Molecular cloning, characterization and expression of lipoxygenase 2 (lox-2) isozyme from Indian soybean [Glycine max (L.) Merrill] cv. Pusa 16

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The consumption of soybean is limited worldwide, despite being highly nutritious and having versatile uses, due to the presence of grassy, beany and rancid off-flavour. The lipoxygenase-2 (LOX-2) is the key enzyme responsible for the production of volatiles released from the beans, which cause off-flavour in soy products. In this study, a 2.6-kb full-length lox2 gene (NCBI accession No. JQ929619.1) was isolated and cloned from soybean (Glycine max L. Merril) cv. Pusa 16. The cloned cDNA sequence of lox2 gene showed the complete open reading frame (ORF) of a putative protein, having 866 amino acids with start codon present at the foremost position and stop codon at the end. The theoretical pI of predicted protein was 6.22. A hydropathy profile calculated from the amino acid sequence resembled those of dicot LOXs, suggesting conservation of the secondary structure of these enzymes. The LOX-2 showed conserved six Histidine residues within a span of 520 to 590 amino acid position, a signature element for the enzyme activity. The lox2 gene was expressed using pET vector in prokaryotic expression system. The recombinant LOX-2 protein was purified after induction with IPTG (isopentyl thiogalactoside). A prominent band of 97 kDa was observed, when affinity purified fractions were analyzed by SDS-PAGE. The purified protein was characterized for the enzyme activity, substrate preference and $K_m$. Inhibitor studies with natural antioxidant molecules present in soybean revealed α-tocopherol to be the most effective inhibitor of LOX-2.

Keywords: Glycine max (L.) Merrill, Soybean, Lipoxygenase, cDNA Cloning, Hydropathy profile, Prokaryotic expression, Recombinant Enzyme kinetics, Inhibitors.

Lipoxygenases (LOXs; Linoleate: oxygen oxidoreductase; EC 1.13.11.12) are class of non-heme iron-containing dioxygenases, widely distributed both in plants and animals. LOX catalyzes the addition of molecular oxygen to polyunsaturated fatty acids (PUFAs) containing a (Z, Z)-1, 4-pentadiene system, leading to two possible unsaturated products — the 9- and 13-hydroperoxides (9- and 13-HPODs). Based on point of oxygen insertion, the different LOX isozymes are classified into two types according to their stereospecificity — the 9-LOX and 13-LOX, oxygenating the ninth and thirteenth carbon of the fatty acid, respectively. In vitro, most LOXs prefer free fatty acids, though it has been shown that esterified fatty acids can also be substrates for LOX in vivo. In plants, linolenic and linoleic acids are the two predominant substrates of LOX. There are three LOX isozymes (LOX-1, LOX-2 and LOX-3) present in mature soybean seeds contributing to about 1% of the total storage protein. Soybean seed LOX isozymes are 94-97 kDa monomeric proteins with distinct isoelectric points ranging from about 5.7-6.4, and can be distinguished by their optimum pH, substrate specificity and product profile that leads to objectionable flavor composition. LOX-1 (94 kDa) exhibits a broad pH activity profile centered on pH 7.0 and displays a moderate preference for producing 9-HPOD. According to the classification based on their primary structure and overall sequence similarity, plant
LOXs are also grouped into two gene subfamilies, type 1 and -2 LOXs. Enzymes designated as type 1 LOXs have a high sequence similarity (>75%) to one another and lack plastid transit peptide. There are 9- as well as 13-LOXs in this class. However, the type 2 enzymes show relatively low overall sequence similarity (<35%) to one another and carry a putative chloroplast targeting sequence. To date, type 2 LOXs have a high sequence similarity (>75%) to one another and lack plastid transit peptide. There are 9- as well as 13-LOXs in this class. However, the type 2 enzymes show relatively low overall sequence similarity (<35%) to one another and carry a putative chloroplast targeting sequence. To date, type 2 LOXs consist exclusively of 13-LOXs

In our preliminary study with soybean (Glycine max) seeds, we have found LOX-2 exhibits significant negative correlation with total antioxidant potential and ascorbic acid content and significant positive correlation with off-flavour measuring parameters. The two isozymes LOX-1 and LOX-3 do not reveal any such antagonistic relationship, indicating that LOX-2 is the key enzyme responsible for the production off-flavour in soybean and products made from it. Hence, targeting LOX-2 by natural antioxidant molecule can be a potential gateway for flavour quality improvement in soybean.

As the three LOXs (LOX-1, -2 and -3) have very similar molecular weight and ionic properties, separation and purification of LOX-2 by traditional chromatographic method is very difficult. Alternative way of obtaining pure LOX-2 can be ectopic expression of the gene and purifying the recombinant protein for further studies. Thus, in the present study, attempts have been made to clone the lox 2 gene using gene-specific primers designed based on sequence data available in the GenBank and characterize and express it in E. coli using an expression vector. Prokaryotic expression is carried out of lox 2 gene by cloning it in pET expression system and induction by IPTG. The recombinant enzyme is tested for LOX-2 specific activity assay. The substrate preference, enzyme kinetics and inhibition of the LOX-2 activity by various natural antioxidant molecules present in soybean seeds have also been studied.

**Materials and Methods**

All reagents used were of analytical quality and were obtained from Sigma (USA), HI-MEDIA and SRL (India) etc.. Molecular biology kits were from Fermentas, Qiagen, etc. The pET protein expression and purification system was from Novas Biotech.

**Seed material**

The cloning and characterization of LOX-2 gene was carried out with soybean (Glycine max) cv. Pusa 16 obtained from Division of Genetics, Indian Agricultural Research Institute. The cultivar was chosen because of its wide acceptability and persistent off-flavour.

**RNA isolation**

Total RNA from developing seeds of soybean was isolated using TRI-Reagent (Sigma) as per the manufacturer’s instruction. Developing seeds (30 DAF) (50-100 mg) were sampled, grounded in liquid N2 and homogenized in 1 ml of TRI-reagent. The homogenate was stored at room temperature for 5 min to permit dissociation of nucleoprotein complexes. Chloroform (0.2 ml) was added to the homogenate and shaken vigorously for 15 s. The aqueous phase containing RNA was transferred to a fresh tube; 0.5 ml isopropanol was added to it, incubated at room temperature for 5-10 min and again centrifuged at 12,000 x g for 8 min at 4°C to precipitate the RNA. The pellet was washed with 75% ethanol (v/v) and dissolved in an appropriate volume of RNase-free water after air drying. The purity and concentration of RNA was checked using spectrophotometer (Specord 200) by taking the absorbance at 260 nm and 280 nm. A ratio of >1.8 of 260/280 showed it to be pure. Separation on 1.5% agarose gel containing 1 µg/ml ethidium bromide and 20 mM guanidine thiocyanate showed two intact bands of 28S and 18S, indicating intactness of the isolated RNA.

**Isolation of cDNA sequence and cloning of lox2 gene**

A two-step protocol was used for isolation of lox cDNA in which in the first step RNA was converted to single-stranded cDNA using RevertAid™ H Minus first cDNA synthesis kit (MBI Fermentas) according to manufacturer’s protocol, followed by the second step in which the single-stranded cDNA was used as template in a PCR reaction using gene-specific primers, lox2F and lox2R (Table 1) designed from lox-2 sequence available in Genbank, using Qiagen long range PCR kit. The PCR product was analyzed on 1% agarose gel along with λHindIII + EcoRI as molecular size marker. A blunt end cloning vector (pJET2.1) was used to clone the amplified PCR products.

The ligation mixture was used for transforming E. coli DH5α cells and recombinants were selected by blue white screening. Plasmid from white recombinant colonies were isolated and designated as pJET.2.1-lox 2. Sequencing of the recombinant
plasmid was done at Chromus Biotech, Bangalore. The DNA sequence obtained was analyzed by NCBI-BLAST to confirm the homology. For sequence analysis, protein sequences of LOX-2 from soybean were collected from GenBank. BioEdit sequence alignment editor version 5.09.04 was used for the analysis of amino acid sequence data. Alignment of the proteins was generated by the package of Clustal X version 1.81. Gonnet series was followed as protein weight matrix for amino acid alignment.

Neighbor joining trees were generated using CLUSTAL X with the default values of multiple alignment parameters. Robustness of phylogenetic tree was assessed from the bootstrap value for each internal node of N-J tree by calculating the 1000 random resampling. Conserved domain protein architecture of LOX-2 protein was modeled using All-IN-ONESEQ-ANALYZER version 1.35.

**Construction of pET28a-lox-2 expression system**

The complete ORF of lox2 was cloned in pET 28a after amplification of the gene using pJET.2.1-lox2 as template for PCR amplification with high fidelity Phusion Taq DNA polymerase (New England Biolabs, Beverly, MA) by the primers lox2F-SacI and lox2R-XhoI (Table 1). The amplicon of approximately 2.6 kb with SacI/XhoI sites was cloned in SacI/XhoI linearized pET28a vector under the control of strong lac promoter. Restriction analysis by SacI and XhoI restriction enzymes was done to confirm cloning of the lox2 gene in vector. Cloning of the gene in proper orientation was also confirmed by PCR and sequencing. Transformation of pET 28a-lox-2 was carried out using E. coli (BL 21) cells.

**Expression and purification of His: LOX-2**

Expression and purification of fusion protein His: LOX-2 were essentially as described with minor modifications. E. coli (strain BL21) cells transformed with pET28a-lox-2, encoding a His-LOX-2 peptide fusion, were grown in the LB-rich medium containing kanamycin and glucose to an OD600,1.0, followed by addition of IPTG to 1 mM as recommended and induced for different time intervals (1, 2, 3 and 4 h) at 37°C; all subsequent steps were performed at 4°C. Cells were collected by low speed centrifugation, resuspended in LEW Buffer (Macherey-Nagel) (50 mM phosphate buffer pH 8.0 containing 300 mM sodium chloride) and stored at -20°C. After thawing, cells were sonicated with the 3 min impulses of 15 s each. After centrifugation at 9000 × g for 30 min, the supernatant was diluted to 50 ml with column buffer. Affinity chromatography using Protino Ni-TED packed columns (Macherey-Nagel) was performed as recommended by the manufacturer. The purified fractions of fusion proteins were analyzed by 10% SDS-PAGE.

**LOX assay and activity calculation**

The LOX activity was determined by a modified method reported earlier. The time course of peroxide formation was measured by the increase of absorbance at 234 nm using Specord 200 Spectrophotometer at 25°C. For LOX-2 activity, 0.2 M sodium phosphate buffer (pH 6.8) was used. To obtain the kinetic curves and determine the curve parameter, the standard protocols were used.

**Inhibition of LOX-2 activity**

The effect of four different inhibitors naturally present in soybean, namely genistein, diadzein ascorbate and α-tocopherol were studied for their LOX-2 specific inhibition. Recombinant LOX-2 was incubated with inhibitor for 5 min in a 10 mm path-length cuvette. The reaction was started by addition of linoleic acid as substrate. Since the inhibitors were dissolved in ethanol (ascorbate and tocopherol) and DMSO (genistein and diadzein), control was run in presence of ethanol and DMSO. For determination of IC50, the inhibitor concentration was varied at constant substrate concentration of 200 µl of 10 mM linoleic acid. The percent inhibition of LOX-2 activity was calculated from the change in absorbance (ΔOD) values at 234 nm at the end of 3 min. Ks was determined by varying the inhibitor and substrate concentrations by Lineweaver-Burk double-reciprocal plot. The data obtained were fitted to a straight line by the method of least squares.

**Results and Discussion**

**Isolation of complete coding sequence (CDS) of lox2**

Specific primers were custom synthesized from sequences of lox2 genes available in NCBI database.
cDNA fragments encoding *lox2* gene sequences were amplified by RT-PCR using QIAGEN® Long Range PCR kit with *lox2* gene-specific primers *lox2F* and *lox2R* (Table 1) and RNA (Fig. 1A) isolated from developing soybean seeds. The amplified product from RT-PCR showed an intact band of ~2.6 kb on agarose gel electrophoresis (Fig. 1B) and was cloned in pJET2.1 vector, followed by transformation of *E. coli* DH5α cells. Recombinants were selected by blue white screening. Plasmids from recombinant white colonies were isolated, digested with *EcoRI* and separated on 1.0% agarose gel along with *λ* DNA cut with *Hind III* + *Eco RI* marker. The restriction of recombinant plasmid showed an insert of 800 bp instead of the expected 2.6 kb (Fig. 1C). This is because pJET 2.1 vector has only one site for *EcoRI* and that is outside of its MCS; also, *lox 2* gene sequence has one site of *Eco RI* and on restriction with *EcoRI* it releases a DNA fragment of 800 bp from the recombinant plasmid. The restriction positive cDNA clone designated as *pJET2.1lox2* was sequenced from Chromus Biotech, Bangalore by primer walking and 2603 bp full-length sequence was submitted to NCBI database (accession No. JQ929619.1).

**In silico analysis of lox2 sequence**

**Homology search**

Homology search using different softwares showed 99% similarity with other *G. max* *lox-2* genes reported in GeneBank at nucleotide level and 99-100% at amino acid level (Fig. 2A). The sequence represented complete ORF coding for a putative protein of 866 amino acids with a molecular mass of 97 kDa and theoretical pl of 6.22. The biochemical characteristics of deduced protein are presented in Table 2. Phylogenetic analysis also revealed that *lox 2* was closely related to other *lox 2* genes reported from various leguminosae plants and distantly related to those from non-leguminous plants (Fig. 2B). The nucleotide sequence of soybean *lox-2* CDNA has been reported earlier and codes for a polypeptide of 865 amino acids with a molecular mass of 97.03 kDa.

**Amino acid composition**

Analysis of amino acid composition showed that predicted protein comprised of highest percentage of hydrophobic amino acids like leucine (10%), valine (7.3%), glycine (6.8%), alanine (6.2%) and isoleucine (5.5%), indicating the membrane-bound nature of LOX and hydrophobic structures sharing important characteristics of membrane-anchored protein. The biochemical characteristics of putative LOX-2 protein are presented in Table 2.

**Table 2—In silico characterization of putative LOX-2 protein**

<table>
<thead>
<tr>
<th>Characters</th>
<th>Values</th>
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<tbody>
<tr>
<td>Length</td>
<td>866 aa</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>97267.41</td>
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<tr>
<td>1 microgram 10.281 pMoles</td>
<td></td>
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<tr>
<td>Molar extinction coefficient</td>
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<tr>
<td>1A(280) corr. to 0.76 mg/ml</td>
<td></td>
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<tr>
<td>1A(280) of 1 mg/ml 1.31 AU</td>
<td></td>
</tr>
<tr>
<td>Isoelectric point</td>
<td>6.22</td>
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<tr>
<td>Charge at pH 7</td>
<td>-8.08</td>
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</table>

Fig. 1—Cloning steps of *lox-2* gene in pJET 2.1 [(A) 1.2% Agarose gel electrophoresis of RNA isolated from developing seeds of *Glycine max* cv. Pusa 16; lanes 1-5, different concentration of RNA; (B) PCR amplification of 2.6 kb cDNA fragment isolated from *G. max* cv. Pusa 16 using *lox2* gene-specific primers and separated on 0.8% agarose gel along with molecular weight markers (1 kb plus, Fermentas); lanes 1-4, PCR product, lane 5, molecular weight marker; and (C) *EcoRI* restriction digestion of recombinant clone *pJET2.1lox2* separated on 0.8% agarose gel (lane 1) along with molecular marker (λ, *Hind III* and *Eco RI*) (lane 2)
total number of negatively-charged residues (Asp + Glu) and positively-charged (Arg + Lys) were 69 and 93, respectively.

**Hydrophobicity profile**

Characterization of hydrophobicity profile using hydrophobic scale provided by Kyte and Doolittle showed 5 prominent hydrophobicity peaks at positions of 80-100aa, 320-340aa, 380-400aa, 700-740aa and 760-800aa in LOX-2 (Fig. 3).

**Multiple clustal W analysis of G. max LOX-2 and functional domain search**

Despite significant differences in behaviour between three isoforms, namely LOX-1, -2 and -3, the amino acid sequences of LOX-1 and -3 showed 67 and 57%
identity to LOX-2 (Fig. 4). Analysis for conserved domain using Simple Architecture Research Tool (SMART) showed two major domains PLAT-LH2 domain at N-terminal and lipoxygenase super family domain at C-terminal, required for catalytic function of LOX-2 in the predicted protein (Table 3). A region of 40 amino acid residues containing a cluster of six His and two Tyr residues was conserved in all three isozymes (Fig. 4). This region is reported to be the iron-binding region and is essential for the enzymatic activity. This was in agreement with earlier reports that LOX-proteins contain highly conserved domains and sequence motifs which are important for the distinct structure and binding of the catalytic iron (LOX-motif: His-X4-His-X4-His-X17-His-X8-His). The tertiary structure in all plant LOXs reveals two domains: the N-terminal beta-barrel (= PLAT (polycystin-1, lipoxygenase, alphatoxin) domain or LH2 (lipoxygenase homology) domain and a catalytic-terminal\(^\text{19}\). His-504 in LOX-1, which corresponds to His-532 in LOX-2, is one of the iron-binding ligands essential for LOX activity. Missense mutation substituting Gln for His-532 resulted in the loss of LOX-2 activity from mature soybean seeds\(^\text{20}\).

### Prediction of trans-membrane helices and protein localization

Analysis did not predict any trans-membrane helices in the putative LOX-2. It was also found that the predicted protein is non-secretary in nature and did not contain either chloroplast or mitochondrial signal peptide. It showed a cytoplasmic localization, which was in accordance with the prediction of absence of trans-membrane domain in the protein.

### Prokaryotic expression of G. max lox2

Soybean seeds contain three LOX isozymes with molecular mass ranging very narrowly from 94-97 kDa, making it very difficult to purify individual isozymes for further characterizations by traditional separation methods. The other simplest way to understand the properties of purified LOX species is to produce it as a single recombinant enzyme by cloning and expressing the gene encoding it in a prokaryotic system like \textit{E. coli}.

The lox2 cDNA of 2.6 kb isolated and characterized in the present study was cloned in a pET expression vector under \textit{lac} promoter to get \textit{pET-His-lox2}. This \textit{pET-His-lox2} plasmid was then used to transform \textit{E. coli} (strain BL21). The pET prokaryotic expression vector was chosen because of its easy purification protocol and presence of small His-Tag, which removed the tedious step of cleaving the tag from purified protein. Out of ten colonies analyzed, only three colonies were found to be positive showing an amplicon of ~2.6 kb (Fig. 5A). Plasmid DNA from the positive colonies designated as \textit{pET-His-lox2} were restricted with \textit{SacI} and \textit{XhoI}.

<table>
<thead>
<tr>
<th>Name</th>
<th>Begin</th>
<th>End</th>
<th>E-Value</th>
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<tr>
<td>LH2</td>
<td>17</td>
<td>176</td>
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<td>Pfam: Lipoxygenase</td>
<td>185</td>
<td>850</td>
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</table>

Table 3—SMRAT analysis of putative LOX-2 protein

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**Fig. 3**—Hydrophobicity profile of \textit{G. max cv. Pusa 16} putative protein using Kyte and Doolittle scale
restriction enzymes to release the 2.6 kb insert of *lox* 2 (Fig. 5B). Initial experiments established that fusion protein with approximate molecular mass of 97 kDa was expressed in *E. coli* (strain BL21) clones containing the *pET-His-lox* 2 on induction with IPTG.

Most of the His-LOX-2 protein existed in soluble fraction when expressed at 37°C. The deduced LOX-2 did not show the presence of any signal sequence, indicating that the protein was not compartmentalised in the cell. The expression of recombinant protein was time-dependent and had maximum induction of protein, when expressed overnight. The optimum time for harvesting the cells was 4-5 h after induction, as the insolubility of fusion protein increased after this time.

Fig. 4—Sequence comparison of *G. max* cv. Pusa 16 LOX-2 protein (gi|388461364) with LOX-1 (gi|351727907), and LOX-3 (gi|351727842) protein from *G. max* reported in NCBI database [The rectangular box represents the sequence differences. Numbers in the left and right margin refer to amino acid residues. The rectangular dark boxes represent the six conserved His residues]
It was found that induction by 1 mM IPTG and 4-5 h incubation time was required to get good amount of recombinant protein (Fig. 5C).

Earlier study has reported induction of the protein in E. coli cells at low temperature like 15°C, but in the present study, good amount of protein was purified from the bacterial cells at 37°C. Recombinant His-LOX-2 protein was purified by one-step nickel-NTA affinity chromatography. About 395 mg total proteins were measured in supernatant from 1 L culture, which yielded ~45 mg His-LOX-2 on purification. The purified protein was dialyzed overnight to remove the salt and other contaminants and then concentrated in a lyophiliser. The activity of purified protein was assayed with the standard assay procedure and confirmed to have same kinetic parameters as native protein.

Biochemical characterization of recombinant LOX-2

The purified protein was used for checking activity and its optimum pH. The protein exhibited its maximum activity at pH 6.8-7.0, as reported in earlier study⁵. The $K_m$ for its two natural PUFA substrates, namely linoleic (18:2) and linolenic acid (18:3) was found to be 19 and 15 µM, respectively (Table 4).

Soybean is a rich source of nutritionally important polyunsaturated fatty acids (PUFAs). But, the presence of high amount of PUFAs makes it prone to lipid peroxidation by LOX isozymes present in the seed. Among the LOX isozymes, LOX-2 has been found to be more active and can also act on membrane-bound PUFAs and also esterified triacyl glycerol⁶. Linolenic acid acts as a better substrate, probably because of the presence of three double bonds and cis,cis-1,4-pentadiene moieties in its structure⁷. Earlier study with different LOX-deficient soybean mutants has suggested LOX-2 mainly responsible for generation of hexanal, a major volatile that contributes to the off-flavour generation through LOX pathway⁸. Kinetic studies confirmed that the recombinant LOX-2 behaved like the native enzyme⁹.

LOX-2 inhibition studies

Four set of naturally active molecules with antioxidant properties, namely — two major isoflavones (diadzein and genistein) and two major radical scavengers (water soluble ascorbate and lipid soluble α-tocopherol) were tried for their LOX-2 inhibitory activity. For inhibition experiments, recombinant LOX-2 protein was incubated with respective inhibitors for 3 min in a quartz cuvette. The reaction was started by the addition of substrate. Figure 6 depicts that all the inhibitors showed inhibition of LOX-2 in a concentration-dependent manner, when incubated with LOX-2 in phosphate buffer (pH 6.8). Among isoflavones, genistein seemed to be a better inhibitor than diadzein, as indicated by lower $K_i$ and IC⁵₀ values. The Lineweaver-Burk plots revealed that all the inhibitors acted by non-competitive manner, i.e. by decreasing $V_{max}$, but the $K_m$ remained unchanged. α-Tocopherol was found to

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**Table 4—Summary of biochemical characterization of recombinant LOX-2 protein**

<table>
<thead>
<tr>
<th>Character</th>
<th>Values</th>
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<tr>
<td>$K_m$ for Linoleic acid</td>
<td>19 µM</td>
</tr>
<tr>
<td>$K_m$ for Linolenic acid</td>
<td>15 µM</td>
</tr>
<tr>
<td>Optimum pH</td>
<td>6.8</td>
</tr>
<tr>
<td>Major inhibitors</td>
<td>α-Tocopherol, Ascorbate</td>
</tr>
</tbody>
</table>

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Fig. 5—Cloning and expression of lox-2 in pET expression system [(A) 2.6 kb colony PCR amplification of lox2 using restriction site attached primers and separated on 0.8% agarose gel along with molecular markers (Hind III and Eco RI). Lane 1, Marker; and lanes 2-4, lox2 amplicon; (B): Restriction digestion of 2.6 kb recombinant clones using SacI and XhoI and separated on 0.8% agarose gel along with molecular markers (Hind III and Eco RI). Lane 1, Marker, lanes 2-4, recombinant lox2 clone; (C): Induction of lox-2 gene in pET expression system using 1 mM IPTG. M, Molecular weight marker; lane 1, pET vector induced alone; lane 2, uninduced pET-lox-2; lane 3, induced pET–lox-2; lane 4, induced BL21 untransformed cells; and (D): Purification of recombinant LOX-2 protein using Ni-NTA affinity chromatography. M, Molecular weight marker; lane 1, uninduced pET-LOX-2', lane 2, induced pET-LOX-2, lane 3, flow through of purification, and lane 4, purified recombinant LOX-2 protein]
be the most potent inhibitor of LOX-2 ($K_i$ and IC$_{50}$ 5 µM and 19 µM, respectively), as compared to the other three (Table 5). This might be due to the fact that $\alpha$-tocopherol is the most effective antioxidant lipid-soluble inhibitor molecule present in the cell$^{23}$.

**Conclusion**

In soybean, LOX genes are well-studied for solving the off-flavour problem, as they can be genetically removed or modified$^{24}$. In plants, lox gene expression is associated with a number of developmental events and induced by environmental changes, notably pathogen attack. PUFA hydroperoxides resulting from the action of LOX are very reactive and give rise to free radicals that contribute to cell death. The hydroperoxides are also converted into more stable, active compounds, including aldehydes and hydroxyl-and epoxy-fatty acids, some of which show anti-microbial activities and jasmonic acid, derived from LOX product 13-hydroperoxy-octadecatrienoic acid$^{24,25}$ is a major signalling molecule. Anti-sense expression of lox gene has shown to enhance susceptibility to pathogen attack$^{25}$. Genetic manipulation of soybean by reducing lox expression to improve flavour quality does not seem to be a feasible approach. Exploitation of enzyme kinetics using various naturally occurring inhibitors is a probable way to reduce the LOX activity. In the present study, the gene encoding one of the most active LOX isozymes LOX-2 was isolated from *G. max*, cloned and expressed in *E. coli* and recombinant LOX-2 was characterized and found to behave like native enzyme. The results obtained on the LOX inhibition by different antioxidant molecules could be further exploited to reduce the off-flavour generation in soybean seeds and soy-products. Increasing the natural anti-oxidant molecules like $\alpha$-tocopherol may result in the decreased activity of LOX-2 in soybean seeds and could be attempted to reduce off-flavour generation.

**Acknowledgements**

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**References**


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**Table 5**—$K_i$ and IC$_{50}$ values for natural antioxidant molecules used for inhibition study of recombinant LOX-2

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>$K_i$ values for inhibition of LOX-2 (µM)</th>
<th>IC$_{50}$ values for inhibition of LOX-2 (µM)</th>
</tr>
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<tbody>
<tr>
<td>Genistein</td>
<td>62 ± 4</td>
<td>102 ± 5</td>
</tr>
<tr>
<td>Diadzein</td>
<td>98 ± 6</td>
<td>122 ± 3</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>42 ± 1</td>
<td>33 ± 2</td>
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<tr>
<td>$\alpha$-Tocopherol</td>
<td>05 ± 1</td>
<td>19 ± 5</td>
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</table>

Fig. 6—Inhibition kinetics of recombinant LOX-2 [Inhibition of recombinant LOX-2 by natural antioxidant inhibitors. The reaction was started by the addition of 100 µmole linoleic acid substrate and enzyme activity was followed spectrophotometrically at 234 nm. All the inhibitors inhibited O, $\bullet$, $\Delta$, $\triangledown$, $\square$ etc LOX-2 in a concentration-dependent manner]