BIOCATALYSIS OF LIPOXYGENASE IN A MODEL SYSTEM USING SELECTED ORGANIC SOLVENT MEDIA

by

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Short Title

Biocatalysis of Lipoxygenase in Organic Media

ABSTRACT

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The biocatalysis of commercially purified soybean lipoxygenase (LOX-1B: EC 1.13.11.12) in ternary micellar and neat organic solvent media, using linoleic acid as a substrate model, was investigated. The organic solvent, used throughout this study, was at different ratios in the ternary micellar system, composed of Tris-HCl buffer solution (0.1 M, pH 9.0) and 10 μM of Tween-40 as the surfactant. The results indicated a 1.4- and 1.7-fold increase in LOX activity when, respectively, 2% iso-octane or 2% hexane was used as the organic solvent in comparison to that in the aqueous medium. The kinetic parameters, including K_m and V_{max} values, the choice of the surfactant, the optimum reaction temperature and the optimum pH, were investigated. The effects of selected parameters, including initial water activity (a_w, 0.23 to 0.75), agitation speed (0 to 200 rpm), reaction temperature (20 to 45°C) and thermal stability of LOX activity in neat organic solvent, were also studied. The experimental findings showed that the K_m and V_{max} values in the ternary micellar system containing 2% hexane was calculated to be 7.7 μmol of linoleic acid and 30.0 nmol of linoleic acid hydroperoxides (HPODs)/mg protein/min, respectively, as compare to that of 20.7 μmol of linoleic acid and 8.3 nmol HPODs/mg protein/min in the neat organic solvent, respectively. The experimental results indicated that the major LOX specific activity, for both aqueous and ternary micellar systems, was measured at pH 9.0, with a minor one at pH 6.0 for the aqueous system and at pH 7.0 for the ternary micellar system. The activation energy (E_a) of the reaction system of LOX was 9.87 kJ/mol or 2.36 kcal/mol. The half-life (T_{50}) for LOX was 27.61, 66.63 and 138.6 min for the aqueous, ternary micellar and neat organic media, respectively.

RÉSUMÉ

M.Sc. Haydar Alturaihi

La biocatalyse de la lipoxygénase purifiée, obtenus à partir de la graine de soja (LOX-1B: EC 1.13.11.12), a été étudiée en milieux micellaire ternaire et en monophasique organique, en utilisant l'acide linoléique comme substrat modèle. Le solvant organique, utilisé dans cette étude, a été utilisé à différentes concentrations dans le système micellaire ternaire, composé d'une solution tampon Tris-HCl (0,1 M, pH 9,0) et 10 µM d'un surfactant, le Tween-40. Les résultats obtenus ont démontré qu'il y a une augmentation de 1,4 et 1,7 fois de l'activité enzymatique de la LOX en utilisant, respectivement, soit du l'iso-octane à 2% ou soit du l'hexane à 2%, comme le solvant organique en comparaison avec celle en milieux aqueux. Les paramètres cinétiques, comportant les valeurs de K_m et de V_{max}, le choix de surfactant ainsi que la température et le pH optimal de la réaction ont été étudiés. Les effets de différents paramètres tels que l'activité initiale de l'eau (a_w) du 0,23 à 0,75, l'agitation du 0 à 200 rpm, la température de la réaction du 20 à 45°C et la stabilité thermique de l'activité de la LOX en milieux monophasiques organiques ont été aussi étudiés. Les résultats obtenus tendent à montré que les valeurs de K_m et de V_{max} en système micellaire ternaire, contenant de l' hexane à 2%, ont été de 7,7 µmol d'acide linoléique et 30,0 nmol d'hydroperyde de l'acide linoléique (HPODs)/mg protéine/min, respectivement, en comparaison à des valeurs de 20,7 µmol d'acide linoléique et 8,3 nmol HPODs/mg protéine/min dans les milieux monophasiques organiques, respectivement. De plus, les résultats expérimentaux ont démontré que l'activité spécifique maximale de la LOX pour les deux systèmes aqueux et micellaire ternaire a été obtenue à pH 9,0, avec aussi une activité minimale à pH 6,0 pour le système aqueux et à pH 7,0 pour le système micellaire ternaire. L'énergie d'activation (E_a) du système de réaction de la LOX était d'une valeur de 9,87 kJ/mol ou 2,36 kcal/mol. La demi-vie (T_{50}) de LOX a été déterminée à 27,61 min dans le milieu aqueux, 66,63 min dans le milieu micellaire ternaire et 138,6 min dans les milieux monophasiques organiques.

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1. INTRODUCTION

In order to meet the consumer needs, growing bodies of scientists are interested to carrying out research work aimed at the production of natural flavors. Understanding the composition and characteristics of flavors is necessary in order to properly appreciate the role of biocatalysis-based processes in helping to supply the demand for natural flavors for their use in food and beverage products (Cheetham, 1997).

Enzymes quite often display chemo-, regio- and enantioselectivity, which makes the catalysis especially attractive for the pharmaceutical and agrochemical areas, where the interest for enantiomerically pure and specifically functional compounds is continuously growing (Carrea and Riva, 2000).

Lipoxygenase (LOX; linoleate: oxygen oxidoreductase; EC 1.13.11.12) is an intramolecular dioxygenase that catalyzes the oxidation of polyunsaturated fatty acids, containing the *cis, cis,* 1,4-pentadiene system, such as linoleic acid, into the conjugated *cis, trans*-diene hydroperoxides (Allen, 1968). The linoleic acid hydroperoxides (HPODs) are considered as the flavor precursors (Schrader *et al*., 2004). The conjugated HPODs can be converted further by hydroperoxide lyase into volatile alcohols, aldehydes and ketones (Kermasha, 2002b; Matsui, 2006).

Fatty acids can be attached to the active site of soybean LOX by two different fashions, either “head first” or “tail first” (Gardner, 1991). Based on these two possible orientations of the substrate, two different isomers of 9- and 13-HPODs are formed. These molecules can be used as aroma precursors for the food industry (Hildebrand, 1989; Hatanaka, 1993). Soybean LOX is mainly composed of three isozymes, LOX-1, LOX-2 and LOX-3, which can be differentiated by their pH optimum values and their ratio of isomers production.

Enzymatic catalysis in non-conventional media has attracted much interest by many industrial and academic researchers. The literatures indicated that numerous enzymes

exhibited new properties in low and neat organic solvent media instead of hydrous (Laane, 1987a; Khmelnitsky *et al*., 1988; Dordick, 1992; Kvittingen, 1994; Piazza *et al*., 1994; Halling, 2000; Klibanov, 2001; Kermasha *et al*., 2001 and 2002a; Vega *et al.*, 2005a,b). In general, enzymes in predominantly non-aqueous environment or low water environment can function provided the essential water layer around them is not stripped off (Aldercreutz and Mattiasson, 1987; Khmelnitsky *et al*., 1988). The addition of water to solid enzyme in organic solvents may increase the enzymatic activity via the enhancement of the polarity and flexibility of the enzyme's active site (Affleck *et al*., 1992; Xu *et al*., 1996). One factor that may contribute toward the enhancement of HPODs formation in microemulsions is the greater O_2 solubility in the organic solvent media (Piazza, 1992). The use of organic solvents also allows better solubility of the hydrophobic substrates (Bell *et al*., 1995, Kermasha, *et al*., 2001) and may serve as an efficient model for enhancing the LOX activity (Nada and Yadav, 2003). The golden rule for the use of non-aqueous media is that non-polar solvents, such as hexane, are much better appropriate than polar ones. This can be explained by the fact that polar solvents, being water-miscible, strip off the essential water layer of the protein (Gupta, 1992).

The most instigating aspects for the biocatalysis in organic solvent media as compared to that in aqueous medium are the solubility of the substrate and stability of the enzyme (Koskinen and Klibanov, 1996). In addition, organic solvent media is preferred over aqueous medium due to the simple recovery of the biocatalyst and a higher thermostability of the active enzyme (Bell *et al*., 1995).

The present work was part of ongoing research in our laboratory aimed at the development of a biotechnological process for the use of LOX and associate enzymes for the production of natural flavors (Kermasha *et al*., 2001 and 2002; Vega *et al.*, 2005a,b and 2006). The specific aim of the present study was to investigate the biocatalysis of commercial purified soybean lipoxygenase type-1B, in a model system, using different reaction media, including, aqueous, ternary micellar environment and neat organic solvent media.

The specific objectives of this research work were:

1. To investigate the activity of a commercially purified soybean lipoxygenase-1B (LOX) in the ternary micellar system, using hexane and iso-octane as the selected organic solvent media.

2. To investigate the effects of different types of surfactants and emulsifiers on LOX activity in the ternary micellar system.

3. To investigate the biocatalysis of LOX in ternary micellar system, in terms of kinetic parameters, including $K_{m,}$ V_{max} and catalytic efficiency, optimum pH, optimum temperature and thermal stability.

4. To optimize the enzymatic activity of LOX in neat organic solvent media, in terms of kinetic parameters, including $K_{m,}$ V_{max} and catalytic efficiency, agitation speed, water activity (a_w), reaction temperature and its thermostability.

2. LITERATURE REVIEW

2.1. Lipoxygenase (LOX)

2.1.1. Definition of Lipoxygenase

Lipoxygenases (LOXs) (E.C.1.13.11.12) are a group of non-heme iron containing dioxygenases which belong to the enzyme class of oxydoreductases (Chikere *et al.*, 2001). They are able of catalyzing in a stereospecific way the addition of molecular oxygen to polyunsaturated fatty acids (PUFAs) containing the *cis, cis*-1,4-pentadiene moiety yielding the 1,3-*cis*-transdiene-5-hydroperoxides. Among the fatty acids with this functionality, linoleic, linolenic and arachidonic acids, are the best known substrates for LOX (Feussner and Kuhn, 1995; Gardner, 1996).

2.1.2. Bioconversion by Lipoxygenase

LOX has been reported to contribute to the biogeneration of volatile flavor compounds from the initial oxidation of lipids (Tressl *et al.*, 1981). The regio-isomeric hydroperoxides can be derived from linoleic acid (octadecadienoic acid hydroperoxides, HPODs), which have been considered as flavor precursors (Wong,1995; Schrader *et al*., 2004), acting as substrates for the subsequent enzymatic activities (Zhang *et al.*, 1992; Bisakowski *et al.*, 1997). One such activity is that of hydroperoxide lyase (HPL), reported to cleave the hydroperoxide, producing a volatile compound such as an alcohol or aldehyde (Kermasha, 2002b; Matsui, 2006). Figure 1 shows the bioconversion of linoleic acid into alcohol and carboxyl compound by the sequential enzymatic activity of LOX and HPL.

2.1.3. Utility and Applicability of Lipoxygenase in Flavor Production

Figure 2 shows that LOX is considered as the primary enzyme in the sequential biocatalytic pathway involved in the production of many desirable flavor compounds (Gardner, 1991; Schrader *et al.*, 2004). The first product in the biocatalytic pathway of soybean LOX is the generation of specific HPODs, 9-HPOD and 13-HPODs, considered as flavor precursors. The subsequent catalytic cleavage by a secondary enzyme, the

hydroperoxide lyase (HPL), HPODs resulted in their conversion into their corresponding oxoacid and volatile flavor compounds, with distinct flavor characteristics. Gardner (1989) reported that the cleavage of 9-HPOD by HPL into 9-oxoacid and the nonenal, 9C-aldehyde, which is a volatile compound, with melon or cucumber flavor, whereas hexanal, 6C-aldehyde, is the product from cleavage of 13-HPOD and it is associated with the apple or grassy flavor.

2.1.4. Sources of Lipoxygenases

Lipoxygenases are present in a wide variety of plant (Gardner, 1991), animal tissues (Yamamoto *et al.*, 2004) and microorganisms (Pinto and Macias, 1996; Bisakowski *et al.*, 1997). LOXs from different sources catalyze oxygenation at different positions along the carbon chain, referred to the positional or region specificity.

2.1.4.1. Plant Lipoxygenase

Lipoxygeases (LOXs) are presented in wide range of biological organs and tissues, especially in grain legume seeds (beans and peas) and potato tubers (Casey, 1998). Soybean is among the most characterized plant LOX from which commercial soybean LOX type-1B is produced. However in other plants such as tomato (Regdel *et al.*, 1994), cucumber (Feussner and Kuhn, 1995) and banana (Kuo *et al.*, 2006) are also rich sources of LOX. Baysal and Demirdöven, (2007) identified that soybean LOX involved in regulation response to plant nitrogen status in both tissue-specific and developmentally controlled patterns. Although the molecular structure of LOX has been reported by Boyington *et al*. (1993a), its physiological roles is not completely known (Siedow, 1991).

2.1.4.2. Microbial Lipoxygenase

LOX activities have been found in fungi namely *Fusarium proliferatum* and *Fusarium oxysporum*, *Geotrichum candidum*, *Sacharomyces cerevissiae* and *Thermoactinomyces vulgaris* (Bisakowski *et al*., 1995 and 1997) and *Penicillium* sp. Including, *Penicillium roquefori* and *Penicillium camemberti* (Perraud and Kermasha, 2000), *Morchella esculenta* (Bisakowski *et al*., 2000) and *Aspergillus niger* (Hall *et al*., 2004). Moreover, LOXs also have been characterized in the algae *Chlorella pyrenoidosa* (Zimmerman and Vick, 1973) and edible mushroom, including *Psalliota bispora* (Husson *et al.*, 2001) and *Agaricus bisporus* (Wurzenberger and Grosch, 1984).

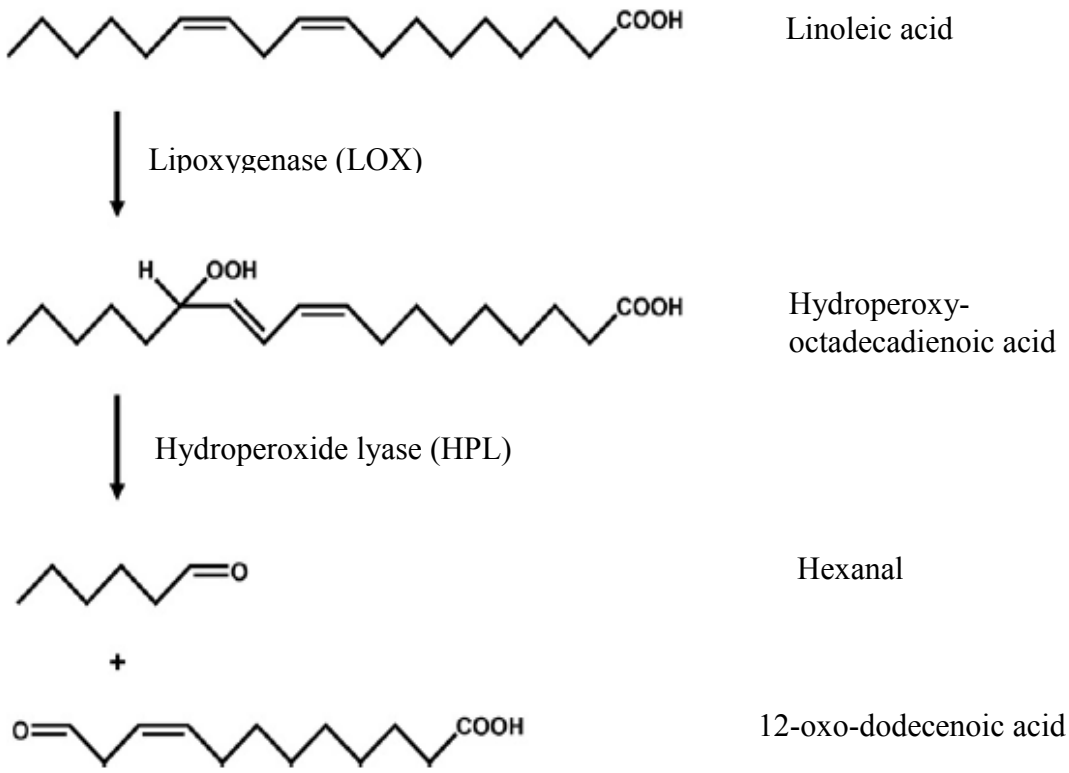

Figure 1. Bioconversion of linoleic acid into short-chain alcohols and carbonyl compounds by sequential enzymatic activities of LOX and HPL.

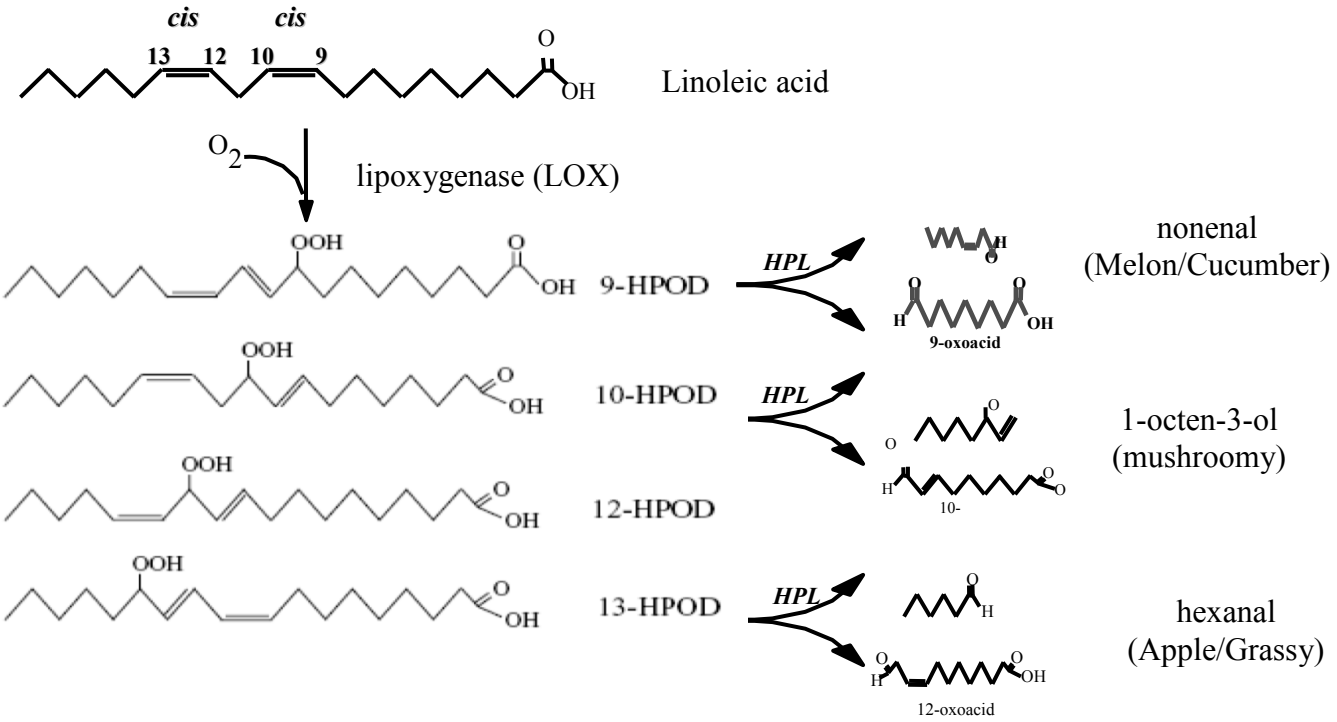

Figure 2. The application of lipoxygenase in the production of aroma.

2.1.5. Structure, Properties, Functions and Catalysis of Lipoxygenase

Lipoxygenases are long, single chain polypeptide of 839 amino acids with molecular weight between 94 and 103 kDa for soybean LOX. The amino terminal domain consists of 146 amino acid residues while the carboxy terminal domain consists of 693 one (Nelson *et al*., 1994). The active site iron is located in the center of the carboxy terminal domain. In non-heme iron enzymes, the oxygen and nitrogen atoms of histidine, iso-leucine, asparagine and glutamine are the most commonly observed ligands (Steczko *et al*., 1992; Minor *et al*., 1993; Nelson *et al*., 1994; Skrzypczak-Jankun *et al.*, 1997; Fox, 1998). Boyington *et al*. (1993b) showed (Fig. 3) that the overall three-dimensional structure of LOX-1B is a combination of 38.0% α-helical content and 13.9% β-sheets content. Oxygenation of naturally occurring PUFAs may proceed enzymatically and/or chemically (Brash, 1999). Both reactions lead to the formation of hydroperoxy PUFAs (Kuhn and Thiele, 1999). The most important difference between two processes is the specificity of the product pattern. Non-enzymatic lipid peroxidation leads to unspecific product mixture consisting of various positional and optical isomers. However, during enzymatic reaction of LOX, PUFAs are usually oxygenated to one specific product isomer which exhibits a high degree of optical purity. Thus, for the large-scale preparation of structurally well-defined oxygenated PUFAs exhibiting positional specificities, LOXs may be used. Liavonchanka and Feussner (2006) showed (Fig. 4) that the metabolism of PUFAs via the LOX-catalyzed step and the subsequent reactions which are collectively named LOX pathway.

2.1.6. Mechanism of Lipoxygenase Reaction

LOXs are multifunctional enzymes because they are catalysts to at least three different types of reactions, including (i) dioxygenation of lipid substrates (dioxygenase reaction) (ii) secondary conversion of hydroperoxy lipids or hydreperoxidase reaction (Kuhn *et al.*, 1987) and (iii) formation of epoxy leuotrienes (leukotriene synthase reation).

It has been shown that LOXs contain one atom of non-heme iron per molecule in their active site (Gaffney, 1996). The iron must be bound directly to functional group of

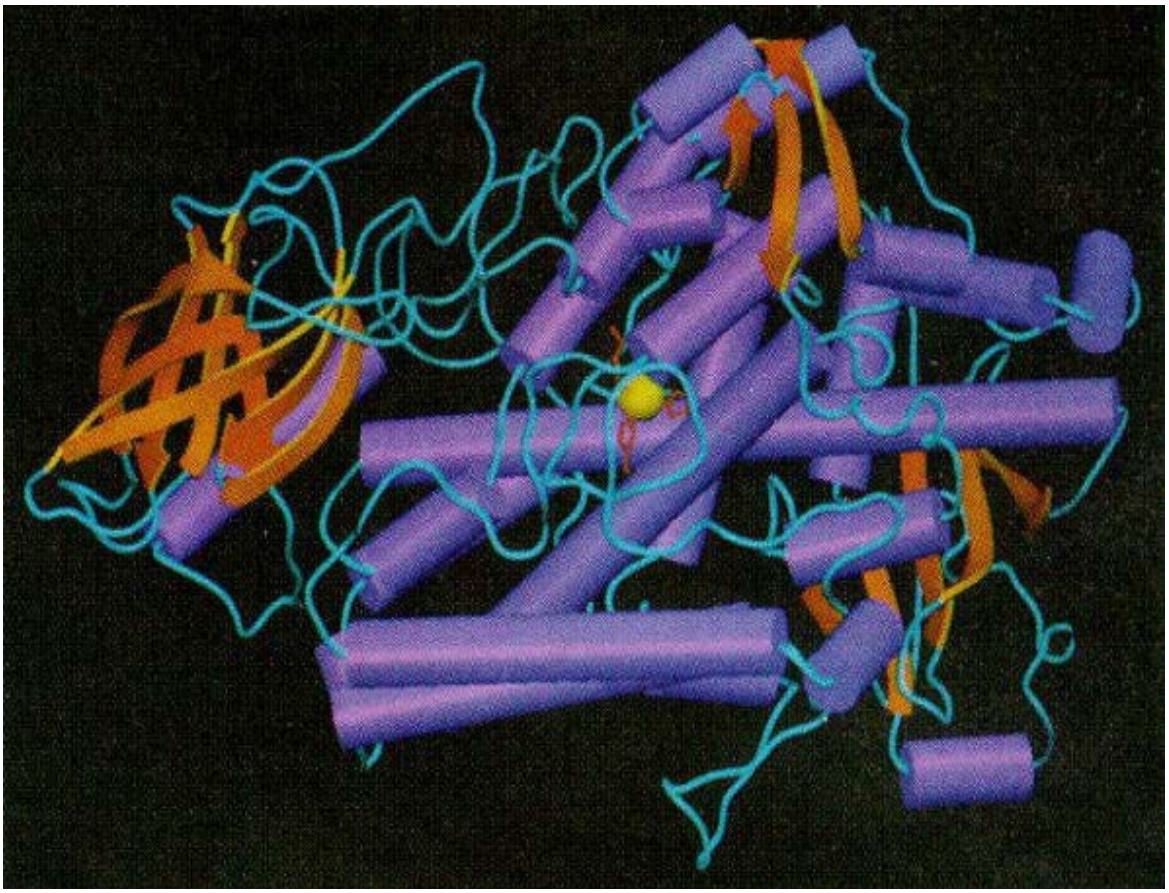

Figure 3. Schematic diagram of the three-dimensional structure of soybean lipoxygenase-1. The α-helices are represented by cylinders, the strands in the β-sheets by arrows, the coils by narrow rods, and the iron by a yellow ball. Only three of the four iron ligands are shown. Domain I, consisting of a single beta barrel, is on the left. Domain II contains the iron, all the helices in the structure, and two small beta sheet structures that lie on the surface of the enzyme. (Boyington *et al*., 1993b)

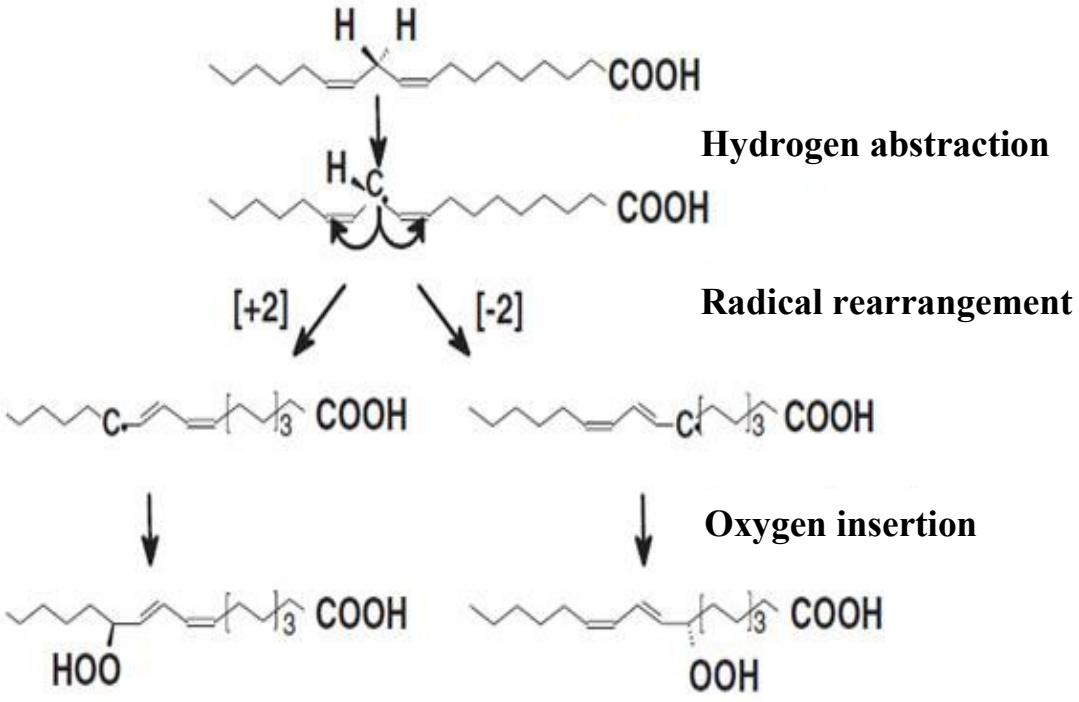

Figure 4. The LOX reaction. The reaction showing the hydrogen abstraction and oxygen insertion.The LOX reaction. The reaction showing the hydrogen abstraction and oxygen insertion. (Liavonchanka and Feussner, 2006)

enzyme since LOXs do not have a heme prosthetic group or an iron-sulphur cluster. In soybean lipoxygenase, the iron atom is in the high spin and exists in one of two oxidation states: Fe^{+2}, the native resting form of LOX (inactive enzyme) or Fe^{+3} (active form). It oscillates between Fe^{+3} and Fe^{+2} during the catalytic cycle (Casey, 1998). In most proposed mechanisms, the lipoxygenase reaction involves two steps: substrate activation and oxygen addition (Feussner and Wasternack, 2002). Neidig *et al*. (2007) demonstrated (Fig. 5) the catalytic cycle of soybean lipoxygenases-1B, the Fe^{+3} form of the enzyme abstracts one electron from the 1,4-*cis, cis*-pentadiene fatty acid substrate and a base abstracts a proton, producing a pentadienyl radical and Fe^{+2} enzyme. Molecular oxygen reacts with the substrate radical to form a peroxyl radical which then abstracts an electron from the metal, regenerating Fe^{+3} and producing a peroxidate anion. Protonation and dissociation from the enzyme allow the formation of the hydroperoxide product (Neidig *et al*., 2007)

2.1.7. Characteristic of Lipoxygenase

Soybean LOX is the most characterized one among other LOXs. Siedow (1991) reported some characteristics (Table 1) of soybean LOX. Moreover, the tertiary structure of soybean has been determined using X-ray crystallography (Boyington *et al.*, 1993a).

2.1.7.1. Isozymes of Lipoxygenase

There are four isozymes (LOX-1, -2, -3a and -3b) that have been isolated from soybeans (Christopher *et al.*, 1970); however, there are very close similarities between LOX-3a and LOX-3b (Siedow, 1991). The first soy isozyme has an optimum pH value of 9.0 and acts only on free polyunsaturated fatty acids producing 9- and 13-hydroperoxides at a ratio of 1:9, respectively (Boyington *et al.*, 1993a). Frazier *et al*. (1973) reported that LOX-1 was used for the bleaching of wheat flour acting as a bread improver and a valuable processing aid during dough development. The second soy isozyme has an optimum pH value of 6.8, it acts on both free and esterified polyunsaturated fatty acids and it forms 9- and 13-hydroperoxide at a ratio of 1:1, respectively (Boyington *et al.*, 1993a). A bleached color can also indicate deterioration in either fresh vegetables, such as yellow French beans or fruits and processed food products, where carotenoids are important natural colorants and antioxidants. Weber *et al*. (1974) reported that type-2 lipoxygenases (LOX-

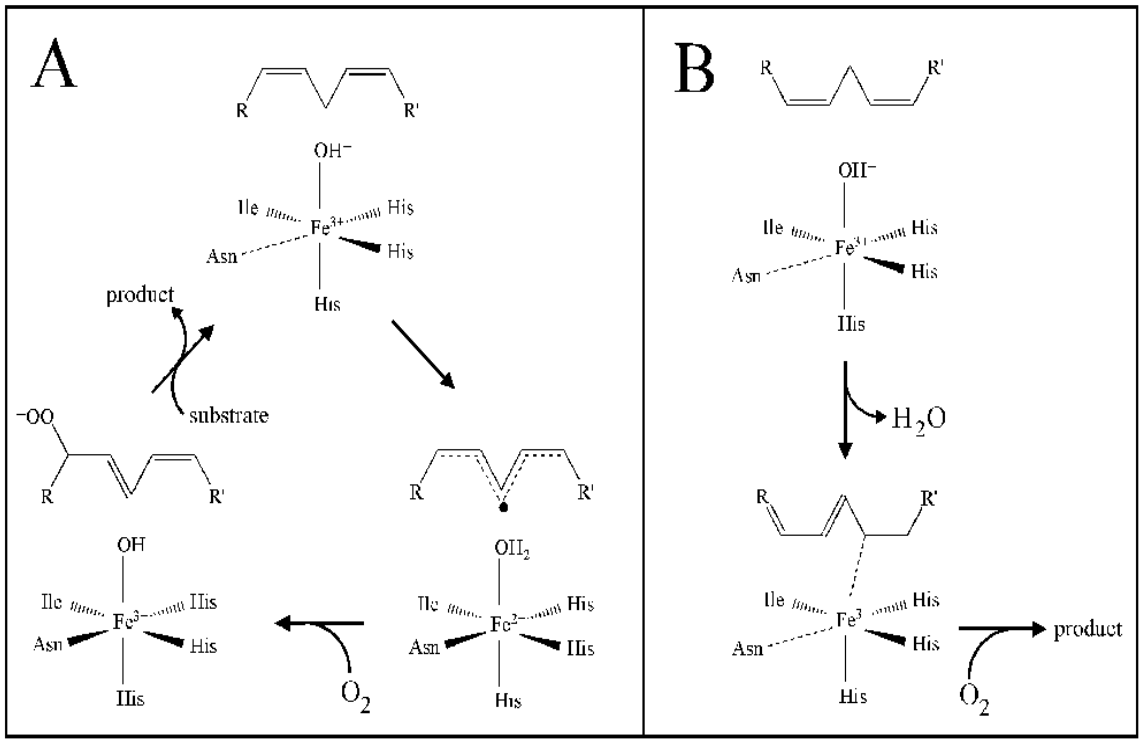

Figure 5. The proposed reaction mechanism for soybean lipoxygenase1B**.** This mechanism involves two steps: (A) hydrogen atom abstraction followed by oxygen addition. (B) Organoiron pathway. (Neidig *et al*., 2007)

2) of soybean, pea and wheat are pigment bleachers in the presence of linoleic acid; however, most of the reported studies for the co-oxidation of carotenoids have been for soybean LOX-1. The third soy isozyme (LOX-3) is similar to isozyme 2, but its activity is inhibited by calcium ions, whereas lipoxygenase-2 is stimulated by the metal (Boyington *et al.* 1993a; Baysal and Demirdöven, 2007).

2.1.7.2. Molecular Weight of LOX Isozymes

Molecular weight (MW) is the ratio of mass of the molecule of interest to 1/12 of the mass of carbon-12. The MW is express in Daltons (Da). Theorell *et al*. (1947) was the first to report the crystallization of soybean with a MW of 100 kDa. Siedow (1991) reported (Table 1) that the three of soybean LOX isozymes are globular, water-soluble proteins that consist of a single polypeptide having a MW of roughly 98,000 Daltons (98 kDa). Nicholas and Drapon (1981) reported the MW of three LOX isozymes (LOX-1, -2 and -3) in bread wheat estimated to be 90, 95 and 110 kDa, respectively. However the MW of LOX from germinating sunflower seeds estimated to be 250 kDa (Leoni *et al*., 1985); in contrast, Matsuda *et al*. (1976) estimated the MW of LOX from *F. oxysporum* to be at 12 to 13 kDa on the basis of ultracentrifugation, SDS-polyacrylamide gel electrophoresis and gel filtration.

2.1.7.3. Isoelectric Point of LOX Isozymes

The migration of proteins is estimated by their isoelectric point (pI) or so called (IEP). pI is a value that can affect the solubility of a molecule at a given pH. Such molecules have minimum solubility in water or salt solutions at the pH which corresponds to their pI. Amino acids which make up proteins may be of positive, negative or neutral charge in nature, to give a protein its overall charge. At a pH below their pI, proteins carry a net positive charge; above their pI they carry a net negative charge. pI is the pH at which a molecule carries no net electrical charge. The isoelectric point is of significance in protein purification because it is the pH at which solubility is often minimal and the point at which protein accumulates (Funk *et al*., 1985). Siedow (1991) reported (Table 1) that the three isozymes of soybean LOX differ with respect to their pI, showing values of 5.68, 6.25 and 6.15 for lipoxygenases-l, -2 and -3, respectively. Matsuda *et al*. (1976) reported the pI for the LOX of *Fusarium oxysporum* to be 9.46 by electrophoresis.

Table 1. Characteristics of lipoxygenase from soybean.

[a]Enzyme characteristics	LOX-1	LOX-2	LOX-3
Molecular Weight (Daltons)	98,000	98,000	98,000
pH optimum	9.00	6.5	4.5-9.0
pI	5.68	6.25	6.15
9/13-HPODs	10:90	50:50	_
Cooxidation activity	low	high	_

[a]Soybean lipoxygenase isozymes differ with respect to their iso-electric points (pI) and pH optimum for activity (Siedow, 1991).

2.1.8. Biocatalyst of Lipoxygenase in Selected Organic Solvent Media (OSM)

Over the past couple of years, there was a tremendous interest by many researchers in development and optimization of LOX biocatalysis in various organic solvent systems as suppose to the conventional system (Piazza 1994; Kermasha *et al*., 2001 and 2002a; Vega *et al*., 2005a,b).

Using organic solvent media for the biocatalytic reactions has been proven to be an extremely useful approach to expanding the range and efficiency of practical applications of biocatalysis (Gupta, 1992; Bell *et al*., 1995; Klibanov, 1997; Kermasha *et al*., 2001; Halling, 2002; Vega *et al*., 2005a; Arriagada-Strodthoff *et al.*, 2007). The main advantages that favor the use of organic solvents in enzyme biocatalysis over aqueous medium, include (a) favoring synthesis over hydrolysis (Bell *et al*., 1995; Halling, 2002); (b) decreasing the possibility of rate-limiting depletion of oxygen co-substrate (Linke, 1965); (c) avoiding water-induced side reactions; (d) enhancing the solubility of hydrophobic substrates (Carrea and Riva, 2000; Kermasha *et al.*, 2002a); (e) recovering the end products (Bell *et al*., 1995); (f) recovering the insoluble biocatalysts for its reuse (Halling, 2002); (g) increasing the biocatalyst thermostability (Ayala *et al.*, 1986; Bell *et al.*, 1995; Carrea and Riva, 2000); (h) reducing the risk of microbial growth (Gupta, 1992); and (i) obtaining more energy efficient downstream processing when volatile solvents are used (Gupta, 1992).

2.1.8.1. Organic Solvent Systems

There are various methods adopted to increase the solubility of hydrophobic substrates in biocatalyzed reactions, where the most obvious and widely employed one is that based on the use of organic solvents (Carrea and Riva, 2000). Kermasha *et al*. (2001; 2002a) reported that the biocatalysis of LOX can be carried out with the use of selected organic solvent system, including water/miscible, biphasic, ternary micellar and neat organic solvent systems. The water/miscible organic solvent system, where small amounts of water miscible solvents commonly used are ethanol, methanol, dimethyl sulphoxide and acetone. The immiscible biphasic system is a mixture of water and water-immiscible organic solvent buffer solution in which the hydrophobic substrate is solubilized. The ternary micellar system is subdivided into two types of micelles which are so called oil in

water (O/W) that is water in low concentration of organic solvents and water in oil (W/O) also called reversed micelles in where aggregates of microemulsions are formed; these microemulsions are composed of either a mixture of the buffer solution and octane or *iso*-octane with Tweens. The neat organic system consists of one of the selected organic solvents, including hexane, *iso*-octane and octane containing traces of aqueous solution of Tris–HCl buffer solution (0.1 M, pH 7.0); the neat organic system is used when chemical reactions which are not feasible in that of conventional system.

2.1.9. Choice of Organic Solvent Media (OSM)

The effect of various solvents on enzyme behavior is an important factor. The choice of organic solvent for a given reaction should be determined by three factors; which are (1) the effects of solvent on the reaction, such as, solubility of the substrates, kinetics and enzyme specificity, (2) the toxicity of the solvent (important for food and pharmaceutical-based processes where compliance with safety and solvent disposal legislation will be a major consideration) and (3) the effect of the solvent on biocatalyst stability (Bell *et al*., 1995).

2.1.10. Effect of Organic Solvent Media on LOX Secondary Structure

Using the spectra Fourier Transform Infrared (FT-IR) and selected organic solvent media including, acetonitrile, methanol, hexane, octane and chloroform, Vega *et al*. (2006) reported (Table 2) that lipoxygenase in organic solvent showed absorbance bands at 1660 and 1653 cm^{-1} slightly shifted from that recorded one in aqueous buffer (1657 and 1648 cm^{-1}). Table 2 shows that the secondary structure of lipoxygenase was similar to that in the powdered form and not to that of aqueous phase. From the FTIR analysis, the authors showed that after the addition of hexane, the ratio of α-helix to β-sheet was increased as compared to that of chloroform, methanol and acetonitrile. Vega *et al.* (2006) also showed that the secondary structure of LOX under the conditions in which it exhibited its highest enzyme activity was predominantly ordered α-helix with, minimal aggregate formation; the intermolecular β-sheet aggregation resulted in a decrease in LOX activity.

Table 2. Percent contributions of the components of the amide I' band to the total integrated absorbance value in the amide I' region (1700–1600 cm^{-1}) of the ATR spectra of lipoxygenase films. The data was calculated using the baseline corrected deconvolved pectra. (Vega *et al*., 2006)

	Percent contribution					
Solvent[a]	α_1[b] (1660-1654)[d]	α_2[b] (1693-1687)[d]	β_1[b] (1693-1687)[d]	β_2[b] (1644-1638)[d]	$\beta_{aggregation}$[b] (1621-1615)[d]	Log*P*[c]
Powder[e]	23	24	16	27	10	NA[g]
Methanol	11	33	ND[f]	29	27	-0.76
Acetonitrile	29	27	6	28	9	-0.33
Chloroform	26	31	4	32	7	2.0
Hexane	31	24	ND[f]	29	15	3.5
Octane	29	29	ND[f]	27	15	4.5

[a] Solvent used to make LOX suspension was evaporated from the surface of the ATR crystal and spectrum of residual film was recorded.
[b]Type of motif associated with the wavenumber (Pelton and McLean, 2000).
[c]Partition coefficient (Log *P*), which is defined as the relative partitioning of a given solvent between octanol and water.
[d]Wavenumber in cm-1 (± cm-1).
[e]No solvent was used.
[f]Band not detected.
[g]Not applicable.

2.1.11. Parameters Affecting Biocatalysis of Lipoxygenase in Aqueous and OSM

2.1.11.1. pH Optimum

The conformation of enzyme and its interaction with a substrate are influenced by the pH of the reaction media, since it is generally consisted of various basic and acidic residues (Ballesteros and Boross, 2000). Generally, enzymatic activities have a narrow range of pH. The optimal pH value for an enzyme depends on several parameters, including sources of enzyme, concentration of buffer and substrate, ionic strength of medium, time and temperature of reaction (Whitaker, 1994). LOX from microorganism has a broader range of pH values in compare to that of plant. The optimal pH of LOX from bacteria and fungal are usually in the pH range of 6.0 to 7.0, including 6.5 for *P. camemberti* (Perraud and Kermasha, 2000), 6.0 for *Fusarium proliferatum* (Bisakowski *et al.*, 1998) and 6.3 for *Sacharomyces cerevissiae* (Shechter and Grossman, 1983).

LOXs generally display maximal activity at pH range of 6.0 to 9.0; however, it becomes inactive at pH greater than 11.0 (Galliard and Phillips, 1971). The major differences among the three soybean lipoxygenase isozymes can be seen at the level of their reactivities. Lipoxygenase-l has a pH optimum for its activity around 9.0, while lipoxygenase-2 shows a sharp pH maximum around pH 6.5 and lipoxygenase-3 displays a broad optimum pH 7 (Axelrod *et al.*, 1981). Lipoxygenase-l shows a marked preference for charged fatty acids and, as such, shows little reactivity with fatty acids that are esterified (i.e. as they would be found in a membrane). Lipoxygenases-2 and -3 are more reactive toward neutral fatty acids but will react with free fatty acids, particularly at pH below 7.0. Related to this, lipoxygenase- l can effectively utilize the sulfate ester of linoleic acid as a substrate and does so with a greater reactivity at pH 6.8 than 9.0 (Siedow, 1991). The decrease in lipoxygenase activity at pH values below 3.0 and above 9.0 may be attributed to changes in enzyme solubility (Vega *et al.*, 2006). However, Satoh (1976) reported the presence of dual pH optima for LOX from the mould *F. oxysporum* at pH 6.0 and 10.0. Iny *et al*. (1993) also reported dual pH optima for LOX from for bacterium *T. vulgaris*, at pH 6.0 and 11.0. Others reported the presence of dual optima for the LOX of *P. camemberti*, at pH 6.5 and 8.0, and *P. roqueforti* at pH 5.5 and 8.0. (Perraud and Kermasha, 2000; Hall *et al*., 2005).

2.1.11.2. Effect of Temperature

In general, changing temperature has an influence on enzyme activity in various ways, including the stability of the enzyme, the affinity of the substrate, the activators and the inhibitors as well as the velocity of conversion of substrate into products (Whitaker, 1994). Reaction temperatures of LOX, from microbial sources, have a broad range from 40 to 60°C, depending on the sources of enzyme. Li *et al*. (2001) reported that the optimal temperature for LOX from *Thermomyces lanuginosus*, a themophilic fungus, was 55°C while the optimal temperature of that from *Gaumannaomyces graminis* is 60°C (Su and Oliw, 1998).However, several LOXs possessed lower optimal reaction temperature, including 15°C for *Gersemia fruticoca* (Mortimer *et al*., 2006), 25°C for *Pleurotus pulmonarius* (Kuribayashi *et al.*, 2002) and 30°C for *P. camemberti* (Hall *et al*., 2008).

2.1.11.3. Types (classes) of Surfactants

Chemically, surfactants are amphipathic molecules having two distinctly different characteristics, polar “head” and non polar "tail", in the same molecule (Warren, 1998). A surfactant molecule has both hydrophilic (water-like) and hydrophobic (water-dislike) characteristics. The hydrophobic group of a surfactant is usually an appropriate hydrocarbon chain that could be a fluorocarbon or siloxane chain (Warren, 1998). The hydrophilic group is polar and it could be either ionic or non-ionic. Since surfactant molecules have both hydrophilic and hydrophobic parts, the most attractive place for them in water is at the surface where the forces of both attraction and repulsion to water can be satisfied. Pavlenko *et al*. (2001) showed (Fig. 6) that surfactants interact to satisfy natural forces of attraction and repulsion between molecules by formation of micelles.

Kermasha *et al*. (2001) reported that in the ternary micellar system composed of a mixture of 96.5:3.5 (v/v) of Tris-HCl buffer solution (0.1 M, pH 9.0), octane and low concentrations of surfactants, including Tweens 20, 40 and 60, showed an increase in LOX activity, whereas a decrease or no change in LOX activity was obtained in the presence of emulsifiers, including Spans 40, 60, 80 and 85. Kermasha *et al*. (2001) also reported that, using the reaction medium composed of Tris-HCl buffer solution (0.1 M, pH 9.0) and iso-octane 96:4 (v/v), the highest specific activity of LOX was obtained in

the presence of 10 μM of Tween 40, whereas a decrease in enzyme activity was observed at low concentrations of Spans 20, 40, 60, 80 and 85.

Surfactants fall in the following classifications according to the nature of the hydrophilic group (Warren, 1998), (1) anionic surfactant in which hydrophilic head is negatively charged such as sodium bis-2(ethylhexyl) sulphosuccinate (AOT) falls into this group; (2) cationic surfactant where hydrophilic head is positively charged; Quaternary Ammonium compounds such as cetyl trimethyl ammonium bromide (CTAB); (3) non-ionic, the hydrophilic head is polar but not fully charged; sorbitan ester thoxylates are in this group, such as Tween 80 and Span 60; and (4) amphoteric surfactant in which the molecule has both potential positive and negative groups or so called a "Zwitterionic" where the charge depends on the pH of the medium; alkyl Betaines, such as cocamidopropyl betaine and amine oxides, are examples of the amphoteric surfactants.

2.1.11.4. Thermal Stability of Lipoxygenase

In conventional media, enzymes lose their catalytic activity as the temperature is raised (Ayala *et al*., 1986). However, those authors reported that enzymes in organic solvents are very stable at high temperatures. Hari Krishna (2002) indicated that this increase in thermalstability of the enzyme in non-conventional media can be explained by the enhanced rigidity of the enzyme structure due to the restricted amount of water in the system. Hall *et al*. (2008) reported that LOX from *P. camemberti* has dramatically decreased in activity upon thermal treatment at 25°C, with residual specific activity (%) of 95.5, 58.8, 27.2 and 0% after 4, 12, 24 and 36 h, respectively. Zaks and Klibanov (1984) reported that pancreatic lipase suspended in organic solvents withstands 100°C for many hours. Ayala *et al*. (1986) suggested that all enzymes in organic solvents acquire thermostability provided, that bulk water is eliminated; hence, the removal of water from the reaction system is directly involved in the rapid formation of incorrect structures and thus in enzyme inactivation. Therefore, a small amount of protein-bound water could be

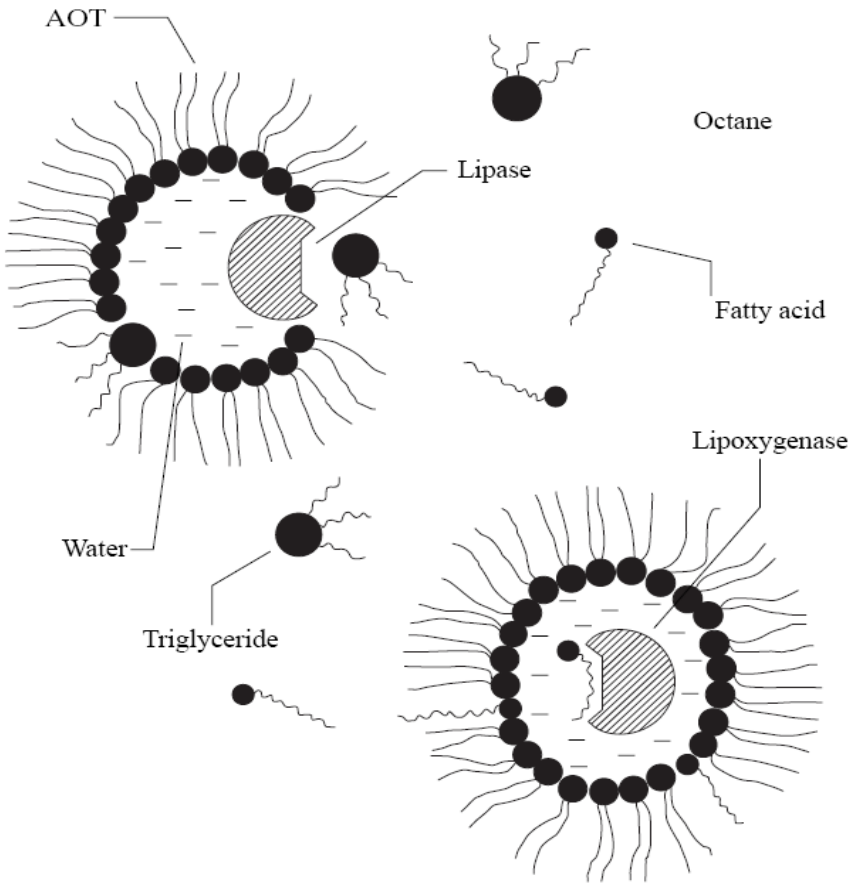

Figure 6. Description of the lipase/lipoxygenase AOT–water–organic solvent in micellar system. (Pavlenko *et al*., 2001)

essential for maintenance of the native protein structure and enzyme activity (Adlercreutz, 2000). The thermal inactivation of the activity of LOX from *P. camemberti* followed first–order kinetics (Hal *et al*., 2008). The main advantage of high temperature of an enzyme could promote its use in food and pharmaceutical industry as well as a shift in the thermodynamic equilibrium in case of endothermic reactions (Iyer and Ananthanarayan, 2008). In addition, the thermostability is also an important factor in determining the processing costs (Illanes, 1999).

2.1.11.5. Enzyme Specificity

One of the important properties of enzymes is their specificity. According to the lock and key model suggested by Emil Fisher in 1894, the specificity of an enzyme and its substrate comes from its geometrically complementary shapes. A few number of enzymes exhibit absolute specificity, where, they can catalyze only one particular reaction. Other enzymes will be specific for a particular type of chemical bond or functional group. In general, there are four distinct types of specificities, including (1) absolute specificity, where the enzyme will catalyze only one reaction, (2) group specificity, where the enzyme will act only on molecules that have specific functional groups, such as amino, phosphate and methyl groups, (3) linkage specificity, where the enzyme will act on a particular type of chemical bond and (4) stereochemical specificity, where the enzyme will act on a particular steric or optical isomer. LOXs are calssified according to their regiospecificity, using either linoleic acid with vegetal LOXs or arachidonic acid with other LOXs (Hornung *et al.*, 2000). LOXs from different sources, catalyses oxygenation at different points along the carbon chain, referred to as "positional" or "regio" specificity, such specificity has significant implications for the metabolism of the resultant hydroperoxides into a number of important secondary metabolites (Hall *et al.*, 2004).

2.1.11.6. Substrate Specificity

The most common substrates for LOX are polyunsaturated fatty acids (PUFAs) containing the *cis, cis*-1,4-pentadiene moiety, such as linoleic, linolenic and arachidonic acids. However, most microbial LOXs showed preferential substrate specificity towards linoleic acid (Perrraud and Kermasha, 2000). Matsuda *et al.* (1976) reported that the LOX

isozyme from *F. oxysporum* contains 13.3-fold higher activity towards linoleic acid as compare to linolenic acid. Iny *et al.* (1993b) reported that the LOX extract from T. *vulgaris* has 100% specificity towards linoleic acid. Bisakowski *et al.* (1997) reported that the different microbial LOX extracts, including *Fusarium oxysporum, Fusarium proliferatum, Saccharomyces cerevisiae and Chlorella pyrenoidosa,* have preference towards linoleic acid, followed by linolenic acid. Perraud *et al*. (1999) reported that the LOX of *Geotrichum candidum* at pH 3.75 prefers linolenic acid as a substrate, whereas at pH 8.0 its preference is shifted toward arachidonic acid. Feussner and Wasternack (2002) showed (Fig. 7) that linoleic acid (LA) is oxygenated either at carbon atom C-9 (9-LOX) or at C-13 (13-LOX) of the hydrocarbon backbone of the fatty acid leading to two groups of compounds, the (9S)- hydroperoxy- and the (13S)-hydroperoxy derivatives of LA. Hall *et al*. (2004) reported that the enriched LOX from *Aspergillus niger* has preferential substrate specificity towards free fatty acids, including linoleic, linolenic and arachidonic acids, with 100, 102 and 85% relative activity, respectively.

In higher plants, the literature Axelrod *et al*. (1981) and Shibata (1995) indicated that the predominant substrates are linoleic (18:2 *n*-6) and linolenic acids (18:3 *n*-3), while Royo *et al*. (1996) reported that mammalian LOXs prefer arachidonic acid (20:4 *n*-6). The oxidation of arachidonic acid by LOX is the source of highly active bioregulators such as leukotriens and lipoxins in animals (Pérez Gilabert and Carmona, 2002). Feussner and Wasternack (2002) showed (Fig. 8) the metabolism of polyunsaturated fatty acids and its derivatives and subsequent enzymatic reaction.

2.1.11.7. Inhibition of Lipoxygenase

Inhibition of LOX-1 by different molecules has generated fundamental investigation to understand the mechanism of the reaction. LOX activity can be inactivated either thermally or chemically. Thermal inactivation of LOX from *Thermomyces lanuginosus* can be achieved at 60°C over a period of 5 min (Li *et al.*, 2001). Whereas the purified LOX-2 activity in the germinating barley was completely lost by heating at 65°C for 5 min (Holtman *et al.,* 1997).

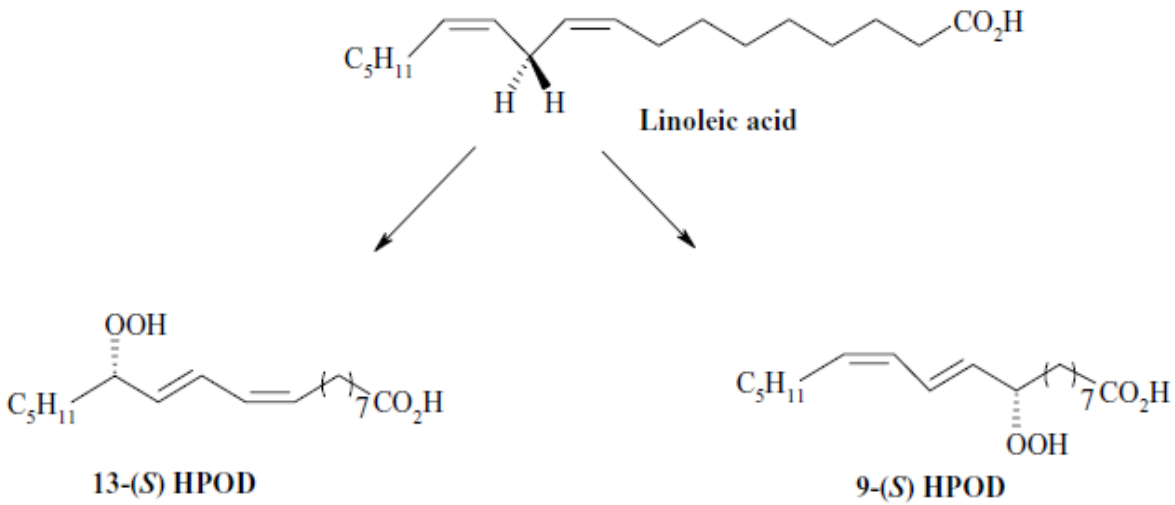

Figure 7. Mechanism of the dioxygenation reaction catalyzed by LOX with linoleic acid as substrate. The two classes of LOX products that are derivatives of PUFAs. The (9S)-hydroperoxy and (13S)-hydroperoxy are considered to be central importance for the production of a plethora of oxylipins found in plants. (Feussner and Wasternack, 2002)

On the other hand LOX chemical inhibitors can be classified into three main categories. Firstly, inhibitors that fixes directly to the active site of the enzyme at the place of substrate, rendering it inactive; eicosatetraenoique can be classified of this type. It transfers the oxygen of the preformed hydroperoxides to the essential methionine of the active site (Kuhn *et al*., 1984) and in some cases can irreversibly inhibit the active sites (Corey *et al.,* 1986).

Secondly, inhibitors that are bind to the iron of active site including iron-chelating agents (Battu and Beneytout, 1992; Wu, 1996). Chelators of Fe^{+2} such as 8-hydroxyquiniline, thioglycollic acid and quinalizarin can bind to the iron site of the enzyme causing either the destruction or a conformational change at the active site, hence, it diminish its activity (Wu, 1996), while chelators of Fe^{+3}, including ethylenediaminetetraacetate (EDTA) and *p*-chlromercurybenzoate, have showed no affect on the activity of LOX from *Fusarium oxysporum* (Bisakowski *et al.*, 1995).

Thirdly, inhibitors that are antioxidants demonstrated to diminish LOX activity, including hydroquinone (HQ), butylated hydroxytoluene (BHT) and *n*-propyl gallate (*n*-PG). Perraud *et al*. (1999) reported that HQ (0.25 mM), BHT (0.10 mM) and *n*-PG (0.10 mM), inhibited the LOX activity from the *Geotrichum candidum* extract at pH 8.0 by 72, 14 and 30%, respectively. Furthermore, Joung Ha and Kubo (2005) showed that 6[8' (Z)-Pentadecenyl] salicylic acid (anacardic acid (C15:1)) inhibited the linoleic acid peroxidation, catalyzed by soybean lipoxygenase-1. Joung Ha and Kubo (2007) indicated that dodecyl gallate inhibited the soybean lipoxygenase-1.

2.1.11.8. End Product Specificity

The specific synthesis is beneficial because different enantiomers or regioisomers could show different sensorial properties (Brenna *et al.*, 2003). Depending on LOX source, substrate and reaction conditions including, temperature and pH, the amounts of the isomers formed can vary (Eskin *et al*., 1977). Hall *et al*. (2004) showed (Fig. 9) the generation of different HPOD region-isomers (9-, 10-, 12- and 13- HPOD) by LOX. Biocatalysis represents a useful tool in catalyzing a large number of stereo- and regio-

selective chemical reactions that can not be easily achieved by the less selective classical synthesis process (Cheetham, 1997).

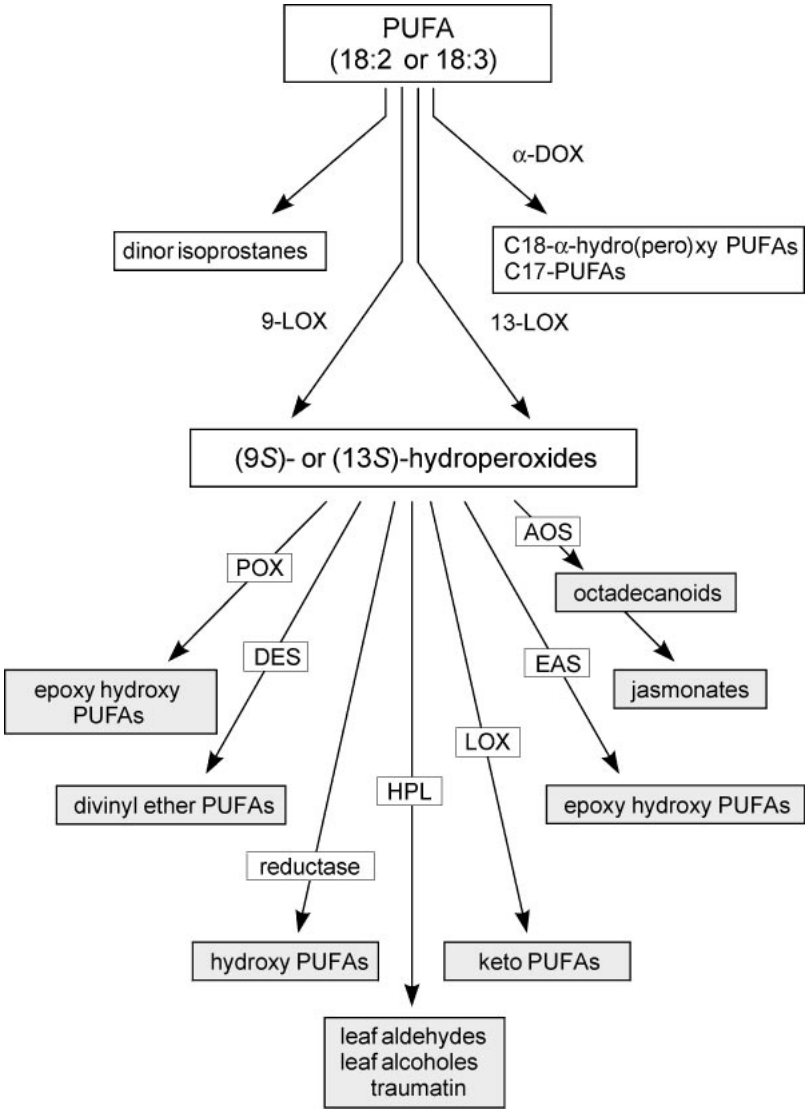

Figure 8. Metabolism of PUFAs and its derivatives. The polyunsaturated fatty acids leads to 9-LOX-derived and 13-LOX-derived hydro(pero)xy PUFAs in plants—the LOX pathway. AOS, allene oxide synthase; DES, divinyl ether synthase; α-DOX, α-dioxygenase; EAS, epoxy alcohol synthase; HPL, hydroperoxide lyase; LOX, lipoxygenase; POX, peroxygenase; PUFAs, polyunsaturated fatty acids. (Feussner and Wasternack, 2002)

Generally, the separation and characterization of the LOX end products are usually accomplished by using high-performance liquid chromatography (HPLC), gas chromatography (GC) and mass spectrometry (MS). The regio-specificity of LOX product formation depends on both positional specificities of the initial hydrogen removal and the subsequent oxygen addition (Kuhn *et al*., 1986). Stereo specificities of microbial LOX from *Penicillium* sp. shown the presence of both (R)- and (S)-enationmers (Perraud and Kermasha, 2000). In contrast, most plant and animal LOXs are usually catalyzing the production of HPODs that has the (S)-configuration; however, (R)-configuration from LOX activity can be found among aquatic invertebrates (Steel *et al.,* 1997).

Initially, Zimmerman and Vick (1973) reported that LOX activity in *Chlorella pyrenoidosa* produced both the 9- and 13-HPOD derivatives at a ratio of (1:4) of linoleic acid. Bisakowski *et al*. (1997) further investigated LOX in *C. pyrenoidosa* and *Agaricus bisporus* showed that the active fractions produced the 9-HPOD derivative together with the 10- and 13-HPOD isomers. Nuñez *et al.* (2000) reported that LOX present in *C. pyrenoidosa* was able to cleave the 13-HPOD into a ω-oxo-fatty acid. The majority of microbial LOXs convert the linoleic and linolenic acids into 9- and 13-HPOD (Perraud and Kermasha, 2000).

2.1.11.9. Chiral Stereo-Specificity

Stereo-specificity of an enzyme is a reaction mechanism that produces different stereo-isomeric reaction products. Although the optical activity of the chemical compound is preserved, the atomic arrangements are different in the space. Using linoleic acid as substrate, soybean LOX type-1B was reported by Brash and Hawkins (1990) to produce 13-HPOD and enantiomeric excesses in favor of the (*S*) form.

2.1.12. Parameters Affecting Biocatalysis of LOX in Neat Organic Media

Klibanov (1997) showed (Table 3) that several physiochemical factors may considerably lower enzymatic activity in neat organic solvent as compared to that in the aqueous medium or even in the ternary micellar system. Moreover, organic solvents are generally lacking water's ability to be engaged in multiple hydrogen bonds and, because of their lower dielectric constants, lead to stronger electrostatic interactions and hence more rigid proteins (Affleck, 1992; Klibanov, 1997).

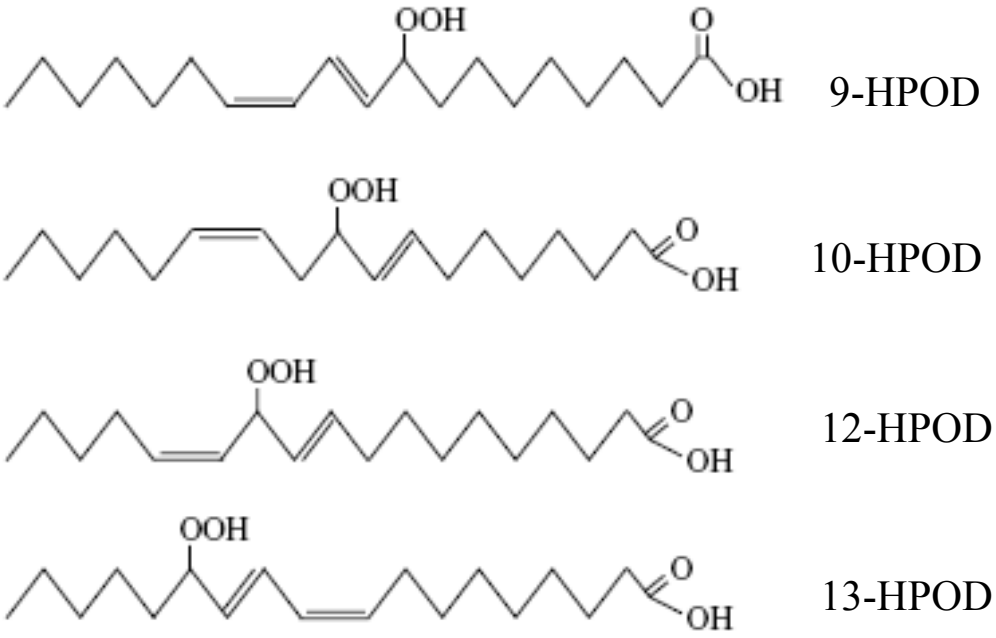

Figure 9. The 9-, 10-, 12- and 13-hydroperoxide (HPOD) regioisomers catalyzed by lipoxygenase. (Hall *et al*., 2004)

2.1.12.1. Agitation Speed

The influence of agitation on the catalytic activity of an enzyme in organic media is considered as one of the main limiting factors (Barros *et al*., 1998; Salis *et al*., 2003). The agitation in the aqueous medium is not considered as a major factor, since the rate of enzymatic reaction is higher than the rate of diffusion of the substrate (Arriagada-Strodthoff *et al*., 2007). However, in two phase mixture, the agitation is required to ensure the availability of the substrate for the enzyme (Adlercreutz, 2000).

2.1.12.2. Water Activity (a_w)

In organic solvent media, water plays a crucial part in maintaining the active enzyme conformation (Rupley *et al*., 1991) and in modulating the equilibrium reaction. It would be therefore expected that enzymes are less thermostable in aqueous medium as compared to that in the non-aqueous one. In non-aqueous media, the water content of the system is best described by the thermodynamic of water activity *(a_w)* (Wehtje and Adlercreutz, 1997).

Water activity *(a_w)* a value of 1.0 in pure water and 0.0 in completely dry systems; hence, dilute aqueous solutions have practical a_w values of 1.0 while a_w of organic solvent media vary widely between values of 0 and 1 (Adlercreutz, 2000).

The water activity of a product can be determined from the relative humidity of the air surrounding the sample when the vapor pressure of the air and the sample are at equilibrium. The sample, therefore, must be in closed space where this equilibrium can take place. Once this occurs, the water activity of the sample and the relative humidity of the air are equal. The measurement taken at equilibrium is called an equilibrium relative humidity or (ERH).

The water content in the reaction media is best measured in terms of it's a_w (Halling, 1994). In aqueous medium, enzymes possess the necessary conformational mobility for an optimal catalysis (Rupley *et al.*, 1991), whereas organic solvent media lacks of water ability to engage in multiple hydrogen bonds (Jeffrey and Saenger, 1994). In addition, it also has the stronger intra-protein electrostatic interactions due to the lower dielectric

constants (Klibanov, 2001); consequently, the enzyme molecules are much less flexible (Affleck *et al.*, 1992; Burke *et al.,* 1993). Therefore, investigating the influence of water content in neat organic reaction media catalyzed by lipoxygenase is an important parameter.

2.1.13. Kinetics of LOX Activity

One of the factors that affect the rate of enzyme activity is the substrate concentration. In order to study kinetics of LOX activity, two parameters must be considered, the maximum velocity of an enzyme reaction (V_{max}) and the substrate concentration [S] at which the rate of the enzyme reaction is at its half maximal velocity ½ V_{max} is called Michaelis constant (K_m). In addition, K_m is an expression of the affinity of an enzyme for a substrate (Whitaker, 1994).

The majority of reports on microbial LOXs indicate that the K_m values are in the range of 0.2 to 0.5 mmol of PUFA substrate. Perraud and Kermasha (2000) reported that K_m values of *Penicillium roquefori* and *Penicillium camemberti* are 0.26 and 0.44 mmol, respectively. Kuo *et al.* (2006) reported K_m value for LOX from banana leaf to be 0.15 mmol. Kermasha *et al*. (2001) reported a K_m value of 5.57 mmol for LOX in ternary micellar system; however, Vega *et al*. (2005a) reported K_m value for soybean LOX to be 14.4 mmol.

The reports of the maximum velocity (V_{max}) of the animal and microbial LOX reaction indicates a range of 50 to 100 nmol hydroperoxide produced/mg protein/min. Bisakowski *et al.* (1995) reported *Chlorella pyrenoidosa* to be 0.401 µmol hydroperoxide produced/mg protein/min and 0.059 µmol hydroperoxide produced/mg protein/min for *Thermoactinmyces valgaris*. Hall *et al.* (2004) reported V_{max} values for microbial LOX from *Asperigillus niger* to be 0.095 µmol hydroperoxide produced/mg protein/min. Kuo *et al.* (2006) reported that LOX V_{max} values from banana leaf was 2400 nmol hydroperoxide produced/mg protein/min. Kermasha *et al*. (2001) reported a V_{max} value of 20.49 µmol hydroperoxide produced/mg protein/min for soybean LOX in ternary micellar system; however, Vega *et al*. (2005a) reported a V_{max} values for soybean LOX to be 5.84 µmol hydroperoxide produced/mg protein/min. The ratio of V_{max} to K_m is termed the

catalytic efficiency. Kermasha *et al*. (2001) reported the enzymatic catalytic efficiency for soybean LOX activity in ternary micellar system to be 3.68. Vega *et al*. (2005a) reported the catalytic efficiency for soybean LOX activity to be 0.41.

2.1.14. LOX Assay

Typically, the methodologies used for the measurement of lipoxygenase activity include direct spectrophotometric assay, polarography and ferrous oxidation.

2.1.14.1 Direct Spectrophotometric Assay

A common technique for the determination of LOX activity in aqueous medium is the spectrophotometric monitoring, which is based on the absorption conjugated diene moiety of polyunsaturated fatty acids containing, a *cis,cis*-1,4- pentadiene moiety obtained by the enzyme activity (Grossman and Zakut, 1979; Axelrod *et al*., 1981).

2.1.14.2. Polarographic Method

The polarographic method is a direct continuous method of measurement of LOX activity based on measuring the uptake of molecular oxygen by the substrate. (Khun *et al*., 1981; Shechter and Grossman, 1983; Iny *et al*., 1993; Kuo *et al*., 1997; Su and Oliw, 1998). The main advantage of this method is the ability to measure the LOX activity in conditions where low pH, type of buffer, emulsifier agent, temperature and nature of activator or where the turbidity generated from the solubility of substrate in aqueous medium prevents the monitoring ability by a direct spectrophotometer assay, since it does not require optically clear substrate for measuring oxygen uptake (Grossman and Zakut, 1979).

Khun *et al*. (1981) described other methods for determination of plant lipoxygenase activity; for instance, the use of MBTH (3-methyl-2-benzothiazolinone), coupled with DMAB [3-(dimethylamino)benzoic acid] in the presence of hemoglobin, which catalyzes

Table 3. Reasons for decrease of enzymatic activity in neat organic solvent media compare to that of aqueous and ternary micellar system. The table refers to crystalline enzymes suspended in neat organic solvent media. (Klibanov, 1997)

Cause	Comments	Remedies
Diffusional limitations	Not as likely as widely claimed	Vigorously agitate enzyme suspensions use small enzyme particles
Active center blockage	Responsible for no more than a few fold activity reduction	If matters, use crystalline rather than amorphous enzyme particles
Unfavorable energetics of substrate desolvation	Severe with hydrophobic substrates; also, likely with natural substrates.	Select the solvent expected to yield unfavorable solvent-substrate interactions
Transition state destabilization	Likely to be pronounced when the transition state is at least partially exposed to the solvent	Select the solvent expected to yield favorable interactions with the transition state
Reduced conformational mobility	Severe in anhydrous, hydrophilic solvents owing to the stripping of the essential enzyme-bound water.	Optimize water activity (*aw*); hydrate the solvent; use hydrophobic solvents; use water-mimicking and denaturing co-solvent additives
Sub-optimal pH situation	May be responsible for activity reduction	Dehydrate from aqueous solution of the pH optimal for enzymatic activity; use organic-phase buffers

the reaction of detection of linoleic acid hydroperoxide, constitutes an assay method capable of detecting lipoxygenase activity in vegetable homogenates.

2.1.14.3. Ferrous Oxidation Assay

Although both methods, direct spectrophotometer and polarographic, are accurate, they are time-consuming and are unsuitable for rapid screening of multiple samples. The Xylenol Orange method (FOX) is a colorimetric method for determination of lipoxygenase activity applicable to high throughput assays. The assay is based on the principle that under acidic conditions a lipid hydroperoxide can oxidize Fe^{2+} to Fe^{3+}, which then oxidizes Xylenol Orange to form a product that absorbs strongly in the visible region (500-600 nm) (Wurzenberger and Grosch, 1984; Nourooz-Zadeh, 1999; Pinto *et al*., 2007). Xylenol Orange colorimetry is considered as an efficient technique used to detect hydroperoxides present in many materials, including animal tissues (Arab and Steghen, 1998; Sodergren *et al.*, 2004), food stuffs (Navas *et al.*, 2004) and LOX enzymatic homogenates (Cho *et al.*, 2006). The ferrous oxidation assay, using Xylenol Orange (FOX) or ferrous thiocyanate, was reported as an alternative to the less sensitive spectrophotometric method for the determination of lipid hydroperoxides (Wurzenberger and Grosch, 1984). Pinto *et al*. (2007) reported that the FOX assay may constitute an easy, inexpensive, and sensitive procedure for the measurement of lipoxygenase activity that only needs generally available apparatus. In addition, the authors describe that the method possesses high linearity and the obtained results are highly reproducible.

3. MATERIALS AND METHODS

3.1. Materials

Commercial purified soybean lipoxygenase (LOX) type I-B (46,000 U/mg solid), Xylenol Orange salt [3,3'-bis(*N,N*-di(carboxymethyl)aminomethyl)-*o*-cresol-sulfonephthalein sodium salt], bovine serum albumin (BSA), sodium bis-2(ethylhexyl) sulphosuccinate (AOT) and cetyl trimethyl ammonium bromide (CTAB) were purchased from Sigma Chemical Co. (St. Louis, MO). Linoleic acid (*cis*-9, *cis*-12-octadecadienoic acid) was purchased from Nu-Check-Prep Inc. (Elysian, MN). A wide range of Spans, including sorbitan monolaurate (Span 20), sorbitan monopalmitate (Span 40), sorbitan monostearate (Span 60) and sorbitan monooleate (Span 80) as well as Tweens, including polyoxyethylene sorbitan monolaurate (Tween 20), polyoxyethylene sorbitan monopalmitate (Tween 40), polyoxyethylene sorbitan monostearate (Tween 60) and polyoxyethylene sorbitan monooleate (Tween 80) were obtained from ICI Americas (Wilmington, DE). Ferrous sulfate of ACS grade as well as Tris(hydroxymethyl)aminomethane (Tris), monosodium dihydrogen phosphate (NaH_2PO_4) and disodium monohydrogen phosphate (Na_2HPO_4) were purchased from Fisher Scientific (Fair Lawn, N.J.). Perchloric acid was purchased from Aldrich Chemical Co. (Milwaukee, WI), whereas sulfuric acid was obtained from LabChem Inc. (Pittsburgh, PA). Water was purified using a Milli-Q plus system (Millipore Canada) to a resistance of 18 MΩ. All other solvents and reagents were ACS grade and were purchased from Fisher Scientific (Fair Lawn, N.J.).

3.2. Methods

3.2.1. Biocatalysis of LOX in Aqueous/Miscible Organic Solvent Media

3.2.1.1 Enzyme Preparation

One mg of solid enzyme (1.42 mg protein) was suspended in 1 mL of Tris–HCl buffer solution (0.1 M, pH 7.3). The enzyme suspension was freshly prepared prior to the enzymatic assay and it was maintained at 4ºC to preserve its activity.

3.2.1.2. Protein Determination

The protein concentration of the soybean LOX was determined according to a modification of the Lowry method (Hartree, 1972), using bovine serum albumin (BSA) as a standard for the calibration curve.

3.2.1.3. Substrate Preparation

Linoleic acid was used throughout this study as the substrate model. To assess the LOX activity in aqueous medium, a final concentration of $4x10^{-3}$ M linoleic acid in the appropriate buffer solution (0.1 M) was prepared according to the procedure described by Kermasha and Metche (1986). For the enzymatic assay in the ternary micellar organic solvent system, the substrate was directly introduced into the reaction medium according to the procedure described previously by Kermasha *et al*. (2001). The substrate-buffer mixture was homogenized by adding 10 µM (11.2 µL/mL) of Tween-40 as a surfactant.

3.2.1.4. FOX Reagent Preparation

The FOX reagent was prepared freshly, since its sensitivity was found to be decreased with time (Piazza, 1992). FOX assay was carried out according to the procedure described by Jiang *et al*. (1991) and optimized in our laboratory by Vega *et al.* (2005b). To prepare the FOX reagent, 50 mM (0.0139 g) of ferrous sulfate and 200 mM (0.0152 g) of Xylenol Orange were solubilized in 200 mL mixture of perchloric acid (850 mM) and deionized water at a ratio of 1:9 (v/v), respectively.

3.2.1.5. LOX Enzymatic Assay

The LOX enzymatic assay was carried out according to the method described by Kermasha *et al.* (2001). A LOX enzyme suspension (1 mg of solid enzyme/mL) was prepared in Tris-HCl buffer solution (0.1 M, pH 7.3). All assays were performed in duplicate trials.

For the enzymatic assay in aqueous medium, a freshly stock solution of linoleic acid $4x10^{-3}$ M was prepared in Tris-HCl buffer solution (0.1 M, pH 9.0). A 100 µL from the substrate preparation stock solution $4x10^{-3}$ M was added to 880 µL Tris–HCl buffer solution (0.1 M, pH 9.0). The reaction was initiated by the addition of LOX enzymatic

suspension (20 µL of enzyme/mL) to the reactor. In parallel, 20 µL of Tris-HCl buffer solution (0.1 M, pH 9.0) was added to the blank trial to adjust the volume to a total of 1 mL. The reaction mixture was stirred for 3 min at 25ºC. Samples of 20 µL were withdrawn continuously during the first 3 min period of the reaction to measure spectrophotometrically at 560 nm the LOX specific activity. In parallel, 20 µL of control trial without LOX was also carried out in tandem of the assay.

For the enzymatic assay in ternary micellar system, a freshly prepared mixture of Tris-HCl buffer solution (0.1 M, pH 9.0), containing either 2% iso-octane or 2% hexane and 10 µM of Tween-40. A volume of 970 µL of the prepared mixture plus 10 µL of linoleic acid (32 µM) was introduced in the reactor, with a total reaction volume of 980 µL. The enzymatic reaction was initiated by the addition of 20 µL of the LOX suspension (1 mg solid enzyme/mL) to the reaction mixture, and was stirred for 3 min at 35^{o}C. The temperature was maintained by using a circulating water-bath. Aliquots of 20 µL were withdrawn from the reactor, where 100 µL of methanol was used to halt the enzymatic activity, followed by 2 mL of the Xylenol Orange reagent. The complex was then agitated for few seconds with Vortex (Genie 2; Fisher Scientific). The mixture was allowed to be settled at room temperature and the color generated being stabilized for 15 min. Using a mixture of 2.0 mL of the Xylenol Orange reagent and 100 µL of methanol, the spectrophotometer was calibrated at 560 nm. After 15 min of the addition of FOX reagent, the absorbance were measured spectrophotometrically at 560 nm, using a Beckman spectrophotometer (Model 650; Beckman Instruments, Inc., Fullerton, CA), and the formation of conjugated diene was then calculated. A blank assay containing all the components in the FOX assay, except the enzyme, was carried in tandem of the trials. Purified HPOD was used as a calibration curve. The LOX specific activity was defined as nmol of conjugated diene hydroperoxides (HPODs) per mg protein per min reaction.

3.2.1.6. Effect of Protein Content on LOX Activity

The effect of different enzymatic protein concentrations on LOX activity was determined. The suspensions of LOX (0 to 80 µg of protein/mL) were freshly prepared in Tris-HCl buffer solution (0.1 M, pH 7.3).

3.2.1.7. Effect of Organic Solvent Concentration on LOX Activity

Organic solvents, including hexane and iso-octane, were used for the LOX biocatlysis at concentration ranging from 0 to 6%. The conversion of linoleic acid into its hydroperoxides by LOX was achieved, at a temperature of 35ºC, in microemulsions medium containing a surfactant, Tris–HCl buffer solution (0.1 M, pH 9.0) and the selected organic solvent.

3.2.1.8. Effect of Surfactants on LOX Activity

LOX activity was assayed in the presence of a wide range of Spans (20, 40, 60 and 80) as well as Tweens (20, 40, 60 and 80). A series of dilutions of each emulsifier and surfactant suspensions (0 to 80 µM) was prepared in the reaction medium that consisted of a mixture of Tris–HCl buffer solution (0.1 M, pH 9.0) and hexane at a ratio of 98:2 (v/v). The effect of the surfactants/emulsifiers on LOX specific activity was investigated.

3.2.1.9. Effect of pH on LOX Activity

The effect of pH on LOX kinetics was investigated by pre-incubating the enzyme at various pH values. The incubation pH value was adjusted by using different buffer solutions (0.1 M), consisting of sodium phosphate (pH 6.0 to 8.0), Tris–HCl (pH 8.5 to 9.0) and sodium carbonate (pH 9.5 to 10.5). The assays were performed either in the aqueous medium with the appropriate buffer solution (0.1 M) or in the ternary micellar system composed of the selected buffer solution, containing 2% hexane and 10 µM Tween-40. The enzyme was added to the reaction and the mixture was stirred mechanically in the small reactor.

3.2.1.10. Effect of Temperature on LOX Activity

The effect of temperature on LOX activity was studied by incubating the enzymatic mixture at a wide range of temperatures (25 to 45ºC). The temperature was controlled by using a circulating water-bath. The enzymatic assays were performed in ternary micellar system, composed of a mixture of Tris-HCl buffer solution (0.1 M, pH 9.0) and hexane at a ratio of 98:2 (v/v), containing 10 µM of Tween-40. An enzymatic assay in the aqueous medium composed of Tris-HCl buffer solution (0.1 M, pH 9.0) was also conducted as a control trial.

3.2.1.11. Effect of Substrate Concentration on LOX Activity

The effect of substrate concentration (0 to 2 mM) linoleic acid on LOX activity was investigated in ternary micellar and neat organic systems, using hexane as the selected organic solvent.

3.2.2. Biocatalysis of LOX in Neat Organic Solvent Media (Neat-OSM)

3.2.2.1. Substrate Preparation

A 10 µL from the stock solution (32 µM) of linoleic acid was withdrawn and dissolved in 970 µL of hexane for the biocatalysis in neat-OSM.

3.2.2.2. Enzyme Preparation

One mg of soybean LOX was suspended in 1 mL of Tris–HCl buffer solution (0.1 M, pH 7.3).

3.2.2.3. FOX Assay in Neat-OSM

The FOX reagent used in neat organic solvent media (OSM) system was prepared as in section 3.2.1.1., except that 200 mL of methanol:water mixture at a ratio of 9:1 (v/v) plus 428 µL of perchloric acid (70%) was added to the mixture. In all experiments a blank was carried out which contains all the components in the sample except the enzyme for determination of the possible activity during the incubation period.

3.2.2.4. Effect of Initial Water Activity (a_w)

One of the parameters that could affect the LOX activity in neat-OSM was the thermodynamic water activity *(a_w)*. Water activity *(a_w)* is defined as the ratio of the partial pressure of water in the system and in the pure state. In neat-OSM, *(a_w)* is a measurement of the distribution of water vapour in the media.

The effect of initial water activity (a_w) on LOX activity was investigated in the range of 0.11 to 0.75. Before the enzyme activity measurement, the selected organic solvent, hexane, was adjusted to the desired a_w by pre-incubating separately in well sealed vessels containing the saturated salt solutions, wetted with deionized water at 25ºC. The wetted salts prepared in the vessels were including, $LiCl_2$ (water activity, a_w = 0.11), $MgCl_2$ (a_w

= 0.33), Mg_2NO_3 (a_w = 0.53) and NaCl (a_w = 0.75). Water activity equilibration was performed over a 72 h period of time at room temperature. Enzyme samples were weighed on a microbalance and hydrated with the same range of water activity (a_w = 0.11 to 0.75) as for the solvents in separated equilibration chambers for 24 h at 4ºC. Enzymatic reactions were carried out as described previously by Vega *et al.* (2005a).

3.2.2.5. Effect of Reaction Temperature

The effect of reaction temperature over a wide range, 20 to 45°C, on the specific activity of lipoxygenase in neat-OSM was investigated.

3.2.2.6. Thermal Stability

The LOX thermal stability in organic solvent media was investigated by incubating 1 mg of the solid purified enzyme in 1 mL of hexane (a_w = 7.5), with a wide range of incubation time (0 to 70 min) at 35°C. Twenty µL of LOX suspension were added to pre-mixture of 970 µL of hexane and 10 µL substrate with constant agitation speed of 200 rpm. The residual LOX activity was measured according to the standard assay conditions. In parallel, a comparative study with the aqueous medium was also conducted. The inactivation constant, k_t, and the half-life, T_{50}, which was defined as the temperature required in a reaction medium to report a 50% decrease in the initial activity of LOX, were determined from the semi-logarithmic plots of the inactivation kinetics, according to the following equation:

$$\ln (A/A_0) = -k_t \cdot T$$

where, T was the incubation time at 35°C; A and A_0 were the LOX activity with a defined incubation time T and without incubation, respectively.

3.2.3. Kinetic Parameters

LOX activity was investigated at a wide range of concentrations (0 to 35 µM) linoleic acid, in the ternary micellar system, composed of a mixture of Tris-HCl buffer solution (0.1 M, pH 9.0) and hexane at a ratio of 98:2 (v/v), containing 10 µM of Tween-40 and in neat-OSM containing hexane. The enzymatic reaction in the ternary micellar system was carried out using the optimized conditions (35°C, 1 mg of solid enzyme and 200 rpm

agitation) and was monitored over the course of a 3 min reaction period. The optimized conditions for the enzymatic reaction in neat-OSM were 35°C, a_w = 0.75, 1 mg of solid enzyme and 200 rpm agitation; the reaction was monitored over the course of a 30 min reaction period. After the enzyme addition, the absorbance at 560 nm was continuously detemined by taking 20 µL aliquot from the enzymatic reaction homogenate. The reaction was halted by the addition of 100 µL of methanol, followed by the addition of 2 mL of FOX reagent. The enzymatic assays were performed in duplicate. The measurements of the enzymatic rate as a function of substrate concentration was plotted (Lineweaver and Burk, 1934) and Michaelis constant (K_m) and maximum velocity (V_{max}) for LOX were calculated from the slope of $1/v$ versus 1/[S]. The lines were drawn to the best fit of experimental points.

4. RESULTS AND DISCUSSION

4.1. Introduction

Previous research work in our laboratory have shown that the biocatalysis of lipoxygenase can be greatly enhanced by using organic solvents rather than conventional aqueous medium (Kermasha *et al*., 2001 and 2002a; Vega *et al*., 2005a,b and 2006). The appropriate solvent for biocatalysis, which has an important effect on the rate of the reaction and on the stability of the biocatalyst (Drauz and Waldmann, 2002) might be chosen on the basis of water activity of the media and the nature of substrate.

4.2. Choice of Solvent

The effect of non-conventional media on the biocatalysis of the commercially purified soybean LOX was investigated. The results (Table 4) show that the LOX specific activity was increased by 1.7-fold when the catalytic reaction was performed in the ternary micellar system, composed of 2% hexane, as compared to that obtained in the aqueous one. The increase in LOX activity may be due to the solubilization of hydrophobic compounds that could alter the enzyme specificity (Klibanov, 1986). Moreover, the hydrophobic solvents have lower affinity for water, required for the activity of the catalyst; hence, the presences of hydrophobic solvents did not strip the essential water around the enzyme molecules (Kvittingen, 1994; Adlercreutz, 2000).

These results are in agreement with those reported by Vega *et al*. (2006), where they showed that both LOX activity and the proportion of α-helical conformation in the overall secondary structure of LOX were increased by 1.54-fold after pre-incubation in hexane solvent as compared to that in aqueous medium.

Moreover, a similar trend was reported for the LOX-1B activity, using a wide range of solvents, in which 4% octane and iso-octane were employed (Kermasha *et al*., 2001). These findings suggest that the presence of the organic solvent, in particular 2% hexane with Tris-HCl buffer solution (0.1 M, pH 9.0), resulted by a relative enhancement of soybean LOX activity.

The highest LOX specific activity of 4.91 nmol of HPODs/mg protein/min was obtained in 2% hexane (log *P*=3.50), with a ratio of activity (RO/A) value of 1.5×10^{-3}, where RO/A is the ratio of the specific activity in the organic solvent to that in the aqueous/miscible organic solvent medium. Although the log *P* of the reaction mixture with 2% iso-octane remained higher than 3.5 as compared to that with hexane, the experimental findings showed a lower specific activity with the most hydrophobic solvent, the iso-octane, as denoted by its higher log *P* value, compared to that obtained in hexane. The results suggest that there was no correlation between the specific activity of LOX and the Log *P* value of the organic solvent. These findings are in agreement with those reported in literature (Van Tol *et al*., 1995).

4.3. Lipoxygenase in Ternary Micellar System

The specific activity of LOX in selected organic solvent media was investigated in terms of the effects of organic solvent, Enzyme concentration, pH of the media, surfactant, reaction temperature, thermal stability and kinetic parameters.

4.3.1. Effect of Reaction Temperature: A Comparison Between Aqueous and Organic Solvents

Using a wide range of organic solvents, previous work in our laboratory (Kermasha *et al.*, 2001), showed that the highest specific activity of LOX was obtained with iso-octane. A comparison between the optimum concentration of hexane for the highest LOX activity in ternary micellar system and that of iso-octane at reaction temperature ranging from 20 to 45°C was investigated. Figure 10A shows a comparison between the aqueous medium and that containing either 2% hexane or 2% iso-octane; it indicates that there was an increase in the optimum reaction temperature for LOX activity from 20ºC in the aqueous medium to 35ºC in the ternary micellar system containing 2% hexane or 2% iso-octane. The results also show that in the ternary micellar system, containing 2% hexane, there was 1.4-fold increase in enzyme activity as compared to that with 2% iso-octane. The specific activity of LOX in the 2% hexane media was 4.91 nmol of HPODs/mg protein/min as compared to that of 3.61 nmol of HPODs/mg protein/min in the 2% iso-octane. However, the results

Table 4. Lipoxygenase specific activity in a reaction medium.

Reaction system[a]	Specific activity[b]
Aqueous	2.86 (± 0.09)[c]
Iso-octane	3.61 (± 0.12)[c]
Hexane	4.91 (± 0.05)[c]

[a]The reaction system was composed of a selected organic solvent and Tris-HCl buffer (0.1 M, pH 9.0) at a ratio of 2:98 (v/v) plus 10 μM Tween-40.

[b]Specific activity was defined as nmol of hydroperoxides of linoleic acid (HPODs) per milligram of protein per minute.

[c]Relative standard deviation of samples was calculated for duplicate trials.

(Fig. 10A) show a decrease in LOX activity at temperature higher than 35^{o}C; the significant decrease in LOX activity might be due to the thermal inactivation of the enzyme.

Figure 10B shows a comparison of the linearity of the plots between the aqueous medium and those containing 2% hexane or 2% iso-octane using the Arrhenius plot, which is the natural logarithm (ln) of specific activity versus the reciprocal of the reaction temperature in absolute temperature (ºK). A quantitative evaluation of the temperature effect over the catalysis reaction can be obtained by calculating the activation energy (Stryer, 1998). The plot of the logarithm of LOX specific activity versus the reciprocal of the reaction temperature (Fig. 10B) shows a straight line, with a correlation coefficient (R^2) of 0.70, 0.88 and 0.99 for the aqueous medium and that containing 2% iso-octane or 2% hexane, respectively.

4.3.2. Effect of Enzyme Concentration

The effect of protein content on LOX activity in ternary micellar system was investigated. Similarly, protein concentration on LOX activity in the aqueous medium of Tris-HCl (0.1 M, pH 9.0) was also conducted as a control trial. The results (Fig. 11) show that the amount of protein required for the optimum LOX activity in the aqueous medium and that of 2% hexane was 20 and 15 µg, respectively, with a specific activity of 3 and 6 nmol of HPODs/mg protein/min, respectively. Increasing the enzyme molecules resulted in an increase in the number of active sites availability for the substrate. However, increasing the enzyme concentration beyond certain level, the rate of reaction will no longer depend upon its concentration; this is due to the enzyme-substrate complex saturation (Khamessan *et al*., 1993). These results are similar to those reported by Kermasha *et al*. (2001). The decrease in the specific activity of LOX at high enzyme loading (Fig. 11) suggests that the system was subjected to mass transfer limitations, where most probably the substrate diffusion towards the enzyme molecules reached its limit (Salis *et al.*, 2003). It is known that in heterogeneous catalysis, the chemical reaction may be limited by the mass transfer of the reagents towards the surface of the catalyst or the external diffusion (Kamat *et al.*, 1992).

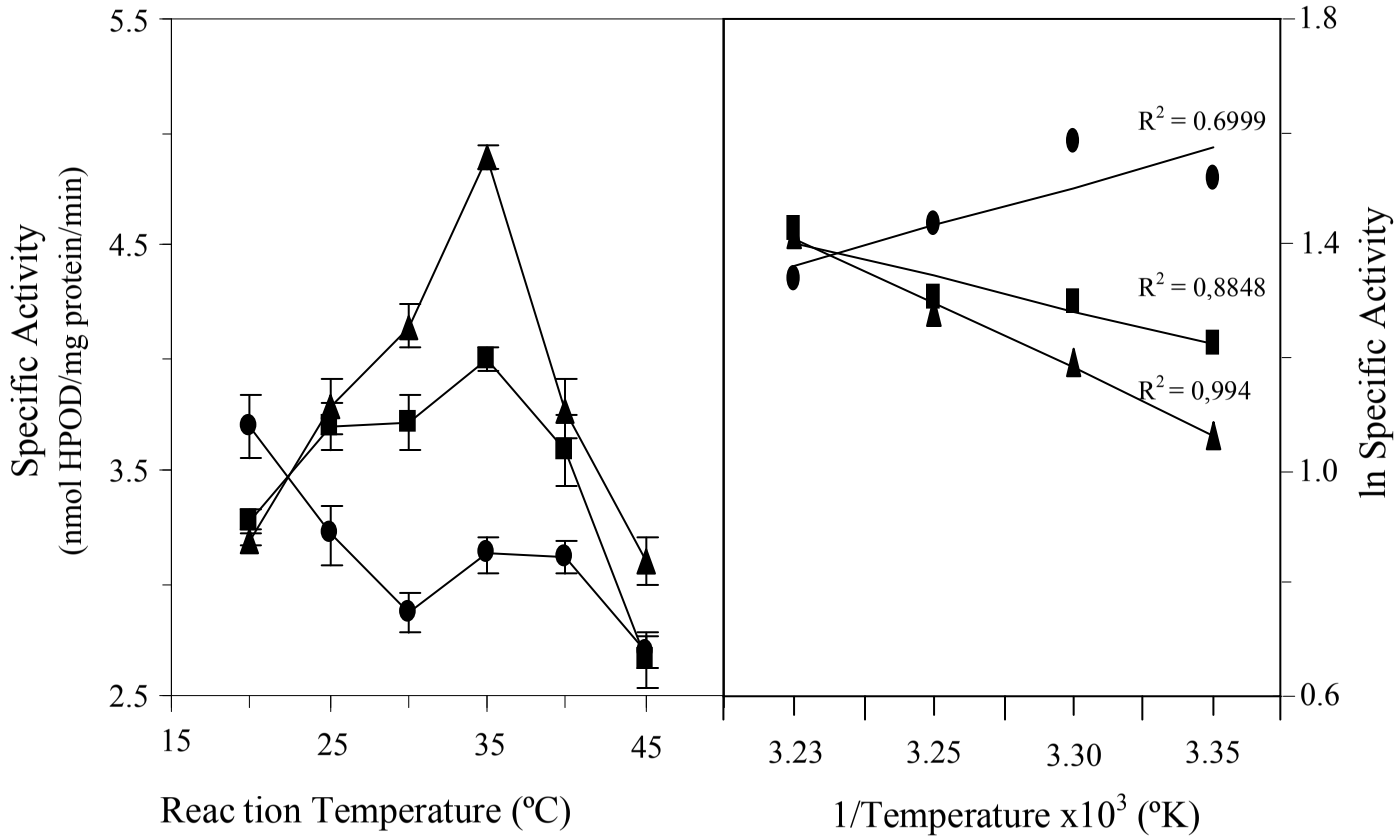

Figure 10. Effect of reaction temperature on the specific activity of lipoxygenase: a comparison between temperature on the specific activity of lipoxygenase: a comparison between (A) aqueous system specific activity of lipoxygenase: a comparison between (A) aqueous system of Tris-HCl buffer lipoxygenase: a comparison between (A) aqueous system of Tris-HCl buffer (0.1 M pH 9.0) comparison between (A) aqueous system of Tris-HCl buffer (0.1 M pH 9.0) (●—●), ternary between (A) aqueous system of Tris-HCl buffer (0.1 M pH 9.0) (●—●), ternary micellar system aqueous system of Tris-HCl buffer (0.1 M pH 9.0) (●—●), ternary micellar system a mixture

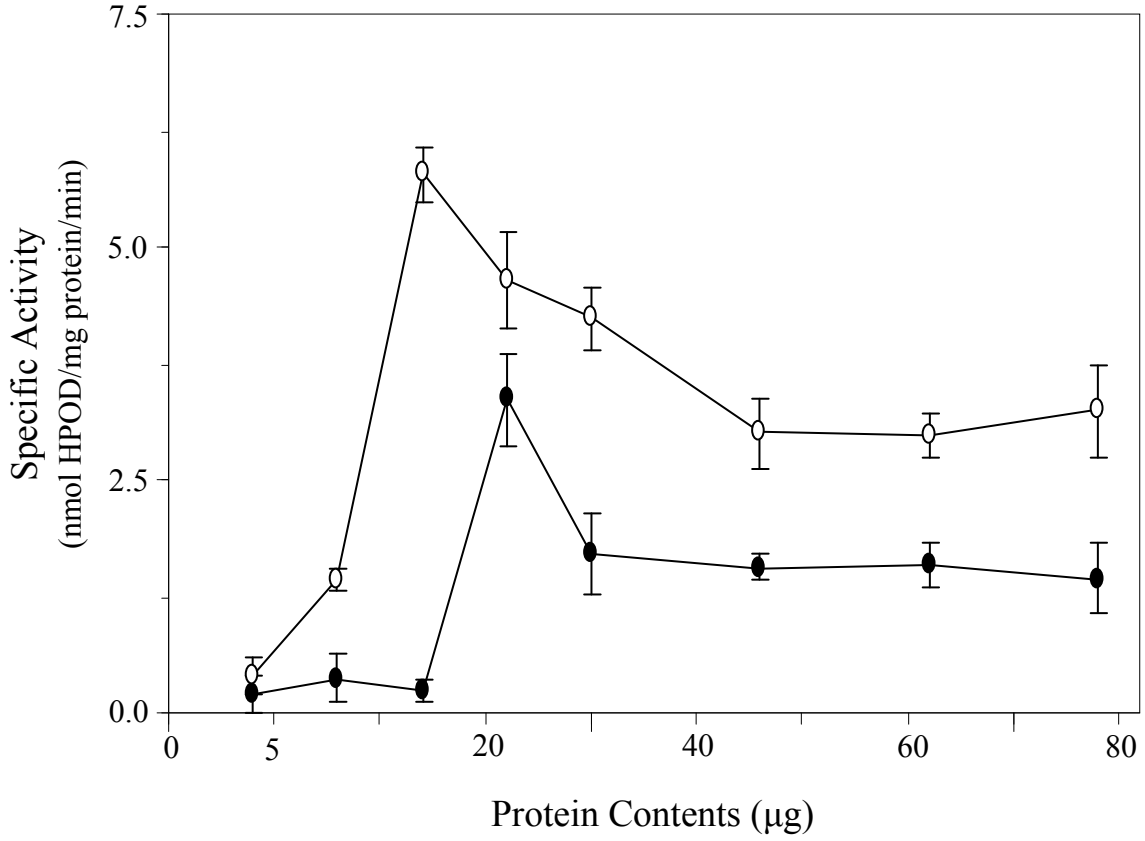

Figure 11. Effect of protein concentration on lipoxygenase specific activity in aqueous medium of Tris-HCl buffer (0.1 M, pH 9.0) (●—●), and in ternary micellar system a mixture (2:98, v/v) of hexane: Tris-HCl buffer (0.1 M, pH 9.0) and 10 µM Tween 40 (○—○).

4.3.3. Effect of pH

The effect of pH on the specific activity of LOX in ternary micellar system, composed of Tris-HCl buffer solution (0.1 M, with a range of pH 7.0 to 10.5) and hexane at a ratio of 98:2 (v/v), containing 10 μM Tween-40, was investigated. The results (Fig. 12) show that the optimum pH value for LOX activity for both aqueous and organic solvent media was 9.0, followed by a further decrease to pH 10.5. Pizza (1992) reported similar pH value of 9.0 for LOX maximal activity. The results (Fig. 12) also show that at pH 7.5, the specific activity of LOX in the ternary micellar system was only 12.5% of that obtained at pH 9.0; however, at pH 7.0 the LOX specific activity in the ternary micellar system was only 5% of that at pH 9.0. Kermasha *et al*. (2002a) reported that the optimum pH for the LOX activity in the ternary micellar system composed of a mixture of 96:4 (v/v) of Tris-HCl buffer solution (0.1 M, pH 9.0) and iso-octane, containing 10 μM Tween-40 was 9.0. Allen (1968) explained that the low activity of LOX at low pH might be due to the insolubility of linoleic acid in this region. Furthermore, the decrease in LOX activity could also be attributed to the secondary structure of LOX at pH values lower than 7 and higher than 9 where different proportions of the corresponding ratios of β-sheets become slightly predominant to those of α-helix ones (Vega *et al*., 2006). The LOX activity profile (Fig. 12) is in agreement with those reported in literature (Asbi *et al*., 1989; Siedow, 1991; Kermasha *et al*., 2002a; Vega *et al*., 2006). Rodakiewicz-Nowak *et al*. (1996) reported that pH has an effect on the intrinsic LOX activity and on the ionization of linoleic acid, where only at sufficiently high value of pH, it is possible to maintain the substrate concentration at value close to the saturation of the enzyme.

4.3.4. Effect of Temperature

The effect of a wide range of temperatures (20 to 45^{o}C) on LOX activity in ternary micellar system, composed of Tris-HCl buffer solution (0.1 M, pH 9.0) and hexane (0 to 6%), containing 10 μM of Tween-40, was investigated. The results (Fig. 13) show that the optimum enzymatic activity of LOX in all concentrations of hexane in the ternary micellar system was 35ºC, whereas that in the aqueous buffer system was at 20ºC. However, increasing the temperature beyond these values resulted by a steady decrease in LOX activity. The trend obtained for the effect of temperature on LOX activity in the aqueous

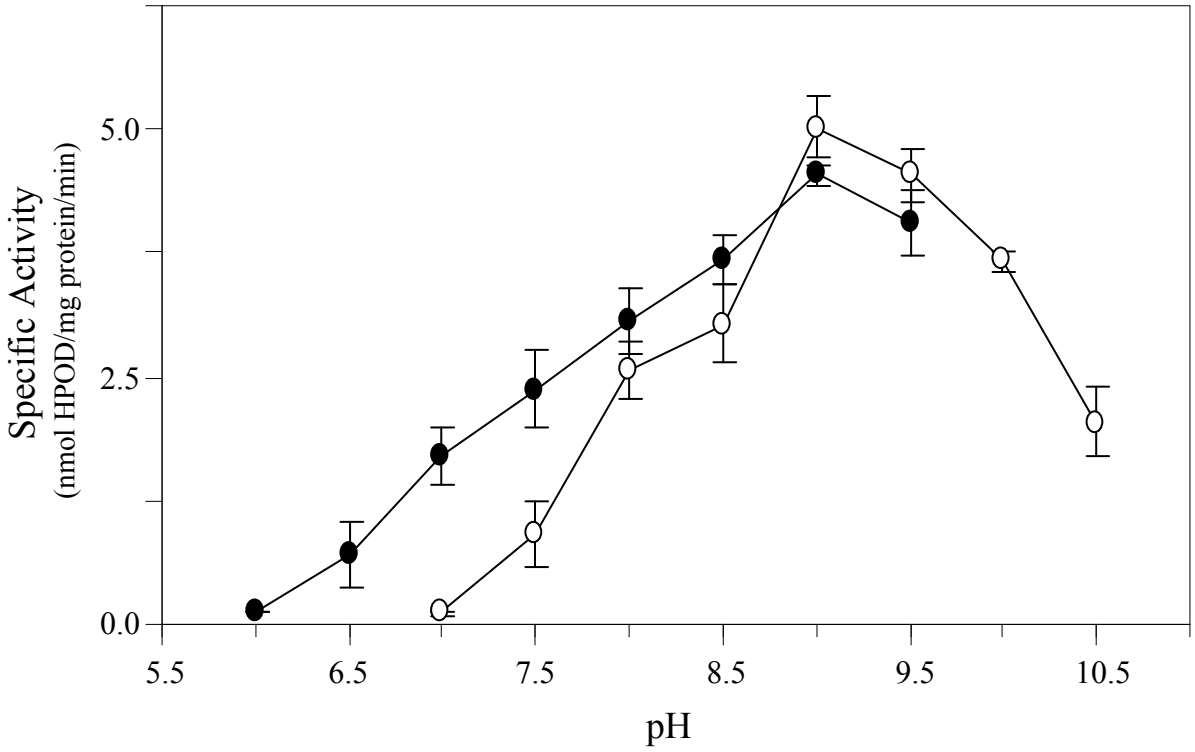

Figure 12. Effect of pH on the specific activity of LOX in aqueous medium of Tris-HCl buffer (0.1 M, pH 9.0) (●—●), and in ternary micellar system a mixture (2:98, v/v) of hexane: Tris-HCl buffer (0.1 M, pH 9.0) and 10 µM Tween-40 with pH between (7.0-11) (○—○).

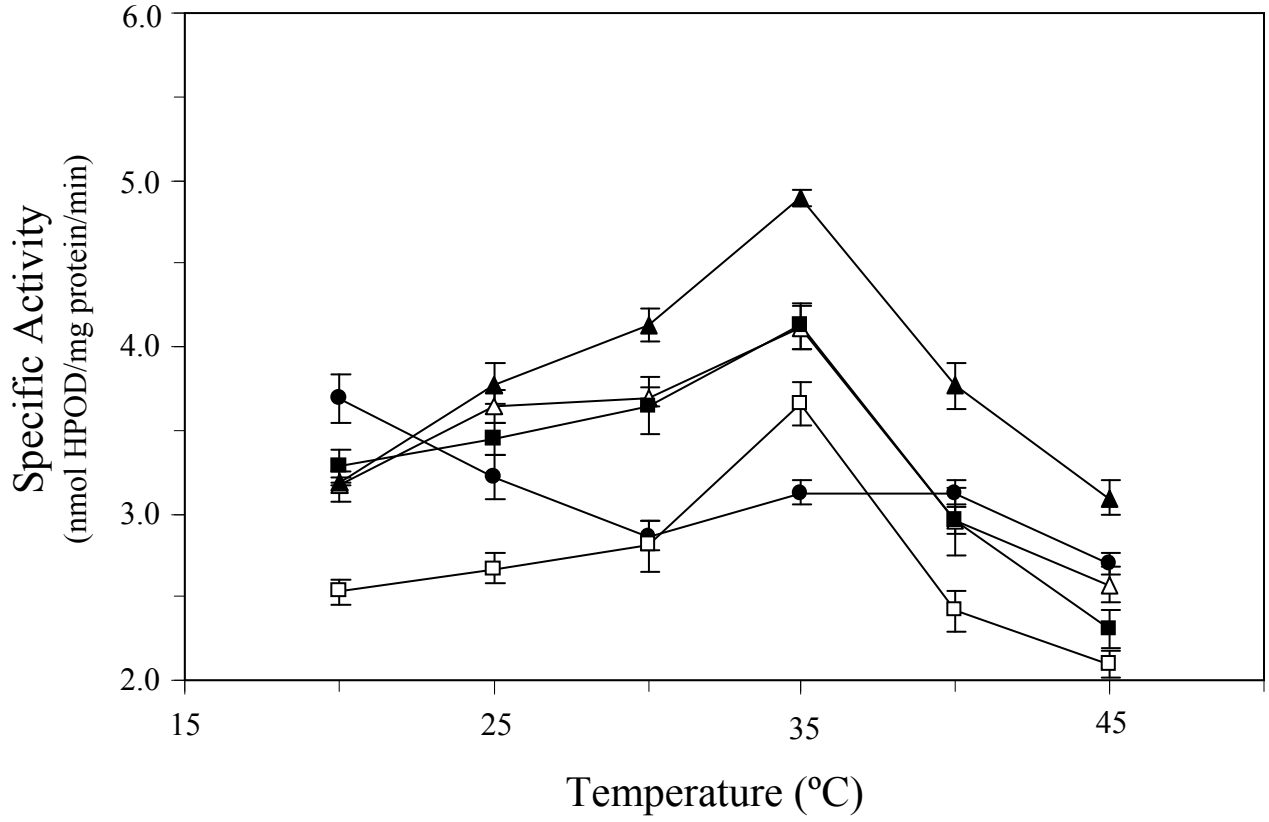

Figure 13. Effect of temperature on lipoxygenase specific activity in aqueous system of Tris-HCl buffer (0.1 M, pH 9.0) (●—●), and ternary micellar system a mixture of Tris-HCl buffer (0.1 M, pH 9.0), 10 μM of Tween-40 in selected hexane concentrations: 1% hexane (Δ—Δ), 2% hexane (▲—▲), 4% hexane (■—■), and 6% hexane (□—□).

medium was similar to that reported by Kermasha *et al*. (2001). Moreover, the results (Fig. 13) show that there was an increase in LOX activity of 1.7-fold at 2% hexane and 1.3-fold for the concentrations of 1 and 4%, whereas at 6% hexane there was a slight increase in enzyme activity of 1.2-fold as compared to that in the aqueous medium. The overall results suggest that the presence of organic solvent in the aqueous medium allows better solubilityof the hydrophobic substrate, which could lead to a higher interaction between the enzyme and the substrate.

4.3.5. Effect of Different Types of Surfactant

The enzymatic activity of soybean LOX in ternary micellar system, composed of Tris-HCl buffer solution (0.1 M, pH 9.0) and hexane (98:2, v/v), containing either an anionic surfactant (AOT: sodium bis-2(ethylhexyl) sulphosuccinate), or a cationic surfactant (CTAB: cetyl trimethyl ammonium bromide), or a non-ionic surfactant (Tween-40: polyoxyethylene sorbitan monopalmitate), was investigated. The results (Fig. 14) show a decrease in the LOX activity when the anionic surfactant was used, followed by that of cationic one. The highest specific catalytic activity of LOX was obtained when the non-ionic surfactant, Tween-40, was used. The specific activity of soybean LOX was 4.91 nmol HPODs/mg protein/min when Tween-40 was used as compared to that of 2.7 and 1.8 nmol HPODs/mg protein/min, for CTAB and AOT, respectively. Sonati and Appu Rao (1996) reported that the non-ionic surfactant, Tween-40, strengthens the hydrophobic environment around cysteine residues, which may enhance the stability of LOX by preventing the specific aggregation due to sulfhydryl oxidation; these authors also reported that non-ionic surfactants may contribute to the prevention of the non-specific aggregation during unfolding and refolding of the enzyme. Hence, the non-ionic surfactant, Tween-40, was used in all subsequent study throughout this investigation.

4.3.6. Effect of Surfactant Concentration

The effect of the surfactants Tweens and Spans, at a wide range of concentrations (0 to 80 μM), on LOX activity in the ternary micellar system of a mixture of Tris-HCl buffer solution (0.1 M, pH 9.0) and hexane at ratio of 98:2 (v/v) was investigated. The results (Fig. 15A) show that a concentration of 10 μM of Tweens 20, 40, 60 and 80 in the reaction

medium, containing Tris-HCl buffer solution (0.1 M, pH 9.0) and hexane at a ratio of 98:2 (v/v), yielded higher LOX specific activity of 3.97, 4.91, 3.97 and 3.10 nmole of HPODs/mg protein/min, respectively. However, the highest specific activity of LOX was obtained with the use of Tween-40. Further increase in the concentration of Tweens resulted in slight increase in LOX specific activity as compare to that obtained with concentration of 10 µM.

Figure 15B shows that in the presence of the reaction media of Tris-HCl buffer solution (0.1 M, pH 9.0) and hexane at a ratio of 98:2 (v/v), containing Spans at concentration of 20 to 80 µM, there was a slight increase in LOX specific activity with 20 µM concentration of Span 40; this slight increase represents 1.2-fold in the LOX activity. However, the presence of wide concentrations (0 to 80 µM) of Spans 20, 60 and 80 in the reaction medium of Tris-HCl buffer solution (0.1 M, pH 9.0) and hexane at a ratio of 98:2 (v/v) resulted by the absence of LOX activity.

The overall results obtained in this study and those reported by Kermasha *et al*. (2001) suggest that in ternary micellar system, the biocatalysis of LOX resulted in lower LOX activity when Spans were used as emulsifiers as compared to that with Tweens as surfactants. These results may be due to many factors, including; (a) the difference in the degree of hydrophobicity of the Tweens and Spans; (b) the presence of surfactant causes an increase in the solubility of both enzyme and substrate at the interface (Nada and Yadav, 2003) as compared to that of in presence of Span emulsifiers; and (c) the length of the hydrophobic tail of Tweens may influence the LOX activity as compare to Spans. Further decrease in LOX activity may be due to its denaturation and hence to its inactivation of enzyme. These results are in agreement with those reported by Kermasha *et al.* (2001).

On the basis of these experimental findings, the most appropriate surfactant, Tween-40 with optimum concentration of 10 µM in the reaction media composed of Tris-HCl buffer solution (0.1 M, pH 9.0) and hexane at a ratio of 98:2 (v/v), was used for further experimental studies for the biocatalysis of LOX.

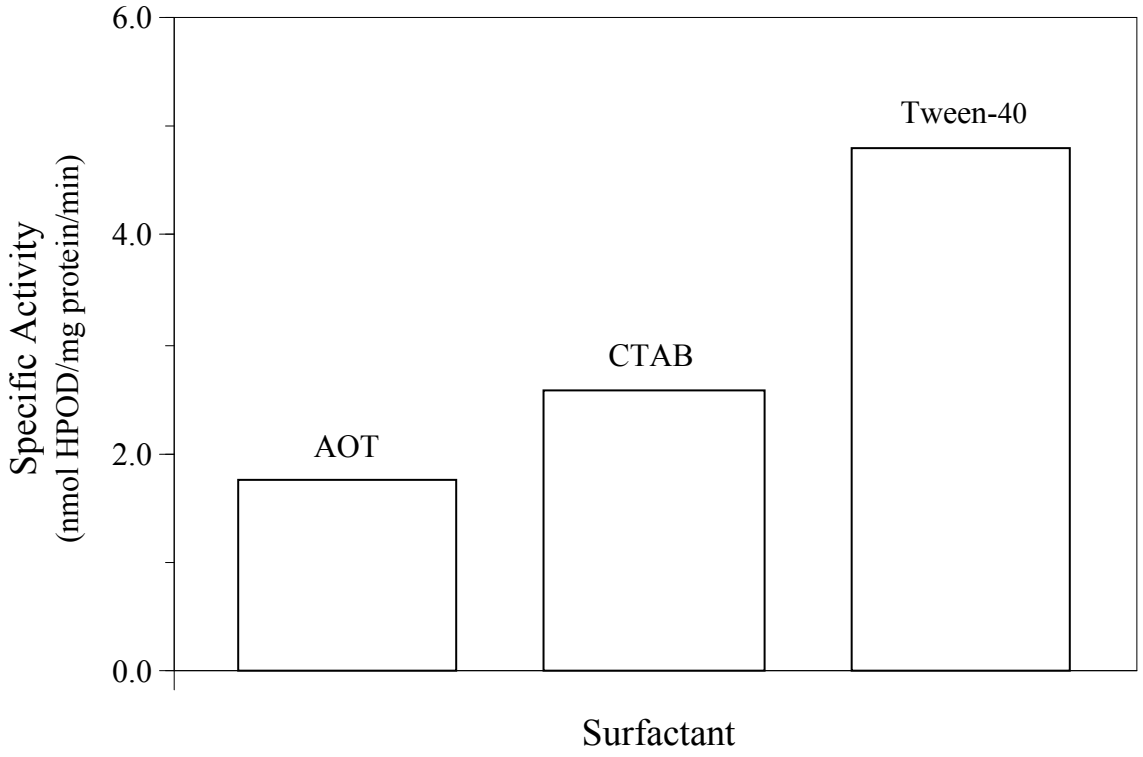

Figure 14. Effect of surfactant types on the specific activity of LOX in a ternary micellar system composed of Tris-HCl buffer (0.1 M, pH 9.0) containing 2% hexane and 10 μM of a surfactant, sodium bis-2(ethylhexyl) sulphosuccinate (AOT), cetyl trimethyl ammonium bromide (CTAB) and polyoxyethylene sorbitan monopalmitate (Tween-40).

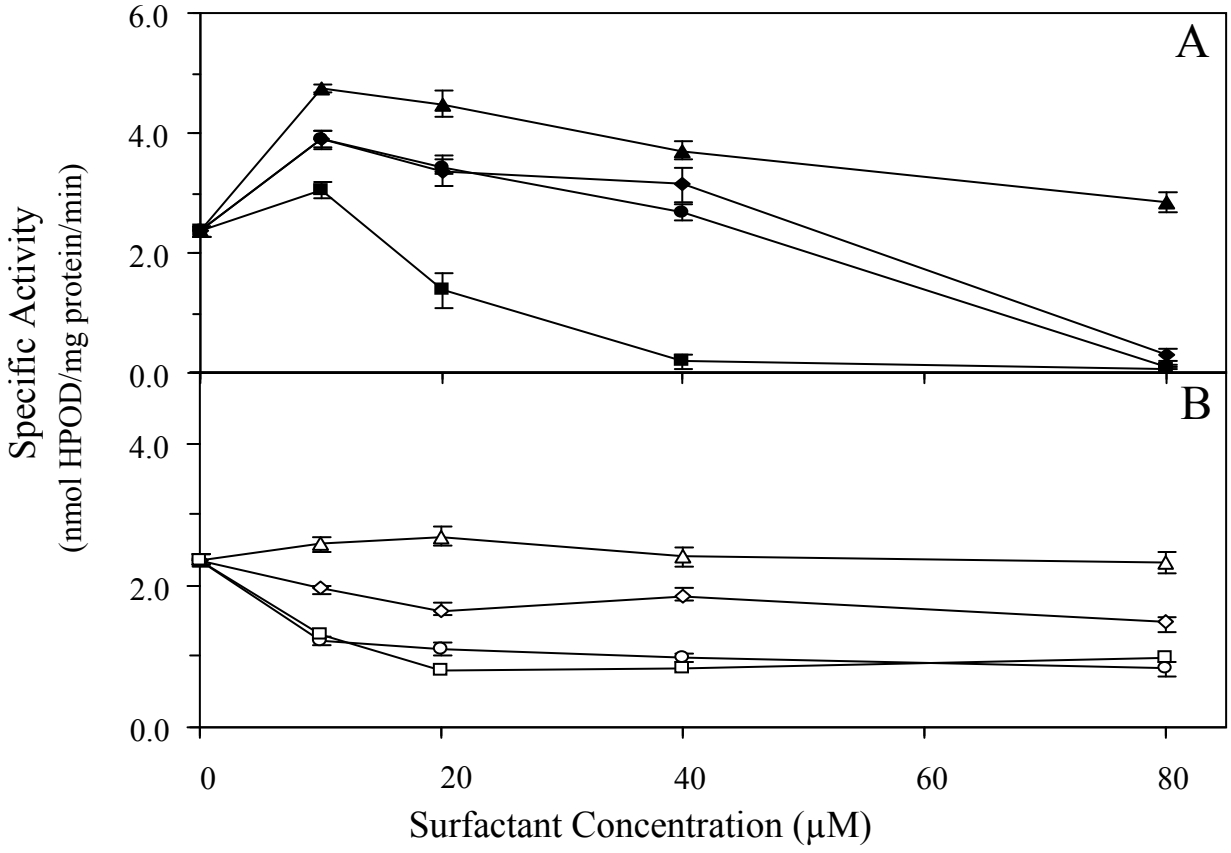

Figure 15. Effect of Surfactant concentration (0 to 80 μM) on LOX specific activity in ternary micellar system a mixture (2:98 v/v) of hexane: Tris-HCl buffer (0.1 M, pH 9.0) and (A) Tweens: Tween 20 (●—●), Tween 40 (▲—▲), Tween 60 (♦—♦) and Tween 80 (■—■); (B) Spans: Span 20 (○—○), Span 40 (Δ—Δ), Span 60 (◊—◊) and Span 80 (□—□).

4.3.7. Thermal Stability in Ternary Micellar System

The thermal stability of soybean LOX in ternary micellar system, composed of Tris-HCl buffer solution (0.1 M, pH 9.0) and hexane at a ratio of 98:2 (v/v) containing 10 µM Tween-40, using optimum temperature of 35ºC, was investigated. The LOX mixture was pre-incubated at 35ºC for periods between 0 to 70 min. The results (Fig. 19A) show that the residual LOX specific activity after incubation at 35ºC for a period of 45 min., was 72.7% of its initial activity as compared to 44.9% for the aqueous medium.

From the semi-logarithmic plots (Fig. 19B), the inactivation constant and half-life (T_{50}) of LOX in aqueous and ternary micellar systems, obtained from equation 1 are summarized in (Table 5), estimated to be 27.61 and 66.63 min, respectively. Hence, the T_{50} of LOX was increased by a factor of 2.4 upon the use of the ternary micellar system. The results (Fig. 19B) also indicate that the thermal inactivation of LOX followed the first-order kinetics behavior as indicated by the linearity of the semi-logarithmic plots.

4.3.8. Effect of Substrate Concentration

As the substrate concentration increases, the rate of reaction also increases until the reaction reaches a saturation or so-called maximal velocity (V_{max}), at which any further increase in substrate concentration will not cause further increase in reaction (Copeland, 2000). The substrate concentration for which the reaction rate is half of V_{max} is termed Michaelis constant (K_m). Enzymes that have low K_m have high affinity to the substrate and act at maximal velocity at low substrate concentration (Drauz and Waldmann, 2002). The results (Table 5) summarize the K_m, V_{max} and catalytic effecincy values for LOX activity in organic solvent media, including the ternary micellar system. The kinetic parameters of K_m and V_{max} values for LOX activity, calculated from Lineweaver-Burk plots of $1/v$ versus 1/[S] using linoleic acid as a substrate at concentrations ranging from 0 to 2 mM and $1/v$ versus 1/[S], indicate (Table 6) that the lowest K_m value was obtained with the ternary micellar system, suggesting that the presence of a surfactant in the ternary micellar system may contributed to better interaction between LOX and its substrate, linoleic acid. The K_m value of 7.7 µmol is in agreement with the results reported by Kermasha *et al.* (2002a) in the ternary micellar system. Similarly, V_{max} values for LOX biocatalysis in the ternary micellar system composed of Tris-HCl (0.1 M, pH 9.0), 2% hexane and 10 µM Tween-40

was 30.0 nmol HPODs/mg protein/min. Although Vega *et al*. (2005a) reported a lower soybean LOX affinity for the substrate; the experimental findings (Table 6) indicate that the V_{max} values are higher than those reported by these authors. The apparent catalytic efficiency of soybean LOX, which is defined as V_{max}/ K_m, was found to be 3.90 min^{-1} in the ternary micellar containing 2% hexane, which is similar to that reported by Kermasha *et al.* (2001) and Vega *et al.* (2005a).

4.4. Lipoxygenase Activity in Neat Organic Solvent

In order to optimize the biocatalytic activity of LOX in neat organic media, the effects of various parameters, including initial water activity (a_w), agitation speed, reaction temperature, thermal stability and substrate concentration, on the enzymatic activity were investigated.

4.4.1. Effect of Initial Water Activity (a_w)

The catalytic activity of enzyme in non-aqueous media increases with the increase in water activity (Ma *et al.*, 2002). For the biocatalysis in neat organic media, the effect of initial a_w values on the specific activity of soybean LOX was investigated. The biocatalyst, LOX, and the solvent, hexane, were pre-equilibrated separately at a wide range of a_w values (0.23 to 0.75).

The specific activity of LOX increased with the increase in the water activity (a_w) up to a 0.53; however, at a_w above 0.53 and up to 0.75, the LOX specific activity was increased only to a small extant (Fig. 16). The results (Fig. 16) also show that an increase in the initial a_w of the reaction system, from 0.23 to 0.75, resulted in 8-fold increase in LOX specific activity. The higher enzymatic activity at a high initial a_w may be due to the need for higher amount of water to maintain the enzyme active conformation for its optimal activity (Klibanov, 1986; Dordick, 1992; Halling, 2002). The initial a_w values for the optimal catalytic activity was reported by Wehtje and Adlercreutz (1997) to be dependent on the nature of surrounding environment of the reaction media.

The overall results suggest that a certain low amount of residual water is needed for enzyme activity. The availability of water in the reaction medium could be an important

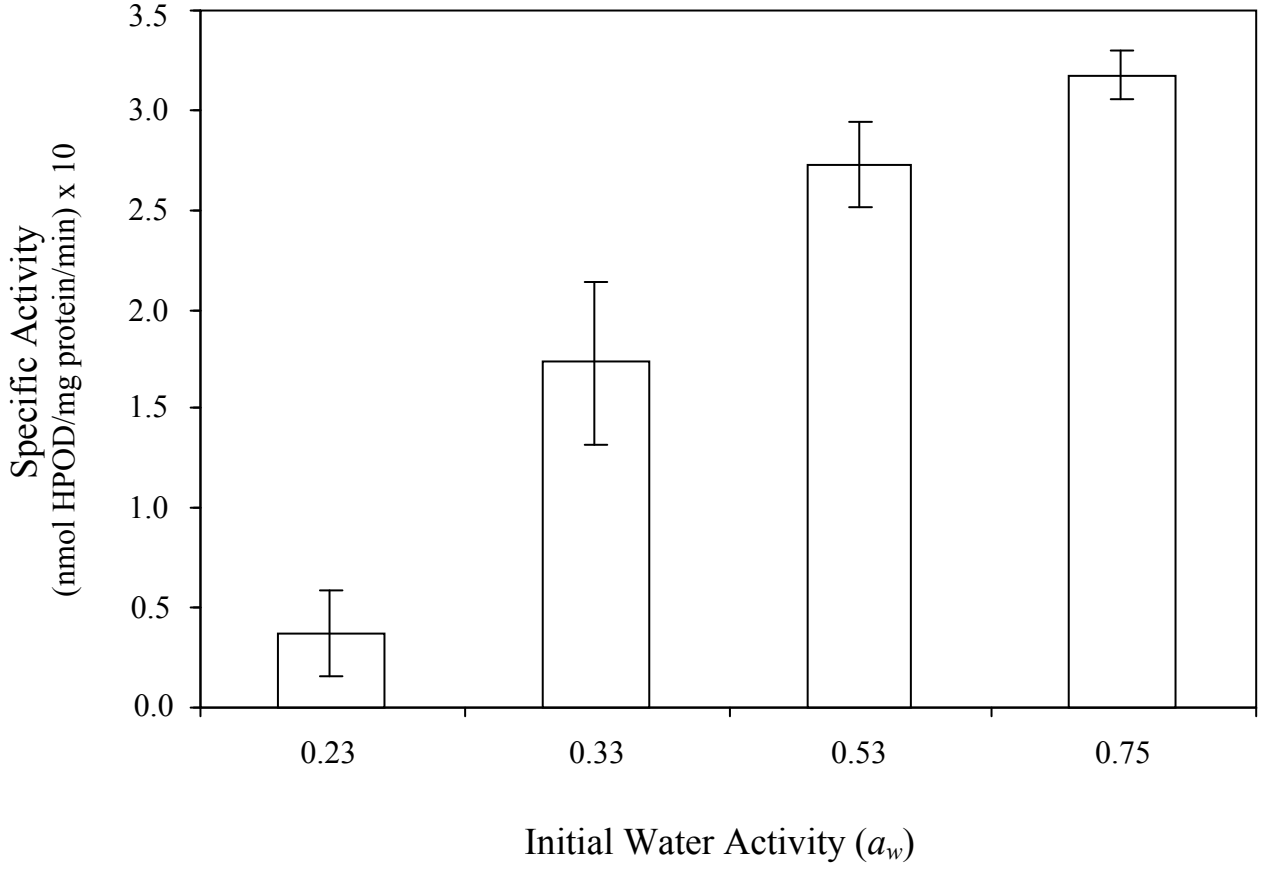

Figure 16. Effect of initial water activity (a_w) on soybean lipoxygenase specific activity in the neat organic solvent.

Table 5. Inactivation constants and half-life parameters of LOX in a reaction medium.

Reaction medium	Thermal parameters[a]	
	k_t (min^{-1}) [b]	T_{50} (min) [c]
Aqueous[d]	0.025	27.61
Ternary micellar[e]	0.010	66.63
Neat organic[f]	0.005	138.6

[a]Parameters were obtained from the linear equation $\ln(A/A_0) = -k_tT$, in which A is the activity at incubation time, T; A_0 is the activity without deactivating incubation period; and k_t is the constant of inactivation of the incubation at 35°C.

[b] Constant of inactivation as determined from the first-order kinetics behavior of the inactivation effect of increasing the incubation time at 35°C.

[c] Defined as the incubation time at 35°C in a reaction medium required to report a 50% decrease in the initial activity.

[d]The aqueous medium was composed of 2% hexane in Tris–HCl buffer solution (0.1 M, pH 9.0).

[e]The ternary micellar system was composed of 2% hexane in Tris–HCl buffer solution (0.1 M, pH 9.0) and 10 μM of Tween-40.

[f]The neat organic was composed of hexane solvent.

parameter for LOX activity in the neat organic media, which plays a critical role in maintaining the active conformation of the enzyme (Adlercreutz, 2000). Based on these experimental findings, the initial a_w value of 0.75 was selected for further experimental work.

4.4.2. Effect of Agitation

The effect of agitation speed on the biocatalytic activity of LOX was investigated. The results (Fig. 17) show that an increase in the agitation speed from 0 to 200 rpm resulted in a 8.5-fold increase in LOX specific activity, from 0.2 to 1.7 nmol HPODs/mg protein/min. Those results are in agreement with those reported by Piazza (1992), where 250 rpm of agitation speed used for the formation of HPODs. The increase in the relative specific activity of LOX with the increase in the agitation speed may be attributed to many factors, including a decrease in the formation of enzyme aggregates, or in the attachment to the walls of the rector (Salis *et al*., 2003) and as well as to a reduction in the layer boundary between the solvent and the catalyst allowing equal distribution of the enzymatic homogenate that lead to a larger portion of the active site to be exposed for the catalytic function and interaction with the substrate, hence, facilitating the product (Barros *et al.*, 1998). Therefore, the agitation speed of 200 rpm was selected for all subsequent investigations.

4.4.3. Effect of Reaction Temperature

The effect of reaction temperature, ranging from 20 to 45°C, on the specific activity of LOX biocatalysis in neat organic solvent media was investigated. The results (Fig. 18A) show that an increase in the reaction temperature from 20 to 35ºC resulted by a 80% increase in relative specific activity of LOX. However, a 75% decrease in LOX activity was obtained when the temperature was increased to 45°C; the dramatic decrease in LOX activity may be due to the thermal inactivation of enzyme. Ayala *et al.* (1986) stated that enzymes lose their catalytic activity as the temperature is raised. Kermasha *et al.* (2002a) showed that the highest specific activity of LOX in a ternary micellar system, composed of a mixture (96:4, v/v) of sodium carbonate buffer solution (0.1 M, pH 9.0) and iso-octane containing 10 μM Tween-40, was 40ºC. However, Vega *et al*. (2005a) have shown that soybean LOX-1 in organic solvent media, containing 10% iso-octane, had a maximum LOX specific activity at 55ºC. The overall experimental finding suggest that the differences

in the optimal temperature for LOX activity between those reported in the literature and these obtained in this study could be due to a better enzyme stability in hexane milieu as compared to that in the other reaction media.

The first part of Figure 18A (20 to 35ºC) follows the Arrhenius law, as indicated in Figure 18B by the linearity of the plot of the natural logarithm (ln) of specific activity versus the inverse of absolute temperature in degree Kelvin ($^{\circ}$K). From the slope of the Arrhenius plot (Fig. 18B); activation energy (E_a) of 9.87 kJ/mol or 2.36 kcal/mol was obtained for LOX catalyzed linoleic acid in neat organic solvent media. Maccarrone *et al*. (2001) reported similar activation energy (E_a) of linoleic acid dioxygenation by LOX-1 in temperatures ranging from 20 to 45°C calculated by Arrhenius diagrams to be 8.3 ± 0.9 kJ/mol.

4.4.4. Thermal Stability in Neat Organic Solvent Media

The thermal stability of soybean LOX in neat organic solvent media was investigated. An aqueous medium was also conducted in tandem. The enzymatic reaction was carried out by incubating 1 mg of the solid enzyme in 1 mL of hexane solution (a_w = 0.75) for a wide range of incubation time (0 to 75 min), with the optimum assay conditions of 35ºC temperature and 200 rpm of agitation speed.

The results (Fig. 19A) show that the residual LOX specific activity in neat organic after incubation at 35ºC for a period of 45 min., was 82.0% of its initial activity as compared to 44.9% for the aqueous medium. The enzymes are generally more thermally stable in organic media than in the aqueous one (Halling, 2002). Ayala *et al*. (1986) reported that when enzymes are placed in organic media they exhibit properties such as enhanced thermostability.

The results (Fig. 19B) show the thermal inactivation of LOX in aqueous, ternary micellar and neat organic media. As shown by the linearity of semilogarithmic plots, the thermal inactivation of LOX was found to follow the first order kinetics behavior. From the logarithmic plots of the thermal inactivation kinetics and (Table 5) show the half-life (T_{50}) of LOX was calculated at 27.61, 66.63 and 138.6 per min for the aqueous, ternary micellar and neat organic media, respectively.

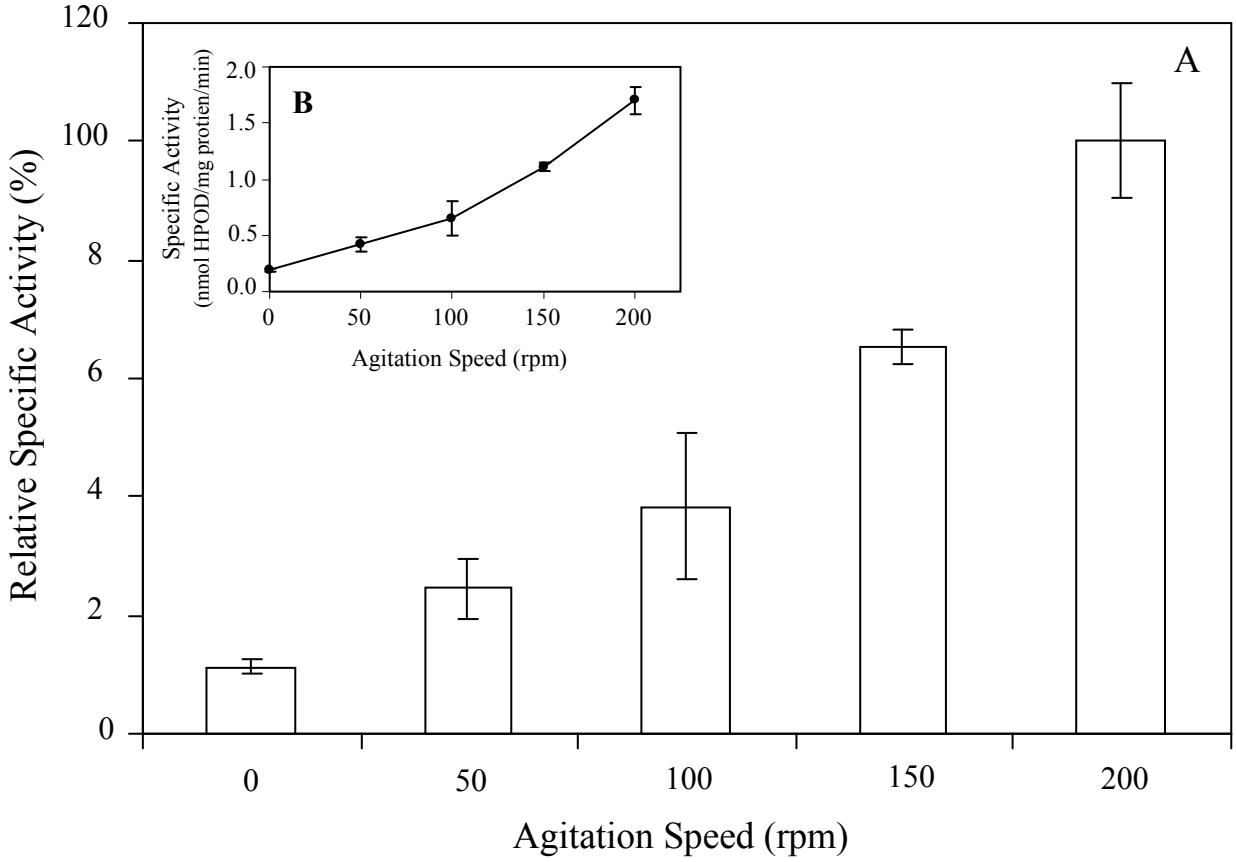

Figure 17. Effect of agitation speed on soybean lipoxygenase activity in neat organic solvent media: (A) Relative specific activity; (B) Specific activity measurements.

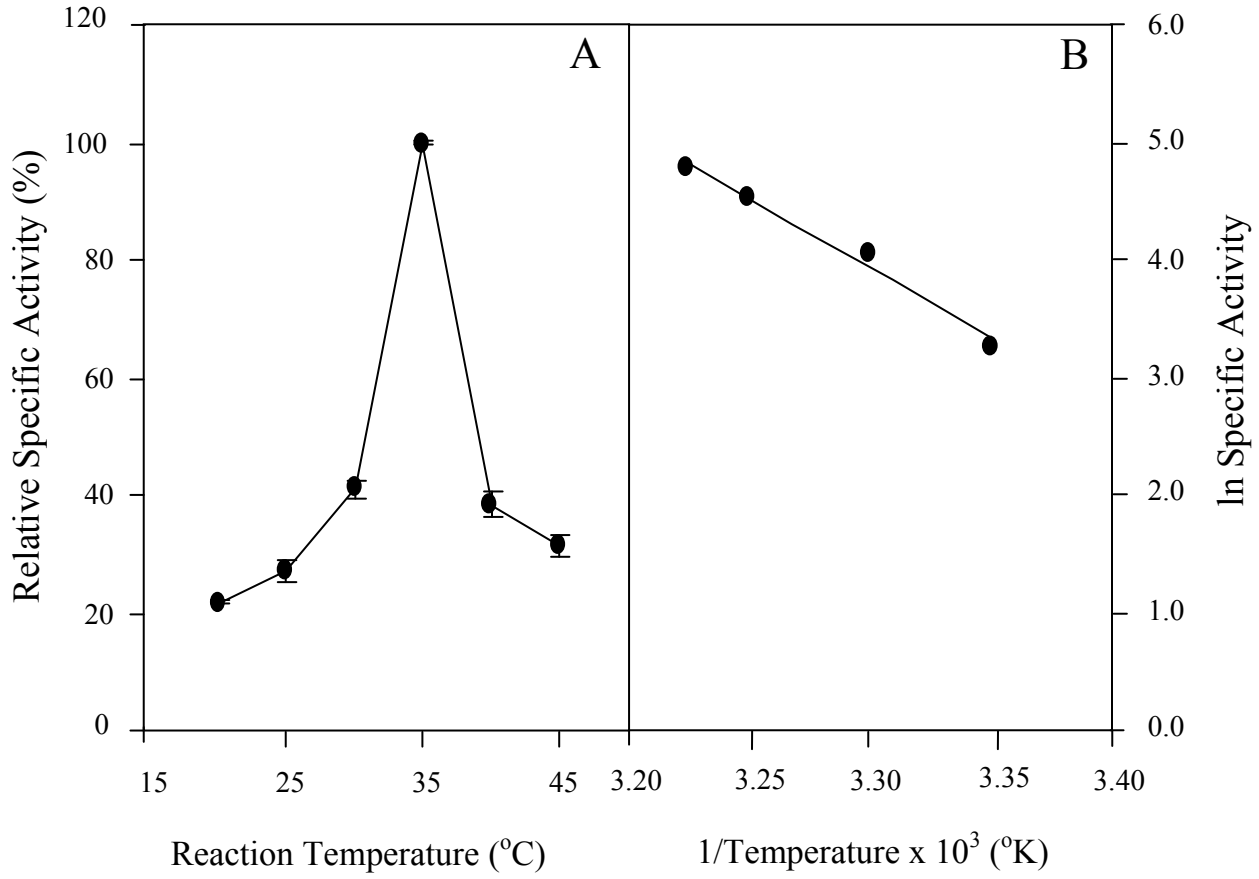

Figure 18. Effect of reaction temperature on lipoxygenase specific activity in the neat organic solvent. (A) relative specific activity versus reaction temperature and (B) Arrhenius plot, where specific activity was determined as nmol HPOD/mg protein/min.

The T_{50} value increased by a factor of 5.0 from the aqueous to the neat organic media and by factor of a 2.1 from the ternary micellar to the neat organic solvent media. Chikere *et al*. (2001) as well as Vega *et al*. (2005a,b) reported the same phenomenon for soybean LOX. However, LOXs from other species, such as potatoes (Park *et al*., 1988) and tomatoes (Anthon and Barrett, 2003), did not display first order kinetic behaviour.

The lower LOX activity obtained in neat organic solvent media as compared to that in the aqueous and ternary micellar ones could be attributed to the change in the secondary structure of LOX. Vega *et al*. (2006) reported that a decrease in LOX activity in organic solvent media was due to the intermolecular β-sheet aggregation. It is also reported that a prolonged soaking of enzyme in organic media causes a change in the structure of molecule preventing the substrate to access the enzyme molecule (Schmitke *et al.*, 1998; Halling, 2002). Furthermore, Larsson *et al*. (1990) reported that the low activity of enzymes, added directly in neat organic, could be explained by formation of clumps which adhered to the walls of the reaction vessels, thereby creating unfavourable mass transfer conditions.

4.4.5. Substrate Concentration

The kinetic parameters for LOX, including K_m, V_{max} and the catalytic efficiency, in neat organic media containing hexane were investigated. The Lineweaver-Burk plots of $1/v$ vs $1/[S]$ (data not shown) displayed a linear relationship, with a correlation coefficient 0.98 for LOX in neat organic solvent media. The results (Table 6) show that the Michaelis constant K_m value for LOX was 20.4 μmol and relatively lower V_{max} value of 8.3 nmole HPODs/mg protein/min was obtained for LOX in neat organic solvent media. Vega *et al.* (2005a) reported the K_m value of 14.4 mM and V_{max} of 5.84 μmol HPODs/mg protein/min for the biocatalysis of soybean LOX in organic solvent media, composed of a mixture of 1,4-Dioxane and hexane at a ratio of 5:95 (v/v), respectively. Kermasha *et al*. (2002a) obtained V_{max} value of 2.27 μmol HPODs/mg protein/min for the biocatalysis of soybean LOX in the ternary micellar system, composed of sodium carbonate buffer (0.1 M, pH 9.0) and 3.5% octane. The discrepancy in V_{max} value for the LOX between the present study and those reported by Kermasha *et al*. (2002a) may be due to differences in the enzymatic assay methods, used for LOX biocatalysis in the neat organic media, or to the conformation changes of the enzyme upon interaction with the neat organic environment.

The catalytic efficiency of LOX, which is defined as the ratio of V_{max}/ K_m, was found to be 0.41 min^{-1} in neat organic solvent media, thus is in agreement with that reported by Vega *et al*. (2005a). The overall result indicated a high K_m value for LOX in neat organic reaction media, suggesting that the low interaction between the enzyme and the substrate may be due to the hydrophobicity of the environment.

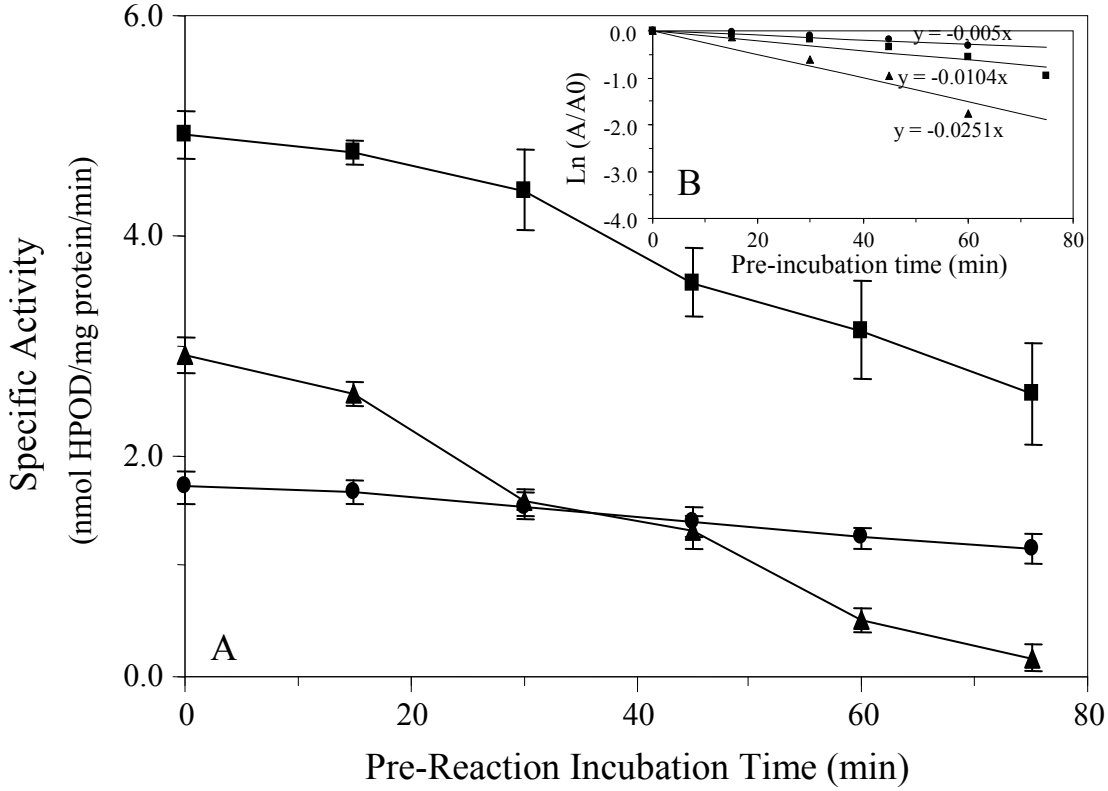

Figure 19. Effect of incubation period (0-75 min), at 35ºC, on thermostability of soybean LOX-1B, in aqueous medium of Tris-HCl buffer (0.1 M, pH 9.0) (▲—▲), in ternary micellar system a mixture (2:98, v/v) of hexane, Tris-HCl buffer (0.1 M, pH 9.0) and 10 µM Tween-40 (■—■) and neat organic solvent media (●—●): (A) Specific activity measurements; (B) First-order kinetics behavior of thermal inactivation effect of LOX in aqueous (▲—▲), ternary micellar (■—■) and neat organic media (●—●).

Table 6. Kinetic parameters for the lipoxygenase activity in ternary versus neat organic solvent media.

Reaction medium	Kinetic parameters		
	K_m[a]	V_{max}[b]	Catalytic efficiency[c]
Aqueous[d]	0.36	18.85	52.36
Ternary micellar[e]	7.70	30.00	3.90
Neat organic[f]	20.40	8.30	0.41

[a]The Michaelis constant, K_m , was defined as μM of linoleic acid.

[b]The maximum velocity, V_{max} , was expressed as nmol hydroperoxides (HPODs)/mg protein/min.

[c]Catalytic efficiency was defined as the ratio V_{max}/K_m.

[d]The aqueous medium was composed of 0.1 M Tris-HCl buffer at pH 9.0 (Kermasha *et al*., 2001).

[e]The ternary micellar system was composed of 2% hexane in Tris–HCl buffer solution (0.1 M, pH 9.0) and 10 μM of Tween-40.

[f]The neat organic was composed of hexane solvent.

5. CONCLUSION

The experimental data obtained throughout this study showed that among a wide range of organic solvents, the purified soybean LOX exhibited higher enzymatic activity in the ternary micellar system, containing hexane as compared to that reported previously in our laboratory with iso-octane or with the aqueous system. Among a wide range of surfactants, including anionic (AOT), non-ionic (Tweens) and cataionic (CTAB) as well as emulsifiers (Spans), Tween-40 was the most appropriate surfactant for the LOX activity in the ternary micellar system. The optimum reaction temperature and the E_a obtained for LOX were in the range of those reported in literature. The thermal inactivation of LOX followed the first-order kinetics behaviour, with the T_{50} higher than that in aqueous medium one. The optimization of LOX biocatalysis in neat organic solvent media showed that an increase in the initial water activity (a_w) of the reaction media, from 0.23 to 0.75, resulted in 8-fold increase in LOX specific activity. The results also showed that the external diffusional limitations were partially overcomed by the increase in agitation speed; an increase in the agitation speed from 0 to 200 rpm resulted in a 8.5-fold increase in LOX specific activity. The kinetics of LOX biocatalysis in neat organic media composed of hexane followed Michaelis–Menton order. The results also revealed that LOX in neat organic solvent media containing hexane had higher half-life values as compared to that in the ternary micellar and in the aqueous medium. In terms of thermal stability, the experimental findings showed that the residual LOX specific activity after its incubation at 35ºC for 45 min, were 44.9, 72.7 and 82% for the aqueous, ternary micellar system and neat organic media, respectively.

The overall results demonstrate that although the biocatalysis of LOX in neat organic media resulted in lower enzymatic activity, its thermal stability was higher than that in the ternary micellar system and to that in the aqueous medium.

The development of this biotechnological approach could lay the ground for the future research using the edible oils for the production of natural flavors.

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