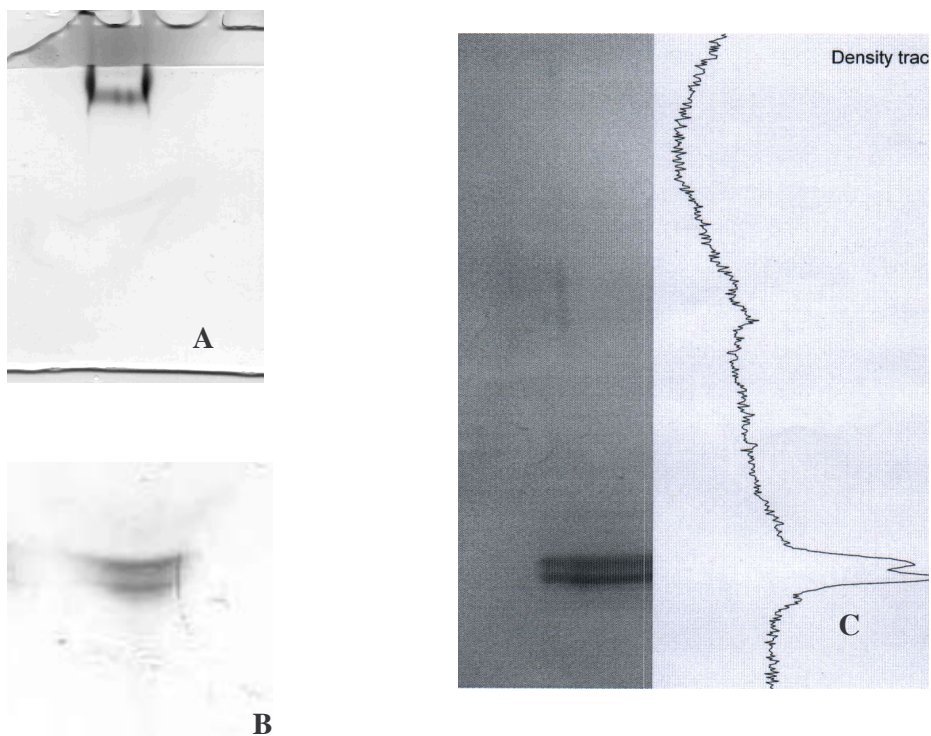


Figure 27: Electrophoresis analysis of HGLOXI. **A: NATIVE PAGE analysis.** Native PAGE was performed according to Laemmli (1970) with 12% separating gel and 3% stacking gel and run at 10 mA for 6 h at 4°C. The concentration of protein loaded was 2 mg / ml. Electrophoretic buffer was 50 mM Tris and 284 mM glycine (pH 8.8). The gels were overrun for half an hour. **B: Urea PAGE analysis.** Urea PAGE was performed with 9% separating gel and 3% stacking gel containing 8 M urea and run at 9 mA for 3 h (Goldenberg, 1997). The concentration of protein loaded was 1 mg / ml. **C: SDS PAGE analysis.** SDS PAGE was performed in 12% separating and 3% stacking gels according to Laemmli (1970). The gel was loaded with HGLOXI and run for 3 hr at 10mA. The purified protein preparation shows two closely moving bands. Density trace by documentation analysis also revealed the presence of two closely moving bands.

All the above gels were stained with coomassie brilliant blue R-250.

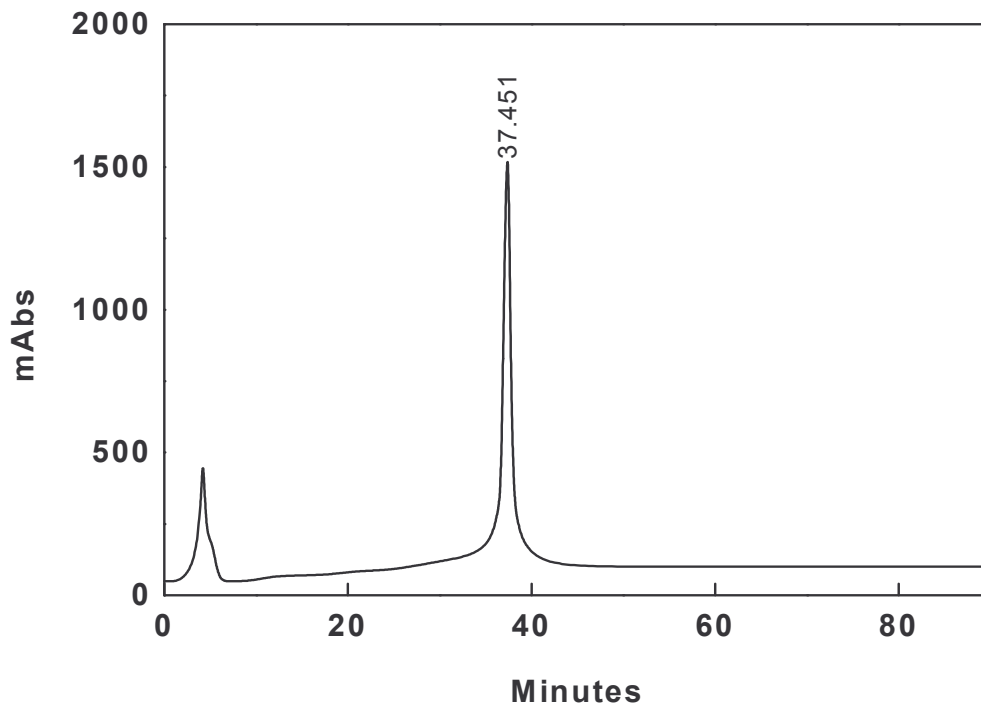


Figure 28: Establishment of the homogeneity of HGLOXI by RP- HPLC.

RP-HPLC of HGLOXI was carried out on a Shim Pak C₁₈ (10 μ , 4.6 mm x 250 mm) column with solvent A (0.1% TFA in water) and solvent B (100% acetonitrile containing 0.1% TFA). The bound protein was eluted by a linear gradient of acetonitrile (50-75%) over a period of 45 min. Protein (20 μ g) was injected at a flow rate of 1 ml/ min. Detection was monitored at 280 nm using Waters[®] 2996 photodiode array detector.

Determination of molecular weight of HGLOXI. The molecular weight of the native enzyme was determined by size exclusion chromatography. Gel filtration was carried out using standard proteins as markers in the molecular weight range of 14, 000 – 150,000 Da. The purified protein had the retention time of 22.72 min (FIG 29) with a molecular weight of ~ 110,000 Da (inset).

MALDI spectrum. The peak fraction of gel filtration on HPLC was collected, concentrated and loaded onto a mass spectrometer. The fragmentation pattern of MALDI - TOF showed two major peaks corresponding to 27,287 and 28,426 Da (Fig. 30). The molecular weights of the subunits, as determined by SDS-PAGE, were 27,100 and 25,900 Da, respectively.

The results of electrophoretic analysis, size exclusion chromatography and mass spectrometry by MALDI-TOF lead to the conclusion that HGLOXI is a tetramer with a molecular weight of 110 kDa. It comprises of two dimer units which inturn possess two unidentical subunits

Characterization of LOX activity. The reaction of HGLOXI with linoleic acid leads to the formation of conjugated diene hydroperoxide, the primary product with absorption maxima at 234 nm. Formation of the products from linoleic acid is found to proceed at a linear rate for at least 1 min, as evidenced from the increase in UV absorption at 234 nm (Fig 31A). A typical sigmoidal curve was obtained at 234 nm (Fig. 31B). In the HGLOXI reaction, ketodiene (absorption maxima at 280 nm) is formed along with the conjugated diene that resembles soya LOX-3 reaction (Axelrod *et al*, 1981). Kinetics of HGLOXI reaction with linoleic acid as substrate at 280 nm is shown in Fig. 31C.

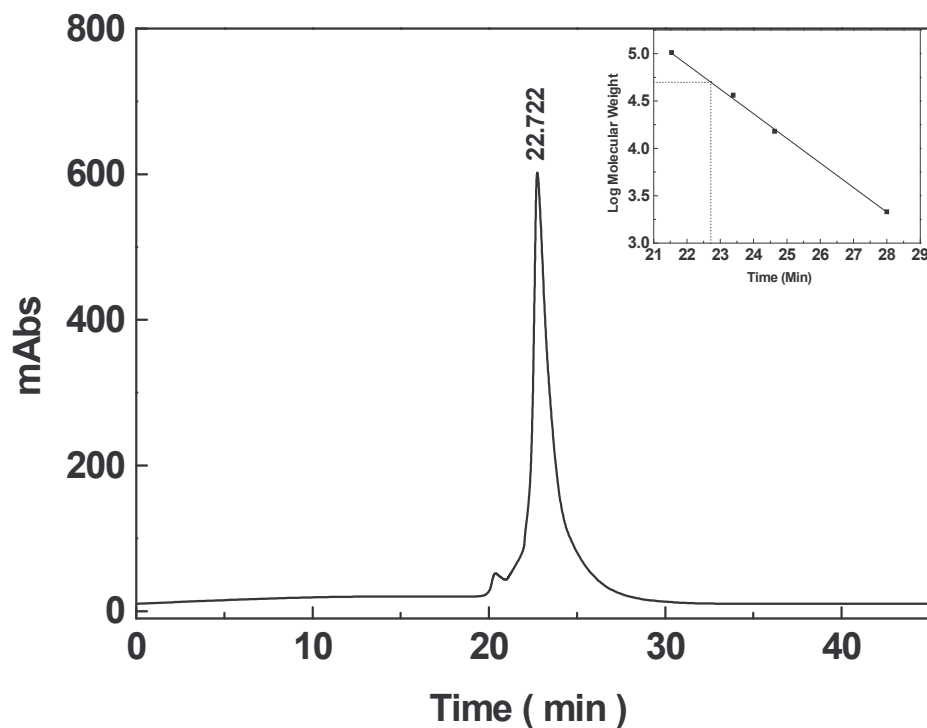


Figure 29: Size exclusion chromatography of HGLOXI by HPLC for homogeneity and determination of molecular weight. Size exclusion HPLC was carried out on a TSK G 2000 SW_{XL} gel column (7.8 mm x 300 mm, 5 μ m) at a flow rate of 0.2 ml / min. Isocratic elution in 20 mM phosphate buffer, pH 6.0 with 0.3 M NaCl was carried out. Detection was at 280 nm using a photo diode array detector. *Inset: Determination of molecular weight.* Molecular weight markers in the range 29,000 - 150,000 Da were run and the molecular weight was determined to be 110,000 Da.

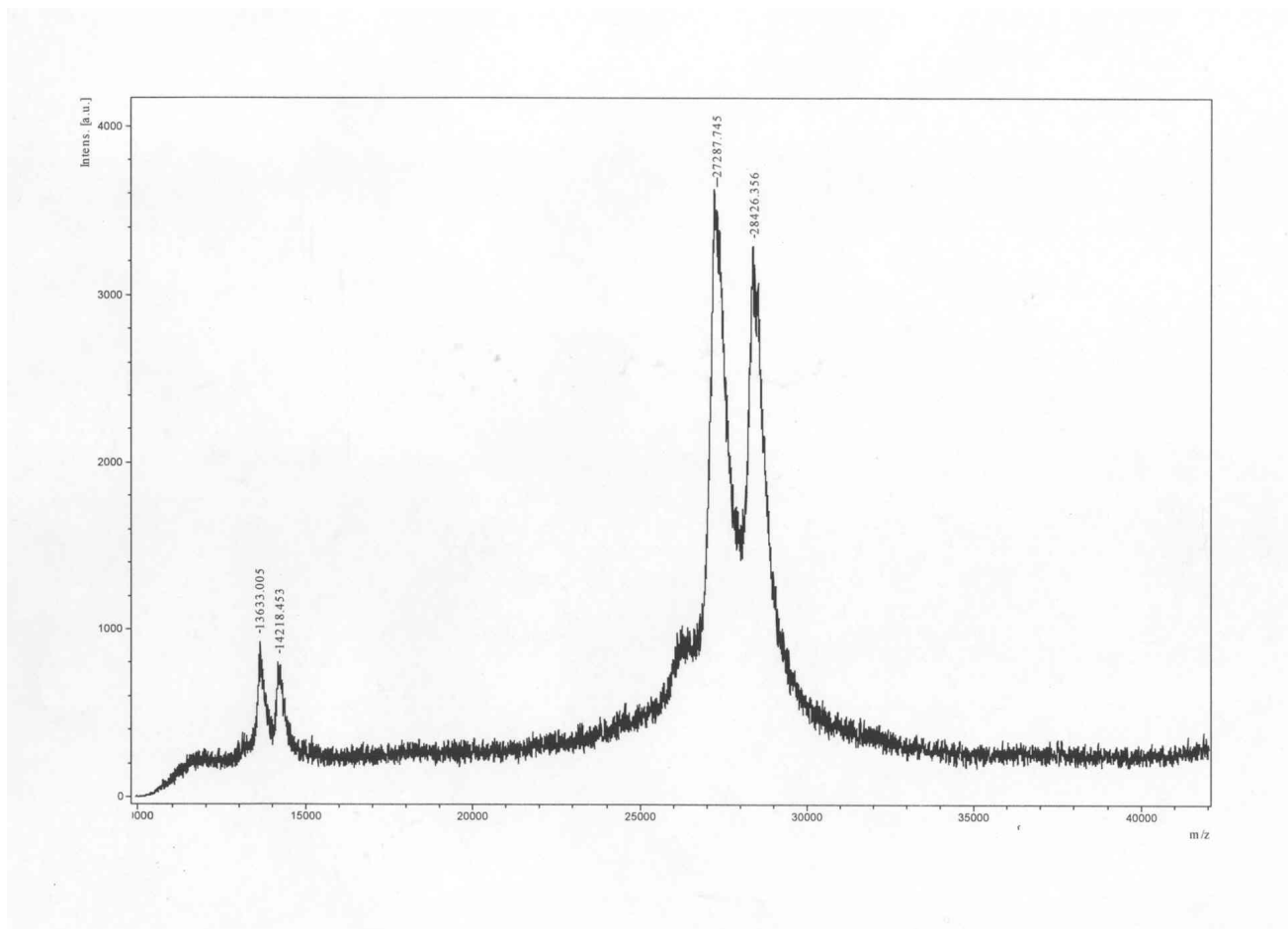
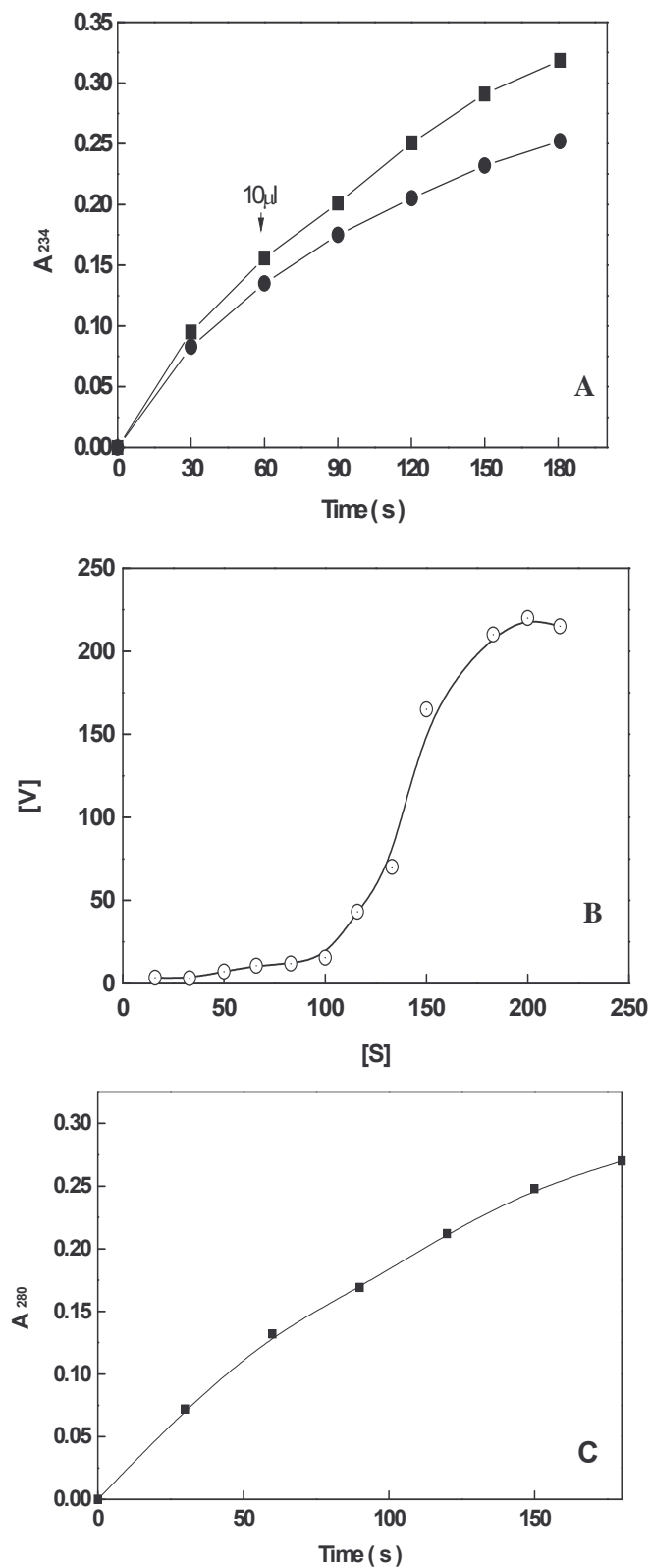


Figure 30: Maldi spectra of HGLOX I showing two subunits. The mass spectrum indicates the presence of two subunits of molecular mass of ~27,287 and ~28,426.



A: Kinetics of HGLOXI at 234 nm. The reaction of HGLOXI with linoleic acid was carried as described under materials and methods. The kinetics was followed for 3 min at 234 nm

—●— enzyme kinetics followed for 3 min,
 —■— 10 μ l of additional enzyme was added after a min and the enzyme kinetics was followed for further two minutes.

B: S vs. V plot of HGLOXI. The concentration of stock linoleic acid (10 mM) in alcohol was used. The reaction mixture contained 0 – 250 μ M linoleic acid in 0.1 M sodium acetate buffer, pH 5.0 containing 2 nM of Tween 20. The assay was performed at 25°C as given under materials and methods.

C: Kinetics of HGLOXI at 280 nm. The reaction of HGLOXI was carried out in 0.1 M acetate buffer, pH 5.0 containing 2 nM Tween 20. 150 μ M linoleic acid was used as substrate in absolute ethanol. The kinetics was followed for 3 min at 280 nm.

Figure 31: Kinetics of HGLOXI

Studies of LOX activity with addition of either 1 mM of Fe^{2+} or Mn^{2+} revealed neither stimulation nor inhibition of enzyme activity.

Optimum pH for activity of HGLOXI. The pH optimum for LOX activity was determined to be 4 – 5, with linoleic acid as substrate (Fig.32). The enzyme was active in acidic pH range 2.5 – 6.0. No activity was observed beyond pH 6.0.

Determination of kinetic constants. The initial velocities of HGLOXI reaction were determined using various concentrations of linoleic, linolenic and arachidonic acid. The apparent Michaelis constant, K_m values for the three substrates were calculated by Lineweaver-Burk plots as shown in Fig 33. K_m for linoleic acid, linolenic acid and arachidonic acid was found to be 166, 200 and 121 μM , respectively (Fig.33). V_{max} was found to be 66.6, 55, 100 $\mu\text{mol} / \text{min}$ for linoleic, linolenic and arachidonic acid, respectively.

Inhibition of HGLOXI activity. The known LOX inhibitors – ETYA, a substrate analogue of arachidonic acid known to be an irreversible inhibitor of lipoxygenases) and NDGA, (which acts as a competitive inhibitor by scavenging hydroperoxy radicals) (Gardner, 1991) were used to study the inhibition of HGLOXI activity. NDGA and ETYA inhibited HGLOXI with the midpoint inhibitor concentration (IC_{50}) of 161 μM and 101 μM , respectively. 4-nitrocatechol which is known to bind to iron enzyme complex (Gardner, 1991) proved to be a poor inhibitor with a midpoint inhibitor concentration of 433 μM (Fig. 34).

Kinetics of co-oxidation of β -carotene. Lipoxygenases are known to cooxidize β -carotene (Grosch *et al*, 1976). The kinetics of cooxidation of β -carotene by the purified protein was followed at 460 nm (Fig. 35).

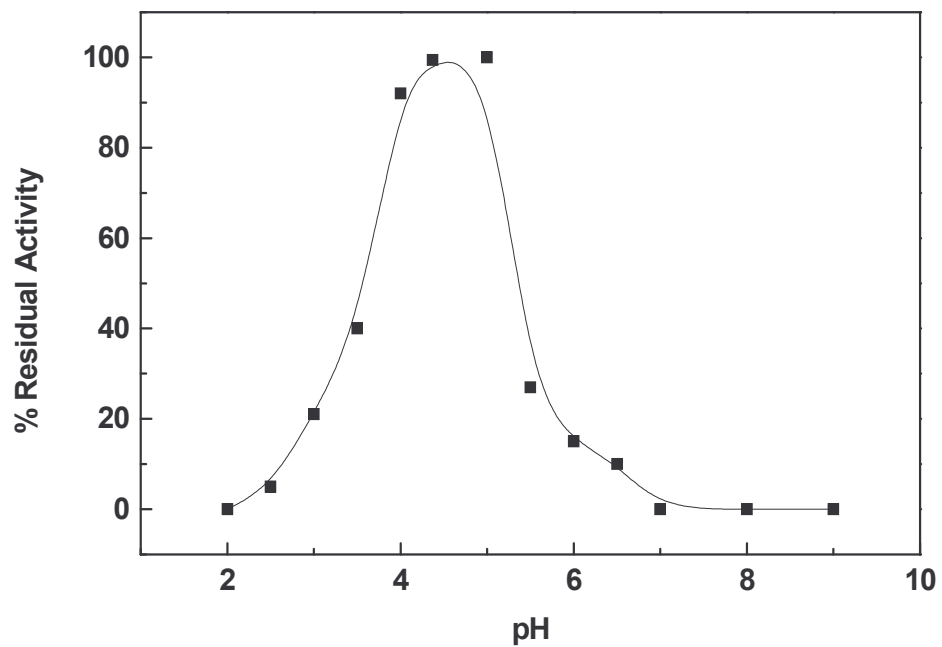


Figure 32: Effect of pH on HGLOXI activity. Buffers were prepared in the pH range of 2 to 9. 0.1 M sodium acetate (pH 2 – 5.5), sodium phosphate (pH 6 – 8) and 0.1M sodium borate buffers (8 – 9) were used for assaying the enzyme. All measurements are the average of 3 estimations.

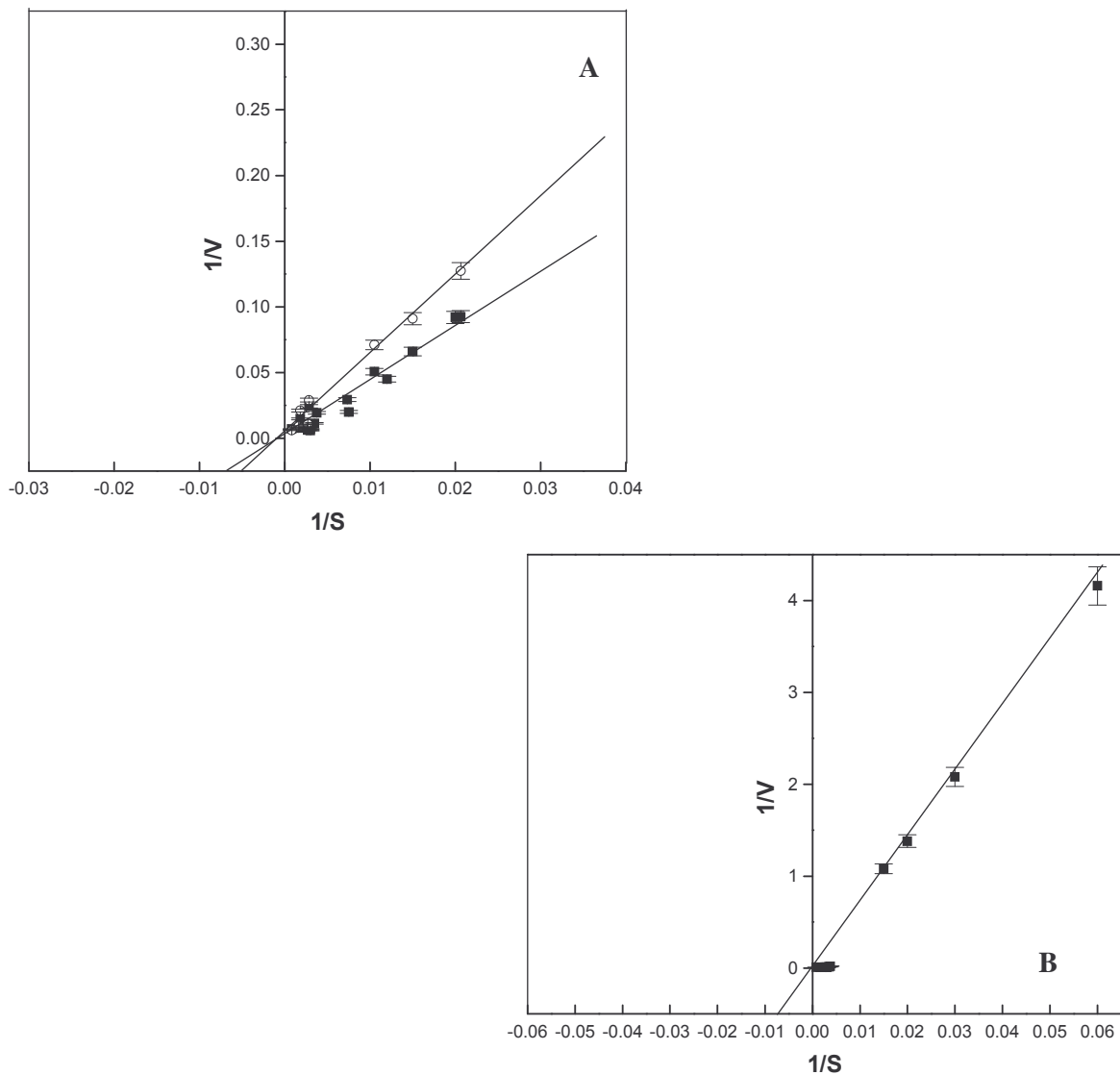


Figure 33: Lineweaver Burk plot of HGLOXI activity with fatty acid substrates. The K_m and V_{max} values of the purified enzyme were determined with linoleic, linolenic (A) and arachidonic (B) acids as substrates in the range of 0 – 275 μM (in 0.1 M sodium acetate buffer, pH 5.0 containing 2 nM Tween-20). The assay was performed at 25°C as given under materials and methods. — ■ — linoleic acid, — o — linolenic acid.

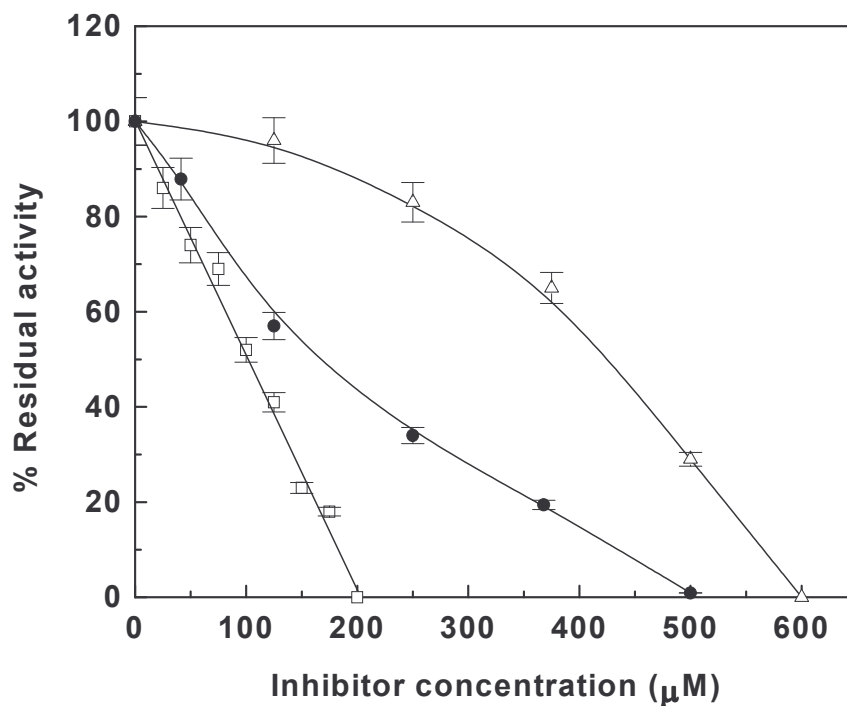


Figure 34: Effect of lipoxxygenase inhibitors on LOX activity of HGLOXI.—△—, 4 - nitrocatechol; —●—, NDGA; —□—, ETYA. NDGA and ETYA stock solutions were in absolute ethanol while 4-nitrocatechol was prepared in buffer (see materials and methods). The enzyme was preincubated with inhibitors for five minutes. Appropriate blanks were used for the experiment. The data shown are an average of 3 experiments.

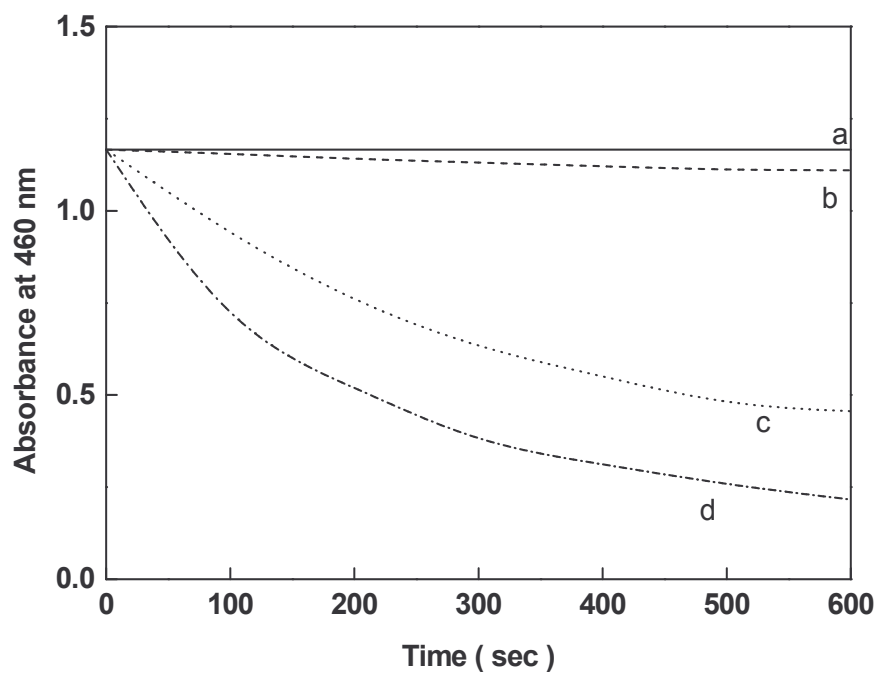


Figure 35: Carotene bleaching activity associated with HGLOXI. Kinetics of cooxidation of β -carotene (460 nm) with different amounts of HGLOXI at pH 6.0 using linoleic acid dispersed with Tween-20 as the substrate: (a) control (b) 5 μg (c) 20 μg (d) 40 μg .

A cooxidation potential of 0.07 was obtained with HGLOXI by relating the carotene bleaching activity (change in absorbance at 460 nm) to the peroxidation activity (change in absorbance at 234 nm) at pH 6.0. HGLOXI was found to be a weak cooxidising enzyme (0.07 cooxidation potential) similar to soya LOX1 (Grosch *et al*, 1976).

Prosthetic group. Atomic absorption spectroscopy revealed the presence of manganese in protein sample. Iron could not be detected in the sample. The Mn concentration was estimated to be 0.673 $\mu\text{g/g}$ protein (Fig 36). Thus the protein contains $\geq 0.67 \mu\text{g Mn/g}$ protein and no iron content. Manganese lipoxygenase from *G.graminis* (Su and Oliw, 1998) appeared to be the only other lipoxygenase enzyme, which is reported to contain manganese as its cofactor (0.5 – 1 mole of Mn per mole of enzyme).

Products of LOX activity of HGLOXI. The reaction products of LOX activity, at pH 5.0 and 25°C using linoleic acid as the substrate, have been analyzed by SP-HPLC. The absorption maxima of the products (234 nm) indicate a conjugated diene. Soya LOX-1, at pH 9.0 and 25°C, exclusively produces 13-HPOD, while potato LOX produces 9-HPOD at pH 5.5 and 25°C (Fig. 37A). Product identification, carried out using straight phase HPLC, revealed two peaks at 11.8 and 14.76 min. Products of LOX activity in the present study were identified to be 13-HPOD and 9-HPOD using soya LOX-1 and potato LOX products as standards, respectively. Profiles obtained for 13-HPOD and 9-HPOD are in the ratio 80: 20 (Fig. 37B).

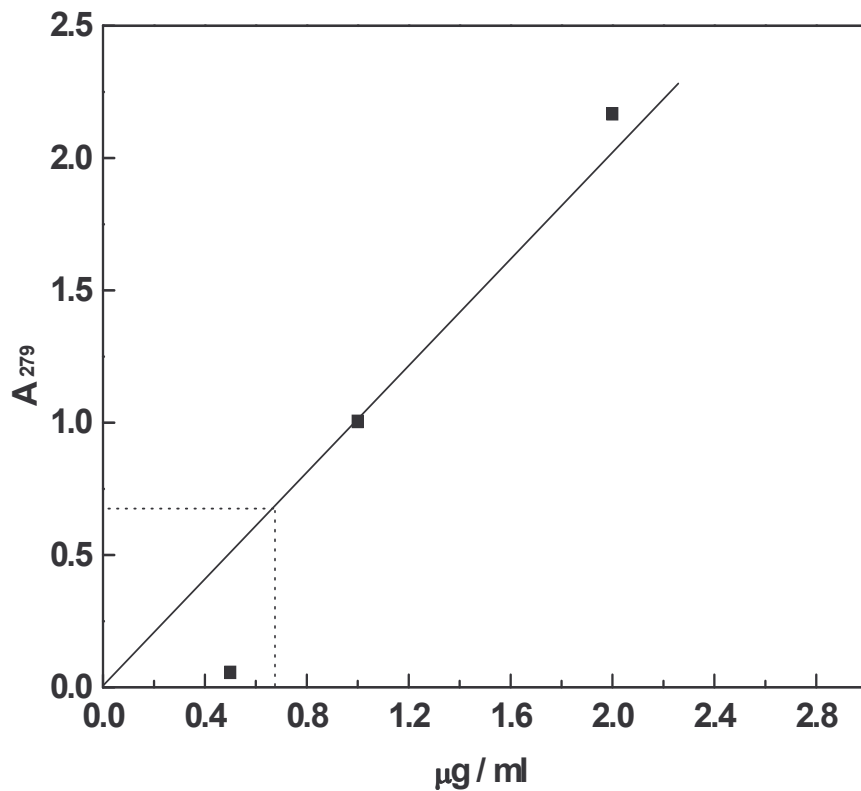


Figure 36: Estimation of Manganese content in HGLOXI by atomic absorption spectroscopy. 1 mg/ml of protein solution was extensively dialyzed against triple distilled water and lyophilized. Manganese solution was used as standard. The absorption was measured at 279 nm.

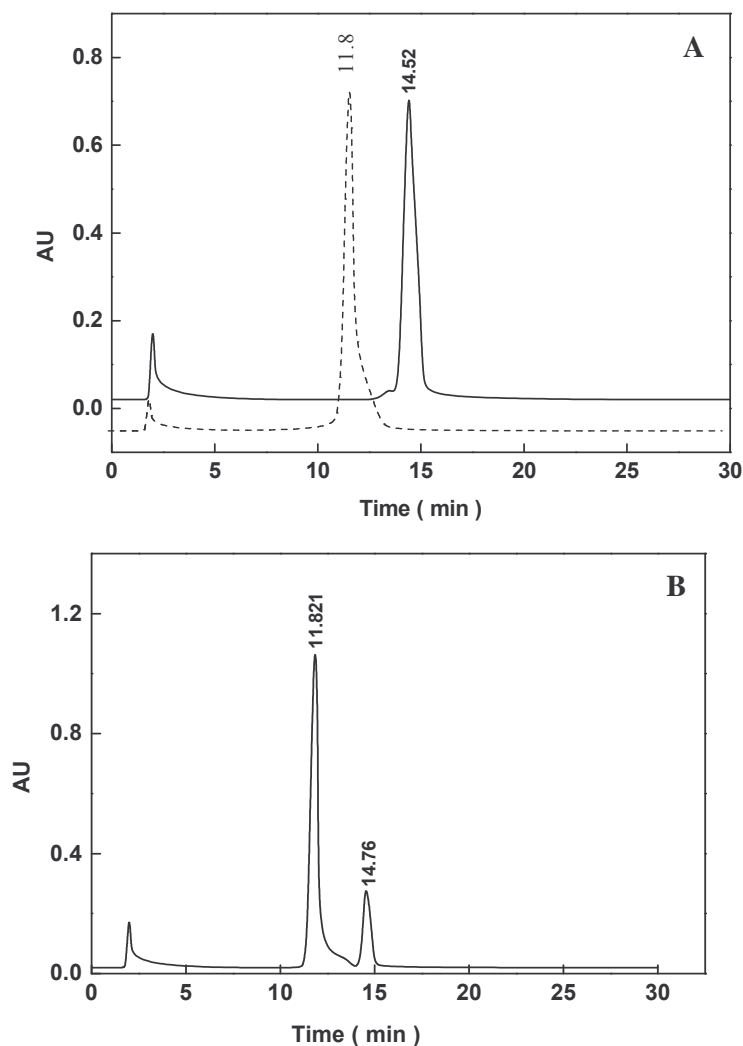


Figure 37: Product analysis of HGLOXI reaction with linoleic acid using SP HPLC. A: 13- HPOD derived from soy LOX action on linoleic acid (11.8 min, dashed line) at pH 9.0 and 9-HPOD derived by the action of potato LOX on linoleic acid at pH 5.5 (14.52 min, straight line) **B:** Products of the reaction of HGLOXI on linoleic acid at pH 5.0. The products of HGLOXI with linoleic acid as substrate was isolated, reduced, methylated and identified by straight phase HPLC on a Sorbax-sil column (4.6 mm x 250 mm, 5 μ particle size). The products were separated by isocratic elution with solvent system n-Hexane/isopropyl alcohol /acetic acid (100:2:0.1) and a flow rate of 1 ml/min. Soya LOX1 and potato LOX were used to get 13- and 9-HPOD, exclusively, which were used as standards

GC-MS analyses of products of LOX reaction with linoleic acid. The GC as well as the fragmentation patterns for products of the reactions of linoleic acid with soya LOX-1, potato LOX and HGLOXI are shown in Fig. 38, 39 and 40. The GC pattern of products of soya LOX-1 showed four peaks, of which, first and second peak belonged to 13- HPOD and 9- HPOD, respectively, while third peak was unreacted substrate and fourth peak was an artifact. The GC pattern of products of potato LOX was similar to that of soya. The second peak, which is prominent than the first, suggested that 9- HPOD is predominant. The GC pattern of the products of HGLOXI was also similar to soya product patterns. The fragmentation pattern of peak 1 of the products (in the present study using linoleic acid as substrate was similar to the fragmentation pattern of 13-HPOD product of soya LOX-1.

The GC patterns of the silylated products of soya LOX-1 and potato LOX on linoleic acid were used as standards. The fragmentation patterns of the silylated derivatives of 13- and 9-HPOD from soya and potato, respectively, were similar. The intensity of the m/z 227 ion was greater than that of the m/z 311 ion in the spectrum of the derivative of 9-HPOD (potato LOX), whereas the reverse was true in the spectrum of the derivative of 13-HPOD (soya LOX-1). The GC-MS pattern obtained for the silylated products of HGLOXI on linoleic acid was similar to that of 13 HPOD as given in Fig. 40B. Prominent ions were observed at m/z 382 (M), 311 [M - 71; loss of $\cdot(\text{CH}_2)_4 - (\text{CH}_3)$] and 227 {due to the ions $\text{Me}_3\text{SiO}^+=\text{CH} - (\text{CH}=\text{CH})_2 - (\text{CH}_2)_4 - \text{CH}_3$ in the spectrum of the derivative of 9-HPOD (Fig.39B) and the ion $[(\text{CH}=\text{CH})_2 - \text{CH}(\text{OSiMe}_3) - (\text{CH}_2)_4 - \text{CH}_3]^+$ in the spectrum of the derivative of 13-HPOD} (Fig. 38B).

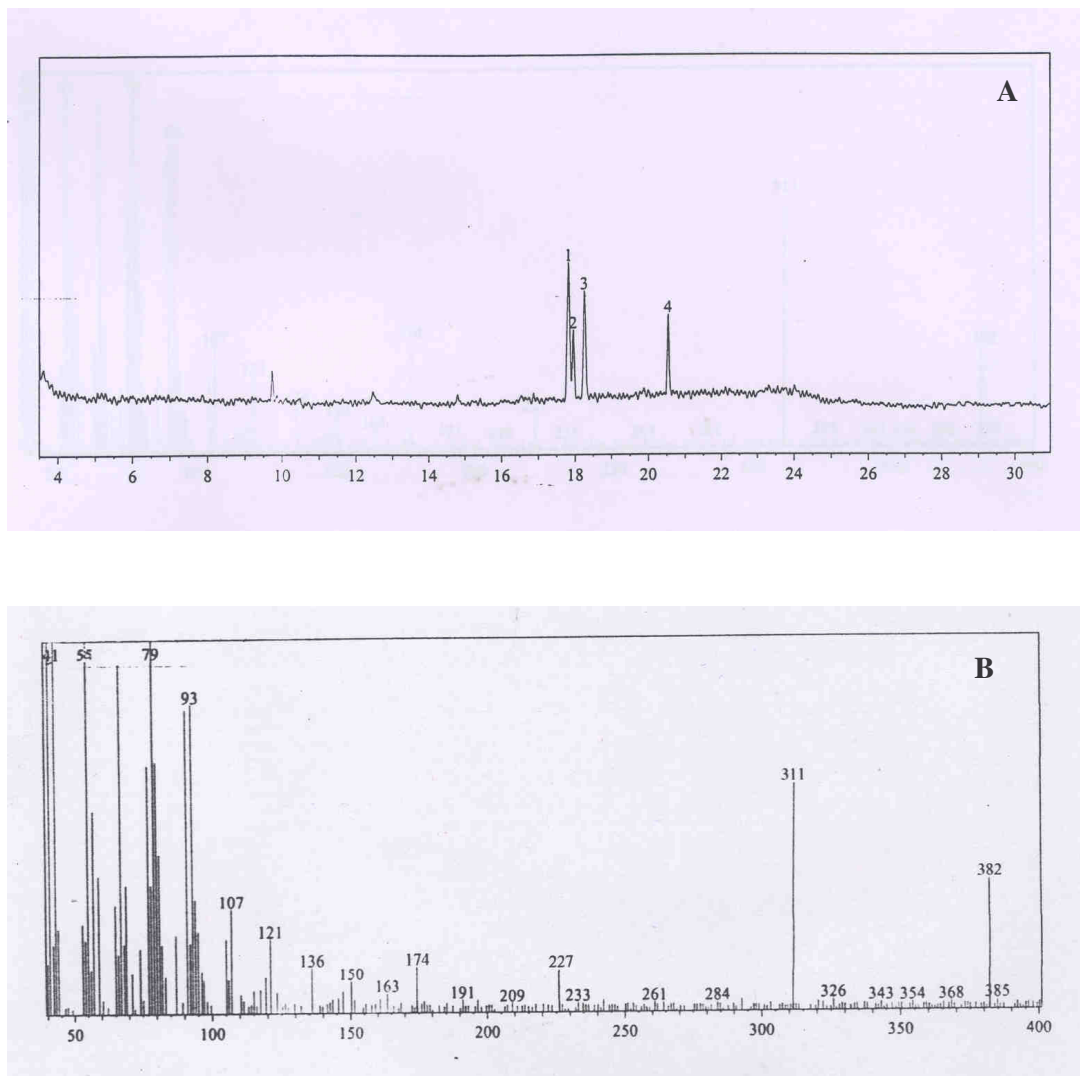


Figure 38: GC MS analysis of products of soya LOX-1 with linoleic acid.

GC-MS analysis of the reaction products (13-HPOD) after hydrogenation with H_2 / Pt and trimethylsilylation with BSTFA. The fragmentation pattern of peak 1 is shown with major ion peaks at 382, 311 and 227. The major ion peak at 311 is prominent when compared to 227. **A:** GC pattern of products of soya LOX-1. **B:** GC spectra of 13 HPOD.

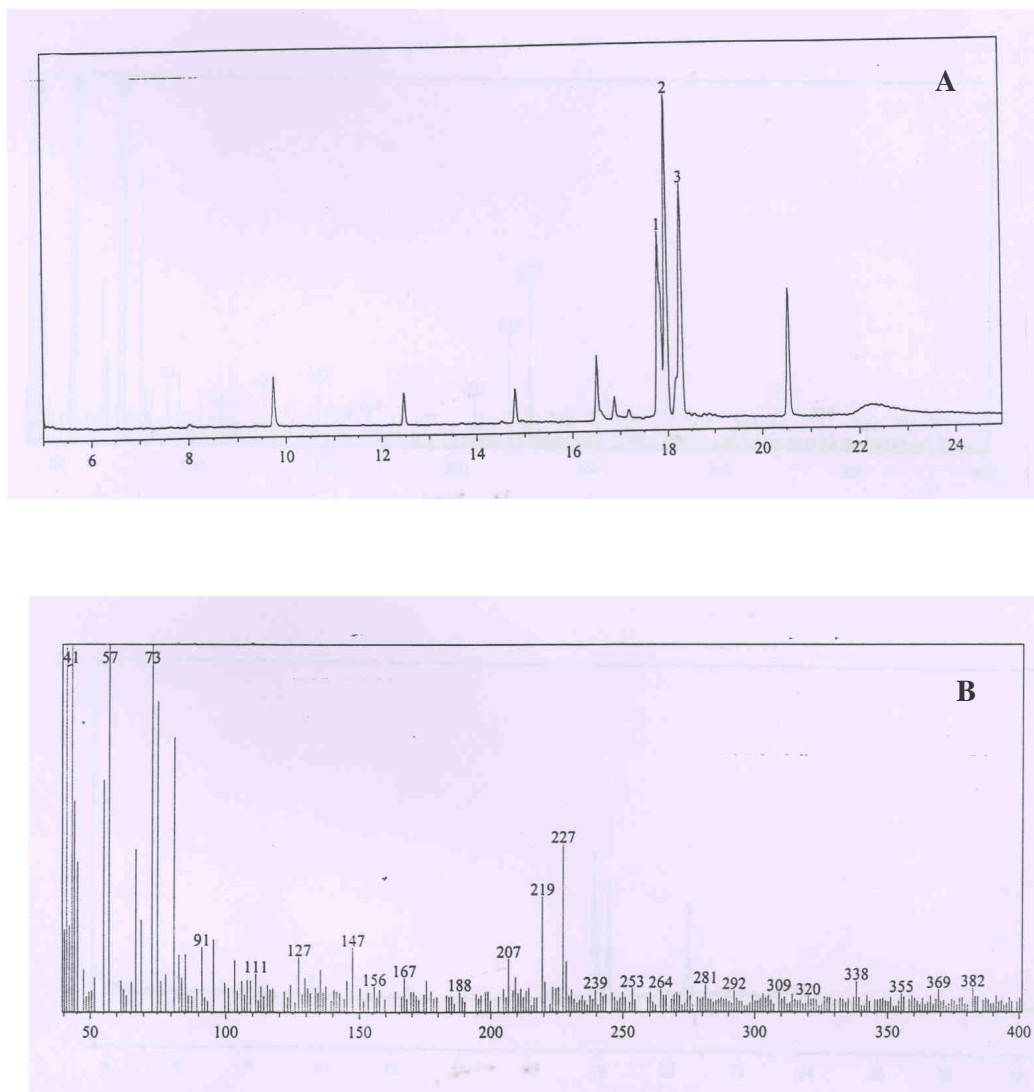


Figure 39: GC MS analysis of products of potato LOX with linoleic acid.

GC-MS analysis of the reaction products (9-HPOD) after hydrogenation with H_2 / Pt and trimethylsilylation with BSTFA. The fragmentation pattern of peak 1 is shown with major ion peaks at 382 and 227. The major ion peak at 227 is a prominent peak. A: GC pattern of products of potato LOX. B: GC spectra of 9 - HPOD.

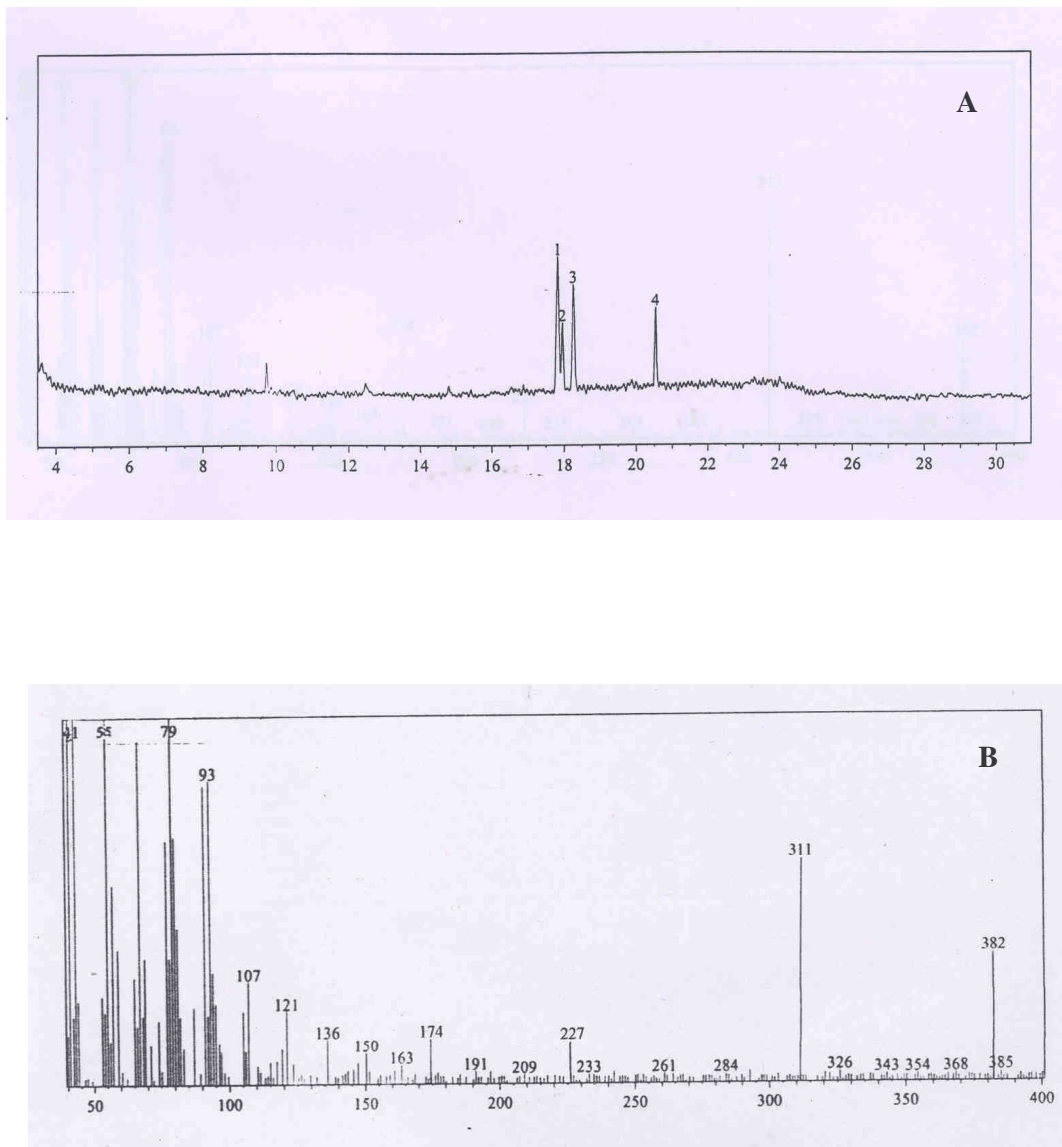


Figure 40: GC MS analysis of products of HGLOXI with linoleic acid. GC-MS analysis of the reaction products (13-HPOD) after hydrogenation with H_2 / Pt and trimethylsilylation with BSTFA. The fragmentation pattern of peak 1 is shown with major ion peaks at 382, 311 and 227. A: GC pattern of products of HG LOX I. B: GC spectra of fragmentation peak 1 corresponding to that of 13HPOD. The GC pattern of HGLOXI and fragmentation pattern of HGLOXI is similar to Soya LOX-1 indicating that it produces 13- HPOD predominantly.

For steric analysis of the products, a chiral phase column was used to identify the stereospecificity of the products. Products of the pea seed LOX-1 (produces 23% of 13(*R*) product) and soya LOX-1 (produces 98% 13(*S*) product) as shown in Fig 41 was used as standards. The standard 13(*S*) product eluted at 26.4 min while the 13(*R*) HPOD methyl ester eluted at 28.4 min (Kuhn *et al*, 1987). In the current study, 13-HPOD methyl ester of the product yielded one major peak with a retention time of 26.3 min and a minor peak at 28.1 min (Fig. 41D). HGLOXI produced 13(*S*) and 13(*R*) HPOD in the ratio of 88:12. Thus the activity of HGLOXI on linoleic acid is comparable to soya LOX-1 which also produces 13(*S*) predominantly.

Spectroscopic studies

Absorption spectra of HGLOXI. The absorption spectra of HGLOXI in 20 mM phosphate buffer, pH 6.0 revealed that the purified protein had an absorbance maximum at 280 nm (Fig. 42A). The purified protein was colorless and lacked significant light absorption between 300 and 600 nm indicating the absence of heme moiety. Concentrated solutions of 1 mg / ml did not have absorbance in the visible range.

Fluorescence spectra. The fluorescence emission spectra of HGLOXI after excitation at 280 nm is shown in fig 42B. The protein was found to have an intrinsic emission maximum at 335 nm, which can be attributed to the tryptophan residues in a non-polar environment.

Circular dichroism spectra. In view of the observed difference in the number of subunits and the prosthetic group, CD measurements were carried out to examine if there was any similarity in structure between HGLOXI and other reported

lipoxygenases. The near UV CD spectra were monitored in the range 320 – 240. The near UV CD spectrum was not well resolved even at protein concentrations of 1-1.5 mg/ ml (42D). A broad peak was observed between 250 and 290 nm. The far UV CD spectra was monitored in the range of 260 – 200 nm. The far UV CD spectrum revealed minima at 230 and 217 nm, establishing predominance of β structure. The far UV CD spectrum for the protein is shown in Fig. 42C. The predominant structure was β sheet comprising of 35% of the whole structure while α helical content was found to be 4.8%, β bend was 22% and random structure comprised 37 % (as deduced by Yang *et al*, 1986). When the secondary structure of HGLOXI was compared with other proteins, it resembled that of *Dolichos biflorus* lectin (Pere *et al*, 1975) rather than any other lipoxygenases.

Thermal stability studies.

Mid point of thermal inactivation. The thermal stability of HGLOXI was studied by determining the midpoint of thermal inactivation. Residual activity of the protein was assayed after incubation at different temperatures in the range (10 – 95°C) followed by rapid cooling to 4°C. LOX activity of HGLOXI was highly stable. 100% activity was retained after 30 min incubation at 65°C. Beyond 65°C, LOX activity was lost rapidly. The midpoint of thermal inactivation, T_m , was found to be 85.5°C (Fig. 43A). The reaction rates (K_r) at temperatures in the range of 78 - 95°C were estimated by plotting residual activity against time as shown in Fig. 43B. The Arrhenius plots (Fig. 43C) of inverse temperature versus the logarithm of velocity were constructed in the range 78 - 95°C for the calculation of inactivation parameters.

FIGURE 41

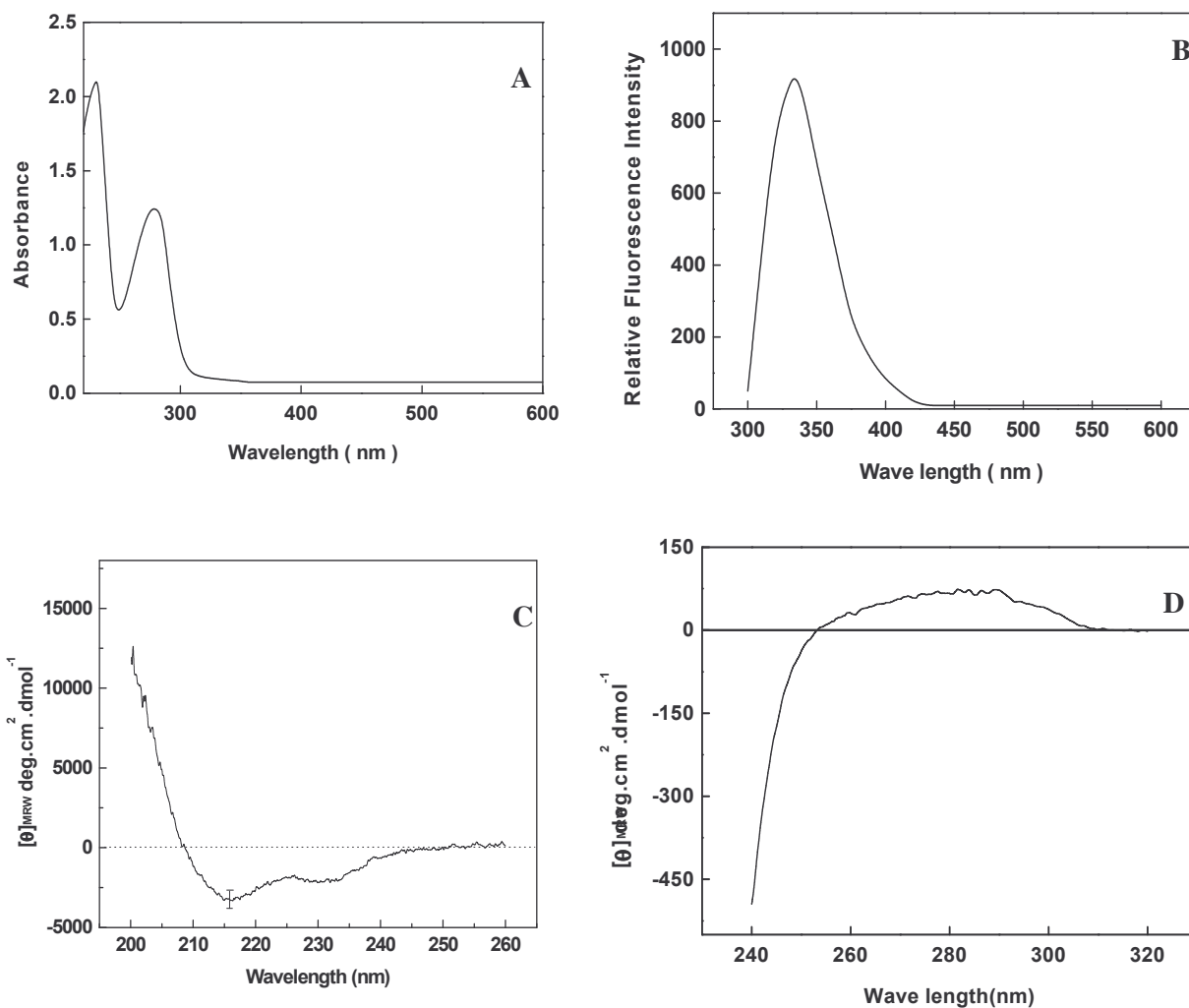


Figure 42: Spectroscopic analysis of HGLOXI. **A:** UV –visible spectra of purified HGLOXI.

Absorption spectrum in 20 mM phosphate buffer, pH 6.0 was recorded from 220 - 600 nm using a 1cm pathlength cell. **B:** Fluorescence spectra of HGLOXI. The bandwidths for excitation and emission monochromators were 5 and 5 nm. The emission spectra was recorded after exciting at 280 nm. Protein concentrations used were 50 mg / ml. Appropriate buffers were used for correcting the baseline. **C:** Far UV CD spectra for HGLOXI. Protein concentration used was 0.42 mg / ml in the far UV region (200 – 260 nm) with a cell path length of 1 mm. **D:** Near UV CD spectra for HGLOXI. The path length of the cell used was 1cm. A protein concentration of 1-1.5 mg / ml was used. Buffer used was 20 mM phosphate buffer, pH 6.0. The measurements were made at 25°C at a scan speed of 10 nm / min. Spectra represented are an average of at least 3 accumulations.

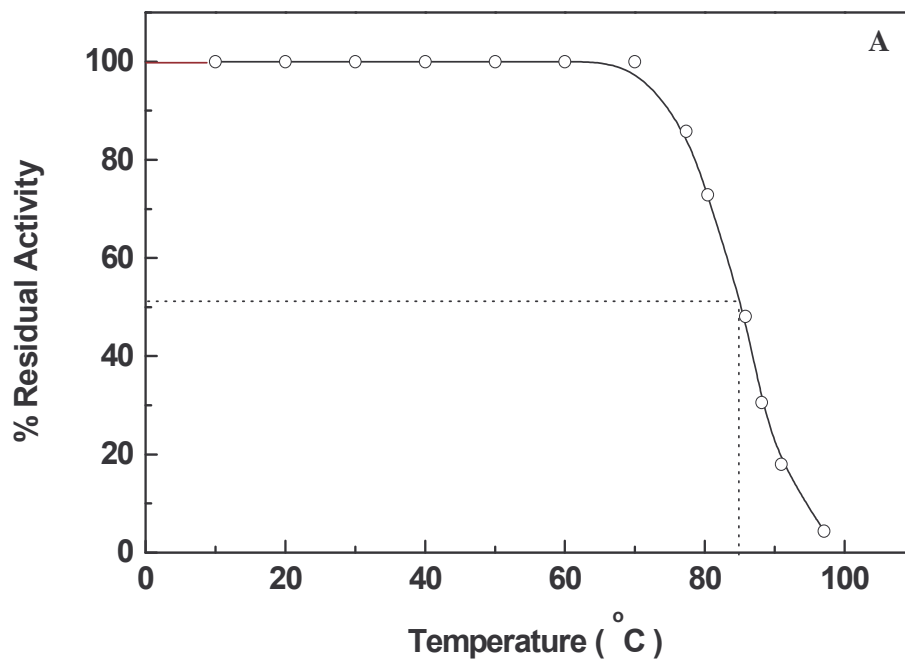


Figure 43: Thermal stability of HGLOXI.

A. Effect of temperature on HGLOXI. The enzyme was incubated at the required temperature in the range of 10 - 95°C in 20 mM phosphate buffer (pH 6.0) for 30 min, cooled to 4°C and assayed for residual lipoxygenase activity as mentioned under materials and methods. The activity of unincubated enzyme was taken as 100% for calculating residual activity.

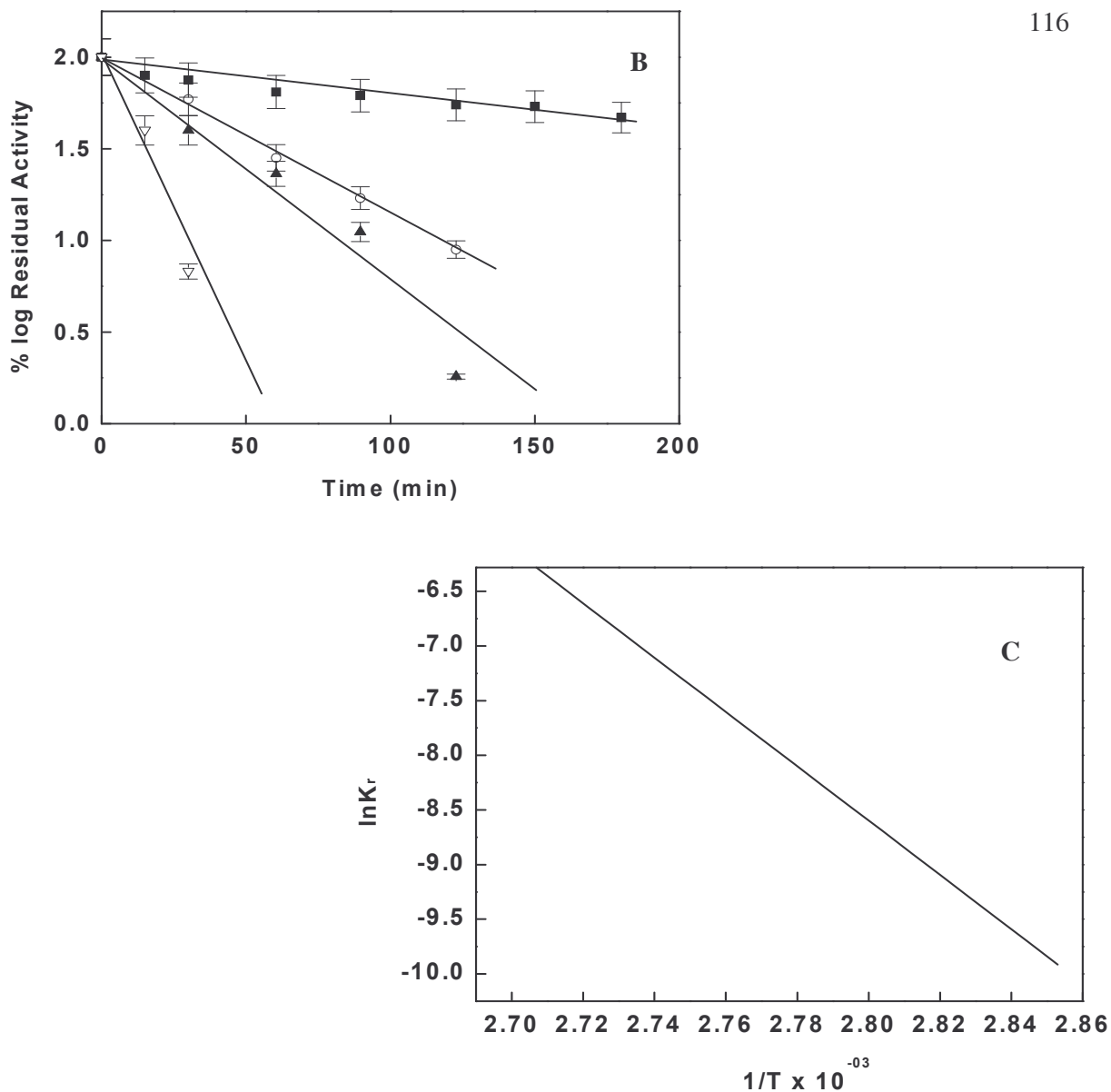


Figure 43. Thermal stability of HGLOXI.

B: *Kinetics of thermal inactivation of HGLOXI.* Samples were incubated at the required temperature in the range of 78 - 95°C in 20 mM phosphate buffer (pH 6.0). Aliquots of the enzyme were drawn at different time intervals, cooled to 4°C and assayed for residual lipoxygenase activity.

—■— 78°C, —○— 82°C, —▲— 88°C, —▽— 95°C.

C: *Arrhenius plot for HGLOXI.* Data from three experimental results incubated in the temperature range of 78 - 90°C was used to construct the Arrhenius plot of the inverted absolute temperature vs logarithm of rate constant and obtain the inactivation kinetics constants.

Thermal inactivation parameters are shown in Table 6.

Table 6: Thermal Inactivation parameters of HGLOXI

Temperature	ΔH^* kcal.mol ⁻¹	ΔG^* kcal.mol ⁻¹	ΔS^* cal.mol ⁻¹ .K ⁻¹
78°C	48.9	25.7	66.1
82°C	48.9	24.8	60.0
88°C	48.9	23.3	71.0
95°C	48.9	23.0	70.5

T_m measurements by circular dichroism. The far UV CD spectra of HGLOXI at 27°C revealed minima at 217 nm and 230 nm suggesting that the native protein had a good amount of β -structure. As the temperature increased, the protein lost its native structure. The structure of HGLOXI was followed at 217 nm in the temperature range of 27°C – 85°C. The molar ellipticity of the protein at 217 nm increased as a function of temperature. There was 47.6% increase in the ellipticity value till 90°C (Fig. 44A and B).

The enzymatic characteristics of HGLOXI are summarized in Table 7.

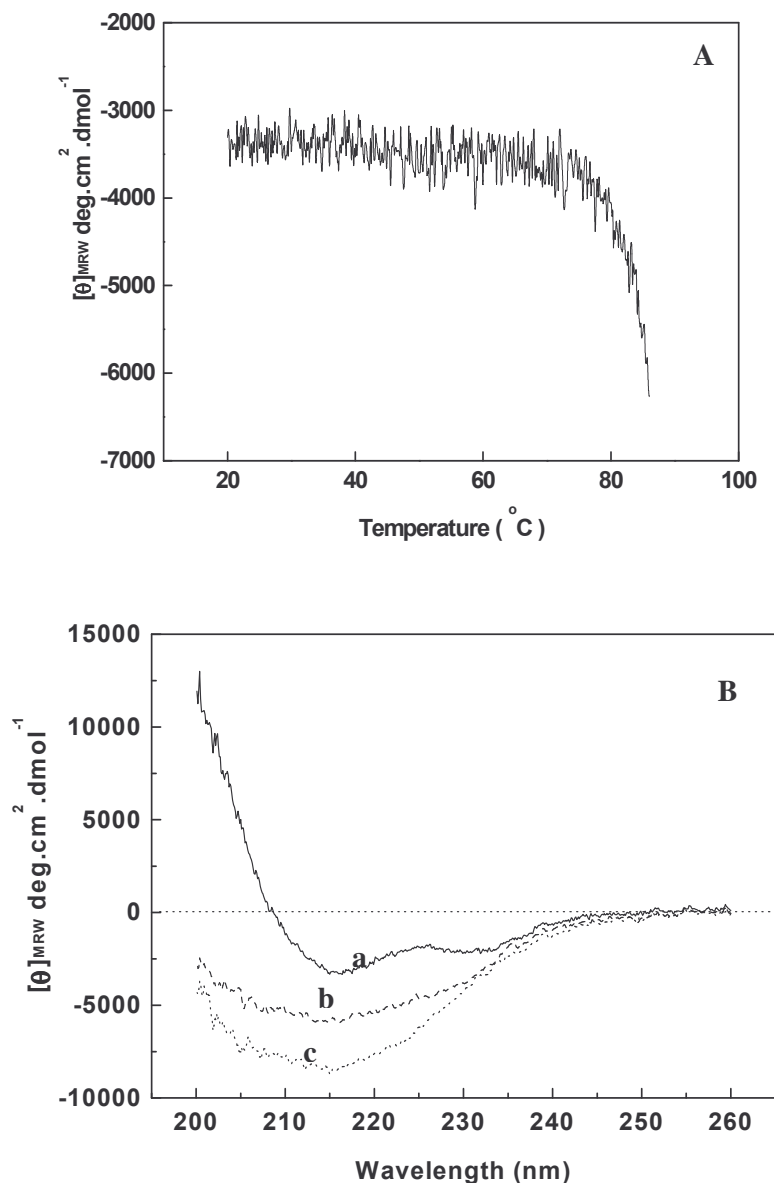


Figure 44: Thermal inactivation studies of HGLOXI by circular dichroism.

A: The purified protein in 20 mM sodium phosphate buffer, pH 6.0 was used to follow the changes in structure at 217 nm using a 1 mm cell in the temperature range 27 - 85°C. Temperature increase at a rate of 1°C/ min was carried out using a peltier attachment (PMH 356WI). A mean residue weight of 115 was used to calculate the molar ellipticity. **B:** Scans of HGLOXI in Far UV range as a function of temperature. The purified protein in 20 mM sodium phosphate buffer, pH 6.0 was used to follow the changes in structure using a 1 mm cell at different temperatures. The mean residue weight of 115 was used to calculate the molar ellipticity. a - 25°C, b - 80°C, c - 85°C.

Table 7: Summary of LOX characteristics of HGLOXI

Molecular weight	~110,000	
pH Optima	4.0 – 5.0	
Substrate	K_m(μM)	V_{max} (μ mol/min)
	Linoleic acid	66.6
	Linolenic acid	55
	Arachidonic Acid	100
Inhibitor	IC₅₀ (μM)	
	NDGA	161
	4-nitrocatechol	433
	ETYA	101
Products produced with linoleic acid	9HPOD : 13HPOD 40 : 60	

Amino acid composition, N-terminal sequence and internal protein sequence data of HGLOXI. The amino acid composition of HGLOXI is presented in Table 8. The protein has very low methionine content and no cysteine. The composition matches very well with that reported for the lectin from *D. biflorus* (Carter and Etzler, 1975a).

HGLOXI, denatured using urea, was subjected to limited proteolysis by trypsin. Trypsin is known to break lys-arg peptide bond. Two prominent tryptic digests were obtained. The two prominent peptides were purified on RP-HPLC using a C₁₈ column (Fig.45). The N-terminal sequence of HGLOXI and the sequence of tryptic digests were determined by Edman degradation. The N-terminal sequence of 30 amino acids and sequence of two internal purified tryptic peptides (~25% of 253 amino acids) were aligned with the *Dolichos biflorus* lectin (PDB no. 1LU1). The N- terminal sequence as well as the two tryptic peptides had 98% similarity to the *Dolichos biflorus* lectin (Fig. 46).

From the amino acid composition analysis, N-terminal sequence, Far UV CD analysis and tryptic digest sequence it appears that HGLOXI is a *Dolichos biflorus* seed lectin having lipoxygenase activity.

Detection of LOX and haemagglutination activities. LOX activity in the protein was detected by activity staining. A part of the band corresponding to LOX activity was cut from the gel and the protein eluted out. This was used to determine haemagglutination activity. The homogeneous preparation showed haemagglutination of 45 ± 5 U / mg protein. Lectin activity was checked at each step of purification of HGLOXI and the LOX / lectin ratio was determined. The

Table 8: Amino acid composition of HGLOXI

Amino acid	Mole (%)	
	Horsegram LOX	<i>Dolichos biflorus</i> lectin*
Asx**	11.2	11.2
Thr	7.4	7.1
Ser	16.4	15.6
Glx ***	7.6	6.4
Pro	4.8	5.0
Gly	6.9	6.4
Ala	10.0	8.8
Val	7.4	7.4
Met	0.18	0.5
Ile	6.1	6.1
Leu	7.9	8.0
Tyr	3.2	3.3
Phe	4.76	4.8
His	1.2	1.2
Lys	3.6	3.9
Arg	2.3	2.4
Cys	0.02	0.0

*(Carter and Etzler, 1975a), **includes both aspartic acid and asparagines
 ***includes both glutamic acid and glutamine

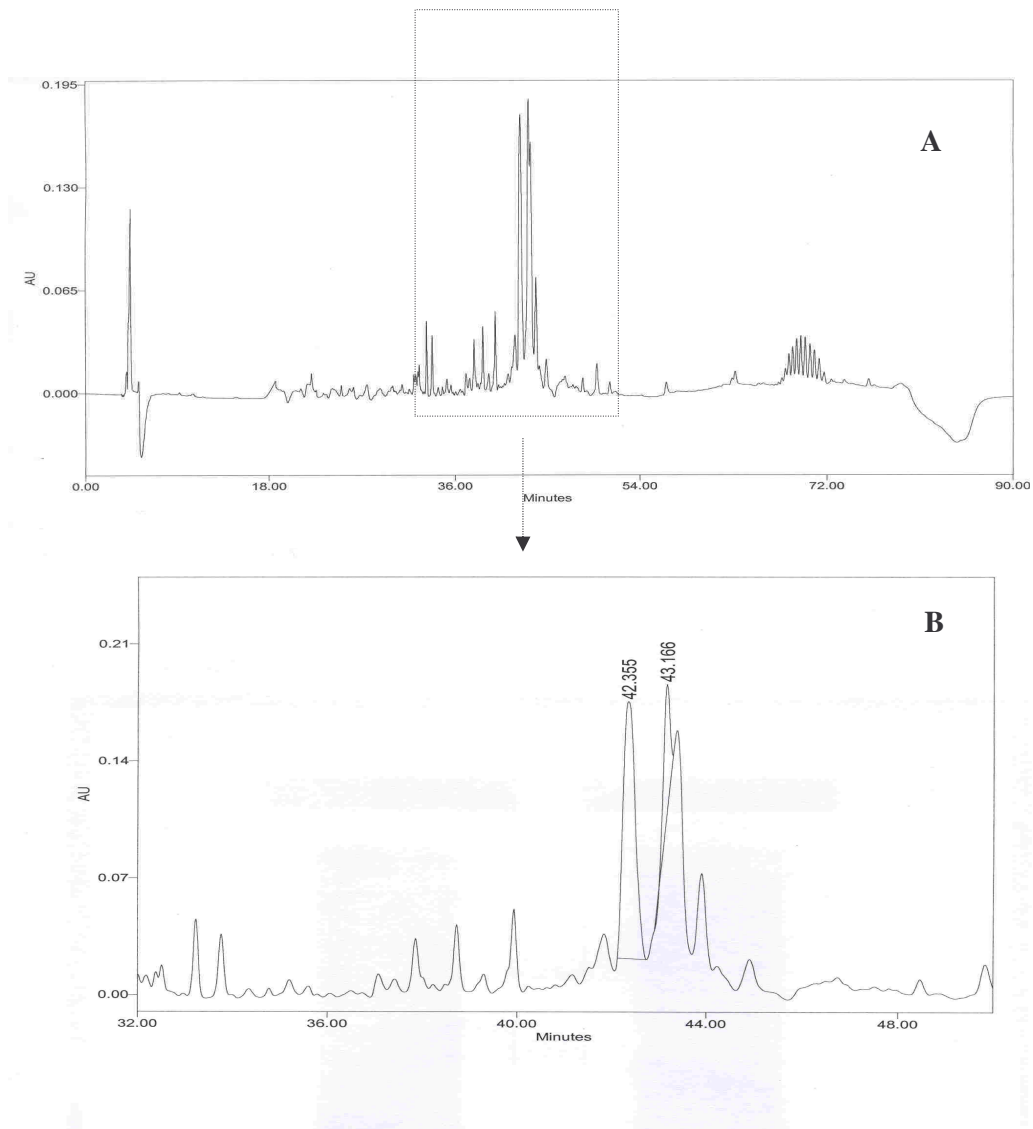


Figure 45: Chromatogram of tryptic digests by RPHPLC. The digest was chromatographed on a Shim Pak C₁₈ (10 μ , 4.6 mm x 250 mm) column and the peptide were fractionated using a gradient of 0.1% TFA in water and 70% acetonitrile in 0.05% TFA at a flow rate of 0.7 ml/min. B. Enlargement of the two major peaks obtained at retention time 42.335 and 43.166. These two peaks were collected and sequenced as described earlier in materials and methods.

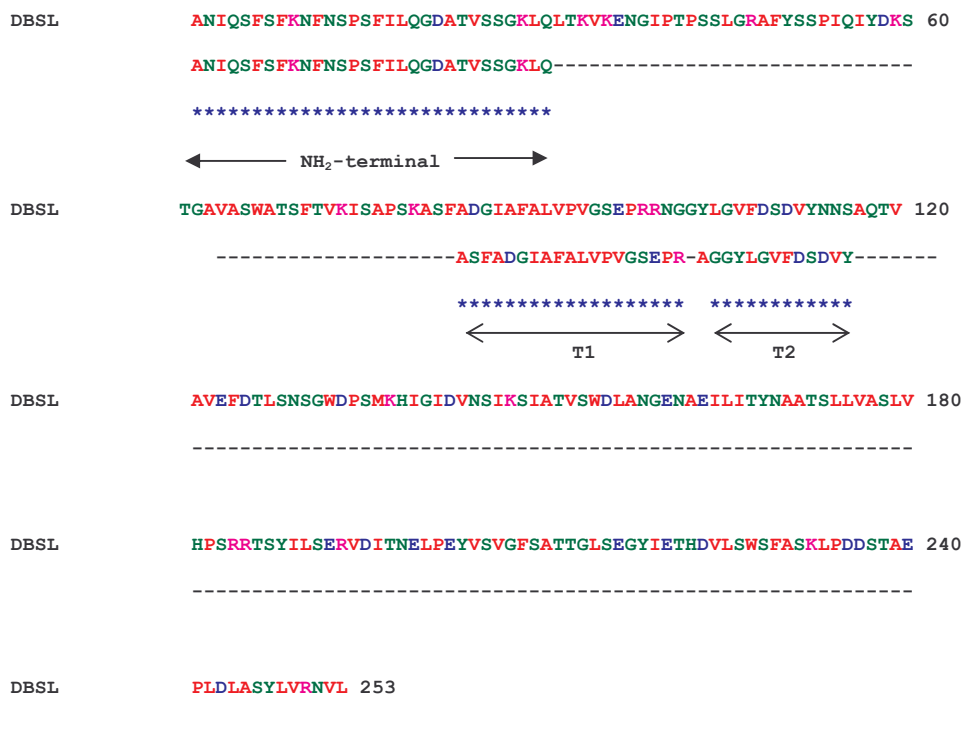


Figure 46: Comparison of the N-terminal sequence and internal peptide sequences of HGLOXI protein with reported sequence of *Dolichos biflorus* lectin. The sequence alignment was carried out by Clustal W version 1.82. T-1 and T-2 are peptides of TPCK-trypsin digest sequence obtained in this investigation. PDB No 1LU1 (*D. Biflorus* seed lectin) (Schnell and Etzler, 1987)

The LOX / lectin ratio, throughout the purification, remained constant especially in the later stages of purification (Table 9). The crude extract had a ratio of 4.8: 1 (LOX: lectin).

Table 9: Purification summary of HGLOXI with lectin activity

	LOX activity (Units/mg protein)	Lectin Activity (Units/mg)	LOX/Lectin ratio
Crude extract	37	7.69	4.8
Sephacryl S-200			
Peak I	48	-	-
Peak II	94	32	2.93
Peak III	50	-	
DEAE- Sephacel	154	51	3.01

n.d. = not determined - = not detected

Among the three peaks obtained from the Sephacryl S-200 column, haemagglutination was associated only with the second peak. The LOX: lectin ratio (2.93:1) remained unchanged after DEAE- Sephacel chromatography, confirming that the protein had both LOX and haemagglutination activity associated with it. Commercial lectin from *D. biflorus* had very weak LOX activity of 8 U/ mg protein. On size exclusion HPLC the commercial lectin and the protein of the present study had the same retention time (co-eluted) of 22.72 min (Fig. 29).

Purification of Dolichos biflorus seed lectin. To further prove that HGLOXI is a lectin having lipoxygenase activity, *Dolichos biflorus* seed lectin purified according to earlier reported conventional purification with slight modifications (Kocourek *et al*, 1977) as given under materials and methods and were checked for lectin and lipoxygenase activity. The protein, from horsegram extract, was subjected to 40-60% ammonium sulfate saturation and ion exchange chromatography. The fractions having lectin activity were pooled, concentrated and subjected to size exclusion chromatography followed by ion exchange chromatography. The purified lectin was found to be a tetramer as reported earlier (Kocourek *et al*, 1977), having two non-identical subunits and the molecular weight was found to be 110 kDa (identical to HGLOXI). The purified lectin was checked for LOX and lectin activity. Haemagglutination activity was found to be 45 ± 5 U / mg protein and lipoxygenase activity was 158 U / mg. The LOX - lectin ratio, throughout the purification, remained constant, especially in the later stages of purification (Table 10).

Identification of cofactor for haemagglutination activity. *Dolichos biflorus* lectin is known to contain manganese as one of its cofactor. The manganese content of the purified protein is found to be the same as reported for *Dolichos biflorus* lectin (Kocourek *et al*, 1977) (Fig.36). However, the manganese content estimated in commercial *Dolichos biflorus* lectin is lower (0.32 μg / g protein). The lower LOX activity in commercial lectin (8 U/mg) can be attributed to the lower content of manganese. However, lectin purified by the reported method (Kocourek *et al*, 1977) is found to have the same amount of manganese content (0.7 mg / g protein) as reported for *Dolichos biflorus* lectin.

Carbohydrate content of HGLOXI. Total carbohydrate content was determined to be 1.3%, which is exactly the same as reported for the *D. biflorus* lectin (Kocourek *et al*, 1977). Protein from the present study and commercial lectin from *D. biflorus* were stained for glycosylation. Both proteins were glycosylated as revealed by the pink bands seen after PA staining (Fig. 47).

Inhibition of haemagglutination activity. Inhibition of lectin activity was carried out using different concentrations of N- acetyl galactosamine, a known competitive inhibitor of *D. biflorus* lectin. Haemagglutination activity of HGLOXI was inhibited by N-acetyl galactosamine with an IC_{50} concentration of 45 μM (Fig.48).

Circular dichroism studies of DBSL lectin. The far UV CD spectra of the purified protein obtained in the present study are very similar to the ones reported for the lectin from *D. biflorus* (Pere *et al*, 1975). The isolated lectin also had minima at 217 and 230 nm very similar to HGLOXI.

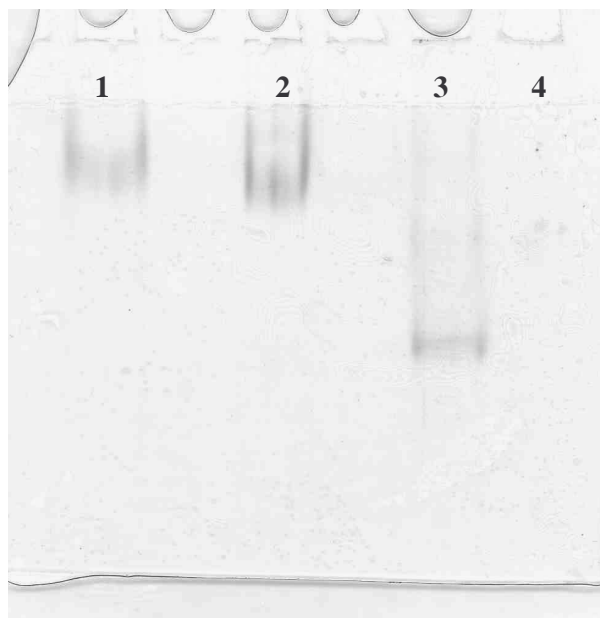


Figure 47: Detection of glycosylation in HGLOXI. The purified protein was subjected to polyacrylamide gel electrophoresis under native conditions. The molarity of the electrophoretic buffer used was 50 mM Tris and 284 M glycine, pH 8.3. Lane 1- purified HGLOXI with lectin activity 1 mg / ml, lane 2 - commercial lectin 1 mg / ml, lane 3 - ovalbumin 1 mg / ml (positive control) and lane 4 - BSA 1 mg / ml (negative control). The gel was immersed in 12.5% trichloroacetic acid (25 – 50 ml / gel) for 30 min followed by incubation with 1% periodic acid in 3% acetic acid for 50 min and stained with fuchsin – sulfite in dark and washed with freshly prepared 0.5% metabisulfite to develop the pink bands indicating the presence of glycosylation.

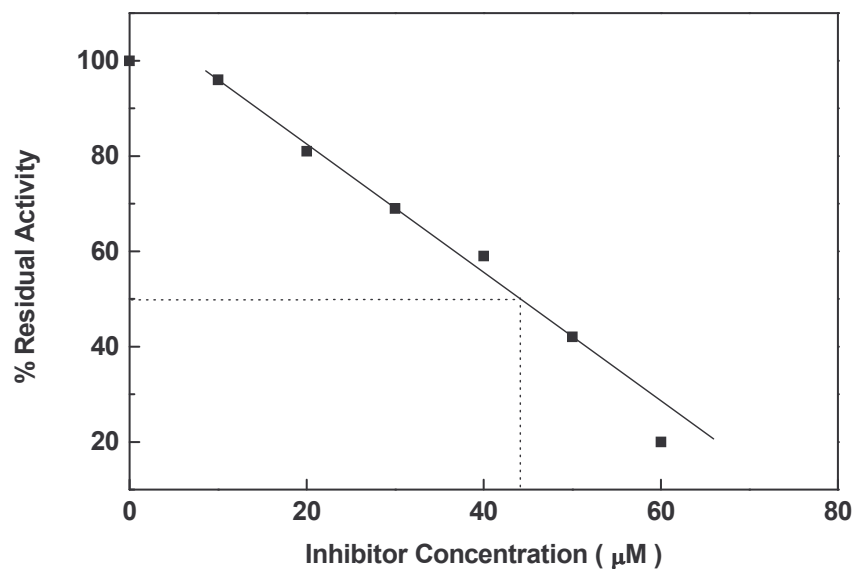


Figure 48: Effect of *D. biflorus* inhibitor on lectin activity of HGLOXI. N-acetyl galactosamine. N-acetyl galactosamine stock solution was prepared in phosphate buffered saline. The protein was preincubated with the inhibitor for five minutes before the assay. Appropriate blanks were used for the experiment. The assay was carried out as described in materials and methods. The data shown are an average of 3 experiments.

Characteristics of the lectin activity of HGLOXI are summarized in Table.11

Table 11: Lectin characteristics of HGLOXI

Molecular weight	~ 110,000 Da
Subunits	2 (Heterodimer)
Carbohydrate content	1.3%
Manganese content	0.68 mole / molecule of protein
Secondary structure	Predominantly β sheet.
Inhibitor	
N-acetyl galactosamine	45 μ M (IC_{50})

The thermal stability of haemagglutination was determined in the range 10 -75°C. 100% activity was retained till 75°C (Fig. 49). However, beyond this temperature, aggregation of protein due to high concentrations rendered it difficult, to continue the experiments.

Identification of LOX activity locus in HGLOXI. To identify the LOX locus as being distinct from the lectin locus of the protein, haemagglutination was assayed in the presence of NDGA (500 μ M) and ETYA (300 μ M) also.

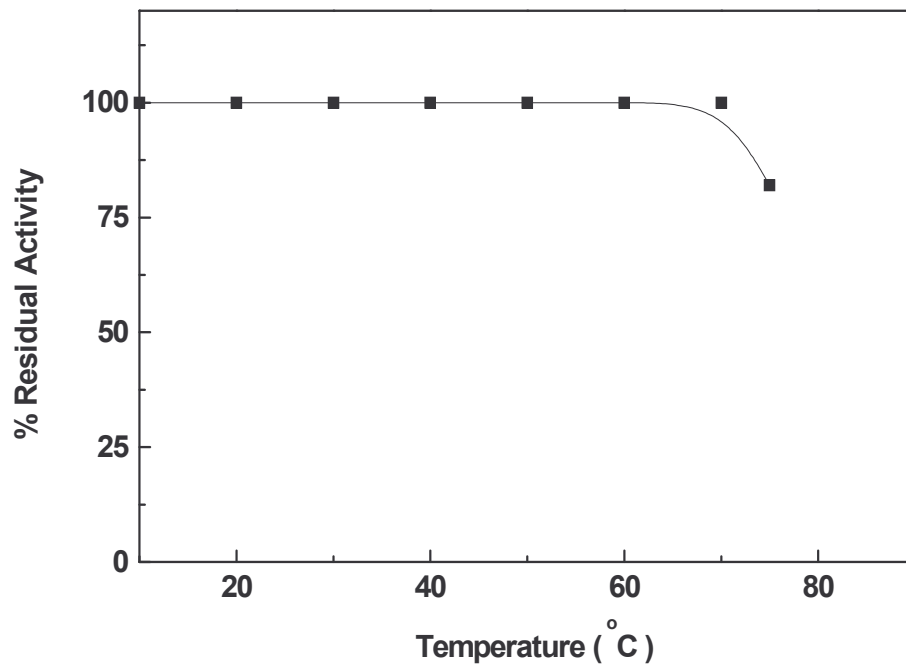


Figure 49: Effect of temperature on lectin activity of HGLOXI. HGLOXI was incubated at the required temperature in the range of 10 - 75°C in 20 mM phosphate buffer (pH 6.0) for 30 min, cooled to 4°C and assayed for residual haemagglutination activity as mentioned under materials and methods. This experiment could not be carried out beyond 75°C as protein aggregation was observed at the concentrations used (2 mg / ml).

There was no effect of either NDGA or ETYA on haemagglutination, in the concentrations used (Fig 50A). N-acetyl galactosamine (500 μ M), an inhibitor of *Dolichos biflorus* lectin, was incubated with protein and the assay for LOX was carried out. N-acetyl galactosamine did not inhibit LOX activity in the concentrations used indicating that the LOX activity locus on the molecule was different from the haemagglutination locus (Fig. 50B).

Polyclonal antibodies of soya LOX-1 and cross – reaction with HGLOXI.

Rabbit antisera were prepared against soya LOX-1 and checked for its crossreactivity with HGLOXI. There was no cross reaction (Fig.51). The antisera did not cross- react with commercial *D. biflorus* lectin or the fractions from the first and the third peak of Gel filtration chromatography (Sephacryl S-200). Further, there is no homology in the lectin sequence as compared to reported plant lipoxygenase sequences.

Fractionation of subunits of HGLOXI. HGLOXI was denatured in urea and the subunits were separated on an ion exchange column as reported earlier by Carter and Etzler (1975a). The separated subunits (Fig. 52A) were checked for LOX activity. Both the subunits had very little LOX activity (6 and 8 units/mg respectively). One of the subunits had haemagglutination activity as already reported (Carter and Etzler, 1975a). The separated subunits were reconstituted and checked for lipoxygenase activity. There was little or no activity observed suggesting that LOX activity is irreversibly lost on fractionation of the subunits. The SDS-PAGE pattern of the unfractionated, separated subunits and reconstituted subunits is shown in Fig. 52B.

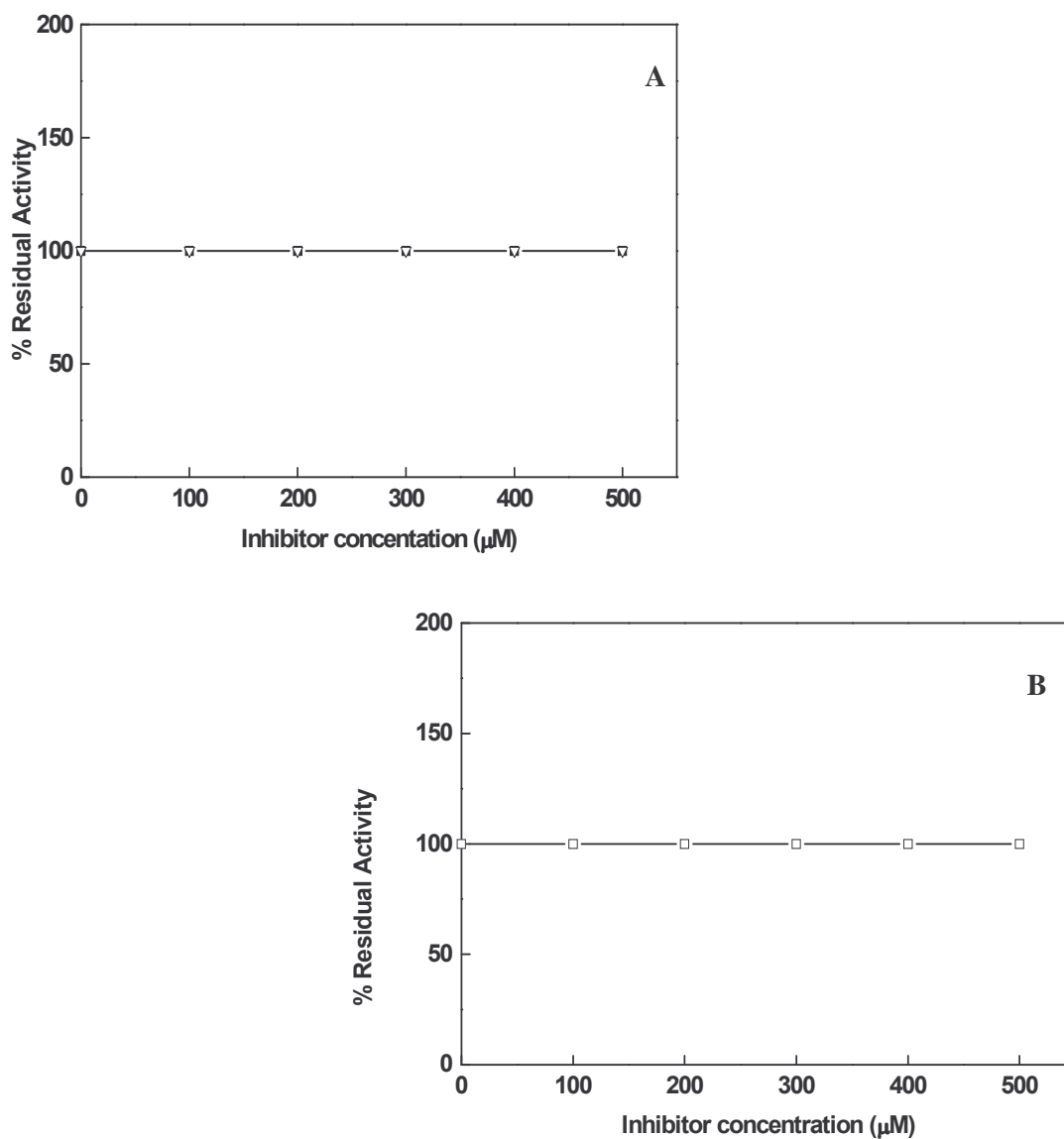


Figure 50: A. Effect of LOX inhibitors on haemagglutination activity of HGLOXI.

—▽— NDGA, —●— ETYA. NDGA and ETYA stock solutions were in absolute ethanol. Appropriate blanks were used for the experiment. Protein was preincubated with the inhibitors for five minutes prior to the assay. **B** Effect of *Dolichos biflorus* lectin inhibitor on lipoxygenase activity of HGLOXI.—□— N-acetyl galactosamine. N-acetyl galactosamine stock solution was prepared in phosphate buffered saline. Appropriate blanks were used for the experiment. The protein was preincubated with the inhibitor for five minutes before the assay.

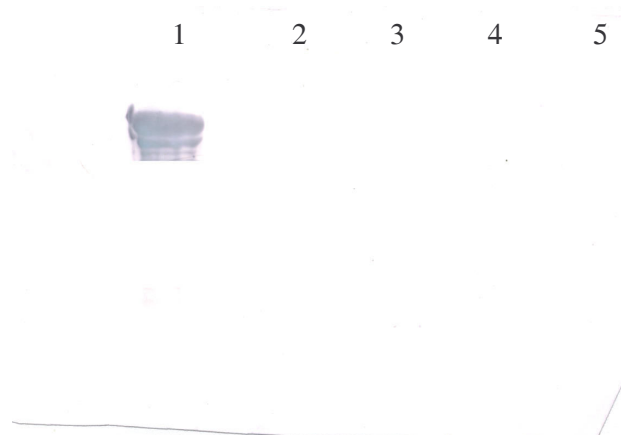


Figure 51: ELISA pattern of interaction of soya LOX antibodies with soya LOX-1, HGLOXI and the 1st and the 3rd peak from gel filtration (Fig 23). SDS-PAGE was carried out using 12% gels, run at 10 mA for 6 h. After electrophoresis, the protein was transferred to a nitrocellulose membrane. The membrane was washed in immunoblot buffer and was incubated with immunoblot containing antibodies against Soya LOXI. The membrane was treated with secondary antibody and incubated in substrate buffer. The alkaline phosphatase activity was detected with a mixture of BCIP and NBT in substrate buffer. Lane 1. Soya LOX I - 60 μ g, lane 2. HGLOX I – 200 μ g, Lane 3. Commercial lectin – 200 μ g, Lane 4. Peak I of Sephacryl S 200 - 200 μ g, Lane 5. Peak III of Sephacryl S 200 – 200 μ g.

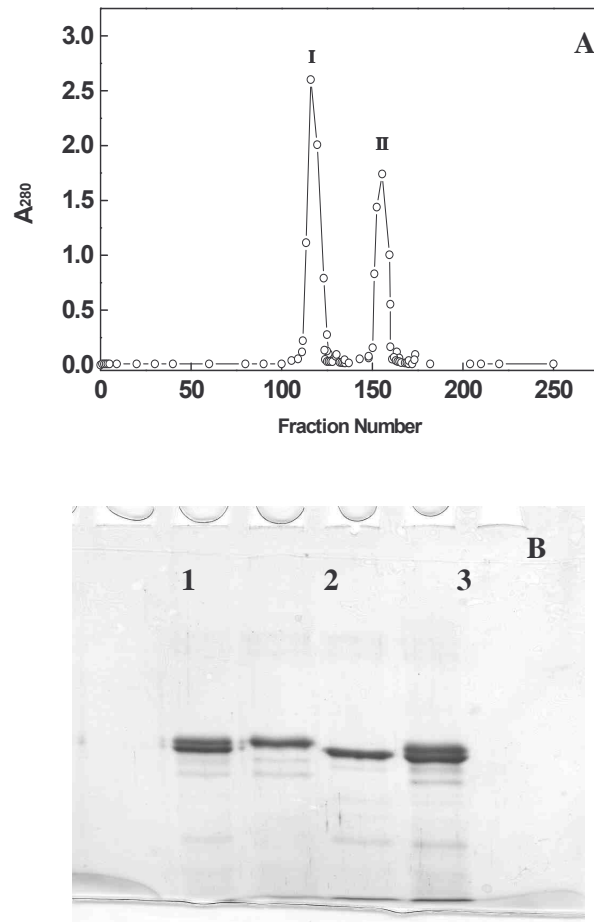


Figure 52: Separation of the subunits of HGLOXI. **A.** The subunits of the protein were separated on a DEAE Cellulose column (2.1 x 7 cm, 25 ml) preequilibrated with 8.0 M urea-0.04 M Tris HCl buffer (pH 7.3) at 25°C. 15 mg of HGLOXI was applied on to the Column and eluted with a linear gradient of 0- 0.075 M NaCl gradient in 0.04 M Tris HCl (pH 7.3) – 8.0 M urea at a flow rate of 10 ml/ h. The pooled fractions were extensively dialyzed against 0.04 M Tris HCl (pH 7.3) containing and 0.02% sodium azide at 4 °C. The fractions were checked for both LOX as well as haemagglutination activity. **B:** *SDS-PAGE pattern of the HGLOXI before and after fractionation of subunits*; Lane 1: HGLOXI from *Dolichos biflorus* – 25 μ g, Lane 2: subunit I of HGLOXI – 20 μ g, Lane 3: Subunit II of HGLOXI – 20 μ g and Lane 4: Reconstituted HGLOXI (by mixing the two subunits in the ratio 1: 1) 15 μ g each.

Fatty acid binding site on HGLOXI. LOX activity of HGLOXI was assayed in presence of adenine, kinetin and ANS, known to bind to the hydrophobic site of the lectin (Hamelryck *et al*, 1999). All the three inhibited LOX activity in a concentration dependent manner. The concentration required for 50% inhibition of LOX activity was 350 μM , 300 μM and 50 μM for adenine (Fig. 53), kinetin and ANS, respectively (Table 12). ANS (50.8 μM) was a competitive inhibitor of LOX activity with the K_m increasing from 161 μM to 333 μM . The V_{max} was not affected (Fig 54 A and B). However, none of the above inhibitors had any effect on soya LOX-1 activity in the concentration range studied (Fig 55).

Fractionation of isoforms of HGLOXI. The isoforms of HGLOXI was separated according to Carter and Etzler (1975b) depending on the extent of glycosylation (Fig 56). The major three fractions obtained showed LOX activity with a specific activity of 158 U/mg suggesting that the extent of glycosylation did not affect lipoxygenase activity.

Table 12: Inhibition of HGLOXI activity with compounds binding to hydrophobic site.

INHIBITORS	IC ₅₀ [μM]
ANS	50.8
Adenine	350
Kinetin	300

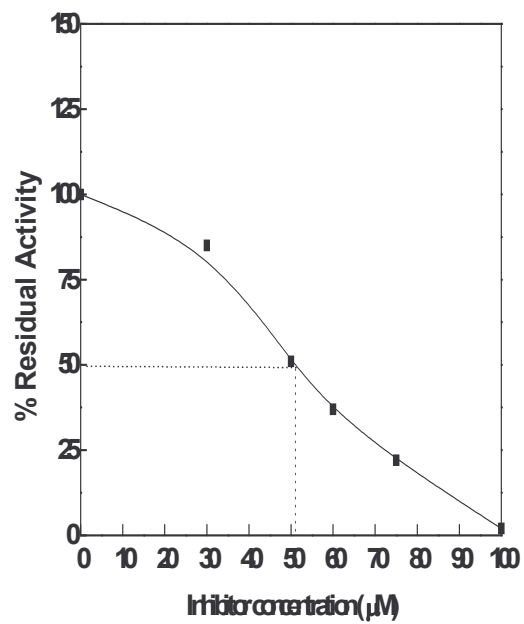


Figure 53: Inhibition of HGLOXI by ANS. IC₅₀ was determined by incubating the inhibitor for five minutes before assaying for activity. Appropriate blanks were used. The data shown is an average of three experiments.

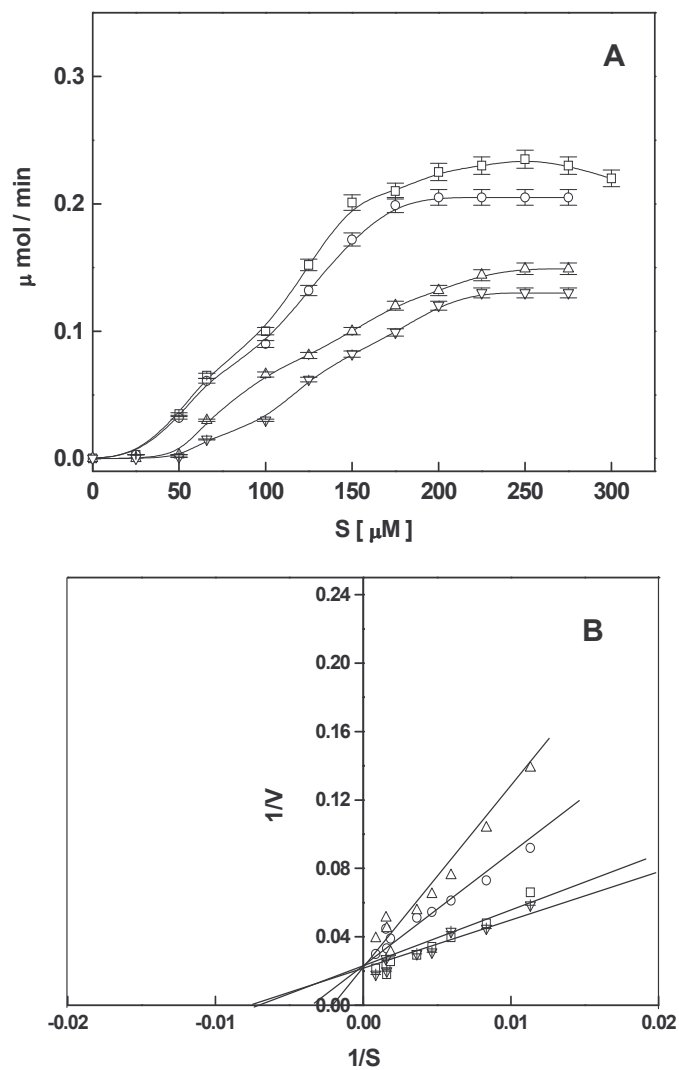


Figure 54: Effect of ANS on LOX activity of HGLOXI. ANS stock solutions were in absolute ethanol. The enzyme was preincubated with inhibitor for five minutes. Appropriate blanks were used for the experiment. The data shown are an average of 3 experiments — \triangle — 50, — \circ — 30, — ∇ — 60 μM , — \square — without inhibitor. A. S vs V plot showing decrease in HGLOXI activity with increased ANS concentration. B. Line weaver Burk plot. KI was determined using double reciprocal plot.

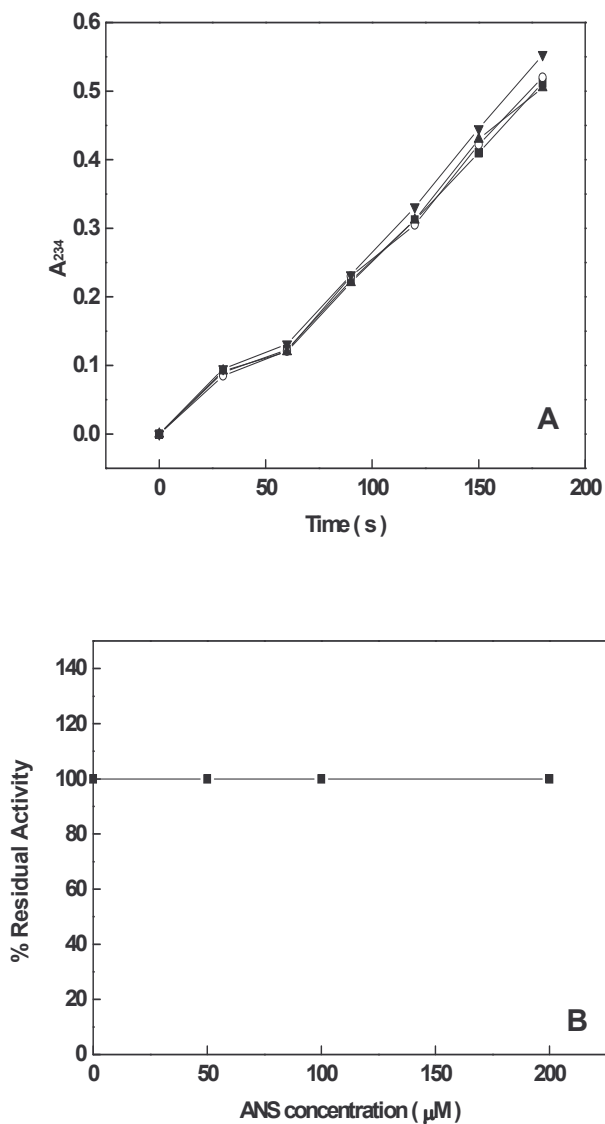


Figure 55. Effect of ANS on soya LOX-1. Soya LOX-1 was preincubated for five minutes with different concentrations of ANS and the residual activity was measured as given under materials and methods. **A.** Kinetics of Soya LOX-1 with linoleic acid as substrate was followed at 234 nm with different concentrations of ANS. — ■ Control, — ○ 50 μM, — ▲ 100 μM, — ▼ 200 μM.

B. Enzyme preincubated without inhibitor was taken as 100 %. Residual activity was calculated.

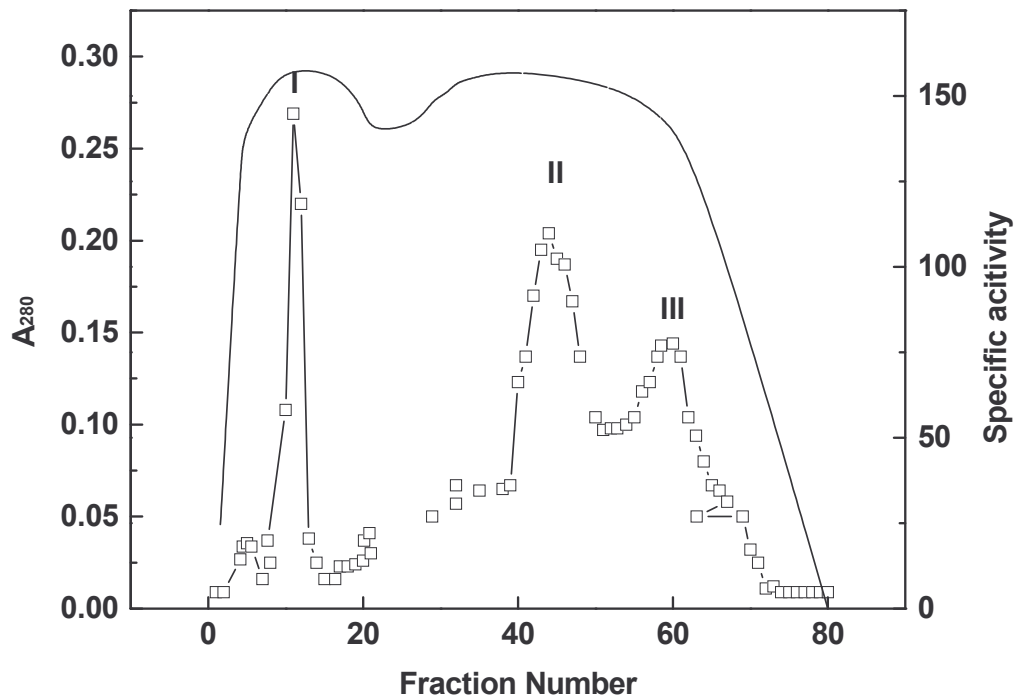


Figure 56: Fractionation of isoforms of HGLOXI on Con A Sepharose.

Fractionation was carried out according to Carter and Etzler, (1975b). HGLOXI was dialyzed against PBS and was loaded onto Con A Sepharose column (10 ml). The column was eluted with a linear gradient of 0-0.3 M α -D-methyl mannopyranoside in PBS. Three major fractions obtained were checked for LOX activity — □ — Abs at 280 nm —

LOX activity.

DISCUSSION

Purification of a protein with LOX activity from horsegram seeds (HGLOXI) to homogeneity was achieved by conventional chromatographic methods. The protein has a specific activity for LOX of 154 U / mg (Table 5) and a pH optimum of 4.0 – 5.0. This is one of the highest specific activities associated with an acidic lipoxygenase from a legume family.

The homogeneity of the preparation has been ascertained by native-PAGE, SDS -PAGE RP-HPLC and size exclusion chromatography. The protein was found to have a molecular weight of ~110,000 Da with two sets of subunits (Fig. 29) of very close mobility. Plant lipoxygenases are known to have molecular weights in the range of 65- 100 k Da. The molecular weight of HGLOXI is in good agreement with the molecular mass of the earlier reported lipoxygenases. The corresponding molecular weights of the subunits as determined by MALDI-TOF were 27287 and 28426 Da suggesting that HGLOXI is a tetramer. Lipoxygenases reported till now comprise single polypeptide chain. HGLOXI is an unusual protein comprising of two non-identical subunits.

From the typical sigmoidal curve of the kinetics obtained at 234 nm, it is clear that an initial concentration of hydroperoxides (products of LOX activity) is necessary for activation of the enzyme (Fig 31B). Earlier reports suggest that a considerable lag has been observed in the kinetics of product formation by soya LOX. In the kinetic scheme proposed by Smith and Lands (1972) suggest that there is a regulatory site on the enzyme. At higher substrate concentration this site is occupied by the reactant resulting in the kinetic lag. The S vs V curves have a sigmoidal shape, indicating that the lipoxygenase activity is very similar to soya LOX-1 with the requirement of hydroperoxides for activation of the molecule (Fig.

31B). The reported K_m for various substrates (Table 7), indicate that this molecule has a good affinity to convert PUFAs to their respective peroxides. In addition to hydroperoxide, HGLOXI gives rise to secondary products absorbing at 280 nm due to the formation of ketodienes (Fig. 31C). The reaction is carried out in an aerobic environment. The formation of ketodienes in a lipoxygenase catalyzed reaction has long been recognized (Gardner, 1991). It can occur aerobically or anaerobically. The ketodienes are known to play a major role in plant defense (Gardner, 1991).

Inhibition studies reveal that HGLOXI behave as a classical lipoxygenase. The classical inhibitors of lipoxygenases like ETYA (substrate analogue) and NDGA (free radical scavenger) inhibit the lipoxygenase activity associated with the molecule with 50% inhibition concentrations of 101 μM and 161 μM , respectively (Table 7). 4- nitrocatechol (binds to iron-enzyme complex) did not inhibit the activity.

HGLOXI is found to be a weak co-oxidising enzyme at pH 6.0 under aerobic conditions (Fig. 35). The pigment bleaching ability of lipoxygenases is shown to differ by several investigators (Klein *et al*, 1984, Sanz *et al*, 1994). Ramadoss *et al* (1978) showed that the capacity of soya LOX-1 to co-oxidize carotene is poor. Soya LOX-1 is a weak co-oxidising enzyme in aerobic conditions but is very effective anaerobically (Garssen *et al*, 1971, Klein *et al*, 1984). The other two major isoenzymes from soya have been individually good in carotene bleaching under aerobic conditions. However a combination of soya LOX-1 and soya LOX-3 or soya LOX-2 and soya LOX-3 are found to be highly effective in promoting bleaching. Preformed 13-hydroperoxide could replace soya LOX-1 in the LOX-1 and LOX-2 combination but 9- hydroperoxide is found to be ineffective

(Garssen *et al*, 1971, Klein *et al*, 1984). According to Klein *et al* (1985), carotene bleaching may be related to the formation of ketodienes in the secondary lipoxygenase reactions as it has been observed that the isoenzymes with the capacity to form ketodienes are also effective in carotene bleaching. However, neither the mechanism of the carotene bleaching nor the aerobic formation of ketodienes is well understood to correlate both the activities.

Positional specificity is known to vary with different lipoxygenases depending on the pH, temperature and other conditions like oxygen level (Gardner, 1991). Products of LOX reaction have been identified as 13-HPOD and 9-HPOD (Fig. 37) in the ratio of 80: 20. In this respect, it is similar to rice lipoxygenase, which is known to produce 13-hydroperoxide predominantly and which is also known to act in acidic pH (Shibata and Axelrod, 1995). The nature of the hydroperoxides has been confirmed by GC-MS data also. The stereochemistry of the hydroperoxides, are identified to be 13(*S*) and 13(*R*) in the ratio of 88:12 (Fig. 41D). Plant lipoxygenases reported until now are known to produce products of *S* stereospecificity (Brash, 1999) predominantly. Both the stereochemistry of the hydroperoxides and the nature of insertion of oxygen across the double bond to produce either 9 or 13-HPOD, indicate that this protein behaves like the classical lipoxygenase molecule (Brash, 1999). The number and structural as well as the functional diversity of lipoxygenase isoforms enable the plant to appropriately respond to environmental challenges (Feussner and Wasternack, 2002). The two classes of lipoxygenase products – (9*S*) hydroperoxy and (13*S*) hydroperoxy derivatives of PUFAs are considered to be of central importance for the production of a plethora of oxylipins found in plants. Lipid peroxidation is common to all biological systems, appearing both in

developmentally as well as environmentally regulated processes of plants. The hydroperoxy polyunsaturated fatty acids, synthesized by the action of various highly specialized forms of lipoxygenases, are substrates of at least seven different enzyme families. Signaling compounds such as jasmonates, 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 (Feussner and Wasternack, 2002). Jasmonates and their octadecanoid precursors act as a master switch in plant development and stress adaptation (Farmer and Ryan 1992).

The CD spectra of the molecule in the near and far UV regions are found to have the characteristic features of β - structure with minima at 217 nm. Near UV CD band is very broad in the region 250 – 290 nm and not well resolved even at high concentrations of protein (1 – 1.5 mg/ ml). The secondary structure of HGLOXI is quite different from soya LOX-1 and rabbit reticulocyte lipoxygenase but similar in structure with *Dolichos biflorus* lectin. Lectin from *D. biflorus* is reported to have two well-resolved peaks in the near UV regions at 265 and 290 nm (Père *et al*, 1975).

Majority of the plant lipoxygenases are reported to have low thermal stability. The T_m for LOX activity associated with protein in the present study is found to be 85°C suggesting that the molecule could withstand high temperatures. The thermal inactivation mechanism was found to follow typical first order reaction kinetics. The corresponding E_a , ΔH^* , ΔG^* and ΔS^* values are given in Table 6. Lipoxygenase activity is highly thermostable with ΔH^* value of 48.99 kcal. Mol⁻¹ compared to 9.9 kcal.mol⁻¹ for soya LOX-1 (Sudharshan, 1996). Inactivation of

both lipoxygenase and haemagglutination is found to follow the same pattern (Fig. 43, 49).

N-terminal sequence of both the subunits is identified to be NH₂-ADIQSFSFKN. The amino acid composition of the protein is very similar to lectin from *D. biflorus* (Schnell and Carter, 1987). The *D. biflorus* lectin is a tetrameric glycoprotein with a molecular mass of 110,000 Da that is composed of two types of subunits, designated subunit I and II. Subunit II (241 amino acid residues) is post translationally formed from subunit I (253 amino acid residues) by the removal of 12 residues from its C-terminus (Hamelryck *et al*, 1999). Since structural studies of the purified molecule have suggested similarities with lectin profile, we looked at LOX: lectin activity (Table.9) during purification was studied, to ascertain the association of LOX activity with the lectin molecule, which remained constant, suggesting that the two activities are associated with the same molecule. The homogenous protein reported here is found to have a haemagglutination of 45 ± 5 U/ mg protein, which is comparable to the commercial lectin from *D. biflorus*.

Subunit I was purified to homogeneity subjected to cleavage by trypsin. Tryptic peptides were separated and subjected to amino acid sequencing. Both the N-terminal sequence (30 residues) and two internal peptide sequences were aligned with *D. biflorus* seed lectin reported in literature (PDB no. 1LU1) (Fig. 46). The above data further corroborate the identification of the protein to be a lectin. Haemagglutination, measured in the presence of the inhibitors of lipoxygenase and LOX activity measured in the presence of *D. biflorus* lectin inhibitor (Fig 50), suggest that the location of lipoxygenase activity is away from the haemagglutination activity on the protein molecule. The separated subunits of the

protein individually have neither haemagglutination nor LOX activity. Efforts to reconstitute the molecule with equimolar concentrations of the subunits have not been successful.

The protein, in the present study has Mn as the prosthetic group. Fe^{2+} and Mn^{2+} are metal ions reported in lipoxygenases from different sources (Brash, 1999, Su and Oliw, 1998). The lectin from *Dolichos biflorus* is reported to contain Mn^{2+} (Kocourek *et al*, 1977). The presence of Mn^{2+} in the lectin molecule may allow it to function as a lipoxygenase molecule, very similar to other manganese lipoxygenases. The mechanism of LOX activity is probably similar to the earlier reported manganese lipoxygenase (Su *et al*, 2000). The catalytic mechanism of Mn LOXs is similar to iron lipoxygenases. The metal center of the latter redox cycles between Fe^{2+} (inactive state) and Fe^{3+} (active state formed by oxidation of lipid hydroperoxides) (Brash 1999, Ruddat, *et al*, 2003) and the mononuclear metal center of Mn-LOX seems to redox cycle between Mn^{2+} and Mn^{3+} in the same way (Cristea, *et al*, 2005). The active form is likely to contain a catalytic base, Fe^{3+} -OH and Mn^{3+} -OH, respectively, which abstracts a bis allylic hydrogen from the fatty acid and forms a carbon-centered radical. This process involves hydrogen tunneling as judged by kinetic isotope effects. The free fatty acid radical reacts with molecular oxygen in a controlled manner relative to the hydrogen abstraction. Antarafacial oxygen insertion is catalyzed by iron lipoxygenases and superficial oxygen insertion by Mn-LOX.

Lectin from *Dolichos biflorus* prepared by the method reported earlier by Kocourek *et al* (1977), is found to possess a lectin activity of 45 U/mg and LOX activity of 150 U/mg (Table 10), against corresponding values of 45 ± 5 U/mg protein and 8 U/mg, respectively, for commercial lectin. The Mn content of

commercial lectin is 0.32 $\mu\text{g/g}$ proteins. The Mn content may have a bearing on LOX activity. One of the unique features of legume lectins is their variable quaternary structure (Hamelryck *et al*, 1999). Although, the structures of their monomers are highly similar, they can associate into a number of different tetramers or dimers. This unique feature of lectin from *D. biflorus* (Hamelryck *et al*, 1999) may explain its ability to bind hydrophobic molecules.

Many of the legume lectins contain multiple hydrophobic sites which bind the fluorescent probes ANS and TNS with varying affinities, typically in the range $10^3 - 10^4 \text{ M}^{-1}$ (Roberts and Goldstein, 1983). The seed lectin (*D. biflorus*) contains two identical sites for binding adenine with affinities ranging from $2 \times 10^5 - 5 \times 10^4 \text{ M}^{-1}$ (Gegg, *et al*, 1992). Cytokinins, plant growth regulators, are reported to compete for the same site with similar affinities (Mok *et al*, 1978). The seed lectin tetramer is composed of two related subunits I and II (subunit II arises from subunit I by post translational proteolytic cleavage at the carboxy terminal end). Lectin has two carbohydrate binding sites per tetramer. There is no interaction between the carbohydrate binding and adenine binding sites (Hamelryck *et al*, 1999).

The rabbit antisera raised against soya LOX-1 did not cross react either with the protein of the present study or the commercial *D. biflorus* lectin. Sequence alignment between the *D. biflorus* lectin and lipoxygenases reported from plant sources did not reveal any significant homology (Fig. 46).

Adenine, kinetin and ANS are known to bind to the hydrophobic site of lectin tetramer. All the three inhibited LOX activity of lectin in a concentration dependent manner and the nature of inhibition with ANS was competitive Table 12 (Fig 54). Hence, the substrate, linoleic acid, must be approaching manganese through the

hydrophobic site of the lectin molecule. Soya LOX-1, which is a classical lipoxigenase, is not inhibited by ANS suggesting that the protein under study is not a classical lipoxygenase enzyme.

The judicious expression of multiple insecticidal proteins that vary in their toxicity mechanisms and manifestations helps plants develop their resistance to pathogen/insects. Plants have a wide array of defense proteins including the proteinaceous protease inhibitors and lectins, induced in response to insect attack (Kessler and Baldwin, 2002). The management of insect pests in agriculture is feasible in a safe and effective manner. Molecular tools give us an opportunity to develop genotypes that carry resistance traits. There is a need to develop appropriate strategies for deployment of transgenics for pest management keeping in view the pest spectrum involved and the effects on non-target organisms in the ecosystem (Ryan, 1990). Thus, the lectin molecule of *D.biflorus* with its associated LOX activity can lead to a mechanism for imparting/ improving pest resistance in other plant crops.

SECTION 3: PURIFICATION AND CHARACTERIZATION OF A LOW MOLECULAR WEIGHT LIPOXYGENASE (HGLOXII) FROM HORSEGRAM SEEDS.

In the previous section, purification and characterization of a dual function protein, HGLOXI, is described. During HGLOXI purification, size exclusion chromatography on Sephacryl S- 200 resolved into three peaks with LOX activity. The third peak was further purified by affinity chromatography to get pure HGLOXII. HGLOXII was found to be of low molecular weight and thermostable.

Protein purification of HGLOXII. A low molecular weight lipoxigenase (HGLOXII) was purified from horsegram seeds by size exclusion chromatography as described earlier (Fig 23) followed by affinity chromatography (Fig.57). The results of the purification are shown in Table 13. HGLOXII has a specific activity of 22 U/ mg and a yield of 0.4% starting from the crude extract. Further a gradient of 0.02 – 0.2 M phosphate was carried out without any protein being eluted. Next, a gradient of 0 – 0.5 M NaCl in 0.2 M sodium phosphate buffer was run and resulted in the elution of protein with LOX activity, which had a specific activity of 60 U/mg. This was not subjected to any further purification. SDS PAGE for homogeneity revealed a single band with molecular weight of ~34 kDa (Fig 58 A & B). The protein eluted at a retention time of 27.827 min (Fig 59 A) when checked for homogeneity by HPLC (size exclusion chromatography). HPLC was carried out using standard proteins as markers in the molecular weight range of 14 – 150 kDa, also confirmed the molecular weight to be ~34 kDa (Fig. 59B).

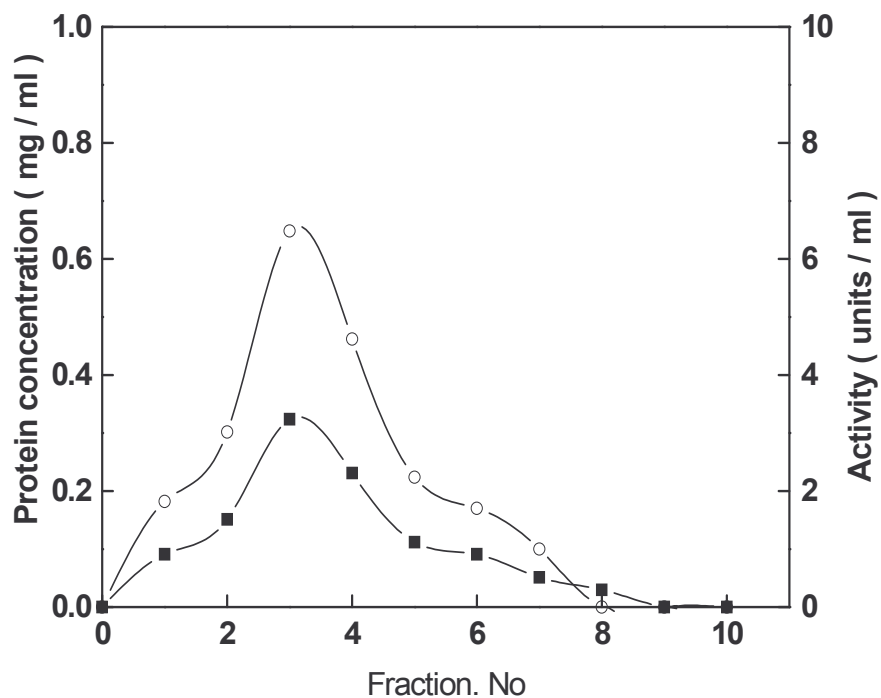


Figure 57: Isolation of HGLOXII by affinity chromatography. Peak III from Sephacryl S-200 column was pooled (Refer Fig.23) and dialyzed against 5 mM sodium phosphate buffer, pH 6.0 and was loaded onto ω -amino hexyl agarose column (10 ml). The column was eluted with 5 – 20 mM sodium phosphate gradient, pH 6.0. Flow rate, 5 ml / hr, Fraction, 0.3 ml / tube. — o .— Activity, — ■.— protein.

Table 13: Summary of purification of HGLOXII

	Protein (mg)	LOX Activity (Units)	Spec.Act (units mg⁻¹ protein)	Yield (%)
Crude extract	120	4500	37	100
Ammonium sulfate (0-70%)	114	3384	31	75
Sephacryl S-200				
Fraction I	21	1080	51	24
Fraction II	16	1560	97	34.6
Fraction III	8	432	54	9.6
ω -Linoleyl Amino hexyl agarose column				
5 – 20 mM phosphate gradient	0.821	18	22	0.4
0 – 0.5 M NaCl gradient	4.16	216	60	4.6

These are the results of a typical purification starting from 2.5g of defatted flour. These values were reproduced in two separate purifications.

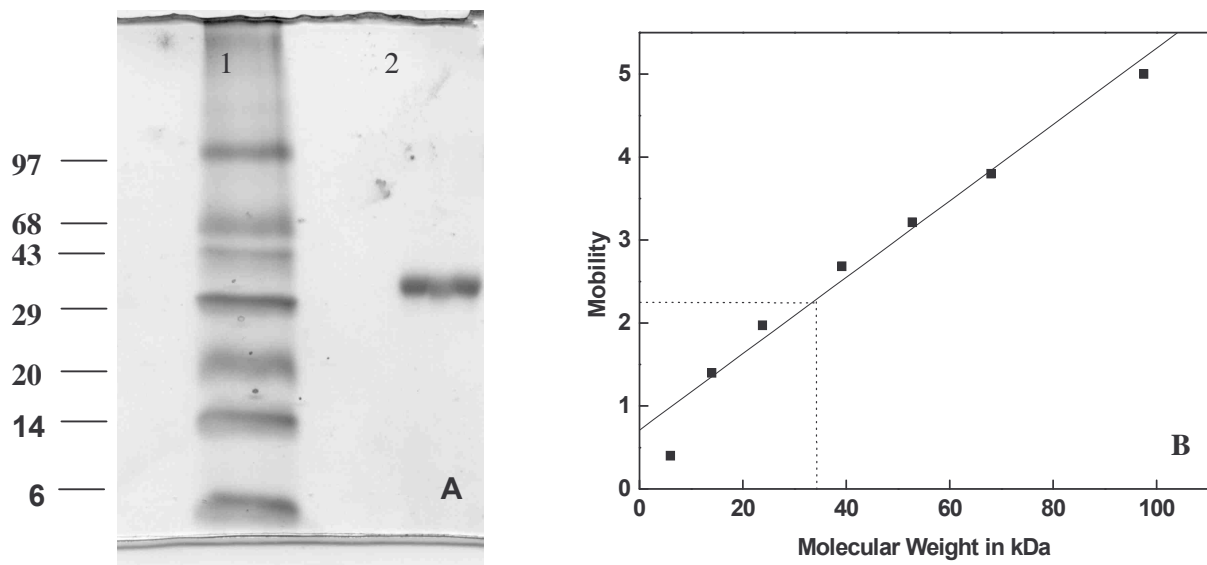


Figure 58: SDS PAGE pattern of HGLOX II. **A** 12% gel was run for the analysis of the purified preparation obtained after affinity chromatography according to Laemmelli (1970). Lane 1, molecular mass markers, Lane 2, 1 mg / ml of HGLOXII. Electrophoresis was carried and the gels were stained with commassie brilliant blue. **B.** Molecular weight determination of HGLOXII. Protein was a single polypeptide chain with a molecular weight of 34 kDa.

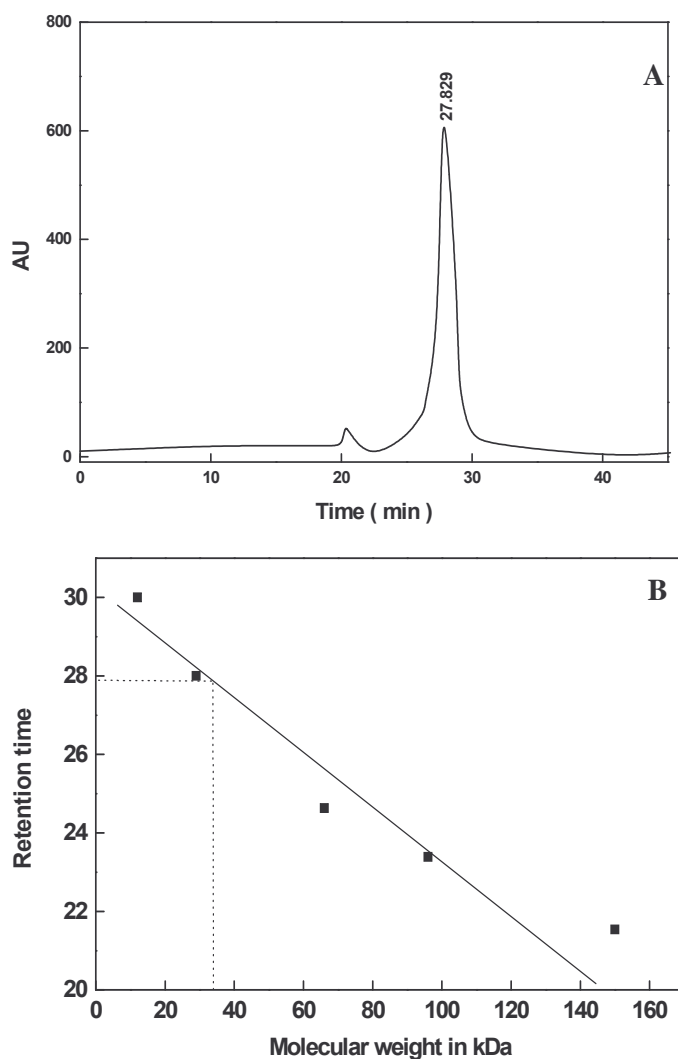


Figure 59: Homogeneity and Molecular weight determination of HGLOXII.

A. Chromatogram of HGLOX II on HPLC gel filtration using TSK G 2000 SW_{XL} gel column (7.8 mm x 300 mm, 5 μ m) at a flow rate of 0.2 ml / min. The column was preequilibrated with 20 mM sodium phosphate buffer, pH 6.8, with 0.3 M NaCl. Elution was carried out with the same buffer. 20 μ g of protein was injected and detection was at 280 nm. **B.** Molecular weight was determined using ADH (150 kDa), Soya LOX-1 (94 kDa), BSA (66 kDa), CA (29 kDa) and lysozyme (14 kDa) as standards.

Characterization of HGLOXII

pH optimum for HGLOXII activity. The pH optimum for LOX activity was determined to be 4.5 – 5 with linoleic acid as substrate (Fig.60). The enzyme was active within the range of 3.5 – 6.0. Kinetics followed at 234 nm suggested that the rate was linear upto one minute (Fig.61). Neither ketodiene formation nor carotene bleaching was observed with this enzyme. The Michaelis constant, K_m , for linoleic acid, linolenic acid and arachidonic acid were found to be 264 μM , 221 μM and 138 μM , respectively (Fig.62 A, B and C).

Inhibition of HGLOXII activity. The LOX inhibitor — 4- nitrocatechol inhibited LOX activity with the midpoint inhibitor concentration (50% inhibition) of 199 μM . The reported binding of 4-nitrocatechol to iron-enzyme complex (Gardner, 1991) result suggested that the enzyme might contain iron as its prosthetic group. NDGA was a poor inhibitor of LOX activity with an IC_{50} of 462 μM (Fig. 63).

Products of LOX activity. The reaction products of LOX activity, at pH 5.0 and 25°C using linoleic acid as the substrate, were analyzed by SP-HPLC. The products, 9- and 13-HPODs had an absorbance maximum at 234 nm, indicating conjugated diene. Soya LOX-1, at pH 9.0 and 25°C, produced exclusively 13-HPOD (Garssen *et al*, 1971), while potato LOX produces 9-HPOD (Galliard and Phillips, 1971) at pH 5.5 and 25°C (Fig 64 A). Product identification carried out using SP-HPLC after reduction with sodium borohydride and methylated with diazomethane revealed two peaks at 11.82 and 14.91 min (Fig 64 B).

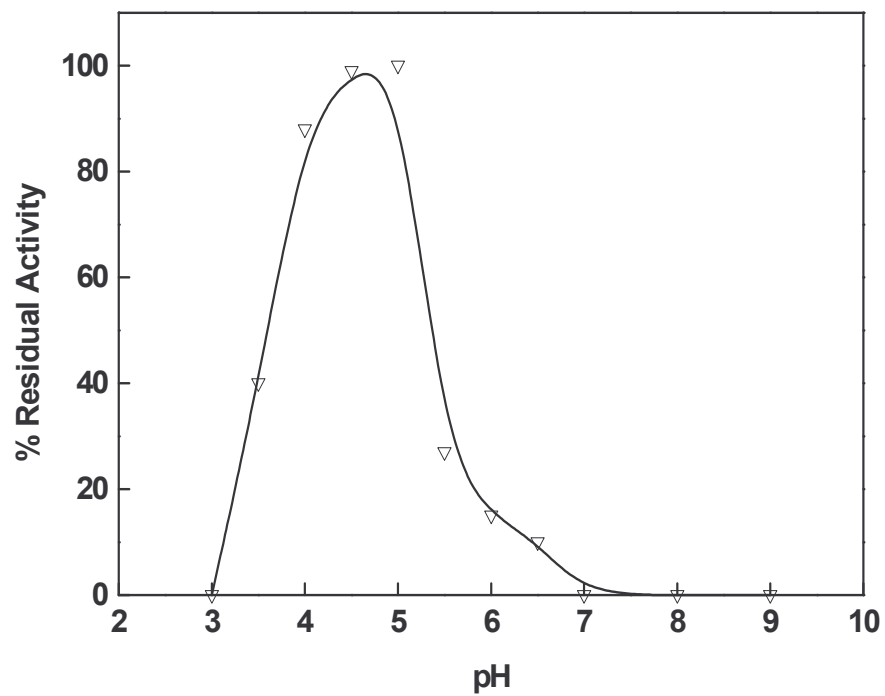


Figure 60: Effect of pH on HGLOXII activity. The enzyme activity was measured with linoleic acid as substrate in different buffers in the range of (2 – 5.5 sodium acetate, 6 – 8 sodium phosphate buffer and 8 – 9.5 borate buffer) with same ionic strength (0.1 M). The assay was carried out as mentioned under materials and methods.

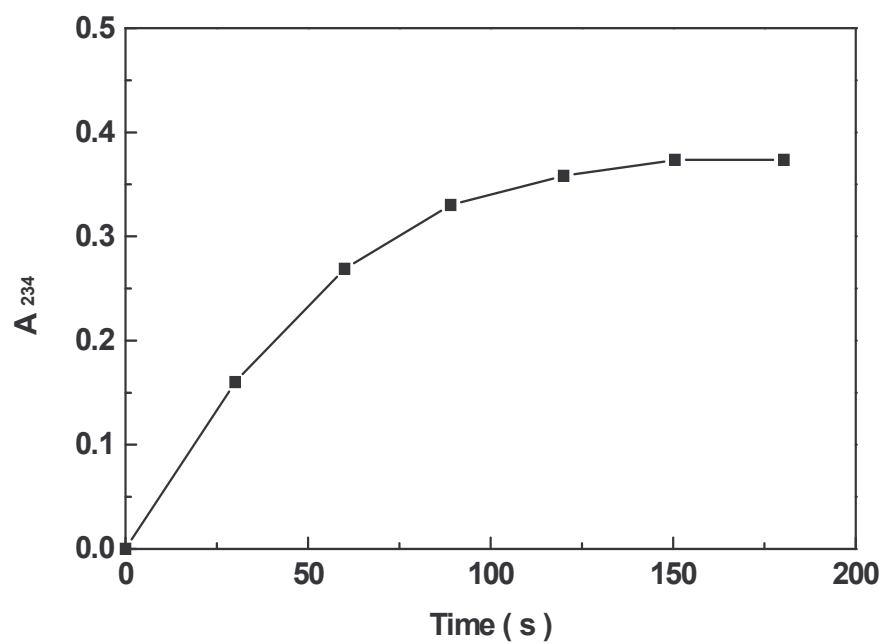


Figure.61: Rate of formation of products absorbing at 234 nm with linoleic acid as substrate. The assay was carried out as described in materials and methods. Kinetics of the reaction was followed for three minutes.

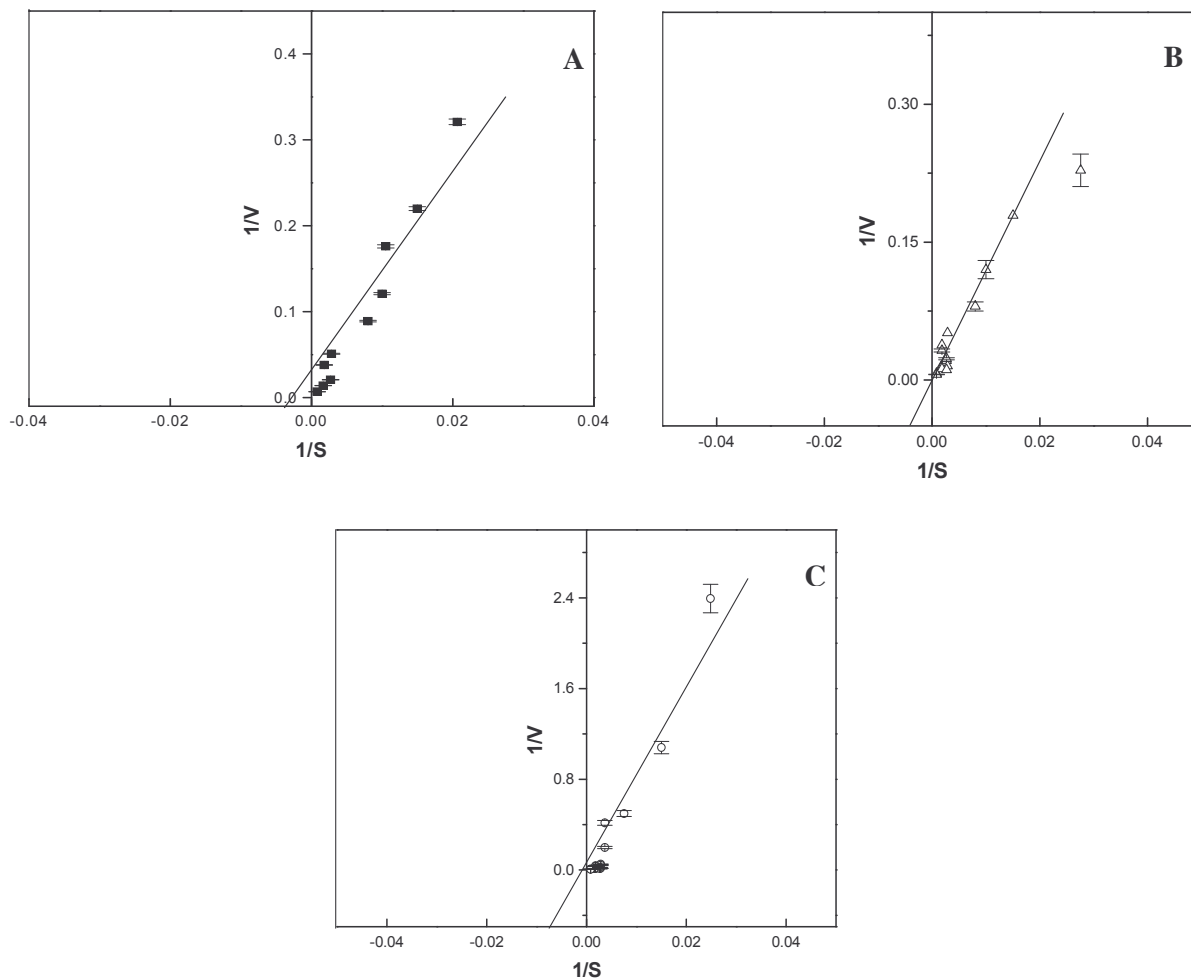


Figure 62: A double reciprocal plot of HGLOXII activity with linoleic, linolenic and arachidonic as substrates. The K_m values of the purified enzyme were determined by Lineweaver- Burk plot with linoleic (A), linolenic (B) and arachidonic (C) acids as substrates in the range of 0 – 275 μM in 0.1 M sodium acetate buffer, pH 5.0 containing 0.1 μM Tween - 20 . Assay was performed at 25°C as given under materials and methods.

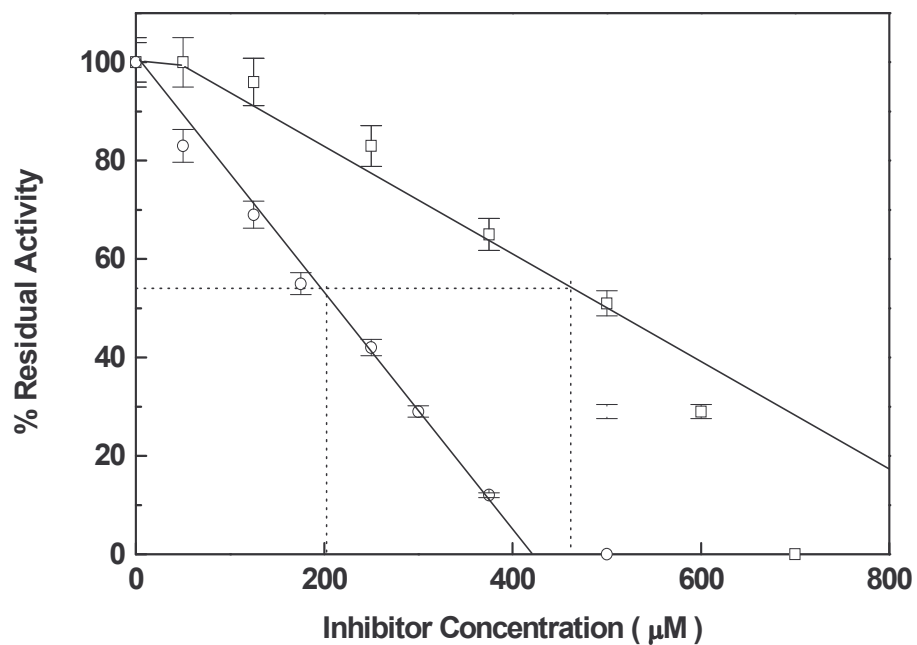


Figure 63: Effect of enzyme inhibitors on the activity of HGLOXII. Effect of NDGA (0- 500 μ M) and 4-nitrocatechol (0 – 500 μ M) on LOX activity was determined. Stock concentration of NDGA was prepared in absolute ethanol and 4-nitrocatechol in 20 mM sodium phosphate buffer, pH 6.8. Appropriate blank solutions were used. The enzyme was preincubated with indicated inhibitor concentration for 5 min and assayed for residual activity as mentioned under materials and methods. — o.— 4 nitrocatechol, — □ — NDGA.

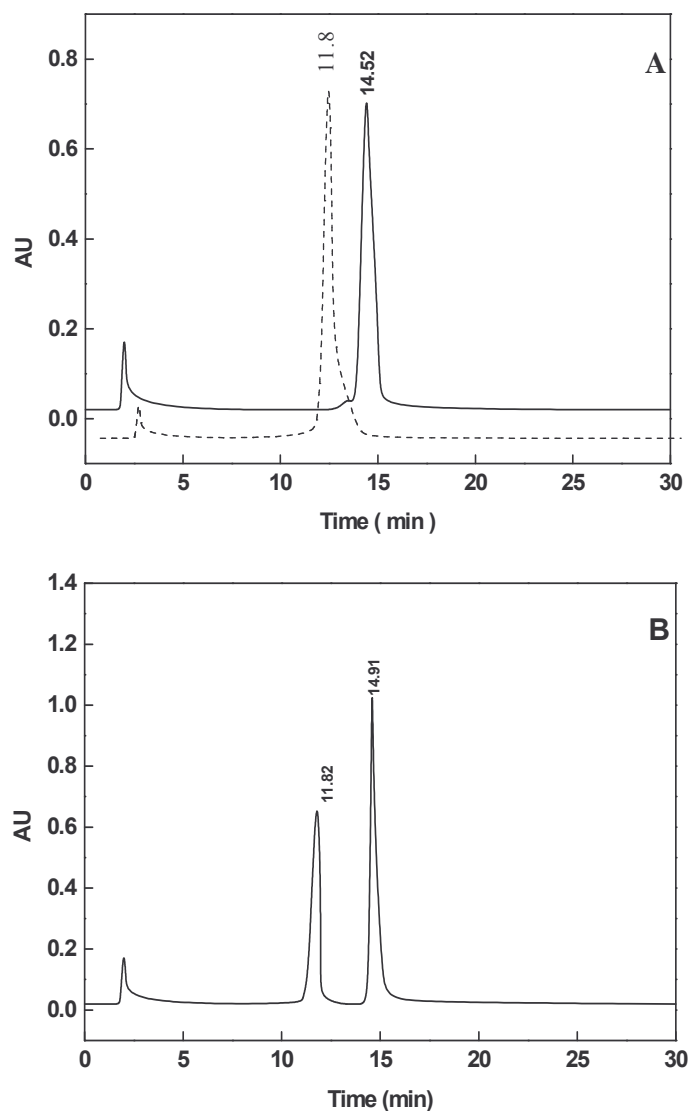


Figure 64: SP HPLC analysis of the reaction products of linoleic acid oxidation catalyzed by HGLOXII. The primary dioxygenation products of linoleic acid oxidation by HGLOXII were reduced with sodium borohydride and eluted with ethyl acetate. The compounds were separated by straight phase HPLC on a Sorbax-sil column (4.6 mm x 250 mm, 5 μ particle size). The products were separated by isocratic elution with solvent system n-Hexane/isopropyl alcohol /acetic acid (100:2:0.1) and a flow rate of 1 ml / min. Soy LOX1 and potato LOX were used to get 13- and 9- HPOD, exclusively, which were used as standards.

The products of LOX activity in the present study were identified to be 13-HPOD and 9-HPOD by using soya LOX-1 and potato LOX products as standards, respectively. Profiles obtained for 13-HPOD and 9-HPOD were in the 25: 75 ratio (Fig.64 B).

Prosthetic group. Concentrated solutions of HGLOXII were colorless (1 mg / ml) and lacked significant light absorption between 300 – 700 nm, suggesting absence of heme in the enzyme. Atomic absorption spectroscopy revealed that the protein sample contained non heme iron. The iron concentration was 0.48 $\mu\text{g}/\text{mg}$ protein (Fig.65). Mn could not be detected in the sample. Therefore, we concluded that the protein contains $\geq 0.48 \mu\text{g Fe}/\text{mg}$ protein as its metal cofactor. Lipoygenases are known to contain 1 mole of iron / molecule of enzyme.

Molecular characteristics of HGLOXII

Spectroscopic studies. The absorption spectra of HGLOXII at a concentration of 1 mg / ml in 20 mM sodium phosphate buffer (pH 6.0) were measured in the range 200 - 600 nm. The purified protein had an absorbance maximum at 280 nm (Fig.66A). The purified protein was colorless and lacked significant light absorption between 300 and 600 nm. Solutions of 1 mg / ml concentrations were also found to be colorless.

The fluorescence emission spectrum for the purified protein obtained by exciting the protein at 280 nm is shown in Fig. 66B. The protein is found to have an emission maximum at 335 nm indicating that tryptophan is in a hydrophobic environment.

Near UV CD spectrum was not well resolved. The far UV CD spectra was monitored in the range 260 – 200 nm. The far UV CD spectrum revealed minima at 230 and 217 nm, establishing predominance of β - structure (80%). The far UV CD spectrum for the protein was as depicted in Fig. 66C. The secondary structure did not match with earlier reported lipoxygenases - plant and animal - such as soya LOX-1 and rabbit reticulocyte lipoxygenases (Spaapen *et al*, 1979, Ludwig *et al*, 1985).

Thermal stability studies. Thermal stability of HGLOXII was measured in the temperature range 4 – 90°C by incubating the enzyme in 20 mM sodium phosphate buffer (pH 6.0) for 15 min, cooled immediately to 4°C and assaying the residual activity. LOX activity of the purified protein was highly thermostable in the range 4 - 90°C. The midpoint of thermal inactivation, T_m , was found to be 74°C (Fig. 67).

Amino acid composition and N-terminal sequence data. Amino acid composition analysis has been carried out following acid hydrolysis at 110°C. The amino acid composition of the purified protein is presented in Table 14. Protein is found to have very low methionine content. The composition does not match with any of the earlier reported plant lipoxygenases. The N-terminal sequence of 8 amino acids is determined and compared with the sequences of other lipoxygenases and was general with other proteins by BLAST search. The sequence of HGLOXII did not match with earlier reported lipoxygenase sequence (Fig. 68) or with any other protein.

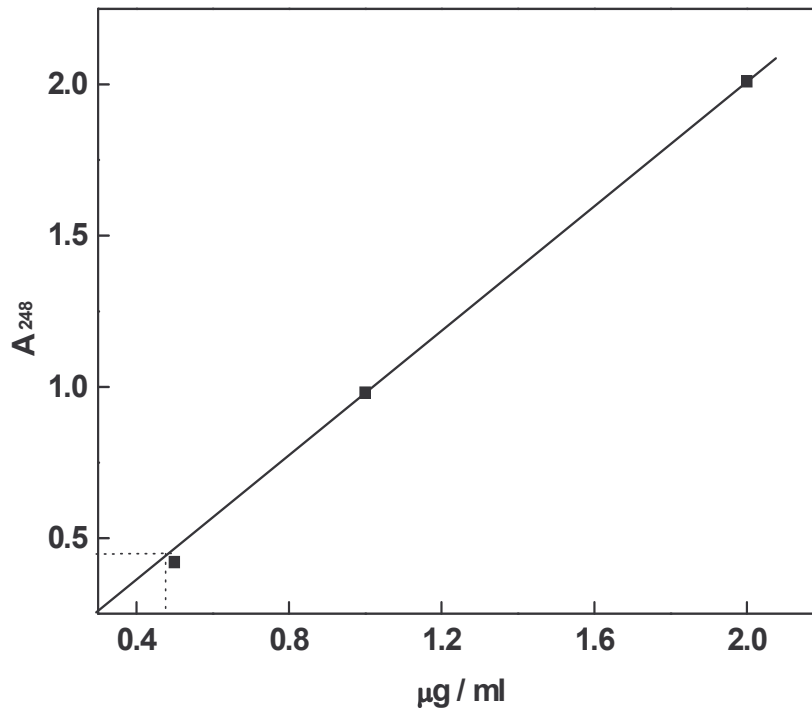


Figure 65: Estimation of iron. 1 mg / ml of protein sample was dialyzed against triple distilled water and the iron content was estimated by atomic absorption spectroscopy. Iron metal solution was used as standard in the range of 0.5-2 ppm.

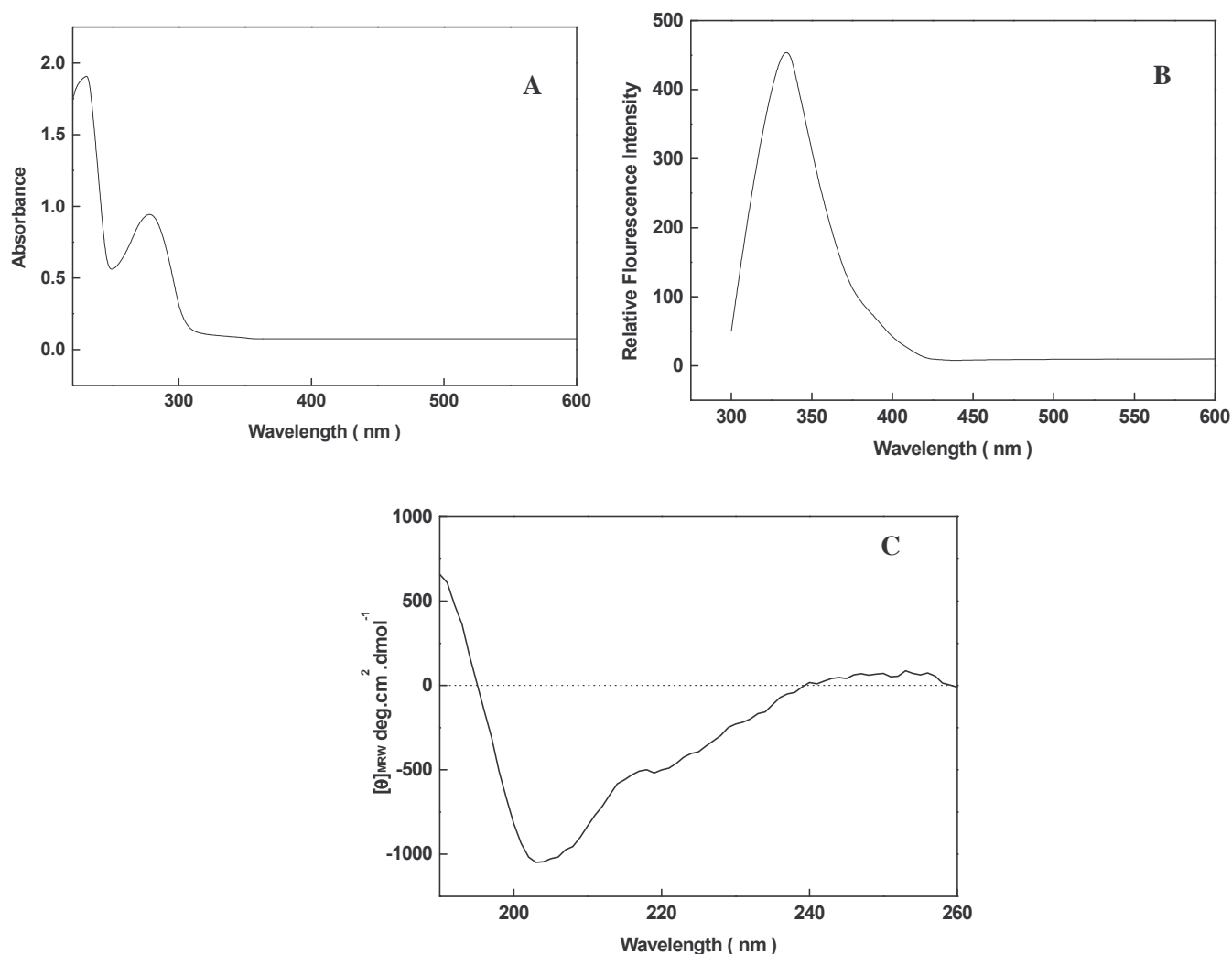


Figure 66: Spectroscopic studies of HGLOXII. **A:** *Light absorption spectroscopy of HGLOXII.* The absorption spectrum of 0.85 mg / ml sample in 20 mM sodium phosphate buffer was recorded from 220 – 600 nm in a 1cm path length cell. **B:** *Fluorescence measurements.* The sample was excited at 280 nm and its emission spectra were recorded in the range of 300 – 600 nm. Excitation and emission slit widths were 5 nm each. Fluorescence quartz cuvette with 1 cm path length was used. The spectrum was recorded at protein concentrations of 0.1 mg / ml.

C: *Far UV CD SPECTRA of HGLOXII.* Buffer used was 20 mM sodium phosphate buffer, pH 6.0. The protein concentration used was 0.51 mg / ml in the far UV region (200 – 260 nm) with a cell path length of 1 mm. Slits were programmed to yield 10 Å bandwidth at each wavelength. Measurements were made at 25°C at a scan speed of 10 nm/ min.

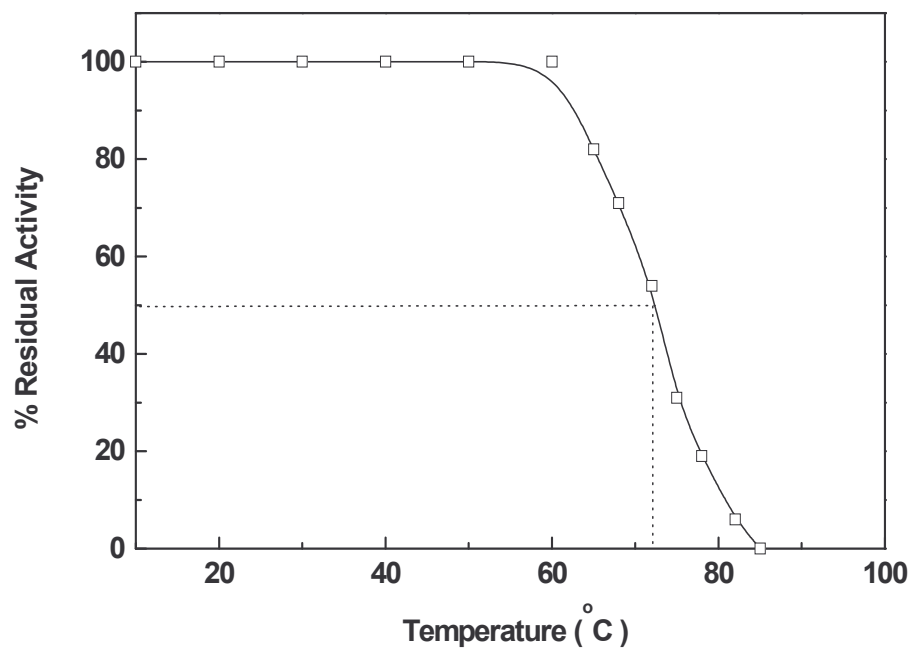


Figure 67: Effect of temperature on HGLOXII activity. HGLOXII was incubated at different temperatures in the range 4 - 90°C for 15 min. Buffer used was 20mM sodium phosphate buffer, pH 6.0. The enzyme was cooled immediately to 4 °C and assayed for residual activity. Activity with unincubated enzyme was taken as 100%.

Table 14: Amino acid composition of HGLOXII

Amino Acid	Mole (%)
Asx*	6.8
Thr	4.4
Ser	13.8
Glx **	14.9
Pro	3.35
Gly	20.6
Ala	7.5
Val	4.4
Met	0.43
Ile	3.07
Leu	5.56
Tyr	0.74
Phe	2.39
His	1.38
Lys	4.8
Arg.	4.09
Cys	1.5

**includes both aspartic acid and asparagines **includes both glutamic acid and glutamine*



Figure 68: N- terminal sequence of HGLOXII.

The N-terminal amino acid for rabbit reticulocyte (PDB No *LOX1*) was glycine while the N-terminal of soya lipoxygenase is methionone (PDB no *1F8N*). The LOX characteristics for the purified protein are summarized in Table 15.

Polyclonal antibodies raised against soya LOX-1: cross – reaction with HGLOXII. Rabbit antisera were prepared against soya LOX-1 and checked for its cross reactivity with purified protein with LOX activity. There was no cross reaction. There appeared to be no homology in the HGLOXII sequence as compared to reported plant lipoxygenase sequences (Fig.69).

Table 15: LOX characteristics of HGLOX II

Molecular weight	~34kDa	
Subunits	Monomer	
pH Optima	5.0	
Substrate affinity	Substrate	K_m
	Linoleic acid	264 μ M
	Linolenic acid	221 μ M
	Arachidonic Acid	138 μ M
Inhibitor	IC₅₀	
NDGA	462 μ M	
4-nitrocatechol	199 μ M	
Products produced with linoleic acid	9HPOD : 13HPOD 75 : 25	
T_m	74°C	



Figure 69: ELISA pattern of interaction of soya LOX antibodies with soya

LOX-1 and HGLOXII. SDS-PAGE was carried out using 12% gels according to Laemmli (1970). After electrophoresis, the protein was transferred on a nitrocellulose membrane. The membrane was washed in immunoblot buffer and was incubated with immunoblot containing antibodies against Soya LOXI. The membrane was treated with secondary antibody and incubated in substrate buffer. The alkaline phosphatase activity was detected with a mixture of BCIP and NBT in substrate buffer.

Lane 1. soya LOX I – 1 mg / ml, lane 2. HGLOX II – 3 mg / ml.

DISCUSSION

Purification of a second lipoxygenase enzyme from horsegram seeds was achieved successfully by gel filtration followed by affinity chromatography. The purified protein had a lower specific activity of 22 U/mg (Table 13) when compared to HGLOXI. The two isoenzymes mainly differ in their molecular mass, subunits, prosthetic group and in the product formed when linoleic acid is used as substrate. SDS PAGE analysis and size exclusion chromatography showed that the protein preparation was homogeneous. It was also found to be a monomer similar to other reported lipoxygenases unlike HGLOXI, which is a heterodimer.

The molecular weight of HGLOXII was determined to be ~ 34 kDa. Plant and animal lipoxygenases reported so far are of high molecular weight (75 – 120 kDa) except one from potato tuber (~ 34 kDa), activity of which has not been characterized (Reddanna *et al*, 1990). Also low molecular weight lipoxygenases have been reported from algal species, *chlorella* and *ulva lactuca* (Nuñez *et al*, 2002, Kuo *et al*, 1997). The molecular weight of HGLOXII is quite consistent with the size determined for LOX reported from potato tuber.

HGLOXII is active in a narrow pH range of 3-5.5 with an optimum pH of 5 in which it resembles HGLOXI. The K_m found for various substrates (Table 15) is found to be higher when compared to earlier reported plant lipoxygenases and HGLOXI.

The classical inhibitor of lipoxygenases, 4- nitrocatechol (Gardner, 1991), which binds to iron-enzyme complex, inhibited the lipoxygenase activity associated with the molecule with 50% inhibition concentration of 161 μ M, (Table 15) suggesting that this enzyme may contain iron as its cofactor unlike the other isoenzyme reported earlier. NDGA, a free radical scavenger is found to be a poor inhibitor. Spectroscopic studies have suggested that this protein lacked heme as

its prosthetic group ruling out the possibility of this enzyme belonging to heme group proteins. Heme proteins are known to bring about lipid peroxidation (Eriksson *et al*, 1970). Plant lipoxygenases contain one atom of iron per molecule of enzyme. HGLOXII unlike HGLOXI contains iron as its prosthetic group. Half an atom of iron was found per molecule of HGLOXII suggesting that this enzyme like other classical lipoxygenases contain iron as its cofactor.

Products of LOX reaction have been identified as 13-HPOD and 9-HPOD (Fig.64) in the ratio of 25:75. HGLOXI produces more of 13HPOD while HGLOXII produces 75% of 9HPOD. LOX is classified into three types according to the positional specificities of the hydroperoxides produced (Galliard, 1980). When linoleic acid is used as substrate, the first type of LOX (like soya LOX-1) specifically produces 13-HPOD. The second type produces both 9 and 13 HPOD (soya LOX-2 and rice LOX). The last type produces specifically 9-HPOD like potato LOX and maize LOX. HGLOXII enzyme belongs to type two lipoxygenases, which produces both 9- and 13- hydroperoxides. 9-hydroperoxides are converted to aldehydes and oxoacids that may have microbial activity, the 13 hydroperoxides are converted jasmonic acid that may play a major role in wound signaling pathway.

The amino acid composition and N-terminal sequence are not matching with HGLOXI, suggesting that these two isoenzymes are not derived from the same gene product nor is it due to proteolytic degradation of HGLOXI. The hydroperoxide products of lipoxygenases have been recognized as versatile reaction intermediates in the production of different fine chemicals in industries (Gardner 1996). Since plant lipoxygenases are stable when compared with animal lipoxygenases, they are used as enzyme catalysts in the production of

leukotrienes and lipoxins, which are involved in regulating specific elicitor response of importance in inflammation and immunity (Samuelsson *et al*, 1987, Gardner 1991). The potential application of lipoxygenase as a versatile biocatalyst in enzyme technology is limited due to its poor stability. Rapid inactivation takes place in both pure as well as crude forms since for most industrial biotransformation biocatalysts are required to be stable under harsher conditions (like exposure to high temperature for a long time) (Gardner, 1996). Thermal stability studies show that HGLOXII is highly thermostable when compared to other earlier reported plant lipoxygenases. This enzyme with its high thermostability may be an answer for the poor stability of lipoxygenases faced by enzyme technology. Horsegram seeds have a better resistance to infestation, when compared with other commercial crops. Hence the insertion of the gene for a highly active thermally stable low molecular weight LOX in susceptible plants could lead to natural insect resistant strains for agricultural and commercial purposes.

SUMMARY AND CONCLUSIONS

The salient features of this investigation are summarized as follows

1. Horsegram has been identified to be the richest source of lipoxygenase activity reported in plants so far. Horsegram seed has 1800 U/g of lipoxygenase activity with a specific activity of 37 U/mg compared to 150 – 350 U/ g in the case of other plant sources. Soyabean, the richest source of lipoxygenase activity so far, has 350 U/ g.
2. The lipoxygenase activity from horsegram is highly thermostable. The activity could be extracted with sodium phosphate buffer, (pH 6.0). The extract, after centrifugation to recover the supernatant, retained 50% activity after heating it for 100 min at 90°C.
3. Horsegram has at least two proteins with lipoxygenase activities – HGLOX1 and HGLOX2. Both these proteins exhibit characteristics that are unusual.
4. HGLOX1 could be purified to homogeneity by conventional chromatography using gel filtration and ion exchange chromatography to a final yield of 25% and 4.1 fold purity. The protein has a specific activity of 154 U/ mg protein and a molecular weight of 110, 000.
5. HGLOX1 activity in horsegram was unusual compared to other plant lipoxygenases in several characteristics:
 - i The protein is a multisubunit protein – a dimer of two unidentical subunits as determined by mass spectrometry. All other plant lipoxygenases are reported to be single polypeptide chains.
 - ii The pH optimum is in the acidic range of 4 – 5.0. Only potato lipoxygenase is reported to have an acidic pH optimum.

- iii The enzyme is highly thermostable retaining 50% activity on incubation at 85.5°C for 30 minutes. Enthalpy (ΔH^*), free energy ΔG^* and entropy ΔS^* of thermal inactivation was 49 kcal.mol⁻¹, 25.8 kcal.mol⁻¹ and 66.1 cal.mol⁻¹.K⁻¹ respectively at 78°C.
- iv HGLOXI was found to have weak cooxidizing activity for β -carotene and was inhibited by classical lipoxygenase inhibitors like NDGA and ETYA.
- v 4-nitrocatechol, an iron chelating substance did not inhibit lipoxygenase activity.
- vi The protein has manganese as the prosthetic group and is devoid of iron.
- vii The protein has no cross- reactivity with antibodies raised against soya lipoxygenase -1.
- viii HGLOXI was rich in β -structure and did not match with other plant or animal lipoxygenases. The near UV CD structure did resolve well.
- ix The amino acid sequence and N- terminal sequence have no similarities to the reported sequences of plant or animal lipoxygenases.
- x The amino acid composition, N-terminal sequence, mass spectra, haemagglutination activity, presence of manganese and carbohydrate contents confirmed the identity of HGLOX1 as seed lectin.
- xi The lectin characteristics of the protein were very similar to that reported earlier. Haemagglutination activity of the protein is 45 U/ mg protein. The lectin activity is inhibited by N-acetyl galactosamine – an inhibitor of haemagglutination activity of *Dolichos biflorus* lectin.

- xii The lectin and LOX activity were located in different loci of the molecule. However, the fractionated subunits exhibit only weak LOX activity (6- 8 U/ mg protein) and haemagglutination activity.
5. The second lipoxygenase activity in horsegram, HGLOXII could be purified to homogeneity by gel filtration and affinity chromatography to a final yield of 0.4% and a specific activity of 22 U/ mg protein. The molecular weight of the protein was 34 kDa as determined by SDS-PAGE and size exclusion HPLC.
 6. HGLOXII resembled plant lipoxygenases in several aspects.
 - i It is a monomeric protein active in the acidic pH range 4.5 – 5.
 - ii It had iron (0.48 μg / mg protein) as the prosthetic group like other plant lipoxygenases.
 - iii The activity could be inhibited by NDGA and 4-nitrocatechol – classical lipoxygenase inhibitors.
 7. HGLOXII had several unusual features that differed from other plant lipoxygenases.
 - i It is rich in β -structure unlike other lipoxygenases. The near UV CD spectra is not well resolved.
 - ii The products of the reaction with linoleic acid are 9 and 13-hydroperoxides in the ratio 25: 75, respectively.
 - iii The enzyme is highly thermostable with a midpoint for thermal inactivation at 74°C.
 - iv The amino acid composition did not match with any reported lipoxygenases. The N-terminal sequence of the protein had no cross reactivity with antibodies raised against soy lipoxygenase 1.

FUTURE DIRECTIONS

On the basis of the data presented here it can be concluded that there are more than two lipoxygenase isoenzymes in horsegram seeds. On gel filtration chromatography three peaks with LOX activity has been found, of which only 2nd and 3rd peak were further purified. Whether the first peak contains a classical lipoxygenase or whether it is closely related if not identical to one of the isoenzymes reported here and whether it has dual function activity is not known. The occurrence of two or more lipoxygenase isoenzymes has also been well studied for seed from other plants. However, the physiological significance of the presence of multiple isoenzymes is unknown so far. Detailed studies on the expression of isoenzymes during germination in different tissues of the seed and on their sublocalization in tissues may help to solve this issue.

The future investigations can be looked into these directions.

1. Studies of HGLOXI with hydrophobic molecules like ANS, ETYA, which binds to fatty acid binding sites, will help elucidate the structure function relationship of the protein.
2. Site directed mutagenesis of the protein ligands involved in catalysis would elucidate the role of these residues (for catalysis), when compared to other classical lipoxygenases and manganese lipoxygenase.
3. Cloning of lipoxygenase will help elucidate its characteristics regarding to structure, function and stability. Insertion of LOX genes into commercial crops to improve the plant resistance to pests.
4. Catalytic mechanism of lipoxygenases- comparison of Mn-LOX and Fe dependent LOXs will help understand this class of enzymes.

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PUBLICATIONS

Patent (1)

Full length paper (2)

Conference abstracts (3 - 6)

1. A process for preparation of thermostable enzyme from Horsegram (*Dolichos biflorus*). **NF-187/04, Del -545/Del/2004.**

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2. Dual function protein in plant defense: Seed lectin from horsegram (*Dolichos biflorus*) exhibits lipoxygenase activity.

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3. Purification and characterization of isozymes from horsegram seeds.

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4. Purification and characterization of a thermostable lipoxygenase from *Dolichos biflorus*.

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5. Thermostable iron containing low molecular weight lipoxygenase from *Dolichos biflorus*: Purification and characterization

S. Roopashree and A. G. Appu Rao*

Abstract published in **FASEB J.** 18, C13

6. Characterization of the lipoxygenase activity associated with lectin.

S. Roopashree, Sridevi Annapurna Singh, Lalitha R. Gowda and A. G. Appu Rao*

Abstract submitted to ASBMB meeting and 9th IUBMB conference
April 1– April 5th, 2006, San Fransisco.