BIOCATALYSIS OF IMMOBILIZED LIPOXYGENASE AND HYDROPEROXIDE LYASE IN ORGANIC SOLVENT MEDIA

Suggested Short Title

BIOCATALYSIS OF LIPOXYGENASE AND HYDROPEROXIDE LYASE

This thesis is dedicated to my family, present and future

ABSTRACT

Ph.D. Mireille Vega

The secondary structure of commercially purified soybean lipoxygenase (LOX) type I-B as well as its immobilization and biocatalysis in organic solvent media (OSM) were investigated. The Fourier transform infrared (FT-IR) spectra of LOX obtained in chloroform, methanol and acetonitrile showed an absorption band at 1617 cm^{-1} indicative of significant protein aggregation, whereas spectra of lipoxygenase in hexane and octane exhibited substantially less aggregate formation. Variable-temperature infrared studies of lipoxygenase in D_2O show that the predominately α-helical structure of the protein undergoes an irreversible transition to intermolecular β-sheet at and above 65°C. The biocatalysis of free and immobilized (Eupergit® C250L/EDA) LOXs was investigated in different mixtures of hexane and a selected cosolvent (95:5, v/v). The results showed a 1.5 and a 1.6-fold increase in the enzymatic activity of free and immobilized LOXs, respectively, using a mixture of hexane and 1,4-dioxane as compared to that in hexane. To determine the enzymatic production of hydroperoxides of linoleic acid in OSM, the xylenol orange (FOX) assay was optimized. An increase in the proportion of methanol from 0 to 75% in the FOX reagent resulted in a 93% increase in the molar absorption coefficients at 560 nm. Moreover, when perchloric acid was used, the source of ferrous ions and presence of denatured LOX had little effect on the sensitivity of the FOX assay whereas sensitivity decreased by 40 and 46%, respectively, with sulfuric acid. In addition, the immobilization of an enriched enzymatic extract of *Penicillium camemberti,* containing LOX and hydroperoxide lyase (HPL) activities, and its biocatalysis in OSM were investigated. The highest immobilization efficiency (173.9 and 694.4% for LOX and HPL, respectively) was obtained with unmodified Eupergit®C250L. The increase in thermal stability of both LOX and HPL activities were obtained by the immobilization of the enriched enzymatic extract on Eupergit®C250L. The thermal inactivation of LOX did not follow first order kinetic behavior but the one of HPL did. Moreover, the biocatalysis of LOX_i and HPL_i in OSM resulted in an increase in their enzymatic activity. The use of 60% iso-octane (v/v) in the reaction medium increased the HPL_i activity by more than 5 fold as compared to that in the aqueous medium. The specific activity of

LOX_i was increased to a maximum of 7.73 nmol HPODs/mg immobilized protein/min with an increase in tetradecane concentration up to 20% (v/v) in the ternary micellar system. Similarly, the addition of tetradecane in the reaction medium increased further the HPL_i activity by 17% as compared to that in 60% iso-octane medium without tetradecane.

RÉSUMÉ

Ph.D. Mireille Vega

La structure secondaire de la lipoxygenase (LOX) de soya purifiée commerciale de type I-B, de même que son immobilisation et sa biocatalyse en milieu de solvant organique (OSM), ont été investiguées. Les spectres infrarouges de transformée de Fourier (FT-IR) de la LOX obtenus en chloroforme, méthanol et acétonitrile ont montrés une bande d'absorption à 1617 cm^{-1} qui indique une agrégation significative de protéines, tandis que les spectres de la LOX en hexane et octane ont montrés sensiblement moins de formation d'agrégats. Les études infrarouges en Variation-Température de la lipoxygénase dans le D_2O ont également démontrées que la structure prédominante en α-hélices de la protéine subit une transition irréversible, à et au-dessus de 65°C, vers les β-feuillets intermoléculaires. La biocatalyse de la LOX de soya libre et immobilisée (LOX_i; Eupergit® C250L/EDA) a été étudiée dans différents mélanges d'hexane et de cosolvent sélectionné (95:5, v/v). Les résultats ont dévoilé des augmentations de 1.5 et 1.6 fois de l'activité enzymatique de la LOX libre et immobilisée, respectivement, en utilisant un mélange d'hexane et de 1,4-dioxane par rapport à celui en hexane. Pour déterminer la production enzymatique des hydroperoxides d'acide linoléique en OSM, la méthode par oxydation ferreuse avec l'orange de xylenol (FOX) a été optimisée. Une augmentation de la proportion de méthanol dans le réactif de FOX de 0 à 75% a eu comme effet une augmentation de 93% des coefficients d'absorption molaires à 560 nm. De plus, quand l'acide perchlorique a été employé, la source des ions ferreux et la présence de la LOX dénaturée n'ont eu que peu d'effet sur la sensibilité de la méthode de FOX tandis que la sensibilité a diminué de 40 et de 46%, respectivement, avec l'utilisation de l'acide sulfurique pour ces mêmes interférences. En outre, l'immobilisation d'un extrait enzymatique enrichi de *Penicillium camemberti*, contenant les activités de la LOX et de l'hydroperoxide lyase (HPL), et sa biocatalyse en OSM ont été étudiées. L'efficacité d'immobilisation la plus élevée (173.9 et 694.4% pour la LOX et la HPL, respectivement) a été obtenue avec Eupergit®C250L non modifié. Une augmentation de la stabilité thermique par l'immobilisation de l'extrait enzymatique enrichi sur Eupergit®C250L des activités de la LOX et de la HPL a aussi été observée. L'inactivation thermique de la

LOX en OSM n'a pas suivi un comportement cinétique de premier ordre; celle de la HPL l'a fait. Par contre, la biocatalyse de la LOX_i et de la HPL_i en OSM a eu comme conséquence une augmentation de leur activité enzymatique. L'utilisation de 60% d'isooctane (v/v) dans le milieu de réaction a augmenté l'activité de HPL_i par plus de 5 fois par rapport à l'activité observée dans le milieu aqueux. L'activité spécifique de la LOX_i a augmentée jusqu'à un maximum 7.73 nmol HPOD/mg protéïne/min avec l'augmentation de la concentration en tétradécane jusqu'à 20% (v/v) dans un système micellaire ternaire. De même, l'addition de tétradécane dans le milieu de réaction a augmenté l'activité de HPL_i de 17% par rapport à celle dans le milieu composé à 60% d'isooctane sans tétradécane.

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CLAIM OF ORIGINAL RESEARCH

1. This is the first study in which soybean lipoxygenase structure-function relationship is studied in organic solvent media.

2. This is the first study in which the FOX assay is optimized to quantify the activity of lipoxygenase and hydroperoxide lyase in organic solvent media.

3. This is the first study in which a microbial extract containing lipoxygenase activity is characterized in organic solvent media. In this study the lipoxygenase activity of *Penicillium camemberti* was immobilized and characterized in terms of activity, stability and kinetics in organic solvent medium, using linoleic acid as model substrate.

4. This is the first study in which an hydroperoxide lyase activity is characterized in organic solvent media. In this study, the microbial hydroperoxide lyase activity of *Penicillium camemberti* was immobilized and characterized in terms of activity, stability and kinetics in organic solvent medium, using the 10-hydroperoxide isomer of linoleic acid as model substrate.

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LIST OF ABBREVIATIONS

ADH	Alcohol dehydrogenase
ATR	Attenuated Total Reflectance
BHT	Butylated hydroxytoluene
CCD	Charged-coupled device
CD	Circular dichroism
DEAE	Diethylaminoethyl cellulose
EDA	Ethylenediamine
FA	Fatty Acids
FOX	Ferric oxidation by xylenol orange
FPA	Focal plane array
FSD	Fourier self-deconvolved
FTIR	Fourier transform infrared
HP	Hydroperoxide
HPD	Hydroperoxide dehydratase
HPETE	Hydroperoxy-eicosatetraenoic acid
HPL	Hydroperoxide lyase
HPLC	High performance liquid chromatography
HPOD	Hydroperoxy-octadecadienoic acid
HPOT	Hydroperoxy-octadecatrienoic acid
IDA	Iminodiacetic acid
IHY	Immobilized HPL_i yield
ILY	Immobilized LOX_i yield
IPY	Immobilized protein yield
LLSD	Laser-light scattering detector
LOX	Lipoxygenase
MDA	Malondialdehyde
NADH	Nicotinamide adenine dinucleotide
OSM	Organic solvent media
PUFA	Polyunsaturated Fatty Acid

ROA	Ratio of activity
SD	Standard deviation
TBA	Thiobarbituric acid
TPP	Triphenylphosphine
Tris	Tris(hydroxymethyl)aminomethane
A_w	Water activity
ε	Molar absorption coefficient

CHAPTER I

INTRODUCTION

Flavor compounds have been produced synthetically since the beginning of the 19th century; nowadays, more than 16,000 volatiles have been identified and chemists can synthesize even the most complex chemical structure (Krings and Berger, 1998). In 2006, the flavor and fragrances market was estimated at 17.9 billion USD (Leffingwell and Associates, 2007). Although nearly 80% of flavor and fragrances used world-wide are obtained chemically, there is an increasing trend for the recovery of endogenous flavors, from raw materials (Tilkari *et al.*, 2007). However, extraction is subjected to various problems, such as the low concentrations of the aroma and the supply's dependence on the season, the weather or the political stability (Serra *et al.*, 2005).

The interest in bioconversion of naturally occurring compounds into natural flavors is constantly rising (Tilkari *et al.*, 2007). Biocatalysts are a tangible substitute to conventional chemical synthesis and catalysts. The advantages of enzyme-catalyzed reactions include their high specificity with unrivalled chiral and positional selectivities, their high activity under mild reaction conditions and high turnover number. As enzymes can be used both in simple and complex bioconversions without tedious molecular group blocking and deblocking steps common in organic synthesis, they are considered an environmentally friendly alternative to chemical synthesis. Their biodegradable character and the labeling of their products as natural have also become important assets (Schmid *et al.*, 2001; Serra *et al.*, 2005).

The use of enzyme in bioprocesses has certainly many advantages. However, some drawbacks of enzyme catalysis include the unsatisfactory stability of the biocatalysts, their difficult isolation and poor productivity (Brena and Batista-Viera, 2006). The immobilization is considered a convenient means of improving enzymes performance and stability. Immobilization could also provide a tool in the recovery of enzymes and in their purification as well as an approach for continuous catalysis in bioreactors (Mateo *et al.*, 2001; Mateo *et al.*, 2002; Hudson *et al.*, 2005).

Vegetable and marine animal oils may be used as sources for the production of high added-value flavor precursors and flavor compounds. The bioconversion of endogenous oils such as marine and edible vegetable oils may prove appealing both from an environment and economical aspect. The bioconversion of such endogenous oils, by biotechnological processes, into economically beneficial and commercially marketable products labeled as "natural" presents a great opportunity for the food industry to meet consumer's demand for healthier foods.

However, substrates such as fatty acids have limited water solubility and therefore their concentrations in biocatalysis are usually lower than in chemical processes, which in turn leads to larger reaction volumes and difficulties in product recovery (Kim *et al.*, 2007). The use of organic solvent media (OSM) may provide with another strategy of improving biocatalysis due to increased substrate and products solubility. In addition, the use of OSM may present changes in enzyme specificity that could lead to the production of a wide range of flavor compounds. The notion that enzymes are only active in aqueous media is no longer valid and their use as catalysts in OSM has gained interest in industrial applications (Hudson *et al.*, 2005).

Lipoxygenases (LOX, EC 1.13.11.12) are an important class of non-heme iron enzymes that catalyze the di-oxidation of linoleic acid and other polyunsaturated fatty acids, containing a *cis,cis*-1,4-pentadiene moiety, into hydroperoxy-fatty acids. These hydroperoxide compounds, which are considered flavor precursors, can be subsequently cleaved by hydroperoxide lyase (HPL) and associated enzymes into flavor compounds, such as ketones, aldehydes and alcohols (Delcarte *et al.*, 2000). HPL activity yields two fragments; a volatile compound and a non-volatile oxoacid (Delcarte *et al.*, 2000) The production of aroma has been recognized as one of the relevant application of the LOX pathway. As HPL is the first enzyme of the LOX pathway to produce volatile compounds, its characterization is also fundamental (Salas *et al.*, 2000). The type of flavor compounds produced depends essentially on the specificities of both LOX and HPL involved (Schrader *et al.*, 2004).

Soybean type 1-B is the most characterized LOX and it is therefore a model of choice; its secondary structure has been determined by X-ray crystallography (Boyington *et al.*, 1993). FTIR analysis could relate the secondary structure of LOX and its relative activity and thus might provide an understanding of LOX behavior in different reaction environment. Modifications in the secondary and/or tertiary structure are determinant in the specificities shown by an enzyme (Pourplancher *et al.*, 1994); given the homology between the sequence reported of more than 50 LOXs across species, the conclusions obtained from soybean LOX should apply to other LOX systems (Prigge *et al.*, 1997).

The optimization of soybean LOX biocatalysis in various non-conventional media, such as monophasic, biphasic and ternary micellar systems, has been reported (Piazza *et al.*, 1994; Kermasha *et al.*, 2002a). Immobilization of soybean LOX has been reported using covalent binding, adsorption and gel entrapment. Covalent immobilization supports, such as oxirane beads (Laakso, 1982; Chikere *et al.*, 2001) and anionic exchanger adsorption supports have been used successfully (Petrus Cuperus *et al.*, 1995). Most studies have, however, been performed in aqueous medium, whereas those in OSM are limited (Piazza *et al.*, 1994; Kermasha *et al.*, 2002a). Furthermore, although the immobilization of soybean LOX and its biocatalysis in OSM have been reported for its purified form (Petrus Cuperus *et al.*, 1995; Pinto *et al.*, 1997; Hsu *et al.*, 2000; Chikere *et al.*, 2001; Santano *et al.*, 2002), there is little information available regarding such investigation for crude microbial enzymatic extracts.

The potential application of LOXs and HPLs are also limited by their poor stability, lacking both in purified and crude forms (Gardner *et al.*, 1991; Chikere *et al.*, 2001). HPL is commonly known as unstable, irreversibly inhibited by its substrate in aqueous medium (Shibata *et al.*, 1995); however, little information was reported on its stability and its biocatalysis in OSM. Although HPL extracts from different plant sources have been immobilized (Gardner *et al.*, 1991; Rehbock and Berger, 1998; Simon *et al.*, 1998; Schade *et al.*, 2003), limited reports on those obtained from microbial sources have been published (Nuñez *et al.*, 1997).

Different methods have been reported for the enzymatic assays of LOX and HPL (Jiang *et al.*, 1991; Perraud and Kermasha, 2000). HPLC analysis has proven to be tedious for the determination of LOX activity (Vega *et al.*, 2005a) and most spectrophotometric methods reported for the determination of LOX activity were developed for an aqueous medium, with the most common being based on the absorption of hydroperoxy-FA containing a *cis,cis*-1,4-pentadiene moiety at 234 nm (Grossman and Zakut, 1979); this method also remains limited by its inability to detect hydroperoxides that lack a conjugated diene chromophore (Surrey, 1964). The ferrous oxidation with xylenol orange (FOX) assay was reported as an alternative spectrophotometric method for the determination of lipid hydroperoxides (Jiang *et al.*, 1991). However, when hydrophobic solvents are mixed with the polar FOX reagent, the reaction assays can become turbid and hence limit the accuracy of the spectrophotometric measurements.

The specific objectives of the research were:

1. To investigate the secondary structure of the purified soybean LOX in various media, using FTIR analysis.
2. To study the immobilization and biocatalysis in OSM of the purified soybean LOX enzyme as a model system.
3. To optimize the FOX assay for the enzymatic assays of LOX and HPL activities in OSM.
4. To investigate the immobilization and biocatalysis in OSM of the enriched enzymatic extract from *P. camemberti* containing LOX and HPL activities.

The present thesis consists of seven chapters. Chapter I provides an introduction to the overall thesis. Chapter II reports on the literature review on LOX and HPL in terms of their role in the production of natural flavors, their assays as well as their immobilization and biocatalysis in OSM. Chapter III describes the FTIR analysis of the secondary structure of soybean LOX in OSM. Chapter IV covers the optimization of the FOX assay of the determination of LOX and HPL activities in OSM. In chapter V are laid out the results of the study of immobilized soybean LOX in OSM. Chapter VI and VII deals with the immobilization and biocatalysis in OSM of LOX and HPL activities, respectively, obtained from *Penicillium camemberti*.

CHAPTER II.

LITERATURE REVIEW

2.1. Flavors and Raw Materials

2.1.1. Definition

Attributed to health-conscious lifestyles, the demand for natural products is rising (Tilkari *et al.*, 2007). In North America, the term "natural flavor" is defined in the Code of US Federal Regulations as follows:

"essential oil, oleoresin, essence or extractive, protein hydrolysate, distillate of any product of roasting, heating or enzymolysis, which contains the flavouring constituents derived from a spice, fruit juice, vegetable or vegetable juice, edible yeast, herb, bud, bark, root, leaf or similar plant material, meat, seafood, poultry, eggs, dairy products, or fermentation products thereof whose significant function in food is imparting flavourings rather than nutrition".

2.1.2. Flavor Bio-Production

Biotechnological processes, using whole cells or enzyme systems, are an attractive alternative to extraction in the production of natural flavors. Fermentation involves the growth of whole cell microorganisms in a medium from which the natural flavor product will subsequently be isolated whereas biotransformation involves the use of whole cells or extracted enzymes to convert an exogenous compound into the desired natural aroma (Whitehead, 1998). Microorganisms such as fungi are the main source of catalysts in bioprocesses as they display a volatile diversity similar to plants (Cheetham, 1997) while being more accessible than plant cells in terms of cost and process parameters such as cultivation time, shearing and product extraction (Serra *et al.*, 2005).

Abraham and Berger (1994) were able to characterize more than 120 volatile flavors, including alcohols, ketones, aldehydes, esters, lactones and phenols, generated using twenty basidiomycete strains. Table 1 lists some important aroma compounds generated by enzymes or whole cells (Krings and Berger, 1998).

Table 1. Aroma compounds generated with the use of biotransformations (Cheetam, 1997, Krings and Berger, 1998).

Compound or group	***Taste***	***Enzyme system[a]***
Benzaldehyde	almond; cherry	*Ishnoderma benzoinum;* β-glucosidase and nitrile lyase
γ-Decalactone	peach, dairy	Yeasts (β-oxidation and lipase) *Candida lipolytica*
Diacetyl	dairy (butter)	*Steptococcus diacetylactis*
Diallylthiosulphonate	garlic	Alliinase
Esters	fruity	Lipases and esterases
Furaneol®	strawberry, pineapple	Rhamnosidases
cis-3-Hexenol *cis*-3-Hexen-1-al	fresh taste (green)	Plant lipoxygenase, hydroperoxide lyase and dehydrogenase
Isothiocyanates	Mustard	Myrosinase
δ-Lactones	various	Yeasts (β-oxidation and lipase)
Linalool	sweet or lavender[b]	*Wolfiporia cocos*
Methyl ketones	dairy	*Penicillium* sp.; *Aspergillus niger*
2-*trans*-6-*cis*-Nonadienal	cucumber	Plant lipoxygenase and associated nzymes
1-Octen-3-ol	mushroom	*Psalliato bispora*[c] *Penicilium* sp.[d] (lipoxygenase; hydroperoxide lyase)
Vanillin	vanilla	*Pycnoporous cinnabarinus*

[a]Enzymes extracted from plants or microorganisms or from whole cell used in the production of the aroma compound.

[b]Depending on chirality.

[c]from Wurzenberger and Grosch, 1984.

[d]from Perraud and Kermasha, 2000.

2.1.3. Green Note Volatiles

The characteristic aromatic profiles of plants depend on a complex mixture of chemicals and on the enzymes involved in the biotransformation (Serra *et al.*, 2005). Linoleic and linolenic acid are present in most plant tissues and are the precursors of a wide range of volatile flavors.

The role of lipoxygenase (LOX) in hexanal formation was first hypothesized in 1943 (Nye and Spoehr); since then, the production of green note aldehydes from plant cells has been reported in many higher plants (Salas *et al.*, 2000). Figure 1 presents the sequential biochemical pathway of LOX which yields the flavor compounds (Schrader *et al.*, 2004).

Products of the LOX pathway in plants are non-volatile ω-oxoacids and volatile unsaturated 6-carbon aldehydes and alcohols, which green-note sensory qualities are sought after (Schrader *et al.*, 2004). Aldehydes containing less than 5 carbon atoms are usually associated with off-flavors, whereas 5 to 10-carbon alkenals impart desirable flavor attributes to fresh plants, such as *cis*- and *trans*-2-hexenals which both possess a green leaf note and *cis*-2-nonenal which is associated with the melon-like odor of cucumbers. Aldehydes obtained from hydroperoxide lyase (HPL) activity in plants can be further modified by the action of isomerases, alcohol dehydrogenase and acyltransferase. These alcohol flavors show, although more delicately, similar odors as their aldehyde counterparts (Gardner, 1995). The metabolism of polyunsaturated fatty acids (PUFAs) containing a 1*(Z)*-4*(Z)*-pentadiene moiety are of particular importance in this study; table 2 (O'Keefe, 1998) presents the model PUFAs, possessing the significant moiety, which can be converted into flavor precursors using LOX. This literature will focus primarily on the flavor molecule obtained from the synergetic action of LOX and HPL.

2.1.4. PUFA Sources

The bioconversion of edible and commercial oils is an attractive choice as these substrates are readily available and in some cases may even be considered waste material and thus are an environmental friendly option (Soares *et al.*, 2000).

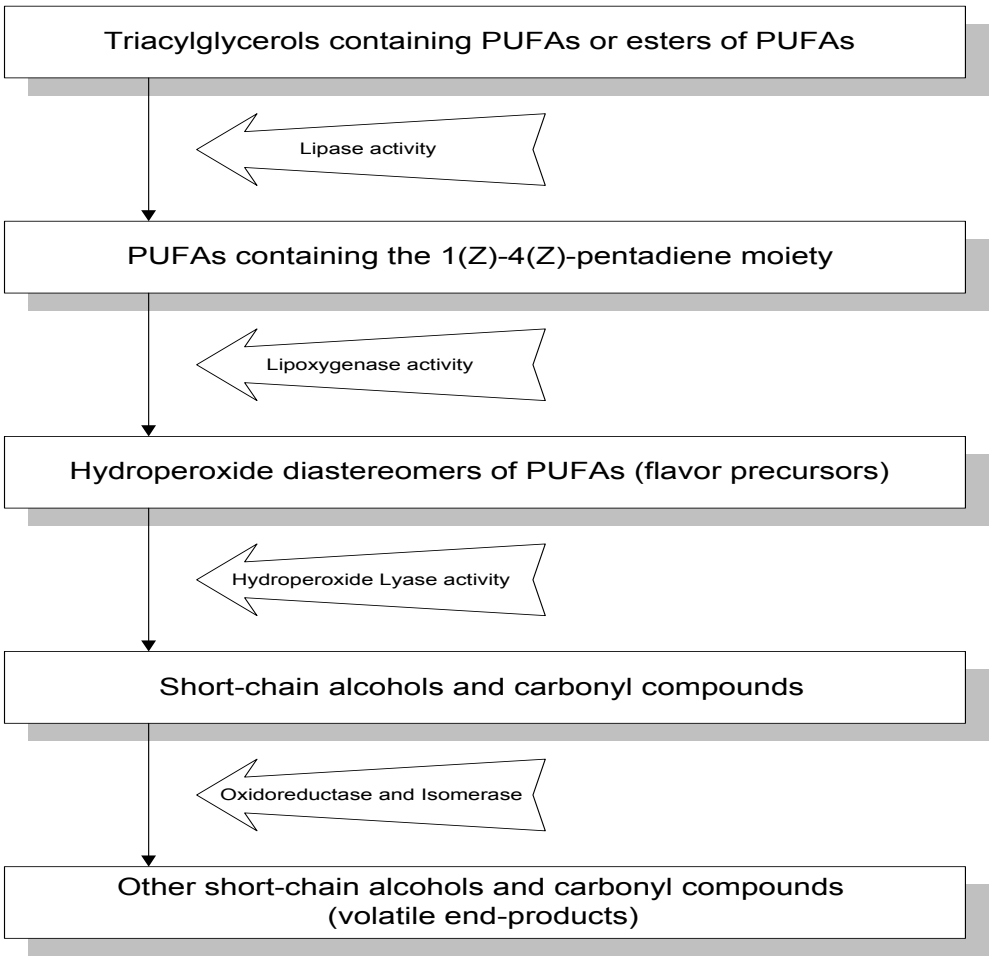


Figure 1. Bioconversion of triacylglycerols and polyunsaturated fatty acids into alcohols and carbonyl flavor compounds by the sequential enzymatic activities of the lipoxygenase pathway.

Table 2. Substrate models for use in the lipoxygenase pathway (O'Keefe, 1998).

Common name	***Systematic name***	***Shorthand name***
n/av	c-7,c-10,c-13-hexadecatrienoic	16: 3n-3
n/av	c-4,c-7,c-10,c-13-hexadecatetraenoic	16: 4n-3
Linoleic	c-9,c-12-octadecadienoic	18: 2n-6
Linolenic	c-9,c-12,c-15-octadecatrienoic	18: 3n-3
γ-linolenic	c-6,c-9,c-12-octadecatrienoic	18: 3n-6
Stearidonic	c-6,c-9,c-12,c-15-octadecatetraenoic	18: 4n-3
Dihomo-γ-linolenic	c-8,c-11,c-14-eicosatrienoic	20: 3n-6
Mead's	c-5,c-8,c-11-eicosatrienoic	20: 3n-9
Arachidonic	c-5,c-8,c-11,c-14-eicosatertaenoic	20: 4n-6
EPA	c-5,c-8,c-11,c-14,c-17-eicosapentaenoic	20: 5n-3
DPA	c-7,c-10,c-13,c-16,c-19-docosapentaenoic	22: 5n-3
DHA	c-4,c-7,c-10,c-13,c-16,c-19-docosahexaenoic	24: 1n-9

2.1.4.1. Animal Fats and Vegetable Oils

Soybean and animal fats make out about half of the 100 million metric tons of oil produced annually, while marine oils and fish oils account for about 2% of the world supply (Bimbo, 1990; Opstvedt *et al.*, 1990; Gunstone, 1999). Animal fats of any source are rich in saturated fatty acids, mainly 16:0 and 18:0 whereas vegetable oils are vastly rich in the PUFA 18:2*n*-6 and the monounsaturated fatty acid 18:1*n*-9, oleic acid (Stauffer, 1996).

2.1.4.2. Fish and Marine Oils

Out of the 17 major fats and oils produced, fish oils come 12th in oil production, after olive oil and before corn oil (Bimbo, 1990). On average, 29% to 32 % of the world landings are converted into fish meal and oil. The raw material used for fish meal and oil production may be considered a waste of no edible value; it could in fact present potential disposal problems (Bimbo, 1990).

Fish oils differ greatly from plant oils or animal fats. Since fishes feed on algae, which are a rich source of 20:5*n*-3 and 22:6*n*-3, the resulting marine oils are a rich source of those PUFAs (Sargent, 1997). On the other hand, blubber and seal meat lipids contain large amounts of highly unsaturated fatty acids (HUFAs). Blubber lipids are made of triacylglycerols at 98.9% with unsaturated representing 87% of the lipids; of those unsaturated lipids, 36.7% are polyunsaturated (Shahidi *et al.*, 1994).

2.2. Biocatalysis of Fatty Acids

Biocatalysis has emerged, in the last decades, as a influential tool for the production of industrial products (Schoemaker *et al.*, 2003). The global market for specialty enzymes was about 2 billion USD in 2004 and is growing by a predicted 5% annually (Hari Krishna, 2002; BCCresearch, 2004). Enzyme catalyzed reactions are carried out under mild conditions of temperature and pH which lowers products isomerization, racemization and epimerization (Carrea and Riva, 2000). Also they display high chemo-, regio- and enantio-selectivity, which is central in the food industry that requires often enantiomerically pure compounds (Serra *et al.*, 2005)

2.2.1. Substrates

Other than stereospecificity, it is the geometric specificity of enzymes that dictates the substrate they catalyze. Although a few selected enzymes are specific to only one compound, most will catalyze a range of related compounds (Kim *et al.*, 2007).

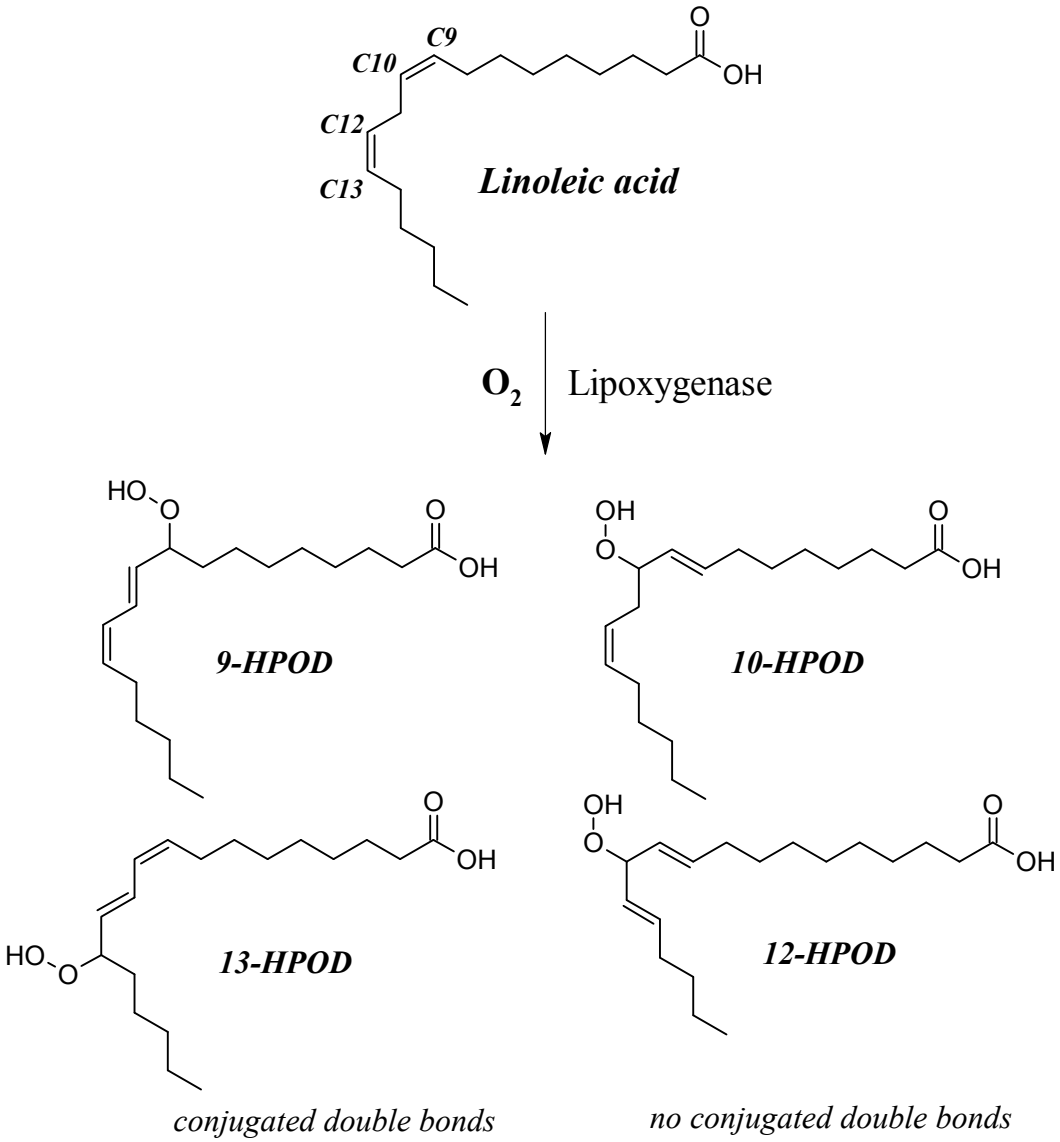
2.2.1.1. Lipoxygenase

Lipoxygenase and associated enzymes convert polyenoic fatty acids, using atmospheric dioxygen as a second substrate, into aldehydes and alcohols. Specifically, lipoxygenase convert the 1*Z*,4*Z* diene moieties of natural PUFAs into 1*Z*,3*E* hydroperoxides (Fig. 2.), which are considered flavor precursors (Schrader *et al.*, 2004). It has been reported, however, that these substrate must be more than 12 carbons long (Hatanaka, 1993).

Although most studies have suggested that the essential characteristics of LOX substrate was the presence of an ω-6 pentadienyl moiety and a carboxyl group, long chain phenol which lack a carboxylic acid moiety may be utilized by soybean LOX, as long as the free phenol group mimicking carboxylic acid, is not blocked (Roth *et al.*, 1998). Other than natural PUFAs, LOX may also catalyze asymmetric oxygenation of unnatural substrates (Nanda and Yadav, 2003).

2.2.1.2. Hydroperoxide Lyase

Flavor precursors produced by LOX are later on converted into volatile flavor compounds by the action of HPL (Schrader *et al.*, 2004). Figure 3 shows the HPL catalyzed cleavage of hydroperoxides (HPs) of PUFAs, the product of the LOX reaction, into aldehydes, alkanes or alcohols and ω-oxoacids (Delcarte *et al.*, 2000; Schrader *et al.*, 2004). It seams that the indispensable configuration of the HPL substrate is a *cis-trans* conjugated diene moiety combined with a *trans*-double-bond next to the (*S*)-HP bearing carbon (Stumpe *et al.*, 2006).



Hydroperoxides of linoleic acid (HPODs)

Figure 2. Bioconversion of linoleic acid into its hydroperoxide isomers using lipoxygenase.

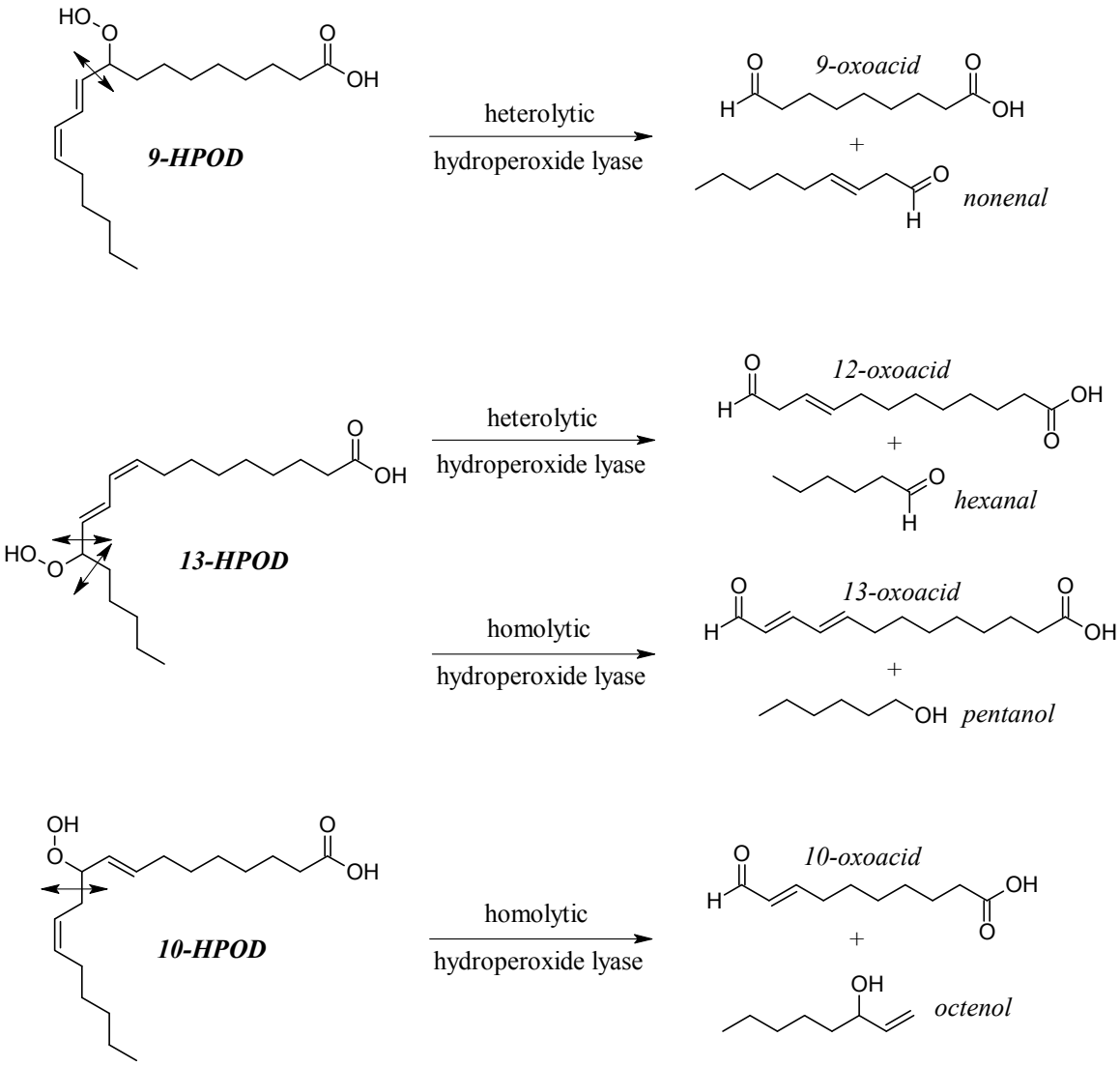


Figure 3. Bioconversion of hydroperoxide isomers of linoleic acid (HPODs) into their corresponding oxoacid and volatile flavor compound by heterolytic and/or homolytic hydroperoxide lyase activity (Delcarte *et al.*, 2000).

2.2.2. Enzyme Sources

2.2.2.1. Lipoxygenase

LOX, an heterogeneous family of lipid peroxidizing enzymes, are widely distributed in different cell types, namely plant cells, fungi and animal tissues (Brash, 1999; Kühn and Thiele, 1999). Although most common in higher plants and animal tissues, LOX have been found in lower marine organisms, such as algae, star fishes, sea urchins and corals as well as in bacteria such as *Pseudomonas aeruginosa* and *S. cellulosum* (Kühn and Borchert, 2002). As LOX preferred substrate are PUFAs with two *cis* double-bonds, they have not been detected in organisms not synthesizing this substrate such as most yeasts, prokaryotes and insects (Brash, 1999).

LOX are found as isozymes, where each LOX isozyme differs on aspects like optimal conditions for enzyme activity as well as substrate and product specificities (O'Connor and O'Brien, 1991). Higher plant and animal cells possess various LOX, with soybean containing as many as eight (Brash, 1999). Although LOX are a heterogeneous family, most functional homologues of the LOX genome across species share 70-95% of their DNA sequence (Brash, 1999)

LOX of higher plants and animal tissues have been thoroughly studied (Siedow, 1991) but less information is available regarding the characterization and the role of LOX in microorganisms (Perraud, 2000). The first reported fat degradation activity in microorganism such as *Penicillium* and *Aspergillus* dates back in 1951 (Mukherjee). Since then, LOX activity has been characterized in fungi and in edible mushroom including *Psalliota bispora* and *Agaricus bisporus* (Wurzenberger and Grosch, 1984; Husson *et al.*, 2001). Many such microorganisms have been studied namely the alga *Chlorella pyrenoidosa* and the fungi *Morchella esculenta, Geotrichum candidum, Fusarium sp.* and *Penicillium sp.* (Bisakowski *et al.*, 1997; Bisakowski *et al.*, 2000; Perraud, 2000).

2.2.2.2. Hydroperoxide Lyase

Since HPL was first purified from watermelon in 1976 (Vick and Zimmerman), it has been reported in many higher plants. If it is assumed that green leaf volatiles, or

oxylipins, originate from HPL activity, it could very well be ubiquitously found in the plant kingdom. HPL activity also has been found in lower plants such as green and red algae, cyanobacteria and mosses (Matsui *et al.*, 1996a).

Two main type of HPLs have been identified depending on their cleaving activity site. Heterolytic HPLs seam to be widely distributed among the higher plant kingdom whereas homolytic HPLs have been reported in lower plants such as fungi, algae and some species of grass (Gardner, 1995). Homolytic cleavage may not be limited to lower plants as this type of activity has also been reported in higher plants such as soybean; it has however been argued that LOX activity could be responsible for homolytic cleavage in soybean under anaerobic conditions (Salch *et al.*, 1995).

Although to this date, no HPL has been isolated from mushroom, HPL activity has been detected in basidiomycetes and some *Fungi imperfecti* like *Penicillium* and *Aspergillus sp.* (Delcarte *et al.*, 2000). Its homolytic activity is responsible for the synthesis of the alcohol 1-octe-3-ol, commonly known as the mushroom alcohol (Wurzenberger and Grosch, 1984; Delcarte *et al.*, 2000). Homolytic cleavage activity and the formation of pentane have also been detected in animal tissues; this activity is however dependant on reductase activity (Vaz and Coon, 1987). And fish could yet be another source of the activity (Gardner, 1991).

2.2.3. Characterization of Soybean LOX-1 and P. camemberti Selected Enzymes

Most of the knowledge gathered on LOX originates from studies of soybean LOX type 1, which was discovered in 1932 (André and Hou). Soybean LOX-1 is a model of choice for the understanding of any LOX as it is relatively easy to obtain and purify, sufficiently stable, its DNA sequence has been cloned, determined and expressed in bacteria and many mechanistic and spectroscopic studies such as X-ray crystallography have been performed using this enzyme (Prigge *et al.*, 1997). Given the homology between the sequence reported of more than 50 LOXs across species, the conclusions obtained from soybean LOX should apply to other LOX systems (Prigge *et al.*, 1997).

2.2.3.1. Effect of pH

The effect of the pH relates to the hydrogen concentration around the enzyme molecule and thus the microenvironment of the enzyme (Ballesteros and Boross, 2000). As the active site of enzymes is generally composed of various basic or acidic amino acid residues, the conformation of the catalytic site as well as its interaction with the substrate is influenced greatly by the pH of the reaction's medium (Ballesteros and Boross, 2000).

LOX-1 has an isoelectric point of 5.68 and displays maximal activity in the small range of pH around 9.0 (Wong, 1995). Microbial LOXs show a broader optimum pH value range with optimal activities for bacteria and fungi LOXs in the range of pH between 6.0 to 7.5 (Perraud, 2000). Two pH optima were found for maximal activity of *Penicillium camemberti*, the major one was found at pH 6.5 and 7.0 using as substrate linoleic and arachidonic acids, respectively, whereas the minor one was at pH 8.0 and 8.5 using the same substrates, respectively (Perraud and Kermasha, 2000).

Mosses HPLs have shown maximal activity around pH 5.0 (Matsui *et al.*, 1996a) whereas higher plants HPLs pH optimum are between 5.8 and 8 (Delcarte *et al.*, 2000). HPLs from algae display slightly higher pH optimum with *Chlorella pyrenoidosa* being most active in a medium environment of pH 6 to 8 (Nuñez *et al.*, 1997).

2.2.3.2. Effect of Temperature

Soybean LOX has been found to exhibit a large kinetic isotope factor concomitant with a small E_{act} which is indicative of low temperature dependence behavior (Knapp *et al.*, 2002). However, Kermasha *et al.* (2001) have shown that soybean LOX-1 displays optimal activity in an aqueous system at 20°C, above which enzyme activity decreases.

Optimal temperatures of LOXs from microorganisms are usually found in the range of 40°C (Perraud, 2000). The study of effect of temperature on *P. camemberti* enzyme stability showed that LOX retained 52% of its activity after 10 min of incubation at 80°C (Perraud and Kermasha, 2000). Upon pre-incubation at 0ºC for 5 h of an extract of *A. bisporus* containing both LOX and HPL activities, the extract, at room temperature, was unable to convert linoleic acid into volatile flavors but HPL activity only decreased

by 14% when 10-HPOD was introduced for transformation into 1-octen-3-ol; thus showing that HPL was stable and that LOX was mostly affected by the pre-incubation (Wurzenberger and Grosch, 1984).

2.2.3.3. Enzyme Specificities

As the building blocks of enzymes are exclusively *L*-amino-acids, these catalysts have unique 3-D shapes which limit the substrate and product they utilize and produce (Voet and Voet, 1995). Biological reactions using enzyme catalysts are highly selective in the substrate molecule they transform as compared to chemical reactions (substrate specificity) and in the isomer product they yield from those molecules (end-product specificity); phenomena of crucial importance in flavor chemistry. For instance, a flavor molecule such as α-aspartame is sweet whereas β-aspartame is bitter (Cheetham, 1997).

Diverse end-products can be obtained from LOX and HPL. The stereo- and regio-specificity of the enzymatic reaction is caused by the inherent chirality of the isozyme used in the process. Generally, higher plant cells will utilize 18C PUFAs to produce oxylipins whereas lower plant cells and animal tissues predominantly transform 20C PUFAs (Brash, 1999). The moss *Physcomitrella patens* was found to utilize 20C PUFAs to yield 8C and 9C aldehydes and alcohols and thus to produce a mixture of plant- and animal-like metabolites (Senger *et al.*, 2005).

2.2.3.3.1. Lipoxygenase

Various factors affect the chemo-, regio- and stereo-specificity of LOX and the orientation taken by the substrate at the active site. Other than the source of the LOX enzyme, the reaction conditions and the nature of the substrate itself, the oxygen concentration has been shown to influence the regiochemical nature of the reaction.

In terms of substrate specificity, LOX may utilize PUFAs or esters, alcohols and halides of fatty acids, which contain a *cis,cis*-1,4-pentadiene structure. Commonly, essential fatty acids such as linoleic, linoleic and arachidonic acids are utilized (Hamberg and Samuelsson, 1967); with most LOX oxidizing the free substrate at a higher rate than the esterified one (Salas *et al.*, 2000). For soybean LOX, linoleic acid has been reported

as the substrate of choice in the catalytic reaction (Axelrod *et al.*, 1981). *P. camemberti* LOX demonstrated similar preferential specificity towards free linoleic acid, followed by free arachidonic acid (Perraud and Kermasha, 2000).

The bioconversion of PUFAs, such as linoleic acid, with LOX isozymes produces different HPs. Plant cell LOXs generally yield 13- and 9-HPs of linoleic acid (HPOD) which are cleaved into C6- and C9-aldehydes, upon HPL action (Hatanaka, 1993). Regiochemistry of the substrate also depends largely on the pH of the reaction's system (Nanda and Yadav, 2003). For soybean LOX, a ratio of 13(*S*)-HPOD to 9(*S*)-HPOD of 19:1 is obtained using linoleic acid as substrate in a system of pH 9.0 whereas the ratio drops to 3:1 at pH 6.0 (Gardner, 1991; Wong, 1995).

Soybean LOX-I produces exclusively the 15S-HPETE from arachidonic acid when at low concentrations whereas it can catalyze 5S and 8S oxygenations at higher concentrations (Brash, 1999). Also, oxygen, the second substrate of the LOX reaction, affect the regiospecificity of soybean LOX depending on its concentration (Nanda and Yadav, 2003). At low oxygen concentration, a ratio of 1:1 13 to 9-HPOD was formed rather than the usual 19:1 ratio observed at higher concentrations.

LOXs from microorganisms yield 9-, 10-, 12- and 13-HPODs (Bisakowski *et al.*, 1997). The production of 10- and 12-HPODs have been reported in different microorganisms including *P. camemberti* (Perraud and Kermasha, 2000). It also has been shown possible for LOX enzyme to display both oxidase and hydroperoxidase activity such as is the case of the moss *Physcomitrella patens* (Senger *et al.*, 2005).

The functional group at the C-1 carbon position of the substrate has also been reported to direct the stereospecificity of the product (Hatanaka, 1993). For instance, soybean LOX-1 produces exclusively 13(*S*)-HPOD from linoleyl alcohol whereas it yields a racemic mixture of the same product using the methyl ester.

Most of the literature is concerned with *S* stereochemistry of the LOX enzyme, although it has been recognized that LOX could also produce HPs of the *R* configuration; manganese LOX yields predominantly 13(*R*)-HPOD (Brash, 1999; Nanda and Yadav,

2003). *R* ans *S* stereo-specificities are said to be dependant upon the oxygenation at the active site, following the antarafacial rule (Nanda and Yadav, 2003).

2.2.3.3.2. Hydroperoxide Lyase

Higher plant HPLs catalyze the cleavage of C-C bonds that are between the HP group and the *E* double bond of the substrate. Plant HPL are specific to 13(*S*)-, 9(*S*)-HPs of 18C linoleic and linolenic acids, or both. HPL specific to 9-hydroeproxide, such as HPL from pear, will yield C9 volatile compounds such as 3-nonenal which has a cucumber/fruity flavor whereas HPL specific to 13-HPs such as watermelon and tomato HPL (Vick and Zimmerman, 1976; Suurmeijer *et al.*, 2000), will produce C6 volatiles such as 3-hexenal which possess green notes. Unspecific HPL such as cucumber HPL will yield a wider range of volatiles (Matsui *et al.*, 2000). All plant HPL, however, could very well be specific; plants showing unspecific activity could in fact present more than one HPL isozyme (Matsui *et al.*, 1989).

Microorganism LOXs, by generating other HPs, may provide HPL and associated activities with different substrates and thus may allow the production of modified flavor profiles. 18C substrates are preferred by mushroom (Wurzenberger and Grosch, 1984; Grosch and Wurzenberger, 1985). Kermasha *et al.* (2002b) have demonstrated the preferential substrate specificity for 10-HPOD over the 9-, 12- and 13- isomers of linoleic acid for HPL of *P. camemberti*; however, only the *S* enantiomer of the racemic mixture of 10-HPOD was utilized (Kermasha *et al.*, 2002b). Although some HPL have been identified as being stereoselective to the *S* configuration, no HPL selective to the *R* configuration has been reported (Gardner, 1995).

The volatile end-products of the HPL and associated activities of *P. camemberti* using 10-HPODs as substrate included 1-octen-3-ol as major product and minor products of hexanal, 1-octen-3-one, 3-octanone, 2-octenal, 2-octen-1-ol and 2,4-decadienal (Kermasha *et al.*, 2002b). Mushroom has been reported to yield many 8C volatiles such as the above mentioned and 1-octanol, 3-octanol, 1,5-octadien-3-one, 1,5-octadien-3-ol and 2,5-octadienal as well as 6C hexanal and 7C benzaldehyde (Delcarte *et al.*, 2000).

2.2.4. Enzyme Structure

Soybean lipoxygenase type 1-B secondary structure has been determined by X-ray crystallography (Boyington *et al.*, 1993). Microbial LOXs and HPLs have been characterized in terms of activity and specificity but their structure is still undetermined.

2.2.4.1. Lipoxygenase

Lipoxygenase (EC 1.13.11.12) is a non-heme, iron containing dioxygenase (Siedow, 1991), with a molecular weight of 94-104 kDa in plant isozymes (Brash, 1999). Although plant LOXs possess about 25% more amino acid residues, which form a second independent domain, they have very similar amino acid sequences to animal LOXs (Minor *et al.*, 1996). The overall structure of soybean LOX-1 is organized into two domains: a β-sheet N-terminal domain, which is absent from mammalian LOX and a large C-terminal domain composed of more than 20 helices including at least six 3_{10}, α or π helices (Minor *et al.*, 1996). The two domains of the protein, the amino terminal of 146 amino acids and the carboxy terminal of 693 amino acid residues, compose the 839 amino acids of the 95 kDa monomer of soybean LOX. Domain I crystallizes as an 8 stranded antiparallel β barrel with an interior core composed of highly hydrophobic aromatic side chains; and domain II, the major domain of the protein, has 23 helices, two antiparallel β-sheets and two distinct sections of π-helix. Domain II helices are mostly parallel or antiparallel to each other and surround a long central helix with both terminal regions exposed to solvent. The secondary structure of LOX is said to be composed of 38% α-helices and 14% β-sheet (Boyington *et al.*, 1993).

2.2.4.2. Hydroperoxide Lyase

Hydroperoxide lyase (EC 4.1.2.-) is a heme-enzyme (Newcomb *et al.*, 2006). HPL heterolytic enzyme, widely distributed amongst higher plants, is membrane bound (Gardner, 1991). Structural studies have further characterized HPL in plants as a heme *b*, homo-trimeric protein of 55 kDa subunits, with 2.2 heme per enzyme molecule (Shibata *et al.*, 1995). The heme could be ligated to the carboxy-terminal cysteine residue at position 441 (Matsui *et al.*, 1996b). Matsui *et al.* (1996b) have elucidated the sequence of bell pepper 480 amino acid residues HPL and classified it into the P450 cytochrome

protein superfamily as a member of the CYP74B subgroup, although it presents a low affinity for CO, does not absorb at 450 nm, does not oxygenate but cleaves and functions without cofactors (Matsui *et al.*, 1996b). CYP74A, the closest subfamily regrouping allene oxide synthase, present a 40% homology with HPL (Delcarte *et al.*, 2000).

Homolytic HPL from *Chlorella pyrenoidosa* was also found associated to the cell membrane with a molecular weight of about 48 kDa (Vick and Zimmerman, 1989; Nuñez *et al.*, 1998) while *Oscillatoria* sp. HPL was reported to bear 56 kDa molecular weight (Andrianarison *et al.*, 1989). A soluble form of the enzyme has been reported in algae (Blee and Joyard, 1996).

2.2.5. Mechanism of Action

Although the mechanisms of the sequential enzymatic activities have been extensively studied in higher plant and animal cells, in microorganisms the biosynthetic pathway leading to the production of flavor compounds from free fatty acids remains unclear (Bisakowski *et al.*, 1997; Knapp *et al.*, 2002).

2.2.5.1. Lipoxygenase

The proposed mechanism of the LOX reaction, while remaining a matter of discussion, may consist of three consecutive steps which are illustrated in Figure 4. Before the reaction can proceed, the enzyme must be activated beforehand by exposure to the HP product of the reaction, which may be present with the unreacted substrate (Haining and Axelrod, 1958). Firstly, one electron is picked up by the Fe^{3+} form of LOX whilst a pro-chiral hydrogen atom is abstracted from a methylene group of the fatty acid carbon chain, forming a fatty acid radical; the LOX enzyme is then in its Fe^{2+} form. This first step is rate-limiting (Knapp *et al.*, 2002). Secondly, radical rearrangement occurs over part of the pentadienyl moiety as the unpaired electron is delocalized. The formation of a dioxygen bridged allyl radical was also suggested (Nelson *et al.*, 1994). The direction of the rearrangement depends upon the adopted conformation of the fatty acid at the active site. The double-bond shift with *cis-trans* isomerization yields conjugation of the *Z,E*-double bond in plant LOX. Thirdly, molecular dioxygen is inserted at the allyl radical forming still another radical intermediate which is then reduced to an anion while

the enzyme is oxidized back into the ferric form. The peroxidate anion will form the HP product upon contact with basic proton (Prigge *et al.*, 1997). It was also proposed that LOX has two binding sites, one for the substrate and one for the product, which could account for the inhibition that occurs at high product concentration (Wu, 1996).

2.2.5.1.1. Iron Coordination

As mentioned, LOXs has been reported to contain one non-heme, non-sulfur iron atom as its only cofactor. Iron may be ligated to histidines ubiquitously found in the catalytic domain of the C-terminal as well as a conserved isoleucine of the N-terminal (Brash, 1999). Site-directed mutagenesis experiments indicated that three of the six highly conserved histidines were required for enzyme activity and thus iron binding (Steczko *et al.*, 1992; Boyington *et al.*, 1993). Minor *et al.* (1996) have reported that of the 35 sequences of plant and animal LOX they compared, these three histidine residues were conserved.

The overall structure of the LOX may suggest how some of the catalytic steps occur. Prigge *et al.* (1997) have suggested that a fatty acid molecule could enter a cavity of the main domain II of LOX after a small movement of the sidechains. Within the cavity, the PUFA may approach the iron atom in such a way that the electron abstracted from the carbon chain is close to one of the unoccupied positions. Molecular oxygen would enter the active site via the tunnel of another cavity and coordinate with iron through another unoccupied position. Thus a total of six ligands would coordinate the iron atom in a distorted octahedral arrangement with reactive site adjacent to one another. Hydrogen abstraction is likely to be aided by the basic hydroxyl group of the water ligand (Minor *et al.*, 1996).

2.2.5.1.2. Antarafacial Rule

The oxygen insertion and the hydrogen abstraction ubiquitously occurs antarafacially to the pentadienyl moiety (Nanda and Yadav, 2003). The antarafacial rule implies that the oxygen molecule is added opposite from the initial hydrogen abstraction. In general, the normal orientation of the substrate in the active site pocket of soybean

LOX type 1 will lead to an attack at the ω-6 olefinic site whereas ω-10 oxygenation is due to opposite orientation of the substrate (Nanda and Yadav, 2003).

2.2.5.2. Hydroperoxide Lyase

HPL activity cleaves HPs of PUFAs into two fragments yielding a volatile compound and a non-volatile oxoacid. Heterolytic type HPL cleaves its substrate between the HP moiety and the double bond carbon, whereas homolytic activity, between the HP moiety and the saturated carbon (Delcarte *et al.*, 2000). The *homo*, or *same* and *hetero*, or *different* –lytic cleavages imply that the atoms that get separated either end up with the same number of electrons or a different number, respectively (Solomon *et al.*, 2003).

The currently accepted mechanism of heterolytic HPL catalysis (Figure 5A) involves several intermediates: an epoxy radical and/or cation is first produced by cleavage of the oxygen–oxygen bond of the HP which undergoes rearrangement to a vinyl ether cation, then. upon attack by water, a vinyl hemiacetal is produced which will yield two aldehyde molecules (Hatanaka *et al.*, 1986; Noordermeer *et al.*, 2001).

It was recently brought forward, however, that the primary intermediate in the heterolytic HPL reaction is the hemiacetal, and the previously accepted mechanism challenged (Grechkin and Hamberg, 2004; Matsui, 2006). The hemiacetal product obtained could be decomposed into an enol and an aldehyde (Grechkin and Hamberg, 2004). As the hemiacetal is said to be the true product of the HPL reaction, this activity would in fact be of an isomerase and not a lyase. It would also appear that the rearrangement of the HP into the hemiacetal occurs homolytically (Grechkin and Hamberg, 2004). The mechanism for homolytic HP lyase is illustrated in Figure 5B.

Matsui *et al.* (1992) have deduced part of the inactivation mechanism of HPL from molecules such as α-tocopherol, which trap free radicals. Although partially inhibiting HPL, they prevent their denaturation (Matsui *et al.*, 1992); the HP substrate may be specifically recognized at the active site and transformed to a hydrophobic radical which concomitantly destroys the essential active site thiol group (Matsui *et al.*, 1992).

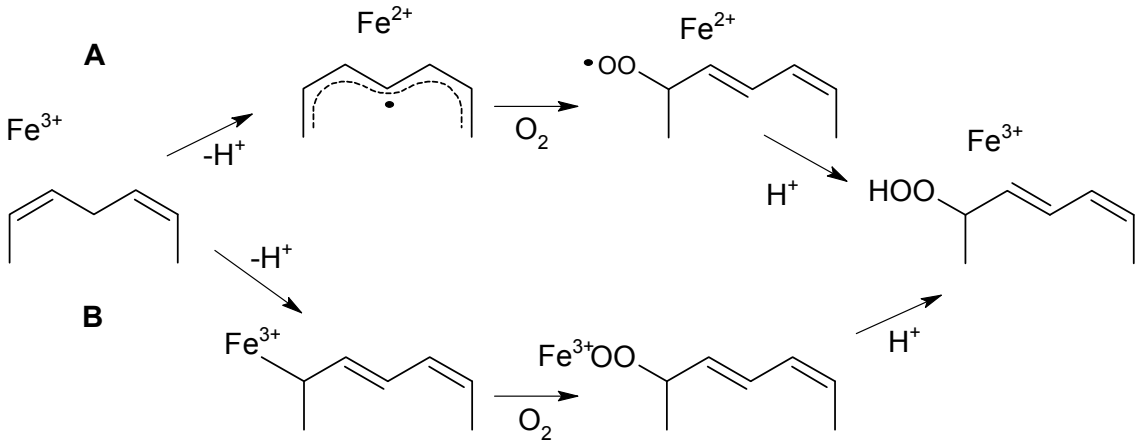


Figure 4. The two proposed mechanism for lipoxygenase activity (Prigge *et al*., 1997).

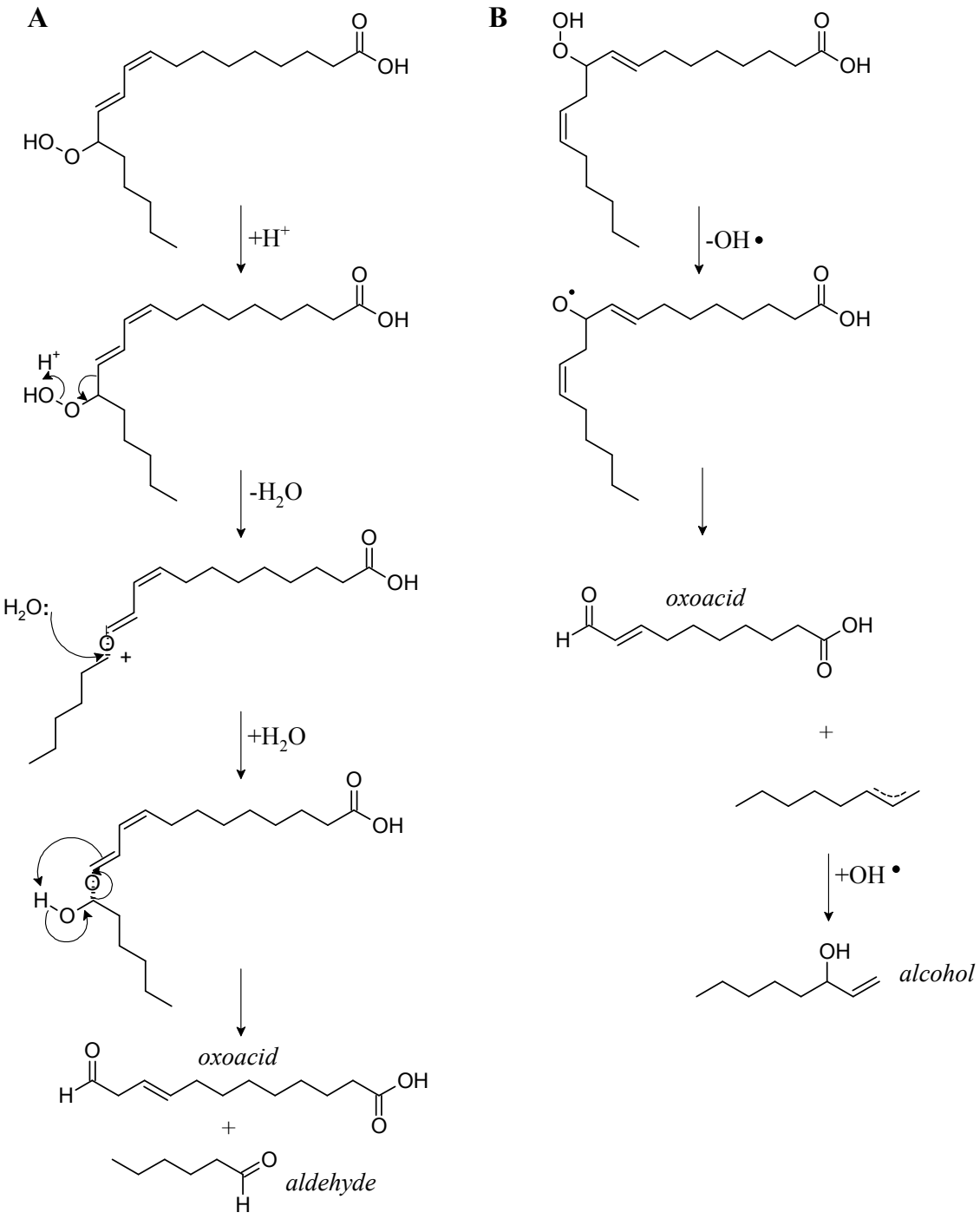


Figure 5. The proposed mechanism of heterolytic (A) and homolytic (B) hydroperoxide lyase activity (Gardner and Plattner, 1984; Wurzenberger and Grosch, 1984; Hatanaka *et al.*, 1986).

2.2.6. Spectrophotometric Assays in Aqueous and Organic Solvent Media (OSM)

To quantify the LOX and HPL activities, different methods have been reported in the literature. These methods are based principally on the quantity of the substrates consumed or on the amount of products generated by the enzymatic reactions. For the LOX reaction, analyzed substrates may include molecular oxygen or fatty acids such as linoleic, linolenic or arachidonic acids; the hydroperoxide (HP) products. For HPL, the HP substrate consumed is mostly used but products such as aldehydes and oxo-acids can also be analyzed. As these molecules possess different physico-chemical characteristics, their method of detection and analysis differ, and may include different spectrophotometric or chromatographic methods.

Spectrophotometric assays are popular means of quantifying any type of activity as they are usually simple and rapid. To measure the LOX activity, direct methods, including on-line kinetics and colorimetric assays, are available. Specifically, the properties of the conjugated diene or the peroxide group of the HP can be utilized in different spectrophotometric methods.

2.2.6.1. Conjugated Double Bonds Absorbance Assay

The absorbance of light at 234 nm is a common tool for the determination of both the LOX and HPL activities in aqueous media. It is based on the ability of the conjugated diene moiety of HP formed from LOX or consumed by HPL, to absorb light at 234nm (Axelrod *et al.*, 1981). For LOX activity, the increase at 234 nm is monitored whereas for HPL, it is its decrease in absorbance that indicates activity (Vick and Zimmerman, 1976).

The main advantage of this spectrophotometric method is that it allows for direct continuous measurements. However, the turbidity of the reaction mixture may be a problem in determining LOX and HPL activity of crude enzymatic extracts (Eskin *et al.*, 1977). Proteins and other compounds present in non-purified extracts often contribute to strong absorption in this region of the UV spectrum. Most of the times, extracts with low enzyme activity can not be measured adequately (Vick, 1991). To increase optical clarity of the solution, an emulsifier, such as polyoxyethylene sorbitan monolaurate (Tween 20), has been used to suspend the fatty acid substrates of the LOX reaction, especially at low

pH values (Kermasha and Metche, 1986). Tween 20 does not interfere in the assay but it can have competitive inhibitory effects when present in higher concentration than that of the fatty acid (Surrey, 1964).

This method has been reported to underestimate LOX activity. Because it is based on the formation of conjugated diene HPs, such as 9- and 13-HPODs; it does not measure the possible formation of other positional isomers, such as 10- and 12-HPODs, which do not contain a conjugated diene moiety (Grosch and Wurzenberger, 1985).

Also, this method is not specific as it does not differentiate between HPL and HP dehydrase (HPD) activities. Both enzymes use up the fatty acid HPs. By incorporating 10 mm KCN, which inhibits the HPD activity, Vick and Zimmerman (1976) overcame this problem. Another factor of discrepancies in the results may come from the oxoacid initially formed. As an example, 12-oxo-9-dodecenoic acid may become isomerized to 12-oxo-10-dodecenoic acid, which has a maximum at 226 nm. This absorption may affect the absorption at 234 nm (Olias *et al.*, 1990).

2.2.6.2. Ferrous Oxidation Assays

The method described above, which was principally developed for plant LOX and heterolytic HPL, may not be suitable for homolytic HPL activity. The spectrophotometric measurement at 234 nm limits monitoring HP such as 10- and 12-HPODs, which lack a conjugated diene chromophore. Ferrous oxidation assays using either xylenol orange or ferrous thiocyanate, have been reported has alternative spectrophotometric methods.

2.2.6.2.1. Xylenol Orange Assay

In acidic conditions, the HPs present oxidize the ferrous ions to their ferric counterparts which complex with the xylenol orange salt to forms a chromophore that absorbs at 560nm (Nourooz-Zadeh, 1999). Two methods of ferrous oxidation of xylenol orange (FOX) have been reported to quantify HP. The FOX1 method has the advantage of being very sensitive to HP present with a molecular extinction coefficient (ε_{560}) of 2.2 X 10^5 M^{-1} cm^{-1} (Wolff, 1994). However, it can only be applied to aqueous systems (Nourooz-Zadeh, 1999). The FOX2 method has thus been developed to measure HPs

present in liposome's or lipoprotein's suspensions and can be applied to lipidic systems (Jiang *et al.*, 1991). It's sensitivity has been found lower than the FOX1 method with ε_{560} of 4.3 X 10^4 M^{-1} cm^{-1} (Jiang *et al.*, 1991).

A major advantage of the FOX methods over the preceding one at 234 nm is the fact that it is not limited to the conjugated diene HP isomers. However, it does not allow for continuous measurements and its sensitivity has been found low although some attempts have been made to increase it. Modifications of the FOX2 method are also available; the use of triphenylphosphine (TPP) reduction allows for a more specific assay (DeLong *et al.*, 2002; Banerjee *et al.*, 2003) and addition of sucrose or replacement of sulphuric acid by perchloric acid have been shown to increase the sensitivity of the method (Deiana *et al.*, 1999; Gay and Gebicki, 2002; Vega *et al.*, 2005b).

2.2.6.2.2. Ferrous Thiocyanate

LOX and HPL activity were also determined by the ferrous thiocyanate assay at 490 nm, which is also specific for the determination of peroxides (Wurzenberger and Grosch, 1984). The ferrous thiocyanate assay has been used for the measurement of the formation of linoleic acid peroxide by a LOX extract from the alga *C. pyrenoidosa* (Zimmerman and Vick, 1973). The reaction is based on the oxido-reduction of $Fe(CNS)_2$ to the colored $Fe(CNS)_3$, detected at 490 nm. The major disadvantage of the ferrous thicyanate method, as for FOX, is that it does not allow direct continuous measurements.

2.2.6.3. Thiobarbituric Acid (TBA)

By heating of the sample in TBA under acidic conditions, a red compound forms, which is thought to be formed by the reaction of TBA with malondialdehyde (MDA). This red chromophore absorbs light at 532 nm with a ε_{532} of 1.56 $X10^5$ M^{-1} cm^{-1} (Yin and Porter, 2003). Methods based on the molecular excitement at 532 nm with fluorescence emission at 553 nm, or the formation of a yellow compound with absorbance at 455 nm, are also used (Yin and Porter, 2003).

Using TBA, the determination of lipoperoxides produced by a LOX activity from the fungus *P. orbiculare* was reported (Nazzaro-Porro *et al.*, 1986). Although very

sensitive, the TBA assays are considered non-specific as compounds other that MDA such as aldehydes, carbohydrates and amino acids, may also yield a red pigment. Furthermore, when using biological systems as samples, artificial oxidation may give a false positive response to the presence of HP and thus this method has been reported to be reliable for simple systems only (Yin and Porter, 2003).

2.2.6.4. Other Spectrophotometric Assays

For LOX activity measurements, the use of *N*,*N*-diethyl-1,4-phenylene-diammonium, substance which react with organic peroxides and forms purple-red species with a maximum of absorbance at 550 nm has been reported (Nazzaro-Porro *et al.*, 1986).

An alternative spectrophotometric assay for HPL activity may be adequate when monitoring plant extracts. This method is based on the ability of yeast alcohol dehydrogenase (ADH) to reduce aldehydes produced by the HPL activity in the presence of the reduced form of nicotinamide adenine dinucleotide (NADH). Thus, HPL can be assayed by coupling with yeast ADH and measuring the loss in absorbance at 340 nm as NADH is oxidized. It has the advantage of not measuring the presence of HPD (Vick, 1991). Also, this assay at 340 nm allows 5 to 10 times higher concentrations of protein extracts as proteins does not interfere as strongly as in the 234 nm region (Vick, 1991). However, the cleavage of HPs by homolytic HPL may result in the production of volatile compounds other than carbonyls (Gardner, 1991), therefore the yeast ADH test may be unsuitable for microbial HPL assays. To measure the activity of microbial HPL, the formation of the oxodiene product can be monitored near 280 nm, a region characteristic for conjugated dienone chromophore (Vioque and Holman, 1962).

2.3. Biocatalysis in Non-Conventional Media

2.3.1. Introduction

As enzymes are the catalysts for living organisms' reactions in water, which is the predominant solvent in living cells, it is obvious that most studies of enzymes have used aqueous environments (Adlercreutz, 2000). Although organic solvent media (OSM) were previously considered as denaturants, the use of enzymes as catalysts in OSM has gained

interest for their potential in industrial applications (Kim *et al.*, 2007). Most of the knowledge to date on OSM enzymology originates from lipases and proteases but applications for other enzymes have emerged (Gupta and Roy, 2004).

2.3.1.1. Definition

Adlercreutz (2000) defines non-conventional media as media with reduced water which contain organic substances, ionic liquids, supercritical fluids or which are solvent free, gaseous reaction media. In opposition, conventional media refers to aqueous solutions. The present discussion will cover the biocatalysis within organic solvent systems; the most commonly used non-conventional media (Adlercreutz, 2000).

2.3.1.2. Classification of Organic Solvent Media

As some organic solvents, such as alcohols, acetone, dimethyl sulphoxide and dimethyl formamide, are miscible with water, these solvents form one-phase systems when dissolved in water. These monophasic systems have the advantage of not displaying mass transfer limitations. However, high concentrations of solvents are often necessary and lower operational stability of the enzyme is reported (Adlercreutz, 2000).

The stability of the biocatalyst can be improved, however, by the use of two-phase organic solvent-water mixtures. These systems are obtained through the use of water-immiscible organic solvents such as hydrocarbons, ethers and esters. Partitioning of the reactions' components is generally obtained as follows: the enzyme and product, depending on solubility, within the aqueous phase and the substrate within the organic phase. This partitioning has the advantage of lowering substrate and/or product inhibition. However, these systems tend to have limited mass transfer and thus call for a higher interfacial area, itself a risk for enzyme inactivation (Adlercreutz, 2000).

Although a minimum amount of water must always be conserved, these two previously described major categories of organic systems can be composed of varying concentrations of solvent and water: from almost no water to almost no organic solvent. Furthermore, surfactants can be introduced in the mixture to prepare microemulsions with micro droplets of oil and water separated by films of surfactant, with the enzyme

normally present in the water phase. Although surfactants may lead to difficult isolation of the reaction product and enzyme inactivation, the very large interfacial area created in these systems increase mass transfer (Adlercreutz, 2000). It was also found that the fatty acid anion of HPOD could act as a surfactant in the system (Piazza *et al.*, 1994).

2.3.2. Advantages of OSM

Different reasons push researchers in using OSM: the use of low water soluble reactants is possible in OSM (Kim *et al.*, 2007), OSM may provide with a mean of changing the specificities of enzymes and thus the possibility of obtaining other valuable flavor compounds, synthetic reactions are favored as opposed to hydrolytic ones, the suppression of unwanted processes such as microbial growth and the increased stability of the biocatalysts (Halling, 2002). Also, the addition of organic solvent to a LOX reaction system decreases the possibility of rate-limiting depletion of oxygen, oxygen being 10 times more soluble in organic solvent than in water (Linke, 1965).

2.3.2.1. Specificity

Although the exact mechanism is not understood, the nature of solvents affect both the enantio- and regiospecificity of enzymes (Gupta and Roy, 2004). The differential solvation of substrates may explain the effect on substrate selectivity (Halling, 2002). Moreover, the conformation and flexibility of a protein in non-aqueous media change (Affleck *et al.*, 1992) although their crystal structure are identical no matter what solvent is used (Fitzpatrick *et al.*, 1994). Thus modifications in the secondary and/or tertiary structure, which are determinant in the specificities shown by an enzyme (Pourplancher *et al.*, 1994), could also explain these variations in specificity.

2.3.2.2. Thermostability

As water is involved in enzyme inactivation, manipulation of its content within the reaction medium influences the stability of the catalyst (Halling, 2002). Lower hydration level of enzyme implies slower conformational changes, which increase the rigidity and thus the stability of biocatalysts (Halling, 2002). Thermostability is also an additional benefit of important significance in the cost of bioprocesses (Illanes, 1999).

2.3.2.3. Solubilization of Substrate and/or Product

Because of limited water solubility of substrates, aqueous biocatalysis leads to large reaction volumes and complicate product recovery (Carrea and Riva, 2000). An advantage of non-conventional media biocatalysis is the increase in solubility of both substrates and products and the possibility of engineering the reaction system in order to more efficiently isolate the products (Kim *et al.*, 2007).

2.3.3. Parameters Affecting Biocatalysis in OSM

2.3.3.1. Quantification of Water

Although an increase in water usually increases the activity of enzymes in OSM, it is the water activity (A_w) more than the water content of a reaction medium that is useful in the quantification of water in enzymatic reaction (Gupta and Roy, 2004). While easier to work with and thus more commonly used than A_w, water concentration does not inform precisely on the hydration level of an enzyme (Adlercreutz, 2000). A_w is so defined that its value is of 1.0 in pure water and 0.0 in completely dry systems; dilute aqueous solutions have practical A_w values of 1.0 while A_w of OSM vary widely between values of 0 and 1 (Adlercreutz, 2000). A good correlation is seen between A_w, enzyme hydration and enzyme activity.

To control A_w of solvents, saturated solutions of some salts can be pre-equilibrated with the different reaction components. These salts release and absorb water at a constant humidity and A_w in the headspace, and thus after incubation with a solvent, equilibrium is attained and the A_w of the solvent is concomitantly reached (Halling, 2002). Specific salts are used for different A_w levels (Gupta and Roy, 2004). Drying agents are convenient for A_w lower than 0.05 whereas the simple addition of water to water-miscible solvents is suitable for A_w close to 1.0 (Halling, 2002).

Variations in the A_w occurs when the bioprocess is producing or consuming water. In situ control of A_w during the reaction can be achieved using pairs of salt hydrates. Salt hydrates can act similarly as buffers to maintain a constant A_w (Adlercreutz, 2000). An alternative method to control in situ A_w is to allow water exchange across a membrane

such as microporous or ultrafiltration membranes or more conveniently, silicone tubing (Halling, 2002).

2.3.3.2. Choice of Solvent

Hydrophilic solvents may have a tendency to 'strip' the water from an enzyme's active site since they are better suited to dissolve that essential water (Hudson *et al.*, 2005). But as the reaction's components are equilibrated to the same A_w, this effect cancels out (Halling, 2002).

The hydrophobicity of solvents partly explains the fact that most non-polar solvent offer the best media for enzyme activity (Hudson *et al.*, 2005). Hydrophobic solvent molecules from the bulk phase show a lower tendency to migrate into the aqueous micro-environment of the enzyme (Halling, 2002). Unlike the dielectric constant which is more an indicator of polarity, *Log P* is a measure of the hydrophobicity of OSM. The *Log P* value is the logarithmic partition coefficient of the solvent in octanol and water.

2.3.3.3. pH

The ionization state of an enzyme is important for biocatalysis. The concept of pH memory was first introduced at the end of the 1980s by Zaks and Klibanov (1988). It is now accepted that suspended enzyme powders in OSM are maximally active if lyophilized at the pH where they are similarly active in aqueous medium (Costantino *et al.*, 1997). Some processes have been shown to 'erase' the pH memory. For instance, buffer counter ions may undergo proton exchange with the enzyme to yield neutral species which are in turn lost during lyophilization (Halling, 2002). Similarly to salt pairs for A_w control, acid-base buffer substances such as trisoctylamine and triphenyl acetic acid can, although tediously, be introduced in the OSM to control the pH of the reaction (Adlercreutz, 2000). Another approach would be the use of buffers that are almost insoluble in the solvent used such as zwitterionic solids (Halling, 2002).

2.3.3.4. Substrate Concentration and Diffusion

When the medium of reaction is changed, the solubility of the substrate is influenced which, in turn, affects the K_m value of the enzyme for this substrate (Halling,

2002). An increase in solubility of the substrate is often concomitant with a decrease of its availability. The solvation of substrate by a solvent may thus influence its ability to interact at the active site and concomitantly influence the substrate specificity of the enzyme (Carrea and Riva, 2000; Kim *et al.*, 2007). A study on horseradish peroxidase has shown that inactivation of the enzyme in solvent was due to the substrate partitioning away from the enzyme to the bulk of the solvent (Akkara *et al.*, 1999).

2.3.4. Activity and Stability of Enzymatic Preparations in OSM

There exist several reasons as to why enzyme activity commonly decreases in OSM, including the loss of essential water at the enzyme active site's surface, a decrease in the polarity of the enzyme's micro-environment, an increase in conformational rigidity of the enzyme's structure, a stabilization of the substrate ground-state in organic solvent as compared to aqueous medium, and/or the loss of activity during enzyme preparation (Lee and Dordick, 2002; Hudson *et al.*, 2005; Brena and Batista-Viera, 2006).

Although immobilization is the strategy most commonly used to stabilize enzyme in organic solvent media, others, such as chemical modification, chemical crosslinking and cross-linked enzymes crystals (CLEC), protein aggregation and protein engineering have been used for increasing the activity of biocatalysts in OSM (Gupta and Roy, 2004; Hudson *et al.*, 2005). Immobilization will be covered in the next part; enzyme preparation as it affects the biocatalytic reaction in OSM is be described bellow.

2.3.4.1. Enzyme Preparation

Suitable preparations for enzyme reactivity in organic solvent media include: enzyme powders, enzyme crystals, covalently modified enzyme dissolved in the solvent and enzyme solubilised by a surfactant or a polymer and in a micro emulsion (Adlercreutz, 2000; Hudson *et al.*, 2005; Sheldon, 2007).

Perhaps the most basic method of using an enzyme in organic solvent is to suspend the powder obtained by lyophilization directly into the solvent (Adlercreutz, 2000). Although simple and rapid, this method does not optimize the efficiency of an enzyme. However, enzyme crystals are the most concentrated form of the catalyst and the

process of crystallization may concomitantly purify a crude enzymatic preparation (Adlercreutz, 2000). Furthermore, the use of glutaraldehyde to crosslink the enzyme crystal has been shown to improve the stability and catalytic activity of such preparation (Margolin and Navia, 2001). CLEs (cross-linked dissolved enzymes) can also be entrapped in a gel matrix for further stability (Cao *et al.*, 2003).

Enzymes can also be solubilized in organic solvents. Generally, solubility is a tradeoff with activity. Enzymes can be covalently modified by polymers such as polyethylene glycol, polystyrene or polyacrylates, which are coupled to their amino groups, to render them soluble in aromatic or chlorinated hydrocarbon solvents (Adlercreutz, 2000). Increased solubility has the advantage of decreasing mass transfer limitations in bioprocesses (Halling, 2002). Although inactivation of the enzyme may occur during the coupling process, covalently modified enzyme can be reused and separated from the product of the reaction after precipitation using a nonpolar solvent (Hudson *et al.*, 2005). Enzyme can also be non-covalently coupled to polymers such as ethyl cellulose and poly(methyl methacrylate); this method prevents the enzyme inactivation which may occur during covalent coupling (Adlercreutz, 2000).

Solubilization of enzymes can be achieved through the use of surfactants, with or without microemulsions. The surfactant dialkyl glucosyl glutamate generally complexes well with enzymes and aerosol OT (dioctylsulfosuccinate, sodium salt) can be used to solubilize chymotrypsin in iso-octane (Paradkar and Dordick, 1994; Adlercreutz, 2000).

2.3.4.1.1. Protection against Lyophilization

In biocatalysis in aqueous medium, the water rehydrates and reverses most of the lyophilization damage. However, the lyophilization effect can not be reversed in pure OSM, and thus the use of additives is common. A wide range of molecules are used as cryoprotectant, including molecules such as sugars, amino acids, polyols and salts; they protect the structure of the enzyme during freezing through preferential exclusion. Lyoprotectants, such as sucrose, protect from the drying by substituting water molecules that are removed and forming H-bonds with the enzyme (Gupta and Roy, 2004).

2.3.4.1.2. Activation of the Enzyme via Excipients and Additives

Several molecules have served as excipients in order to activate the enzyme preparation for biocatalysis in OSM, such as non-buffer salts, crown ethers, cyclodextrins, acids and bases, molecular imprinters and denaturants and surfactants. They sometimes are concomitantly used against lyophilization damages.

2.3.4.2. Enzyme Modification and Protein Engineering

As enzymes have evolved for a particular function in vivo, within a complex metabolic network, this restricts their use in an industrial environment. Mutant enzymes can be screened for specific applications (Gladilin and Levahsov, 1998). Site-directed mutagenesis is based on the knowledge of the primary, secondary and tertiary structure of an enzyme. Mutations are introduced within the structure of the protein to increase stability and activity in OSM. Contrary to directed evolution, the structure and mechanism of an enzyme must be known. Directed evolution consists in introducing random mutations, errors introduced over many generations. The candidate of the first generation which shows the best characteristics, taking into account that one feature is not evolved at the expense of another, is subjected to another generation. The final enzyme should be more adapted to a new environment. Various choices are offered to the scientist wishing to create a DNA library from which a candidate will be screened: error-prone PCR, combinatorial mutagenesis, *In vitro* combination, DNA shuffling, random-priming recombination, to name a few (Hari Krishna, 2002).

2.3.5. LOX and Associated Enzymes in OSM

Many research groups have studied the effect of OSM on soybean LOX-1 since the 1990s (Parra-Diaz *et al.*, 1993; Piazza *et al.*, 1994; Hsu *et al.*, 1997; Hsu *et al.*, 1998; Kermasha *et al.*, 2001; Kermasha *et al.*, 2002a; Vega *et al.*, 2005a). However, to this date, no report of similar studies using microbial LOX and HPL is found in the literature. Table 3 summarizes the most significant findings on LOX in OSM. More studies in OSM have been reported using immobilized LOX; they are presented in the next section.

Table 3. Summary of literature concerned with LOX in OSM.

Enzyme and source[a]	***Optimal system***[b]	***Results***[c]	***Research Group***[d]
IMM Soybean LOX	93:5:2 hexane:dioxane:buffer pH 9.0	1.6 fold increase in activity as compared to in hexane alone	Vega *et al.*, 2005
IMM Soybean LOX	ternary micellar system 4% iso-octane in buffer pH 9.0 with Tween 40	218-272 nmol HPODs formed per mg protein per min	Kermasha *et al.*, 2002
Soybean LOX	49:1 solvent:buffer pH 9.0	2.6, 2.2 and 1.8 fold activity with iso-octane, octane and hexane, resp.	Kermasha *et al.*, 2001
IMM Soybean LOX	oxygen-saturated iso-octane (10%) in buffer pH 9.0 at 25°C	IMM-LOX 60% more active than in aqueous medium	Hsu *et al.*, 2000
IMM Soybean LOX	15% oxygen-saturated iso-octane in buffer pH 9.0	IMM-LOX 3.5 fold more active than in aqueous medium	Hsu *et al.*, 1998
IMM Soybean LOX	71% 1,1,2-trichlorotrifluoroethane in buffer pH 9. 0 at 15°C	22.2 μmol HPODs formed per mg imm-LOX in 1 hr	Piazza *et al.*, 1994
Soybean LOX	water-saturated octane (99%) in buffer pH 9.0 at 15°C	9-15.2 μmol HPODs formed per mg protein in 3 hrs	Parra-Diaz *et al.*, 1993
Soybean LOX	microemulsions of solvent and surfactant Solvent was 2,2,4-trimethylpentane in buffer pH 9.0 Surfactants were Nonidet P40 and Triton X-35	nearly quantitative conversion	Piazza, 1992

[a]The prefix IMM is used when the enzyme was immobilized; the support will be further discussed in the next section.
[b]Composition of the organic solvent medium used.
[c]Results most significant to the present review
[d]Reference.

2.4. Enzyme Immobilization

2.4.1. Advantages of Immobilized Enzymes

Using immobilized enzymes increases the stability of biocatalysts both in terms of operational activity and storage time. Other advantages include a better control of the process, more flexibility in reactor design as well as the possibility of reusing the enzymes and thus reducing the cost of the process (Illanes, 1999; Hudson *et al.*, 2005). As the immobilized enzyme does not usually mix with the product, the use of immobilization also facilitates product recovery (Pedersen and Christensen, 2000).

Also, an immobilized biocatalyst can be used in different ways, creating new possibilities: into a solid-phase enzyme reactors (packed bed and open tubular) for use in continuous flow processes, onto membranes incorporating sensors such as potentiometric enzymes and as solid-phase enzyme films in disposable kits (Sheldon, 2007).

2.4.2. Definition and Classification

When first introduced as an immobilization method in 1916 (Nelson and Griffin), animal charcoal was used to immobilize invertase. Since then, a vast range of compounds and methods have been used in order to immobilize enzymes, or cells, onto solid supports, with immobilized glucose isomerase probably being the largest scale application (Pedersen and Christensen, 2000). In general, immobilization has been accepted as a convenient mean of improving enzymes performance and stability (Brena and Batista-Viera, 2006).

Immobilization can be defined as the confinement of enzymes or whole cells, with retention of catalytic activity, which allows for physical separation from the mixture of substrate and product for continuous biocatalysis and reuse (Pedersen and Christensen, 2000; Brena and Batista-Viera, 2006).

2.4.2.1. Covalent Binding and Physical Adsorption

Covalent and physical adsorption binding are considered the conventional methods of immobilization. In general, they both lower activity but increase the stability

of biocatalysts (Heichal-Segal *et al.*, 1995; Brena and Batista-Viera, 2006). Both methods utilize insoluble matrix.

Alumina and charcoal were the first supports to be used and thus adsorption binding was the first type of immobilization technique developed (Scragg, 1988). Different types of physical adsorption exist, depending on the nature of the support, namely hydrogen bonding, hydrophobic interaction and ionic forces. DEAE (diethylamino-ethyl) cellulose and CM (carboxymethyl) cellulose are good examples of anion and cation exchangers used, respectively (Scragg, 1988); adsorption being carried out through ionic forces. Adsorption can also be performed using hydrophobic polymers. They typically are acrylic, polypropylene or divinilbenzene-styrene based. Thirdly, adsorption is sometimes performed using silica, through H bonds (Pedersen and Christensen, 2000; Brena and Batista-Viera, 2006).

The main advantages of physical adsorption techniques over covalent binding are their simplicity of use and their high initial yield. As they are not as harsh on proteins as covalent binding treatment, physical adsorption techniques does not affect the 3-D conformation of protein but as they create weaker bonds; the protein has a tendency to desorb from the support (Scragg, 1988). On the other hand, adsorption allows for reuse of the support (Pedersen and Christensen, 2000).

Covalent attachment of enzymes to carrier is typically used when long term binding is wanted. Covalent bonds are formed between the amino or carboxyl groups present in an enzyme and insoluble support materials including porous glass, ceramics, stainless steel, sand, charcoal, cellulose, synthetic polymers such as nylon, and metallic oxides like TiO_2 (Scragg, 1988). The degree of attachment is also an important parameter in covalent coupling in terms of stability and retained activity. Multipoint attachment can result in a more rigidly immobilized enzyme and thus in a more stable biocatalyst (Pedersen and Christensen, 2000; Brena and Batista-Viera, 2006).

2.4.2.2. Enzyme Entrapment

Enzymes can be entrapped within a polymer (or gel entrapment), by micro-encapsulation and using semi-permeable membranes (Worsfold, 1995; Sheldon, 2007). Gel entrapment, into a 3-D polymer network, is widely used for whole cell immobilization but toxicity of the support on biocatalyst limits its industrial use on enzymes (Pedersen and Christensen, 2000).

Although higher activities then conventional immobilization methods are retained, drawbacks of gel entrapment are their narrow bore and brittleness which increase diffusional limitations (Heichal-Segal *et al.*, 1995; Sheldon, 2007). Different polymers are used in this process, including natural collagen, gelatin, agar, agarose, chitosan and cellulose and synthetic polyacrylamide, methacrylates, polyethylene-glycol, polyurethane and epoxy resin. Because alginate can be polymerized under mild conditions, at room temperature, it is commonly used.

Sol-gel matrices have also been used; they entrap enzymes into an amorphous SiO_2-network (Pedersen and Christensen, 2000); microencapsulation has the advantage of yielding immobilized enzymes with high activity due to the increase surface area of the polymer (Sheldon, 2007). Enzymes or whole cells can also be entrapped within a permeable membrane or an hollow fiber unit (Sheldon, 2007). Membranes are for use in bioreactors where the actual immobilization process may involve physical adsorption or covalent binding onto hollow fiber (Pedersen and Christensen, 2000). Fixed-beds and microporous membrane reactors allow for continuous reaction and product removal (Carrea and Riva, 2000).

2.4.2.3. Carrier Free Immobilized Enzymes

Cross-linking is a method used for preparing enzymes for use in OSM. It can also be used as an immobilization method using multi-functional reagents (Worsfold, 1995). Enzymes can cross-link to one another to form larger insoluble particles called CLECs (cross-linked enzymes crystals) (Cao *et al.*, 2003). CLECs display simplicity of preparation, high concentrated enzyme activity as compared to carrier immobilized enzymes and low production cost since there is no need for expensive carrier (Cao *et al.*,

2003; Sheldon, 2007). Most commonly used multi-functional cross-linking reagents are glutaraldehyde, dimethyladipimidate and dimethyl suberimidate, through amino groups and aliphatic diamines activated with carbodiimides through carboxyl groups (Scragg, 1988).

2.4.3. Effect of Immobilization on Kinetics and Properties of Enzymes

2.4.3.1. Stability

Multipoint attachment and immobilization increase the rigidity of an enzyme, limit conformational changes and increase the stability of enzymes (Cao *et al.*, 2003; Brena and Batista-Viera, 2006).

2.4.3.2. Stearic Hindrance

The activity of immobilized enzyme is often lowered due to stearic hindrance, which is caused by the covalent attachment of some enzymes that impede access to the substrate. The use of spacers on the support material as well as moderate loading of the enzyme on the carrier limit this phenomenon (Scragg, 1988; Carrea and Riva, 2000).

2.4.3.3. Diffusional Limitations

Diffusional limitation lowers enzyme activity of immobilized preparation. External diffusional limitations take place because of mass transfer of substrate from the bulk solution to the immobilized enzyme's boundary layer of water whereas internal limitations are caused by the difficult diffusion of substrate into the support material into the micro-environment of the enzyme preparation. To increase activity, the enzyme particle size can be reduced, the substrate concentration increased, the porosity of the support material improved and the boundary layer depth decreased (Hudson *et al.*, 2005).

2.4.4. Immobilized LOX and Associated Enzymes

Microbial LOX immobilization has not been reported and only one publication can be found on the immobilization of microbial HPL (Nuñez *et al.*, 1997). On the other hand, numerous papers have been published on the immobilization of soybean LOX using various types of supports such as covalent binding (Laakso, 1982; Chikere *et al.*, 1998; Chikere *et al.*, 2001; Vega *et al.*, 2005a), adsorption (Parra-Diaz *et al.*, 1993;

Piazza *et al.*, 1994; Petrus Cuperus *et al.*, 1995) and physical entrapment (Pinto and Macias, 1996; Hsu *et al.*, 1997; Hsu *et al.*, 1998; Shen *et al.*, 1998; Hsu *et al.*, 2000). However, most studies have only been performed in aqueous media; immobilized soybean LOX has been reported in a ternary micellar system (Kermasha *et al.*, 2002a) and in the presence of organic solvent (Piazza *et al.*, 1994; Vega *et al.*, 2005a). Table 4 and 5 summarize the studies performed on immobilized LOX and HPL, respectively.

Table 4. Summary of literature concerned with LOX immobilization.

Enzyme and source	***Immobilization Support***[a]	***Type***[b]	***Results***[c]	***Research Group***[d]
Soybean LOX-1	modified Eupergit®C250L *OSM*	CB	increase in activity and in stability to heat and co-solvent	Vega *et al.,* 2005
Soybean LOX-1	DOWEX®50W4-200 *OSM*	PA(i)	stability and up to 4 cycles possible	Kermasha *et al.*, 2002
Soybean LOX	oxirane acrylic beads *BIORECTOR*	CB	protection against H_2O_2 inactivation 50% of initial activity after 4 cycles	Santano *et al.*, 2002
Soybean LOX-1 Soybean LOX-2	Fractogel®EMD Azlacone Fractogel®EMD epoxy Eupergit®C oxirane beads	CB	half-life increased 20 fold with immob. performed in low ionic strength buffer	Chikere *et al.*, 2001
Soybean LOX	alginate sol-gel matrix *OSM*	GE	IMM-LOX has increased activity using dilinolein over free while physical and chemical characteristics similar to free preparation	Hsu *et al.*, 2000
Soybean LOX	sol-gel matrix *BIOREACTOR* (calcium alginate or phyllosilicate)	GE	equivalent to five continuous stirred tank reactors (CSTRs)	Hsu *et al.*, 1999
Soybean LOX	cross-linked phyllosilicates *OSM*	CL	60% protein leaching after 20 hr But IMM-LOX conserved initial activity	Hsu *et al.*, 2000
Soybean LOX	cross-linked phyllosilicates	CL	Storage stability for more than 3 months Increased substrate diffusion	Shen *et al.*, 1998
Soybean LOX-1 Soybean LOX-2	Fractogel®EMD Azlacone	CB	IMM-LOX-1 showed better stability than IMM-LOX-2 with 90% of initial activity retained after 40 days at room T.	Chikere *et al.*, 1998

Table 4. Continued.

Enzyme and source	*Immobilization Support*[a]	*Type*[b]	*Results*[c]	*Research Group*[d]
Soybean LOX-1 Soybean LOX-2	calcium alginate beads	GE	Retains activity >25 days at room T. activity restored to free LOX levels with glycerol or organic solvent treatment	Hsu *et al.,* 1997
Potato tuber LOX-5	oxirane acrylic beads	CB	90% activity preserved for 30 days 60% residual activity after 9 cycles	Pinto *et al.,* 1997
Soybean LOX	polyacrylamide gel *BIOREACTOR* derivatized with glutaraldehyde	GE	catalytic efficiency slightly lowered but increased stability at -70 and 4°C	Pinto and Macias, 1996
Soybean LOX-1	DEAE anion exchanger cellulose	PA(i)	10-15 μmol HPODs min^{-1} g^{-1} immobilization efficiency of 86 to 89%	Petrus-Cuperus *et al.,* 1995
Soybean LOX	carbonyl-diimidazole act. polymer *OSM*	GE	no data available on immob. effect easier product separation	Piazza *et al.,* 1994
Soybean LOX	carbonyl-diimidazole act. polymer *OSM*	GE	Retained full initial activity in buffer but 60% in OSM after 7 cycles 10 X stability as compared to free LOX	Parra-diaz *et al.,* 1993
Soybean LOX	agarose (cyanogen act. Sepharose) *BIOREACTOR*	GE	75% activity recovered 6 months stability 0.6 mg HPODs ml^{-1} wet gel hr^{-1}	Laakso, 1982

[a]The symbol *OSM* indicates that OSM was also used in the study whereas *BIOREACTOR* implies the use of continuous product removal from the reaction system.

[b]CB for covalent binding, PA(i) for physical absorption by ionic interactions, GE for gel entrapment

[c]Results most significant to the present review

[d]Reference.

Table 5. Summary of literature concerned with HPL immobilization.

Enzyme and source	***Immobilization Support***[a]	***Type***[b]	***Results***[c]	***Research Group***[d]
Carnation petals, Tomato leaves and Strawberry LOX and HPL	Ca^{2+} alginate matrix *BIORECTOR*	GE	112X hexanal production as compared to endogenous yields over 30 min.	Schade *et al.,* 2003
Tomato LOX and HPL	not immobilized but *BIOREACTOR*	--	hollow-fiber ultrafiltration and product Removal using solid phase microextraction Bioreactor operation stable for 1 week	Cass *et al.,* 2000
Spinach HPL	Akrilex®C polyacrylamide	CB	64% of initial activity after 5 cycles 360 mU g^{-1} dry gel	Simon *et al.,* 1998
Mung beans HPL (*Phaseolus radiatus* L.)	UltraLink® Iodoacetyl gel	CB	86% activity retained after 18 days 1.3 U ml^{-1} gel	Rehbock and Berger, 1998
Chlorella HPL (*pyrenoidosa, fusca*)	Affi-Gel®501	CB	no activity loss after 5 cycles no loss of activity after 4 months storage	Nunez *et al.,* 1997
Soybean leaf HPL	free extract comparison	--	Half-life of 1.5days on ice	Gardner *et al.,* 1991

[a]The symbol *OSM* indicates that OSM was also used in the study whereas *BIOREACTOR* implies the use of continuous product removal from the reaction system.

[b]CB for covalent binding, GE for gel entrapment.

[c]Results most significant to the present review

[d]Reference.

CHAPTER III.

FOURIER TRANSFORM INFRARED STUDY OF LIPOXYGENASE CONFORMATION IN ORGANIC SOLVENT MEDIA

3.1. Abstract

The secondary structure of commercially purified soybean lipoxygenase (EC 1.13.11.12) was investigated in selected monophasic organic solvents, including chloroform, methanol, acetonitrile, hexane and octane. The Fourier transform infrared (FTIR) spectra of the enzyme obtained in chloroform, methanol and acetonitrile showed an absorption band at 1617 cm^{-1} indicative of significant protein aggregation, whereas spectra of lipoxygenase in hexane and octane exhibited substantially less aggregate formation. Variable-temperature infrared studies of lipoxygenase in D_2O show that the predominately α-helical structure of the protein undergoes an irreversible transition to intermolecular β-sheet at and above 65^oC. Chemical imaging technology employing an FTIR spectrometer equipped with an infrared microscope and a focal-plane array detector was used to examine the changes in the secondary structure of lipoxygenase at the water-hexane interface in the presence and absence of substrate. The secondary structure of lipoxygenase at the hexane-water interface was comparable to that of the structure of lipoxygenase in D_2O after exposure of lipoxygenase solution to hexane.

3.2. Introduction

The use of enzymes as catalysts in organic solvent media (OSM) is gaining interest for its potential industrial applications (Gupta and Roy, 2004). Reactants having low solubility in water can be used with OSM, and solvent engineering in enzyme biocatalysis allows optimization of reaction yield and changes in specificity (Carrea *et al.*, 1995). Ongoing research in our laboratory is aimed at the development of biotechnological processes involving biocatalysis by lipoxygenases in OSM (Kermasha *et al.*, 2001; Kermasha *et al.*, 2002a; Vega *et al.*, 2005a). Lipoxygenase (EC 1.13.11.12) is an important class of non-heme iron enzymes that catalyze the di-oxidation of linoleic acid and other polyunsaturated fatty acids containing a *cis,cis*-1,4-pentadiene moiety to their corresponding hydroperoxides, which are considered flavor precursors (Wong, 1995). The hydroperoxide products have been recognized as reaction intermediates in the

production of various biomolecules, aroma compounds and fine chemicals (Gardner, 1991). Soybean lipoxygenases are of three types (Siedow, 1991); type 1-B is the most well characterized and its secondary structure has been determined by X-ray crystallography (Boyington *et al.*, 1993).

Modifications in the secondary and/or tertiary structure are determinants of the specificities shown by an enzyme (Pourplancher *et al.*, 1994). In an X-ray crystallographic study (Fitzpatrick *et al.*, 1994), proteins suspended in different media were found to show similar structures after crystallization, but X-ray crystallographic data cannot account for a protein's behavior and conformation in solution (Klibanov, 1997); crystal forces may induce a structure that differs from the conformation adopted in solution (Schweitzer-Stenner, 2001). Spectroscopic techniques such as NMR, circular dichroism (CD), FTIR, Raman, and fluorescence spectroscopy offer versatile and complementary means of determining protein structure in solution (Bloemendal and Johnson, 1995; Jung, 2000; Pelton and McLean, 2000). The advantage of FTIR spectroscopy lies in its ability to investigate the secondary structure of proteins under a variety of physicochemical conditions, including at the water-solvent interface. The use of FTIR imaging systems, equipped with an IR microscope and a focal plane array (FPA) detector, allows for the simultaneous acquisition of hundreds of FTIR spectra from contiguous portions of a sample, with a spatial resolution on the order of 5.6 μm (Kirkwood *et al.*, 2004), making FTIR imaging a useful technique for the elucidation of the substrate, enzyme and product partitioning in OSM reaction media.

The present work is aimed at investigating the conformation of lipoxygenase type I-B by FTIR spectroscopy in aqueous medium as a function of pH and temperature as well as in various organic solvents and at the interface in biphasic systems.

3.3. Material and Methods

3.3.1. Materials

Commercial purified soybean lipoxygenases type I-B (394,100 U/mg protein) was purchased from Sigma Chemical Co. (St-Louis, MO). Linoleic acid (*cis*-9, *cis*-12-octadecadienoic acid) was obtained from Nu-Chek Prep Inc. (Elysian, MN). All solvents

used were of HPLC grade or more and were purchased from Fisher Scientific (Fair Lawn, N.J.), except for deuterium oxide (99.8 atom%), which was obtained from Aldrich Chemical Co. (Milwaukee, WI). Boric acid was obtained from BDH (Toronto, ON) whereas NaOH, citric acid, sodium citrate, KCl and HCl were obtained from Fisher Scientific.

3.3.2. Substrate Preparation

The substrate model used throughout the present study was linoleic acid. For the enzymatic assay in aqueous medium, stock solutions of the substrate at a concentration of 4 mM were prepared using Tween 20 as surfactant according to the procedure described previously (Kermasha and Metche, 1986), whereas for that in OSM, a 70 mM stock solution was prepared directly in the OSM (Vega *et al.*, 2005a).

3.3.3. Enzyme Preparation

Purified soybean lipoxygenase type I-B was dialyzed to remove the stabilizer and salts from the commercial preparation. The enzyme suspension (5%, w/v) was prepared in Tris-Deuterium hydrochloride (DCl) buffer solution (0.1 M, pD 9.2). Protein concentration was determined according to a modification of the Lowry method (Hartree, 1972), using bovine serum albumin (Sigma Chemical Co.) as a standard for the calibration curve.

The effect of pre-incubation of lipoxygenase in hexane on its activity in aqueous medium was also determined. In the pre-incubation step, 20 μL of lipoxygenase suspension were shaken for 5 min with 0.98 mL hexane. The aqueous phase containing the enzymatic fraction was recovered by centrifugation (1,000 xg, 1 min) and subjected to FTIR analysis and lipoxygenase activity determination in aqueous medium.

3.3.4. Enzyme Assay in Aqueous Medium

Lipoxygenase activity in Tris-HCl buffer solution (0.1 M, pH 9.0) was assayed spectrophotometrically using a pre-incubated (25°C, 3 min) reaction mixture containing 150 μL of a 4.0×10^{-3} M substrate solution, the enzymatic extract (10 μg of protein) and a sufficient amount of the appropriate buffer solution to adjust the final volume to 1 mL.

Lipoxygenase activity was demonstrated by the increase in absorbance of conjugated diene hydroperoxides at 234 nm (Surrey, 1964) using a Beckman DU-650 spectrophotometer (Beckman Instruments Inc., San Ramon, CA). The specific activity was defined as μmol of conjugated diene hydroperoxides produced per mg of protein per min, using a molar extinction coefficient of 25,000 M^{-1} cm^{-1} (Vick, 1991).

3.3.5. Enzyme Assay in Organic Solvent Media

The reaction medium consisted of the selected organic solvent and a limited amount (2%) of Tris-HCl buffer solution (0.1 M, pH 9.0). The total reaction volume was 1 mL. The enzymatic reaction was initiated by the addition of 20 μL of the lipoxygenase suspension (2.5 to 15 mg protein/mL) to 100 μL of the substrate stock solution and 880 μL of the OSM. The reaction mixture was stirred for 30 min at room temperature. Lipoxygenase activity in OSM was demonstrated by the decrease in the residual substrate peak area obtained by HPLC analysis (Perraud and Kermasha, 2000; Vega *et al.*, 2005a) and quantification of residual linoleic acid was determined by HPLC analysis according to the method described by Vega *et al.* (2005a), using an Alphabond silica normal-phase column (300x3.9 mm, 5 μm; Alltech Associates, Deerfield, IL) with a Beckman Gold HPLC system (Model 126, Beckman Instruments Inc., San Ramon, CA) coupled to a laser-light scattering detector (LLSD, Varex Corporation, Burtonsville, MD) and fitted with a computerized data handling integrated delivery system. The LLSD detection was performed at 75°C, with a nitrogen flow rate of 40 mL/min. Injection was carried out with an automatic injector (Beckman Gold autosampler 507) fitted with a 50 μL loop. Elution was conducted with an isocratic mobile phase, consisting of hexane:2-propanol:acetic acid (993:7:1, v/v/v), at a flow rate of 1 mL/min. Calibration curve were constructed using different concentrations of linoleic acid standard.

3.3.6. Fourier Transform Infrared Spectroscopy

Infrared transmission spectra were recorded using a Nicolet model 8210 Fourier transform infrared (FTIR) spectrometer (Nicolet Instrument Corp., Madison, WI) equipped with a deuterated triglycine sulfate detector (DTGS). A transmission cell with two 13 × 2 mm CaF_2 windows and a 50 or 90 μm Teflon spacer was employed. ATR spectra were recorded using an ABB-Bomem Work IR FTIR spectrometer (ABB

Bomem, Quebec City, Qc) equipped with a single bounce ATR accessory from Pike Technology (Madison, WI). FTIR imaging was performed using an Excalibur FTIR spectrometer equipped with a UMA 600 microscope and a 16 × 16 FPA liquid-nitrogen cooled mercury cadmium telluride detector (Varian, Randolph, MA). All three FTIR spectrometers were purged with dry air from a Balston dryer (Balston, Lexington, MA). All spectra were recorded by co-adding 512 scans at 4 cm^{-1} resolution. Each sample was analyzed in triplicate.

For the elucidation of protein secondary structure, the amide I region of the infrared spectrum (designated throughout as amide I' when the solvent is D_2O) was subjected to Fourier self-deconvolution (FSD) with a resolution enhancement factor (k) of 1.8 and a bandwidth of 27 cm^{-1}. The area A under the FSD spectrum between 1700 and 1600 cm^{-1} and the area A_x under each band component defined by the limits in Tables 6 and 7 were measured, and the percent contribution of each band component was calculated as $A_x/A \times 100$. To examine changes in secondary structure as a function of temperature and pH, FTIR spectra were recorded from aliquots (100 μL) of saturated lipoxygenase solution in D_2O-buffer placed in a 90 μm CaF_2 transmission cell. For the variable-temperature studies, the temperature of the cell was regulated (±0.1°C) with an Omega temperature controller (Omega Engineering, Stamford, CT); the temperature was incremented in 5°C intervals, and the sample was allowed to equilibrate for 15 min at each temperature before spectral acquisition.

3.3.7. Effect of Organic Solvent Media on Lipoxygenase Secondary Structure

The organic solvent media used for the FTIR studies were acetonitrile, methanol, hexane, octane and chloroform. Transmission spectra of lipoxygenase suspended in these solvents were acquired using a 50 μm CaF_2 transmission cell. ATR spectra were recorded by depositing an aliquot (0.5 mL) of lipoxygenase in each solvent (20%, w/v) onto the surface of a single-bounce ATR accessory and allowing the solvent to evaporate; the FTIR spectrum of the protein film was then recorded.

3.3.8. Lipoxygenase Distribution and Conformation in Micellar Mixture

The interface of a mixture of a lipoxygenase solution in Tris-DCl buffer (0.1 M, pD 9.2) and hexane, with and without the substrate linoleic acid, was imaged by focal plane array-FTIR spectroscopy in the transmission-reflection mode using a flow-through cell built in-house. The cell assembly consisted of a CaF_2 window covering a polished aluminum block into which a 25-μm T-shaped groove had been cut. The Tris-DCl buffer and hexane were mixed by injecting them simultaneously into opposite sides of the T-shaped groove. All images were acquired in accordance with the operating procedure described by Kirkwood *et al.* (2004) except that 512 scans were co-added at a resolution of 4 cm^{-1} over a range of 4000-950 cm^{-1} and were ratioed against a background spectrum recorded by reflecting the IR beam off the surface of the Al block.

3.4. Results and Discussion

3.4.1. Effect of Temperature on Lipoxygenase Activity and Structure

The effect of temperature on the secondary structure of lipoxygenase was studied by variable-temperature-FTIR spectroscopy in Tris-DCl D_2O-buffer (0.1 M; pD 9.2). Figure 6 shows the deconvolved infrared spectra in the amide I' region. Bands between 1628 and 1640 cm^{-1} are associated with β-structures, while bands between 1647 and 1654 cm^{-1} are associated with α-helical portions (Pelton and McLean, 2000). At 65°C a decrease in the absorbance value of the α-helical band at 1647 cm^{-1} and the appearance of a new band at ~1620 cm^{-1} were observed, indicative of unfolding of the protein and formation of intermolecular β-sheets (Ismail *et al.*, 1992). The absorbance value of the ~1620 cm^{-1} band increased with increasing temperature. Upon cooling to room temperature, the FTIR spectral changes remained, indicating that the formation of intermolecular β-sheets was irreversible; a slight shift in the band maximum from 1620 cm^{-1} at 95°C to 1618 cm^{-1} at 25°C was attributed to an increase in the hydrogen bonding forces at lower temperature (Ismail *et al.*, 1992). No enzyme activity was detected after the protein had been subjected to the heating-cooling cycle.

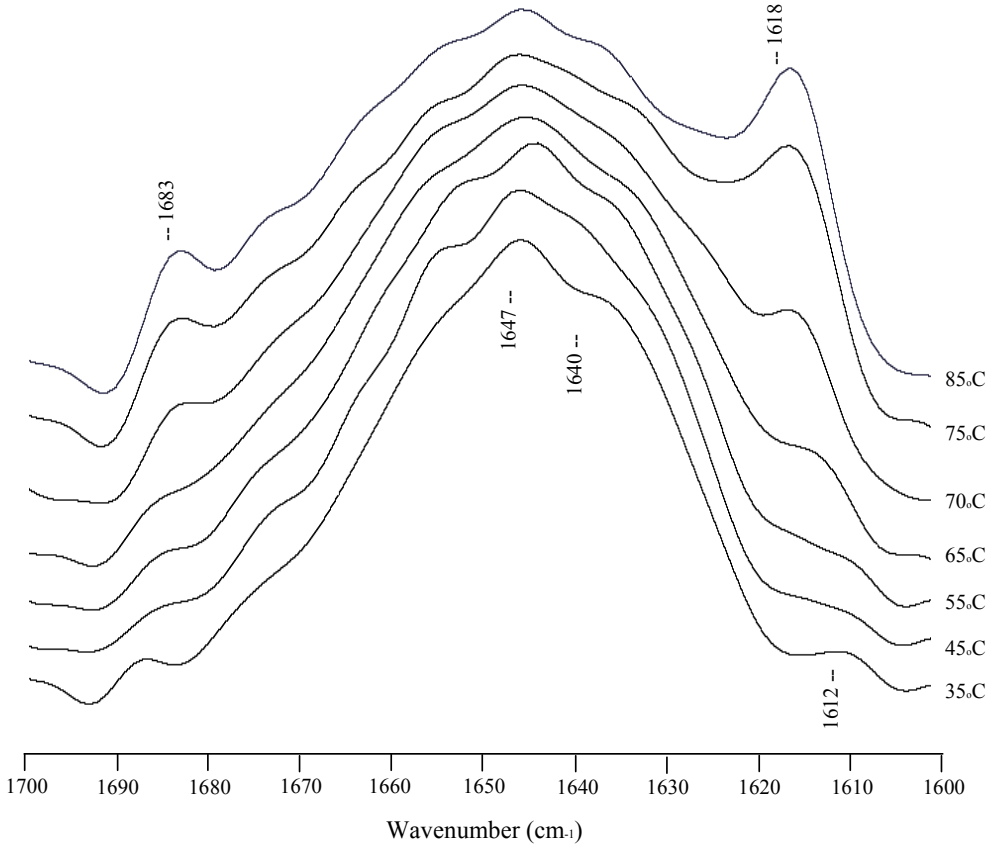


Figure 6. Stacked deconvolved spectra in the amide I' region of 10% of lipoxygenase (w/v) in D_2O-Tris-DCl buffer at pD 9.2 as a function of increasing temperature. The solution was allowed to equilibrate for 15 min at each temperature before spectral acquisition.

3.4.2. Effect of pH on Lipoxygenase Secondary Structure

The effect of pH on lipoxygenase solubility was examined by measuring the integrated absorbance value in the amide I' region (1700-1600 cm^{-1}) of the FTIR spectra of saturated lipoxygenase solutions in D_2O buffer solutions with pD values ranging from 2.2 to 10.2. Figure 7 shows a plot of the integrated absorbance value as a function of pD, which indicates maximal solubility at pD 2.2 followed by a sharp drop at pD 4; in addition, within the range of pD 4-10.2 there is a small maximum at pD 9.2. The maximal solubility at pD 2.2 may be attributed to the molten globule structure of lipoxygenase. The increase in lipoxygenase solubility at pD 9.2 coincides with the maximum enzyme activity obtained at pH 9.0 (Siedow, 1991). Hence, the decrease in lipoxygenase activity at pH values >3 and <9 may be attributed to changes in enzyme solubility.

Lipoxygenase contains approximately 36% β-sheet and 42% α-helix (Shibata *et al.*, 1987; Boyington *et al.*, 1993), corresponding to a ratio of β-sheet to α-helix of 0.86. From the spectra of lipoxygenase in buffer solutions with pD values ranging from 2.2 to 10.2 (data not shown), the relative proportions of the selected secondary structure motifs, in the different pD environments, were calculated. Figure 8 shows that lipoxygenase displayed a higher proportion of α-helical than β-sheet motifs in all the pD environments except at pD 10.2, where β-sheet became slightly predominant. The experimental results (Fig. 9) yield ratios of β-sheet to α-helix of 0.66, 0.53, 0.55, 0.80, 0.74 and 1.09 for pD values of 2.2, 4.2, 6.2, 8.2, 9.2 and 10.2, respectively. Hence, the values closest to the ratio of 0.86 reported in the literature were displayed at pD 8.2 and 9.2, which correspond to the pH values at which lipoxygenase is most active.

3.4.3. Effect of Organic solvent Media on Lipoxygenase Structure

Table 6 shows that intermolecular β-sheet aggregation absorption bands account for approximately 28, 25 and 25% of the integrated absorbance value in the amide I region when the enzyme is suspended in chloroform, acetonitrile and methanol, respectively. However, the aggregation bands account for only 14% of the integrated absorbance value in the amide I region when LOX is suspended in octane whereas there is no evidence of aggregation when the enzyme is suspended in Tris-HCl buffer. Since

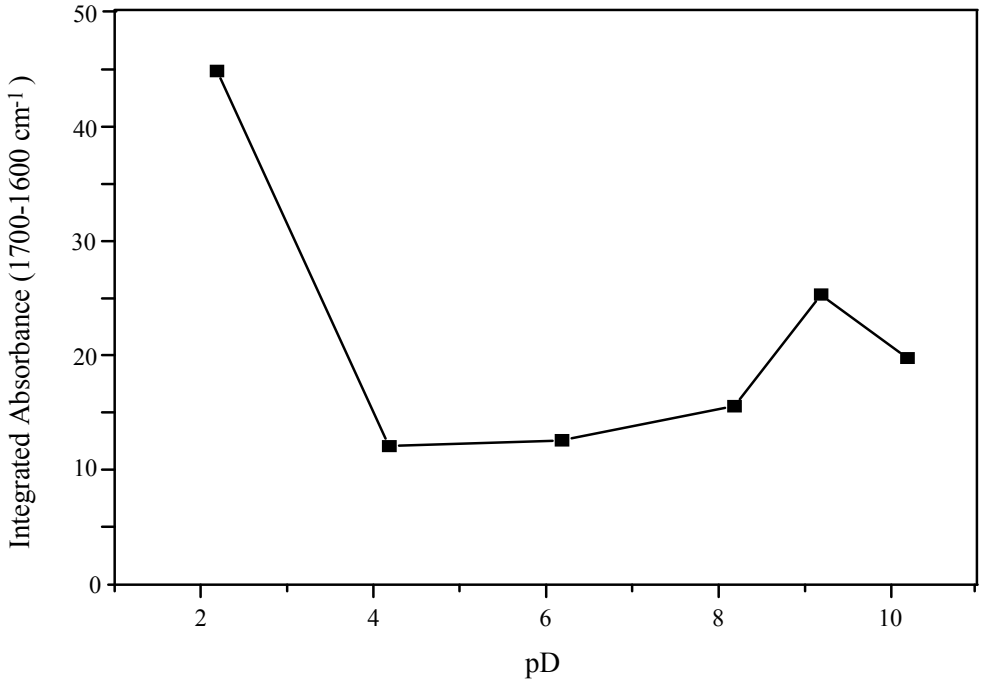


Figure 7. Integrated absorbance value of the amide I' region (1700-1600 cm^{-1}) in the Fourier self-deconvolved (FSD) spectra of saturated lipoxygenase solutions in D_2O buffer solutions as a function of pD.

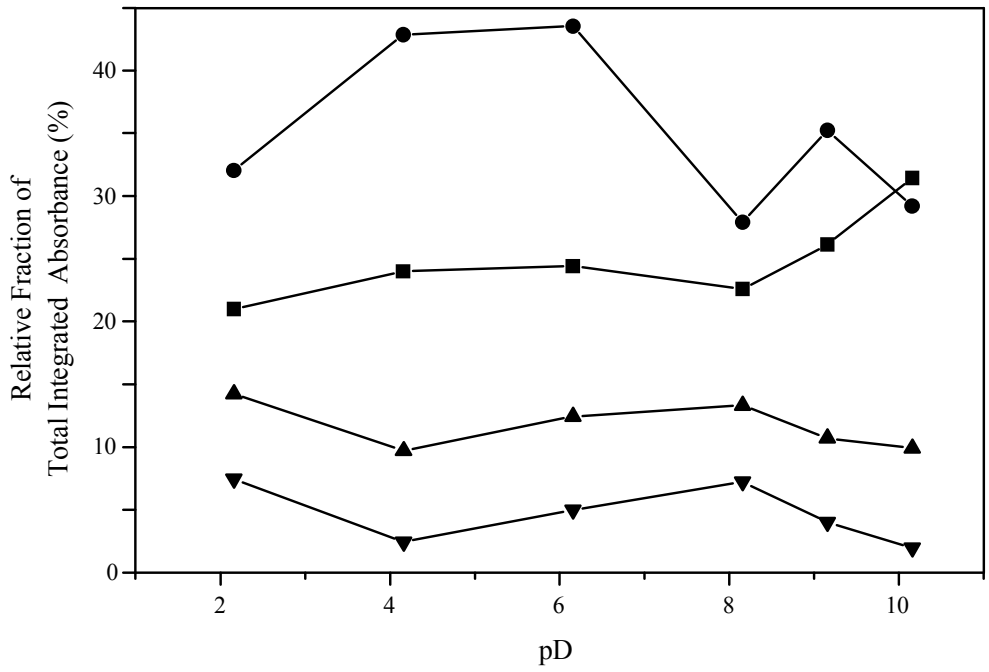


Figure 8. Percent contributions of the components of the amide I' band at 1636 cm^{-1} (—■—), 1647 cm^{-1} (—●—), 1671 cm^{-1} (—▲—) and 1682 cm^{-1} (—▼—). in the Fourier self-deconvolved spectra of lipoxygenase as a function of pD. For each component band, a bandwidth of 6 cm^{-1} was used to calculate its percent contribution to the total integrated absorbance value in the amide I' region (1700 - 1600 cm^{-1}).

Table 6. 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 transmission spectra of lipoxygenase suspensions. The data was calculated using the baseline corrected deconvolved spectra.

	Percent contribution					
	α_1[b]	α_2[b]	β_1[b]	β_2[b]	$\beta_{aggregation}$[b]	
Solvent[a]	(1660-1654)[d]	(1651-1645)[d]	(1693-1687)[d]	(1644-1638)[d]	(1621-1615)[d]	Log *P*[c]
Buffer[e]	34	33	ND[f]	33	ND[f]	NA[g]
Methanol	28	ND[f]	ND[f]	47	25	- 0.76
Acetonitrile	16	ND[f]	10	49	25	- 0.33
Chloroform	ND[f]	62	11	ND[f]	28	2.00
Octane	21	22	10	33	14	4.50

[a]Pure solvent used to make LOX suspension/solution.

[b]Type of motif associated with the wavenumber (Pelton and McLean, 2000).

[c]Log *P* which is defined as the partition coefficient expressing the differential solubility of the solvent in octanol and water.

[d]Wavenumber in cm^{-1} ($\pm 3 cm^{-1}$).

[e]Buffer was Tris-DCl (0.1 M, pD 9.2).

[f]Band not detected.

[g]Not applicable.

lipoxygenase was found to have optimal activity in aqueous solutions and in solvents with low polarity such as hexane and octane (Kermasha *et al.*, 2001), it is likely that the presence of significant amounts of intermolecular β-sheet can be linked to enzyme inactivation.

Griebenow *et al.* (2001) demonstrated that dehydrated enzymes are most active in organic solvents when their structure and molecular mobility are similar to those in water. The data in Table 6 indicate that the relative proportions of the secondary structure motifs of lipoxygenase in octane correspond most closely to the conformation of the enzyme in aqueous medium. The 1641 cm^{-1} band, assigned to β-sheet structure, represents ~33% of the integrated absorbance value in the amide I region in both buffer solution and octane as compared to ~48% in methanol and acetonitrile whereas it was not detected in chloroform. The two α-helical bands at 1657 and 1648 cm^{-1} observed in buffer solution are also observed in octane whereas only the band at 1657 cm^{-1} is observed in methanol and acetonitrile and only the band at 1648 cm^{-1} is observed in chloroform. Thus, among the solvents examined, octane appeared to cause the least alteration of the secondary structure adopted by lipoxygenase in aqueous medium. This finding is consistent with that reported by Griebenow and Klibanov (1996), in which anhydrous organic solvents tended to render the protein structure rigid to changes. Hydrophilic solvents tend to cause more structural variations by stripping the water from the surface of the protein molecule, forcing hydrogen bonding to increase between the side chains, the carbonyl groups or the peptide backbone of the protein rather than the water molecules.

For the ATR/FTIR analysis, lipoxygenase films were obtained by the evaporation of the organic solvents. The lipoxygenase films showed absorbance bands at 1660 and 1653 cm^{-1} slightly shifted from 1657 and 1648 cm^{-1} recorded in aqueous buffer by transmission FTIR. Since the absorptions in the amide I region are attributed mainly to the C=O stretch vibration (Pelton and McLean, 2000), which is greatly affected by hydrogen bonding with solvent molecules, the observed shifts may be caused by the changes in the H-bonding environment. Films deposited from all organic solvents showed an aggregation band at around 1620 cm^{-1} (Fitzpatrick *et al.*, 1994). Table 7 shows that the secondary structure of lipoxygenase in the films was similar to that in the powdered form

and not to the conformation of the enzyme in aqueous environment; these results are in agreement with those reported by Dong *et al.* (1996) who stated that the initial solid structure of a protein is not indicative of its final structure in the liquid medium.

Despite the manifold studies that have been performed on enzymes in organic solvents, it is still unknown whether activity differences in different solvents can be accounted for by a change in protein secondary structure. In order to correlate the activity of lipoxygenase with its conformation in OSM, the dry protein was first suspended in aqueous buffer solution (0.1 M Tris-HCl, pD 9.2) followed by the addition of hexane and shaken for 5 min to allow the formation of micelles and to facilitate the contact between the enzyme and the organic solvent. The FTIR analysis (Fig. 9) was performed before and after this treatment on the D_2O-buffer fraction. Figure 9 shows that the ratio of helices to β-sheet, as indicated by the relative areas of the amide I' band components around 1647 and 1636 cm^{-1}, increased slightly after the addition of hexane. There was also little or no evidence of intermolecular β-sheet aggregation as minor changes were observed around 1620 cm^{-1} when hexane was used. The lipoxygenase activity of the aqueous fraction was also tested after treatment with hexane and was found to be enhanced by a factor of 1.54 as compared to the non-treated lipoxygenase. Relative standard deviation was below 9% for all samples. The overall results provide additional indication that the maximal abundance of helical conformation is required for maximal catalytic activity of this enzyme.

3.4.4. Micellar System distribution

Figure 10A shows an optical microscopic image of the micelles formed at the interface of a mixture of 5% lipoxygenase protein in D_2O-Tris buffer (w/v) and hexane containing 7 mM linoleic acid as the substrate. The image shows that the hexane micelles are approximately 100 μm in diameter.

The "chemical images" that can be generated using FTIR imaging technology provide useful information with regard to the location of individual compounds in an emulsion. Figure 11B represents the chemical image generated by plotting the absorbance value of the band due to hexane at 1378 cm^{-1} in each spectrum recorded by the 16 × 16

Table 7. 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 spectra.

	Percent contribution					
Solvent[a]	α_1[b] (1660-1654)[d]	α_2[b] (1651-1645)[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]Log *P* which is defined as the partition coefficient expressing the differential solubility of the solvent in octanol and water.

[d]Wavenumber in cm^{-1}($\pm 3cm^{-1}$).

[e]No solvent was used.

[f]Band not detected.

[g]Not applicable.

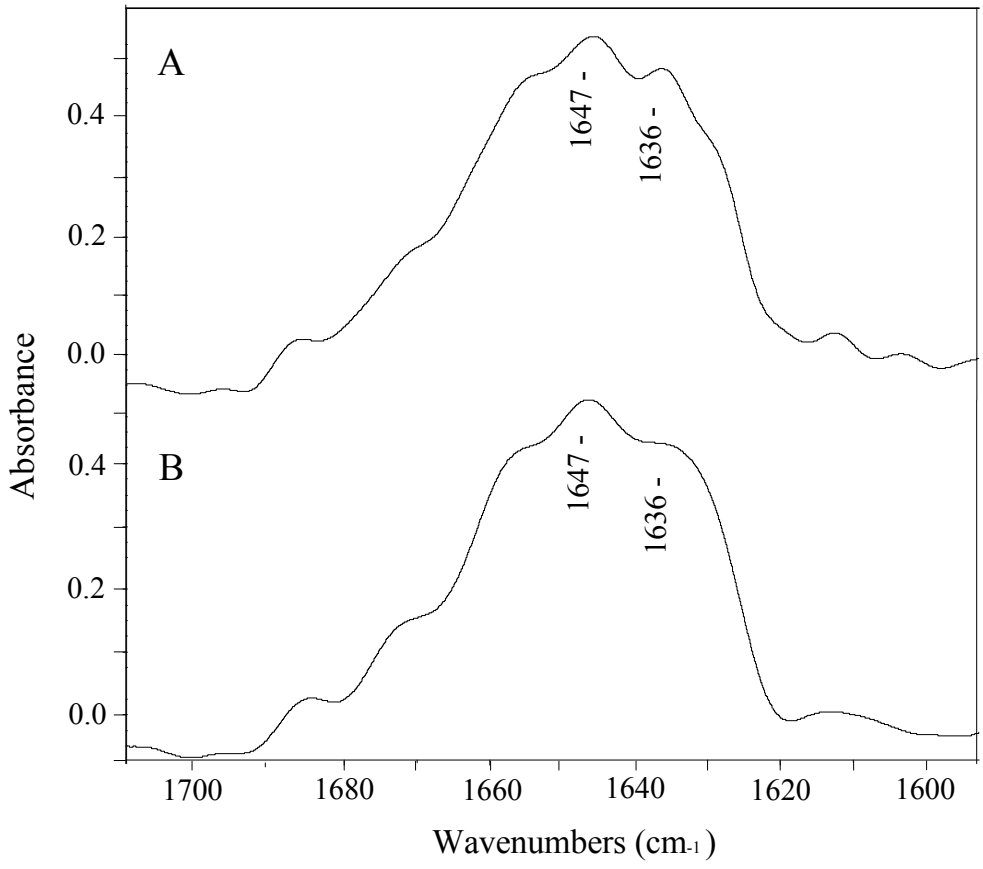


Figure 9. Fourier self deconvolved (FSD) spectra of lipoxygenase in Tris-DCl buffer (0.1 M, pD 9.2) before (A) and after (B) exposure to hexane.

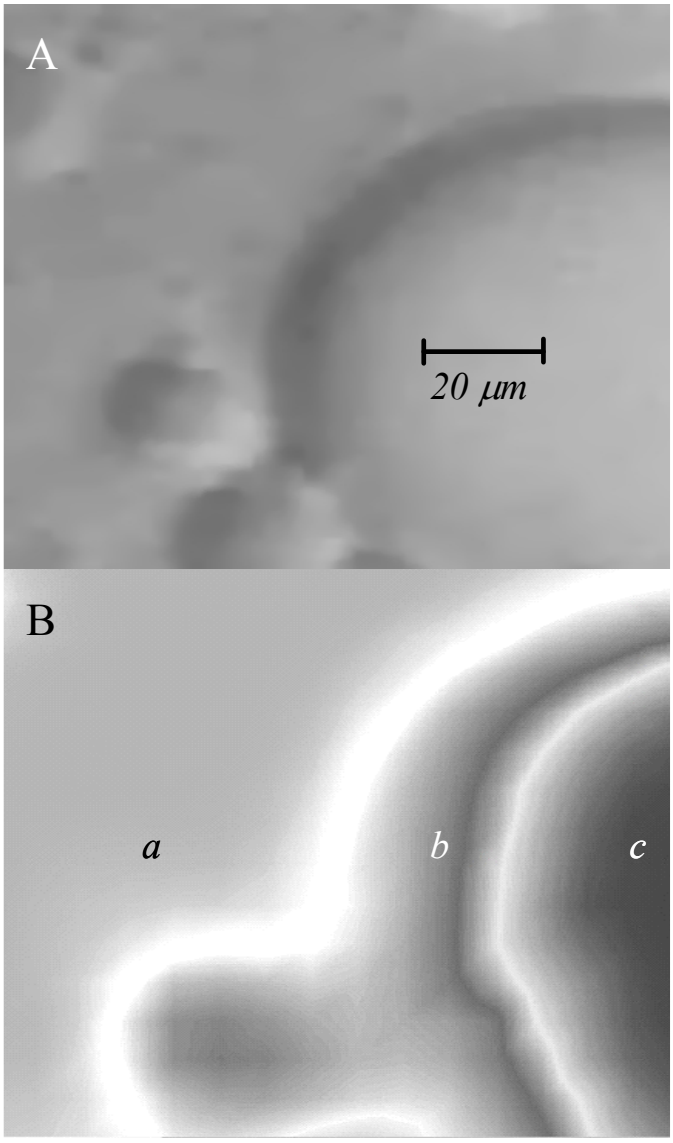


Figure 10. Images of micelles formed by mixing hexane and D_2O-Tris-DCl buffer (0.1 M, pD 9.2) containing lipoxygenase and linoleic acid. (A) Optical microscopic image of the micelles recorded using a charged-coupled device (CCD) camera through a 15× objective. (B) Chemical image generated by plotting the absorbance value of the absorption band of hexane at 1378 cm^{-1} of each spectrum recorded by the 16 × 16 array focal-plane-array (FPA) detector: (*a*) D_2O phase, (*b*) interface, and (*c*) hexane micelle.

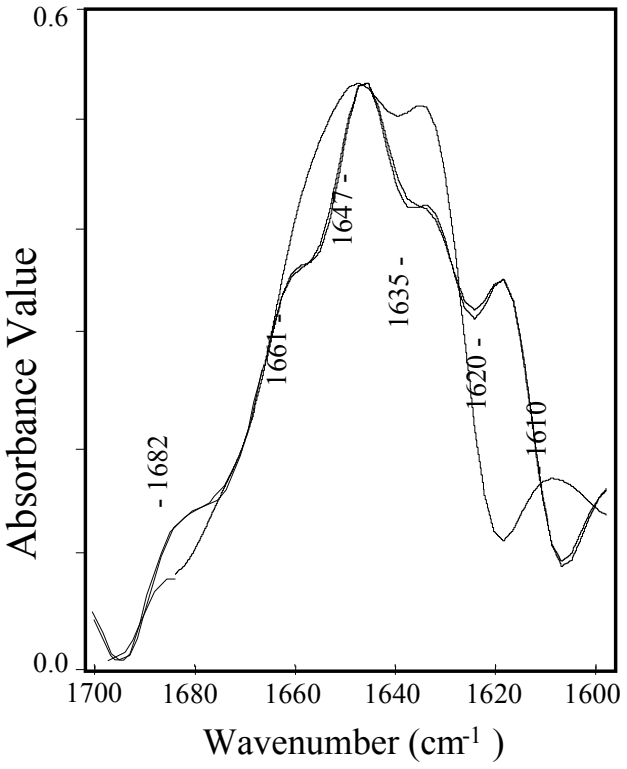


Figure 11. Overlaid Fourier self-deconvolved (FSD) spectra obtained by averaging selected pixels from the hyperspectral image of lipoxygenase within the D_2O phase (—) and at the D_2O-hexane interface (—). The FTIR spectrum of lipoxygenase in D_2O buffer (not exposed to hexane) is also shown for comparison (---).

array FPA detector. This chemical image shows clearly the pure hexane micellar phase, the D_2O phase (delineated by the lack of the hexane absorption band), and the interface of the two solvents. Based on the nominal spatial resolution (5.6 μm), the estimated width of the interface is 20 μm, which is much larger than expected; however, the actual spatial resolution was not determined experimentally, and this estimate is considered an upper limit. Additional chemical images (data not shown) were obtained by plotting the absorbance values of bands due to linoleic acid (substrate), protein (amide I' band) and D_2O. The overall results provided a better understanding of LOX biocatalysis, in terms of the area of the interface where the enzyme-substrate reaction takes place. Furthermore, the data shows that the bioconversion of linoleic acid to its hydroperoxides by the action of lipoxygenase in the micellar system had to occur at the interface; the D_2O fraction contained lipoxygenase without substrate whereas the micelles contained the substrate without the enzyme. The use of organic solvent media in enzyme biocatalysis provides solubility of substrates and end products, hence preventing the diffusion limitations, as is the case with aqueous medium (Klibanov, 1997).

Figure 11 shows the amide I' region of spectra of LOX at the interface and in the D_2O phase. The spectral profiles of LOX at the interface and within the D_2O phase are very similar and display a much lower proportion of anti-parallel β-sheet (band at ~1635 cm^{-1}) than in the spectrum of a D_2O solution of LOX as well as a strong aggregation band at 1620 cm^{-1}, which is absent in the spectrum of a D_2O solution. Although more protein aggregation occurred when the substrate was added, the α-helical content was still significantly high, demonstrating the need for this motif for proper catalytic function.

3.5. Conclusion

The results gathered showed that the secondary structure of LOX under the conditions in which it exhibits its highest enzyme activity is predominantly ordered α-helix with minimal aggregate formation. The intermolecular β-sheet aggregation induced by the use of heat as well as by organic solvents resulted in a decrease in LOX activity. Furthermore, it was shown that both LOX activity and the amount of α-helical conformation were increased by pre-incubation with hexane solvent.

CHAPTER IV

STATEMENT OF CHAPTER IV LINKAGE

Chapter IV reports on the immobilization of the purified soybean LOX, used as a model system, onto modified EupergitC®250L and its biocatalysis in OSM. The FTIR analysis of the secondary structure of the purified soybean LOX as well as the changes in its conformation in OSM (Chapter III) provides insight as to the structure-function relationship of LOX.

CHAPTER IV.

STABILITY OF IMMOBILIZED SOYBEAN LIPOXYGENASE IN SELECTED ORGANIC SOLVENT MEDIA

4.1. Abstract

The immobilization and biocatalysis of commercially purified soybean lipoxygenase (LOX) type I-B (EC 1.13.11.12), were investigated in organic solvent media (OSM). The results showed that the highest immobilization efficiency of LOX, 30.6 and 29.3%, were obtained with DEAE-cellulose and modified Eupergit® C250L supports, respectively. The biocatalysis of free and immobilized (Eupergit® C250L/EDA) LOXs was investigated in different mixtures of hexane and a selected co-solvent (95:5, v/v). The results showed a 1.5 and 1.6 increase in the activity of free and immobilized LOXs, respectively, using a mixture of hexane and 1,4-dioxane as compared to that in hexane alone; however, co-solvents, including 2-octanone, 2-heptanone, 2-butanone and cyclohexanone, displayed an inhibitory effect on LOX activity. In the mixture of 1,4-dioxane and hexane, LOX activity was dependent on the co-solvent concentration, which was increased with 1,4-dioxane up to 5% (v/v). The threshold 1,4-dioxane concentration (C_{50}) and the incubation period (T_{50}) at which 50% of the maximal enzyme activity was obtained, for the free and immobilized LOXs were 6.7 and 8.9% (v/v) of 1,4-dioxane and 9.1 and 17.0 min, respectively.

4.2. Introduction

Lipoxygenases (LOX, EC 1.13.11.12) are ubiquitously found in various animal tissues (Yamamoto *et al.*, 2004), plant cells (Gardner, 1991) and microorganisms (Perraud and Kermasha, 2000). LOXs are an important class of non-heme iron enzymes that catalyze the specific di-oxidation of polyunsaturated fatty acids containing a cis,cis-1,4-pentadiene moiety to hydroperoxy fatty acids; these hydroperoxides are regarded as flavor precursors as they can be subsequently cleaved by hydroperoxide lyase and associated enzymes to flavor compounds such as ketones, aldehydes and alcohols (Fauconnier and Marlier, 1997). The production of aroma chemical has been recognized as one of the relevant application of lipoxygenase (Gardner, 1996; Chikere *et al.*, 2001). In addition, hydroperoxide compounds have been considered valuable intermediates for

chemical synthesis and as important precursors to a number of physiological effectors such as leukotrienes and lipoxins (Gardner, 1991; Fauconnier and Marlier, 1997).

Biocatalysis of LOX in organic solvent media (OSM) may decrease the possibility of rate-limiting depletion of oxygen co-substrate, since oxygen is more than 10 times more soluble in organic solvent than in water (Linke, 1965). Furthermore, the biocatalysis of enzymes in non-aqueous media may provide a better solvation of the hydrophobic substrate, an easier recovery of the products and of the insoluble biocatalyst, and a higher thermostability of the active enzyme (Bell *et al.*, 1995). Over the past few years, the optimization of LOX biocatalysis in various non-conventional media, such as monophasic, biphasic and ternary micellar systems, was investigated (Piazza *et al.*, 1994; Kermasha *et al.*, 2001); however, the biocatalysis efficiency of LOX in these systems was limited by its poor stability (Chikere *et al.*, 2001).

Enzyme immobilization has been proven a convenient mean of improving enzyme's performance and stability (Mateo *et al.*, 2002). In addition, it offers other advantages such as easy recovery of the enzyme allowing for a repeated use of the biocatalyst and the possibility of continuous catalysis in bioreactors (Bickerstaff, 1997). Immobilization of soybean LOX has been studied using covalent binding (Chikere *et al.*, 2001; Santano *et al.*, 2002), physical adsorption (Petrus Cuperus *et al.*, 1995) and gel entrapment (Pinto and Macias, 1996; Hsu *et al.*, 2000). In addition to the fact that most of reported research work was carried out in aqueous medium, the literature did not provide an overall conclusion for the stability of immobilized LOX in OSM (Piazza *et al.*, 1994; Kermasha *et al.*, 2002a).

The present work is part of ongoing research in our laboratory aimed at the development of biotechnological process for the use of LOX and associate enzymes for the production of natural flavors (Kermasha *et al.*, 2001; 2002a). The specific aim of the present study was to evaluate selected supports for the immobilization of soybean LOX used as a model system and its biocatalysis in selected OSM, in terms of immobilization efficiency as well as enzyme activity and stability.

4.3. Material and Methods

4.3.1. Materials

Commercial purified soybean LOX type I-B (394,100 U/mg protein; or 47.29 μmol oxidized linoleic acid/mg protein/min) and DEAE cellulose (diethylaminoethyl cellulose) were purchased from Sigma Chemical Co. (St-Louis, MO). Linoleic acid (*cis*-9, *cis*-12-octadecadienoic acid) was purchased from Nu-Chek Prep Inc. (Elysian, MN). Silica gel support was purchased from Silicycle (Quebec City, Qc) whereas Affigel 10 (active ester agarose) was purchased from BioRad (Hercules, CA). Eupergit®C and Eupergit®C250L (oxirane acrylic beads) were a kind gift from Rohm Pharma (Darmstadt, Germany). Dowex®50WX4-200 (anionic ion exchange resin), ethylenediamine (EDA) and iminodiacetic acid (IDA) additives and Tris(hydroxymethyl)aminomethane (Tris) were obtained from Aldrich Chemical Co (Milwaukee, WI). All solvents used were of HPLC grade or more and were purchased from Aldrich Chem. Co. except for hexane, iso-octane and 1,4-dioxane, which were purchased from Fisher Scientific (Fair Lawn, N.J.).

4.3.2. Preparation of the Modified Epoxy Supports

Different epoxy supports were prepared according to a modification of the method reported by Mateo *et al.* (2001). One gram of wet *Eupergit®C* or *Eupergit®C250L* support was suspended in 10 mL of EDA aqueous solution (5%, v/v) or in 5 mL of a 1.8 M IDA suspension at room temperature under continuous stirring. The modified epoxy/EDA and epoxy/IDA supports, were washed with an excess of deionized water, after 15 min and 5 h of stirring, respectively.

4.3.3. Protein Determination

Protein concentration for free and immobilized LOXs as well as other fractions was determined according to a modification of the Lowry method (Hartree, 1972). Bovine serum albumin (Sigma Chemical Co.) was used as a standard for the calibration curve.

4.3.4. Immobilization of LOX

Prior to immobilization, the enzyme was dialyzed in order to remove the stabilizer and salts from the commercial preparation. The investigated supports, including DEAE-cellulose and modified *Eupergit*®, were washed twice with Tris-HCl buffer (0.1 M, pH 9.0). Immobilization of LOX was carried out by adding 1 mL of the enzyme suspension prepared in Tris-HCl buffer (0.1M, pH 9.0) to the wet support to obtain final loadings ranging from 10 and 100 mg protein/g wet support. Enzyme-support mixtures were stirred at 4°C until the protein equilibrium was reached, which corresponded to incubation time of 2, 0.5, 3, 0.75 and 16 h for AffiGel®10, DEAE cellulose, Dowex®50W, silica gel and Eupergit® supports, respectively. The supports, containing the adsorbed enzyme, were recovered by centrifugation (2,000 xg, 2 min), washed with 1 mL of buffer to remove unbound proteins and stored wet at 4°C. To evaluate the immobilization yield and efficiency, the supernatants and wash solutions were subjected to protein content determination and LOX assay.

The immobilized protein yield (IPY) and the immobilized LOX yield (ILY) were calculate from the immobilized protein and immobilized LOX activity, respectively, divided by total initial protein and total LOX activity before immobilization, respectively, multiply by 100. The selectivity factor was defined as the specific activity of the free enzyme over that of the supernatant recovered after immobilization. The immobilization efficiency was calculated by dividing the specific activity of the immobilized LOX over that of the free one, multiplied by 100.

4.3.5. LOX Assay in Aqueous Medium

The LOX activity was measured, in triplicates, according to the modified procedure of Perraud and Kermasha (2000). Prior to each enzymatic reaction, a stock solution of linoleic acid (4 mM) was freshly prepared in the Tris-HCl buffer as described previously (Kermasha and Metche, 1986).

For the free LOX, enzyme suspensions (0.05 to 0.10 mg protein/mL) were prepared in the Tris-HCl buffer solution. The reaction medium contained 150 μL of the substrate stock solution which was adjusted to 980 μL with the Tris-HCl buffer solution

and pre-incubated at 25°C. The reaction was initiated by the addition of 20 μL of the enzymatic suspension (1 to 2 μg protein/mL reaction). The absorbance was monitored continuously over a period of 3 min. The LOX activity was calculated from the slope of the linear portion of the plot of absorbance-versus-time of hydroperoxides of linoleic acid (HPODs) products and expressed in μmol conjugated diene hydroperoxides/mg protein/min.

On the other hand, for the immobilized enzyme, LOX assay was performed as for the free one with certain modifications. The reaction medium contained 150 μL substrate stock solution and 550 to 750 μL of the Tris-HCl buffer was pre-incubated to 25^{o}C. The enzymatic reaction was initiated by the addition of 100 to 300 μL of enzyme suspension containing 0.07 to 2.00 mg wet immobilized LOX preparation (0.1-7.3 μg protein). The reaction mixture was stirred for 5 min and centrifuged (2,000 xg, 2 min) to halt the reaction and to remove the enzyme. Supernatant absorbance was measured at 234 nm. Immobilized LOX activity was calculated from the slope of the plot of hydroperoxide concentration versus the amounts of solid immobilized enzyme and expressed as μmol conjugated diene HPs/mg immobilized enzyme/min.

The absorbance of the reaction mixtures was measured spectrophotometrically at 234 nm (Surrey, 1964), using Beckman DU-650 spectrophotometer (Beckman Instruments Inc., San Ramon, CA). The hydroperoxide product concentration was calculated from the molar extinction coefficient of 25,000 M^{-1} $cm^{-1.}$ for HPODs (Vick, 1991). Control reactions without LOX were carried out in tandem with the assays.

4.3.6. LOX Assay in Organic Solvent Media

LOX biocatalysis in OSM was carried out, in triplicate, according to a modification of the method of Kermasha *et al*. (Kermasha *et al.*, 2002a). Prior to the enzymatic assay in OSM, a stock solution of 70 mM linoleic acid was freshly prepared in hexane.

For the free enzyme, LOX suspensions (2.5 to 15 mg protein/mL) were prepared in the Tris-HCl buffer solution. The enzymatic reaction was initiated by the addition of

20 μL of the enzyme suspension (0.05 to 0.3 mg protein) to 100 μL of substrate stock solution and 0.88 mL of hexane. For the immobilized LOX assay, the enzymatic reaction was initiated by the addition of 100 μL of substrate stock solution to the immobilized LOX (12.5 to 50 mg wet support containing 34 to 134 μg protein) suspended in 0.88 mL of hexane and 20 μL of the Tris-HCl buffer.

Control reaction without LOX were carried out in tandem with the reaction under the same conditions. The reaction mixture was incubated at 25°C under gentle agitation for 30 min. The free and immobilized LOXs were then removed by centrifugation (2,000 xg, 2 min) and the supernatant was subjected to high-performance liquid chromatography (HPLC) analysis. LOX activity in OSM was calculated from the slope of the oxidized linoleic acid versus enzyme concentration.

4.3.7. HPLC Analysis

Quantification of residual linoleic acid was determined by HPLC analysis according to a modification of the method described by Perraud and Kermasha (2000), using an Alphabond silica normal-phase column (300 x 3.9 mm i.d., 5 μm; Alltech Associates, Deerfield, IL) with a Beckman Gold HPLC system (Model 126, Beckman Instruments Inc., San Ramon, CA) coupled to a laser-light scattering detector (LLSD, Varex Corporation, Burtonsville, MD) and fitted with a computerized data handling integrated delivery system. LLSD detection was performed at 75°C, with a nitrogen flow rate of 40 mL/min. Injection was carried out with an automatic injector (Beckman Gold autosampler 507) fitted with a 50 μL loop. Elution was conducted with an isocratic mobile phase, consisting of hexane:2-propanol:acetic acid (993:7:1, v/v/v), at a flow rate of 1 mL/min. Calibration curve were constructed using different concentrations of linoleic acid standards.

4.3.8. Effect of Co-Solvent on LOX Activity in Organic Solvent Mixtures

A comparative study of the effect of selected co-solvents in hexane, on the activity of free and immobilized LOXs, was performed. The co-solvents were 2-butanone, cyclohexanone, 1,4-dioxane, 2-heptanone and 2-octanone, with Log *P* values

of 0.29, 0.96, –1.10, 1.80, and 2.40, respectively. The LOX activity was assayed using the standard conditions described previously for the enzymatic assay in OSM.

4.3.9. Effect of 1,4-dioxane Concentration on LOX Activity in OSM

The effect of 1,4-dioxane co-solvent concentration on the activity of free and immobilized LOXs was investigated by varying its proportion from 0 to 10% in hexane medium. LOX activity was assayed using the standard conditions described previously for the enzymatic assay in OSM. A quantitative determination of LOX inactivation by 1,4-dioxane was obtained by calculating the first order inactivation constant, k_c, and the concentration of co-solvent required to decrease the LOX activity by 50% (C_{50}) using the following linear equation:

$$\ln (A/A_0) = -k_c \cdot C \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (a)$$

where, C was the concentration of 1,4-dioxane in hexane medium; A and A_0 were the LOX activity at C concentration of 1,4-dioxane and without 1,4-dioxane, respectively.

4.3.10. Thermal stability of LOX in OSM

The thermal stability of LOX activity was investigated by incubating 20 μL of free and immobilized LOX suspensions with 0.88 mL of the 1,4-dioxane/hexane mixture (5:95, v/v) for a wide range of incubation time (5 to 40 min) at 40°C. The residual LOX activity was then measured according to the standard assay conditions. The inactivation constant, k_t, and the half-life, T_{50}, of the free and immobilized LOXs were determined from the semi-logarithmic plots of the inactivation kinetics, according to the following equation :

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

where, T was the incubation time at 40°C; A and A_0 were the LOX activity with a defined incubation time T and without incubation, respectively.

4.3.11. Kinetics parameters of LOX in OSM

The effect of substrate concentration on the specific activity of free and immobilized LOXs in OSM was investigated using a wide range of linoleic acid concentrations (0 to 14 mM).

4.4. Results and Discussion

4.4.1. Immobilization of LOX

Commercially purified soybean LOX was immobilized by physical adsorption and covalent binding on different types of supports. AffiGel®10, Eupergit®C and Eupergit®C250L as well as the modified epoxy supports were used for their covalent chemical bonding ability, whereas DEAE cellulose, Dowex®50W and silica gel for their ionic and hydrophobic adsorption. The results (Table 8) show that the immobilized protein yield (IPY) and immobilized LOX yield (ILY) were strongly dependent on the nature of the support. The immobilization of LOX on silica gel resulted in the lowest IPY (11.9%), whereas the lowest ILY (13.6%,) was obtained with the use of Eupergit®C/EDA. The highest IPY (57.5%) was obtained when Eupergit®C250L was used as support, while the highest ILY (69%) was obtained with AffiGel®10. The experimental findings also indicate that the immobilization of LOX on most of the investigated supports resulted in a lower ILY than IPY, except for AffiGel®10, Eupergit®C/IDA and silica gel; the latest supports showed a capacity to immobilize the LOX preferentially as compared to the other proteins present in the enzymatic preparation. The differences between IPY and ILY values may be due to differences in the adsorption selectivity onto the support between the soybean LOX and the other proteins.

The selectivity factor can be used as a measure of the adsorption selectivity (Mateo *et al.*, 2001). Among all the investigated supports, AffiGel®10, Eupergit®C/IDA and silica gel showed a selectivity factor higher than 1.0 (Table 8), which confirmed the higher affinity of soybean LOX for those supports as compared to the other proteins. Similarly, the differences in affinity and/or selectivity might be related to different supports (Clark, 1994). From the values of the selectivity factor, it can also be seen that

Table 8. Soybean lipoxygenase immobilization parameters, using selected covalent and adsorption supports.

Support	IPY (%)[a]	ILY (%)[b]	Selectivity factor[c]	Immobilization efficiency (%)[d]
AffiGel®10	51.0	69.0	1.66	4.26
DEAE cellulose	46.0	43.9	0.87	30.6
Dowex®50W	34.1	21.7	0.80	0.64
Silica gel	11.9	31.7	1.13	1.14
Eupergit®C	40.2	20.6	0.69	5.24
Eupergit®C/IDA	25.0	39.6	1.20	9.86
Eupergit®C250L	57.5	33.1	0.59	5.19
Eupergit®C250L/EDA	33.8	13.6	0.80	29.3
Eupergit®C250L/IDA	49.6	33.5	0.77	6.39

[a]The immobilized protein yield (IPY) was determined as the relative percentage of immobilized protein to that of the total initial protein. An enzyme to support ratio of 1:100 was used.

[b]The immobilized LOX yield (ILY) was calculated as the relative percentage of the total activity of the immobilized enzyme to that of the free enzyme treated under the same conditions.

[c]The selectivity factor was defined as the ratio of the specific activity of the free enzyme over that of the supernatant, obtained by filtration and centrifugation after immobilization.

[d]The immobilization efficiency was defined as the relative percent specific activity of the immobilized enzyme compared to the free one.

the pre-treatment of the epoxy supports with EDA and IDA resulted in an increase in the adsorption selectivity of LOX onto those supports. These results are in agreement with those reported by Mateo *et al*. (2001) who have shown that the selectivity of adsorption could be increased when the concentration of the chelate groups, such as the epoxy groups, were low.

Although AffiGel®10, Eupergit®C/IDA and silica gel showed a preferential capacity for the immobilization of LOX as compared to other proteins, these supports did not yield high immobilization efficiency (Table 8). The highest immobilization efficiency was obtained with Eupergit®C250L/EDA (29.3%) and DEAE-cellulose (30.6%). Petrus Cuperus *et al.* (1995) has investigated the LOX stability in aqueous medium by its immobilization on DEAE-cellulose. In addition, epoxy supports were used previously for the immobilization of soybean (Chikere *et al.*, 2001; Santano *et al.*, 2002) and potato LOXs (Pinto *et al.*, 1997) as well as for other enzymes such as acylases (Mateo *et al.*, 2001; 2002). The results (Table 8) also indicate that the immobilization of LOX by adsorption on silica gel and Dowex®50W altered greatly the enzyme activity with an immobilization efficiency of 1.14 and 0.64%, respectively; the low recovered LOX activity upon immobilization may be attributed to the changes in enzyme conformation, steric hindrance at the active sites and substrate diffusion limitations (Clark, 1994).

The overall results show that the most appropriate supports for immobilization, in terms of LOX residual activity, were Eupergit®C250L/EDA and DEAE-cellulose. However, experimental work (data not shown) indicated that the effect of support on the partitioning of the substrate was more important with DEAE-cellulose than that with Eupergit®C250L/EDA. Based on these findings, Eupergit®C250L/EDA was used for the immobilization of LOX throughout this study.

4.4.2. Effect of co-solvent on LOX activity

One of the limitations of LOX biocatalysis in OSM is the insolubility of hydrophilic hydroperoxide end products and the relatively more hydrophobic fatty acids in the same reaction medium. In addition, Siedow (1991) reported that the accumulation of hydroperoxides in the microenvironment of the enzyme has an inhibitory effect on

LOX activity. To overcome the solubility restriction, the effect of presence of selected co-solvents in hexane medium (5:95, v/v) on LOX activity was investigated (Table 9). The choice of co-solvents was made because of their capacity to solubilize hydroperoxides and of their miscibility with hexane.

Using Eupergit®C250L/EDA as support, the specific activity of free and immobilized LOXs in hexane medium was found to be 0.20 and 0.36 μmol/mg protein/min, respectively, whereas that in aqueous buffer medium it was 18.02 and 5.28 μmol/mg protein/min, respectively (Table 9). These experimental findings indicate that the ratio of activity (ROA) of LOX in organic solvent over that in aqueous medium was 0.01 and 0.05, respectively, for the free and immobilized LOXs. Pencreac'h and Baratti (2001) reported a wide range of ROA values for wide range of commercial lipase preparations, with most of them were under 1.0. The experimental results (Table 9) also indicate similar ROA for LOX, with values higher for the immobilized enzyme as compared to the free one; these findings could be explained by an underestimation of the activity of the immobilized enzyme in aqueous medium, as a result of the diffusion limitations of the hydrophobic substrate (Pencreac'h and Baratti, 2001).

Table 9 also shows that the addition of most co-solvents, including butanone, cyclohexanone, heptanone and octanone, to the hexane medium resulted in a decrease in the specific activity of LOX, which could indicate their denaturating effect on enzyme activity (Carrea and Riva, 2000). However, the denaturating effect of co-solvents was less pronounced with the immobilized LOX than with the free one, which may be owing to the multi-point covalent attachment (Mateo *et al.*, 2002) of the immobilized enzyme onto the support, leading to lesser conformational changes. On the other hand, the results (Table 9) demonstrate that the addition of 1,4-dioxane as co-solvent increased the specific activity of soybean LOX by 1.6-time. These findings suggest that the presence of 1,4-dioxane may have decreased the end-product partitioning effect by accelerating its removal rate from the micro-environment of the enzyme, which is a limiting step in the conversion of linoleic acid into its HPODs. The presence of 1,4-dioxane in the reaction medium may prevent the LOX deactivation by the HPOD product (Siedow, 1991). Although the Log *P* values of the solvent mixtures are close, the LOX showed variable

Table 9. Activity of free and immobilized LOX in different mixtures of co-solvent and hexane.

Organic co-solvent[a]	Specific activity[b]		Log P[d]
	Free LOX	Immobilized LOX[c]	
Aqueous medium[e]	18.02 (±2.59)[f]	5.279 (±0.506)[f]	-
Hexane only	0.204 (±0.028)[f]	0.363 (±0.043)[f]	3.50
Octanone	0.121 (±0.015)[f]	0.210 (±0.010)[f]	3.45
Heptanone	0.090 (±0.010)[f]	0.213 (±0.016)[f]	3.41
Butanone	0.031 (±0.005)[f]	0.260 (±0.025)[f]	3.25
Dioxane	0.304 (±0.041)[f]	0.577 (±0.084)[f]	3.12
Cyclohexanone	-[g]	0.037 (±0.460)[f]	3.33

[a]The organic phase consisted of mixture of the defined co-solvent with hexane (5:95, v/v); the reaction mixture was composed of the organic phase and a limited amount of Tris-HCl buffer (0.1 M, pH 9.0) at 98:2 (v/v).

[b]Specific activity expressed as μmol of linoleic acid hydroperoxides (HPODs)/mg protein/min.

[c]Enzyme was immobilized on Eupergit C250L (Rohn Pharma) support treated with ethylenediamine, with an enzyme to support ratio of 1:10.

[d]Log P of the mixtures were calculated according to the empirical formula log P mixture = $X_1 \log P_1 + X_2 \log P_2$ where X_1 and X_2 are the mole fractions of solvents 1 and 2 (Hilhorst *et al.,* 1984).

[e]The aqueous medium was composed of Tris-HCl buffer (0.1M, pH 9.0).

[f]Standard deviation of triplicate trials.

[g]Absence of activity for this samples.

specific activity. Higher enzymatic activity is usually expected with the concomitant increase of solvent hydrophobicity, which is due to a decrease in the solvent ability to strip the water layer from the surface of the enzyme (Carrea and Riva, 2000). In addition, the molecules of the polar solvents are more likely to be present in the micro-environment of the enzyme (Griebenow and Klibanov, 1996). Bell *et al.* (1995) reported that in addition to the physical properties of the solvent, their molecular structure played an important role in enzyme activity. Enzyme-solvent interaction may alter the active conformation of the enzyme by a direct molecular binding of the solvent onto or near the active site of the enzyme, hence resulting in a denaturating effect (Carrea and Riva, 2000).

4.4.3. Effect of 1,4-dioxane co-solvent concentration on LOX activity

Figure 12 shows the effect of 1,4-dioxane proportion in hexane medium on LOX activity. The results indicate that the specific activity of free and immobilized LOXs was increased to its maxima of 0.304 and 0.577 μmol of oxidized linoleic acid/mg protein/min, respectively, with a concomitant increase in 1,4-dioxane concentration up to 5% (v/v). The increase in LOX activity could be explained by an increase in the hydroperoxide end-product solvation which may decreased their micro-environmental effect and hence their inhibition role (Siedow, 1991). However, a further increase in 1,4-dioxane proportion to 10% resulted in a 88 and 57% decrease in the specific activity of free and immobilized LOXs, respectively; this decrease in enzyme activity may be owing to changes in the conformation of LOX by stripping off the essential water layer surrounding the enzyme molecule (Griebenow and Klibanov, 1996). The presence of specific interactions between co-solvent molecules and the enzyme may also account for the low specific activity in higher proportions of 1,4-dioxane (Arroyo *et al.*, 2000).

Figure 12B shows a straight line with correlation coefficient of 0.99 and 0.97 for the free and immobilized LOXs, respectively, indicating hence that the enzyme inactivation by 1,4-dioxane followed first order kinetic behavior. First order kinetic of enzyme inactivation by co-solvents in monophasic aqueous mixtures has been reported for aminotransferase (Moreno and Fagain, 1997; Boross *et al.*, 1998) and acylase (Arroyo *et al.*, 2000).

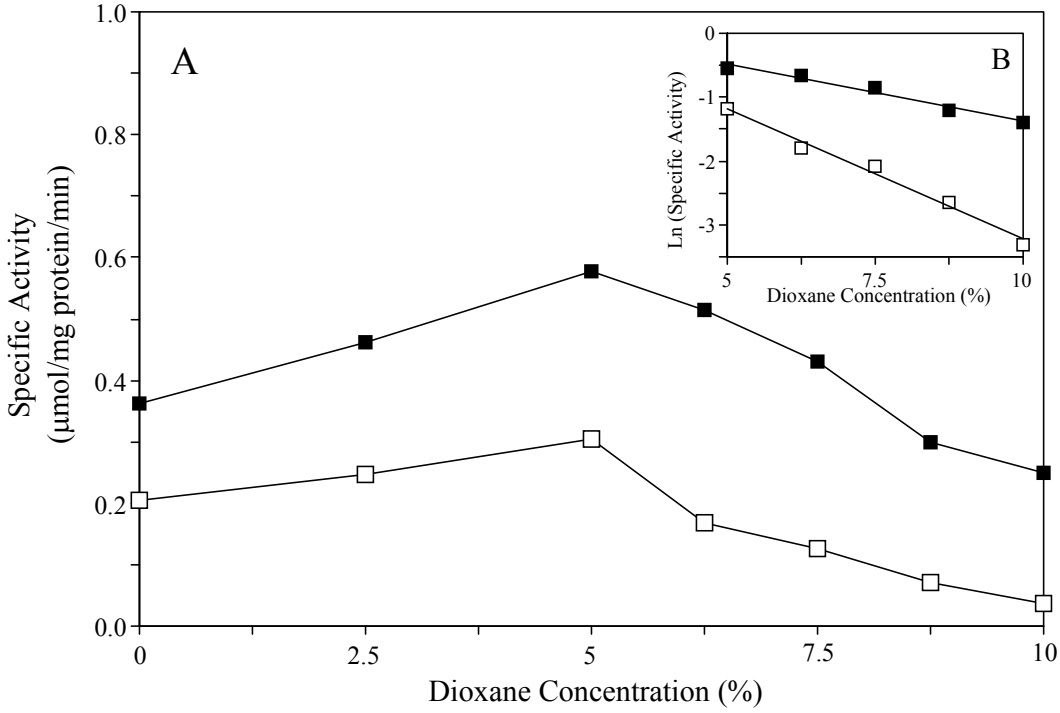


Figure 12. Effect of 1,4-dioxane co-solvent on the activity of free (□) and immobilized (■) soybean lipoxygenase enzyme in hexane medium: (A) specific activity measurements; (B) first order kinetics behavior of deactivation effect of co-solvent concentration.

A quantitative determination of LOX inactivation by the co-solvent 1,4-dioxane was obtained by calculating the inhibition factor (C_{50}). The C_{50} (Table 10) was estimated to be 6.7 and 8.9% for the free and immobilized LOXs, respectively; these findings suggest that 1,4-dioxane may display a higher denaturating effect on the free enzyme than on the immobilized one. The low denaturating effect of 1,4-dioxane on immobilized LOX may be explained by multipoint covalent attachment of the immobilized LOX on the support, which may have led to less conformational changes (Mateo *et al.*, 2002).

4.4.4. Thermal stability of LOX in OSM

The thermal stability of LOX in the 5:95 (v/v) 1,4-dioxane/hexane mixture of was investigated at 40°C. The results (data not shown) indicated that after 40 min of incubation, the free and immobilized LOXs retained only 4.4 and 28.5% of their initial activity, respectively. From the semi-logarithmic plots, the inactivation constant and half-life of free and immobilized LOXs were estimated (Table 10). The half life (T_{50}) of LOX increased by a factor of 2 upon immobilization on Eupergit®C250L/EDA. The results (Fig. 13) also indicate that the thermal inactivation of both enzyme preparations followed first order kinetic behavior, as indicated by the linearity of those plots. Similarly, Chikere *et al*. (2001) reported that soybean LOX type I-B, immobilized on epoxy supports in borate buffer (0.2 M, pH 9.0), showed first order kinetic. By contrast, the kinetic inactivation of LOXs from tomato (Anthon and Barrett, 2003), beans (Indrawati *et al.*, 1999) and potatoes (Park *et al.*, 1988) did not follow first order kinetics. Park *et al.* (1988) have demonstrated that the non-first-order kinetic behaviour of a crude enzyme preparation was owing mainly to the presence of more than one LOX species with different thermal stabilities.

4.4.5. Kinetics parameters of LOX in OSM

To compare the catalytic efficiency of free and immobilized LOXs, their kinetic parameters were determined in the 1,4-dioxane/hexane mixture. The Lineweaver-Burk plots of $1/v$ versus 1/[S] (data not shown) displayed a linear relationship with correlation coefficient of 0.97 and 0.98, respectively, for the free and immobilized LOXs, which indicate a Michaelis-Menten kinetic behavior.

Table 10. Inactivation constants and half-life parameters for free and immobilized LOX in dioxane:hexane mixtures.

Parameter	Free LOX	Immobilized LOX[a]
Co-solvent inactivation[f]		
k_c ($\%^{-1}$)[b]	0.4064	0.1786
C_{50} (%)[c]	6.7	8.9
Thermal inactivation[g]		
k_t (min^{-1})[d]	0.0759	0.0408
T_{50} (min)[e]	9.1	17.0

[a]Enzyme was immobilized on Eupergit C250L (Rohn Pharma) support treated with ethylenediamine, with an enzyme to support ratio of 1:10.

[b]Constant of inactivation as determined from the first-order kinetics behavior of the inactivation effect of increasing concentration of 1,4-dioxane co-solvent.

[c]Defined as the concentration of solvent required for half of the initial activity.

[d]Constant of inactivation as determined from the first order kinetics behavior of the inactivation effect of increasing incubation time at 40°C.

[e]Defined as the incubation time at 40°C in dioxane:hexane (5:95. v/v) required to report a 50% decrease in the initial activity.

[f]Parameters were obtained from the linear equation $\ln (A/A_0) = -k_c \cdot C$ where A is the activity at concentration of dioxane C. A_0 represents the activity at the optimal 5% dioxane concentration and k_c is the constant of inactivation of the dioxane effect.

[g]Parameters were obtained from a linear equation $\ln (A/A_0) = -kt \cdot T$ where A is the activity at incubation time T. A_0 represents the activity without deactivating incubation period and kt is the constant of inactivation of the incubation at 40°C.

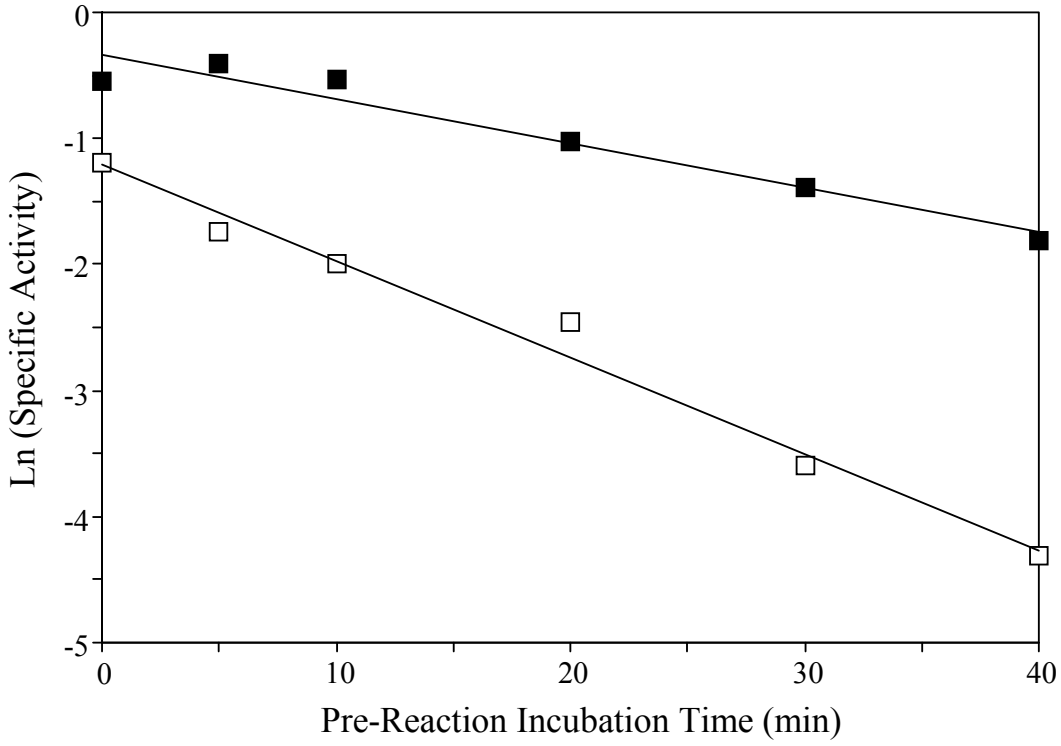


Figure 13. Thermal inactivation of free (□) and immobilized (■) soybean lipoxygenase in a reaction medium mixture of 5:95 (v/v) 1,4-dioxane/hexane.

Table 11. Kinetics parameters of LOX activity in 5% 1,4-dioxane in hexane (v/v).

Parameter	Free LOX	Immobilized LOX[a]
K_m	14.40	36.00
V_{max}	5.84	4.94
Catalytic efficiency[b]	0.41	0.14

[a]Enzyme was immobilized on Eupergit®C250L (Rohn Pharma) treated with ethylenediamine. The free LOX was treated under the same conditions as the immobilized LOX, without support.

[b]Catalytic efficiency was defined as the ration of V_{max} over K_m.

The kinetic parameters, K_m and V_{max}, for the free and immobilized LOXs, (Table 11) were estimated from the Lineweaver-Burk plots. The results indicate that the K_m values were determined as 14.4 and 36.0 mM for the free and immobilized LOX, respectively.

The immobilization of LOX onto Eupergit®C250L/EDA increased the K_m value by approximately 2.5. Bindhu and Abraham (2003) has attributed the increase in K_m values for peroxidase to the increased hydrophobicity of the enzyme which may have altered the interaction with the substrate. In addition, the decrease in enzyme affinity for the substrate may be owing to the immobilization process, which tends to cause conformation changes, steric hindrance and substrate diffusion limitations (Clark, 1994). However, Kermasha *et al.* (2002a) reported lower K_m for LOX after its immobilization, with values as low as 0.06 mM in octane medium.

The results (Table 11) show that the V_{max} values for the free and immobilized LOX were, respectively, 5.84 and 4.94 μmol HPODs/mg protein/min. Kermasha *et al.* (2002a) obtained relatively lower V_{max} value (2.37 μmol HPODs/mL/min) for the immobilized soybean LOX on_Dowex®50W in iso-octane; however, these authors reported much higher V_{max} (29.68 μmol HPODs/mL/min) for the free enzyme. The discrepancy in $V_{\max}$ values of the free LOX between the present study and those reported by Kermasha *et al*. (2002a) may be explained by the apparent character of V_{max}. In order to compare the kinetics data for both free and immobilized LOXs, the present study treated the free enzyme under the same conditions as for the immobilized one, which implied continuous stirring for 16 h. Because the free LOX is poorly stable in aqueous medium (Chikere *et al.*, 2001), the lower V_{max} value may be due to a partial denaturation of the enzyme.

The apparent catalytic efficiency, which is defined as V_{max}/K_m, was found to be 0.41 and 0.14 min^{-1} for the free and immobilized LOX, respectively. Although Kermasha *et al.* (2002a) reported higher affinity for the immobilized soybean LOX in octane medium, the experimental findings (Table 11) indicate that the V_{max} values are higher than those reported by these investigators. The catalytic efficiency of soybean LOX in

octane medium (Table 11) are thus similar to those reported by Kermasha *et al.* (2001; 2002a).

4.5. Conclusion

The experimental data obtained throughout this study showed that the immobilization of soybean LOX increased its activity and stability in monophasic organic solvent media as compared to the free one. In addition, the use of co-solvent in the reaction medium increased the enzyme activity. Moreover, the relationship between LOX activity and both co-solvent concentration and thermal inactivation of the enzyme followed first order kinetic.

CHAPTER V

STATEMENT OF CHAPTER V LINKAGE

Chapter V summarizes the experimental results of the optimization of the xylenol orange (FOX) assay, in terms of sensitivity and interferences, for the determination of LOX and HPL activities in OSM. The development of the FOX assay for its use in OSM was an essential objective, since the HPLC analysis used throughout the research work in Chapter IV had proven tedious for the determination of purified LOX activity.

CHAPTER V.

OPTIMIZATION OF ENZYMATIC ASSAY FOR THE MEASUREMENT OF LIPOXYGENASE ACTIVITY IN ORGANIC SOLVENT MEDIA

5.1. Abstract

Lipoxygenases (LOX; EC 1.13.11.12) are an important class of non-heme iron enzymes that catalyze the di-oxidation of polyunsaturated fatty acids to hydroperoxy-fatty acids which can be measured by the xylenol orange method (FOX). To determine the enzymatic production of these fatty acids in organic solvent media, the FOX assay was optimized using the standard cumene hydroperoxide. An increase in the proportion of methanol from 0 to 75% in the FOX reagent, resulted in a 93% increase in the molar absorption coefficients at 560 nm. In addition, the presence of linoleic acid in the cumene hydroperoxide sample enhanced the formation of the FOX complex, resulting in a 50% increase in the sensitivity of the method. Moreover, when perchloric acid was used, the source of ferrous ions and presence of denatured LOX had little effect on the sensitivity of the FOX assay whereas sensitivity decreased by 40-46% with sulfuric acid. The overall results demonstrated that the modified FOX assay may be used for the precise and accurate measurement of hydroperoxy fatty acids obtained by LOX activity in organic solvent media.

5.2. Introduction

Lipoxygenases (LOX; EC 1.13.11.12) are an important class of non-heme iron enzymes that catalyze the regio- and stereo-specific di-oxidation of polyunsaturated fatty acids (PUFAs) containing a *cis,cis*-1,4-pentadiene moiety to hydroperoxy-fatty acids (FA), considered as precursors of flavor compounds. In addition, LOXs are ubiquitously found in plants, microorganisms and various animal tissues, where they are implicated in physiological activities (Gardner, 1991).

Most spectrophotometric methods reported for the determination of LOX activity were developed for an aqueous medium, with the most common being based on the absorption of hydroperoxy-FA containing a *cis,cis*-1,4-pentadiene moiety at 234 nm (Grossman and Zakut, 1979); however, this method remains limited by its inability to

detect hydroperoxides that lack a conjugated diene chromophore (Surrey, 1964). The ferrous oxidation assay, using xylenol orange (FOX) or ferrous thiocyanate, was reported as an alternative spectrophotometric method for the determination of lipid hydroperoxides, with the latter assay showing lower sensitivity compared with the FOX assay (Wurzenberger and Grosch, 1984) and used for the quantification of high amounts of lipid hydroperoxides.

The FOX assay is based on the oxidation of ferrous ions (Fe^{2+}) by hydroperoxides into their ferric counterparts (Fe^{3+}), which, in turn complex with the xylenol orange salt to form a chromophore that absorbs at 560 nm (Nourooz-Zadeh, 1999), with a molar absorption coefficient (ε_{560}) of 4.5 x 10^5 M^{-1} cm^{-1} for lipid hydroperoxides in a methanol-based reagent (Wolff, 1994). To measure hydroperoxides in liposome or lipoprotein suspensions, Jiang *et al*. (1991) also used methanol in the preparation of the FOX reagent. In addition, the specificity of the FOX assay to measure lipid hydrperoxides in an extract can be improved by the addition of triphenylphosphine (TPP) (DeLong *et al.*, 2002; Banerjee *et al.*, 2003), and its sensitivity can be increased by the use of sucrose (Deiana *et al.*, 1999) or the replacement of sulfuric acid by perchloric acid (Gay and Gebicki, 2002).

Over the last ten years, LOX biocatalysis in organic solvent media (OSM) has gained great interest (Piazza *et al.*, 1994; Kermasha *et al.*, 2001; 2002a). Numerous advantages are associated with biocatalysis in non-conventional media, including a decrease in the rate-limiting depletion of oxygen, changes in enzyme specificities as well as increases in the thermo-stability of enzymes and the solubility of hydrophobic substrates (Siedow, 1991). Non-polar solvents are generally considered as appropriate reaction environments for biocatalysis in organic solvent media (Laane *et al.*, 1987; Piazza *et al.*, 1994; Kermasha *et al.*, 2001; 2002a); however, when such solvents are mixed with the polar FOX reagent, the reaction assays can become turbid and hence limit the accuracy of the spectrophotometric measurements. In addition, the FOX method can have a certain degree of interference due to the presence of LOX, a non-heme iron protein that could potentially complex with xylenol orange.

The overall objective of the present study was to optimize the FOX assay in order to rapidly and accurately quantify hydroperoxides, obtained by LOX activity, in OSM, and to evaluate its performance as an enzymatic assay. The specific objective was to optimize the FOX assay in terms of various parameters including the methanol/water ratios, degree of solvent degassing, hexane/FOX reagent ratios, the presence of different reagent components including BHT, sulfuric acid or perchloric acid in combination with ferrous sulfate or ammonium ferrous sulfate, and its specificity and substrate selectivity.

5.3. Material and Methods

5.3.1. Materials

Commercial soybean LOX type I-B (131,000 U/mg solid, with a specific activity of 15.72 μmol oxidized linoleic acid/min/mg solid), xylenol orange salt (3,3'-bis(N,N-di(carboxymethyl)aminomethyl)-*o*-cresol-sulfonephthalein sodium salt), butylated hydroxytoluene (BHT; 2,6-di-tertbutyl-*p*-cresol) and cumene (1-methylethyl)benzene) hydroperoxide were purchased from Sigma Chemical Co. (St-Louis, MO). Linoleic (*cis*-9,*cis*-12-octadecadienoic), α–linolenic (*cis*-6,*cis*-9,*cis*-12-octadecatrienoic) and arachidonic (*cis*-5,*cis*-8,*cis*-11,*cis*-14-eicosatetraenoic) acids were purchased from Nu-Check-Prep Inc. (Elysian, MN). Ammonium thiocyanate, ammonium ferrous sulfate hexahydrate and ferrous sulfate of ACS grade as well as tris(hydroxymethyl)aminomethane, HCl, triphenylphosphine (TPP) and all the solvents used were purchased from Fisher Scientific (Pittsburgh, PA). Meso-tetraphenyl-porphine copper(II) and perchloric acid were purchased from Aldrich Chemical Co. (Milwaukee, WI) whereas sulfuric acid was obtained from LabChem Inc. (Pittsburgh, PA). Sodium phosphate (Na_2HPO_4) was purchased from ACP Chemicals (Montreal, Qc). The LC-Si cartridges were obtained from Supelco (Bellefonte, PA).

5.3.2. FOX Assay

The FOX assay was carried out according to a modification of the procedure described by Jiang *et al*. (1991). To prepare the FOX reagent, methanol and deionized water were degassed independently for 10 min using a Bransonic ultrasonic cleaner (Model 3510, Bransonic, Danbury, CT). The FOX reagent was composed of 250 mM Fe^{2+} ions, 25 mM sulfuric acid and 100 mM xylenol orange in a methanol/water mixture

(90:10, v/v) and used within 4h after preparation. FOX assays were performed, in triplicate. FOX assays were initiated by the addition of 25 μL of hydroperoxide sample in hexane to 2.0 mL of the FOX reagent. Ethanol (96%) was then added to the mixture to complete the volume to 2.1 mL. The mixture was stirred with a Vortex (Genie 2, Fisher Scientific) for 10 sec and stirred again every 5 min for a total reaction time of 15 min. Absorbance was measured after 20 min of reaction time at 556 nm against a blank assay containing all the components in the FOX assay except the hydroperoxide, using a Beckman spectrophotometer (Model 650, Beckman Instruments, Inc., Fullerton, CA). A calibration curve was used based on a wide range of concentrations of cumene hydroperoxide, ranging from 0.025 to 0.4 mM (Jiang *et al.*, 1991).

5.3.3. Optimization of FOX Assay

5.3.3.1. Methanol/Water Ratio and Degassing

FOX assays were optimized by varying the methanol/water mixture ratios, including 0:100, 45:55, 60:40, 75:35 and 90:10 (v/v), of the FOX reagent. The stability of the FOX reagent was also assessed by degassing the water and methanol for different time periods ranging from 0 to 15 min.

5.3.3.2. Effect of BHT and Linoleic Acid

The effect of 4 mM BHT on the FOX assay was also investigated in the absence and presence of 7 mM linoleic acid. BHT as well as linoleic acid were prepared in methanol and added to the hydroperoxide samples before the FOX assay. To ensure that an increase in the sensitivity of the FOX assay was due to the presence of linoleic acid in the samples and not its potential auto-oxidation, all samples were blanked against a constant concentration of linoleic acid.

5.3.3.3. Effect of Acid and Fe^{2+} Source

The effects on the FOX assay of perchloric acid as well as sulfuric acid at a final concentration of 25 and 85 mM, respectively, and either ammonium ferrous sulfate or ferrous sulfate as a Fe^{2+} source at a final concentration of 250 μM were also investigated in the presence and absence of denatured LOX (3.2 μg). Denaturation was achieved by

incubating LOX in Tris-HCl buffer (0.1 M, pH 9.0) for 5 min at 100°C. The denatured enzyme was added to the hydroperoxide sample before the FOX assay.

5.3.4. Effect of TPP Reduction on the Specificity of FOX Assay

The specificity of the FOX assay was assessed, using TPP, according to the procedure described by Nourooz-Zadeh (1999). Calibration curves of cumene hydroperoxide, in the presence and absence of TPP, were obtained as follows. A 25 μL methanol solution, with and without 10 mM TPP, was added to an equal amount of the sample. The mixture was stirred for 30 min before the addition of 2 mL of FOX reagent. Ethanol was added to complete the volume to 2.1 mL. Samples were analyzed spectrophotometrically at 560 nm and the difference in absorbance between the TPP-treated and the untreated samples was used to determine the measurements of lipid hydroperoxides. The TPP reduction effect was also investigated for hydroperoxide samples containing 7 mM linoleic acid.

5.3.5. Selectivity of the FOX Assay

To investigate the substrate specificity of the assay, standard curves of different FA hydroperoxides were prepared using the modified FOX method with perchloric acid and ferrous sulfate in the reagent preparation. In order to prepare the standard curves, 14 mM linoleic acid was also added to all samples.

5.3.6. Preparation of Fatty Acid Hydroperoxide Standards

Following the procedure described by Schieberle *et al*. (1984), photooxidation of linoleic, linolenic and arachidonic acids yielded their corresponding hydroperoxides, HPODs, HPOTs and HPETEs, respectively. The reaction medium was composed of 1 g linoleic, linolenic, or arachidonic acid, 22 mL of benzene and 2 mg of meso-tetraphenyl porphine as a sensitizer. Photooxidation was performed at 15°C by bubbling a stream of O_2 into the reaction medium, which was stirred for 70 min while being subjected to a 500 W halogen light, through a 1 cm layer of deionized water used to filter IR radiation. The separation of hydroperoxides from the residual FA was performed following the method of Toschi *et al*. (1995), using LC-Si cartridges. Photooxidized and cumene

hydroperoxides were quantified with the ferrous thiocyanate assay as described by Wurzenberger and Grosch (1984).

5.3.7. LOX Assay in Organic Solvent Media

The enzymatic reaction was initiated by the addition of 20 μL of LOX suspension (8 to 24 μg protein in 0.1 M Tris-HCl buffer; pH 9.0) to 0.98 mL of the hexane reaction mixture, containing 7 mM linoleic acid. The mixture was gently stirred at room temperature. At different time intervals (0 to 5 min), an aliquot was withdrawn and the concentration of HPOD was quantified, according to the modified FOX assay using perchloric acid and ferrous sulfate in the reagent preparation. Aliquots of 25 to 200 μL were taken from the 1 mL reaction medium and analyzed for the presence of HPOD. All experiments were performed in triplicate. After mixing and transferring the FOX assay mixture to the spectrophotometric cells, a sufficient period of time (5 min) was allowed for the dissipation of the formed emulsion. In addition, freshly prepared solutions of linoleic acid and the respective HPOD were used to obtain the standard curves.

5.4. Results and Discussion

5.4.1. Preparation of Reaction Medium for FOX Assay

Depending on the nature of the sample, different ratios of methanol/water in the FOX reagent have been used to enhance hydroperoxide solubility and avoid turbidity in the assay (Grau *et al.*, 2000; Eymard and Genot, 2003). The results (Fig. 14) show that an increase in the proportion of methanol from 0 to 75% (v/v) in the FOX reagent produced a 93% increase in the molar absorption coefficients (ε_{560}) for cumene hydroperoxide; however, further increases in the proportion of methanol to 90% (v/v) slightly decreased the ε_{560} value from 197,250 to 181,780 $M^{-1}cm^{-1}$. These experimental findings clearly indicate that the presence of a high proportion of methanol enhanced the sensitivity of the FOX assay, which could be attributed to an increase in the solvation of hydroperoxide (Gay *et al.*, 1999a).

Since the occurrence of turbidity at lower methanol proportions (≤60%) may have affected the validity of the assay, as indicated by the lower correlation coefficients (r^2) for the linear regression of the calibration curves (≤0.95), the volume of the hydroperoxide

sample prepared in hexane was increased from 25 to 200 μL. Although an increase in the ratio of non-polar hexane to polar xylenol reagent could have potentially produced turbidity, the experimental results (data not shown) demonstrated the absence of turbidity with the 200 μL sample volume, as indicated by the corresponding r^2 value of 0.997; this high value validated the selection of using a methanol/water ratio of 90:10 (v/v) in the reagent for the FOX assay. Similarly, Eymard and Genot (2003) used a methanol/water mixture at a ratio of 91:9 (v/v) for quantifying fish lipid hydroperoxides. However, the experimental data (Fig. 14) do not show a relationship between cumene hydroperoxide and absorbance for methanol content below 60%. The absence of such linear relationship may be due to a lower solubility of cumene hydroperoxide in low concentrations of methanol. Nevertheless, Hermes-Lima *et al*. (1995) used a lower methanol concentration (10%) for the determination of extracted lipid hydroperoxides from animal tissues.

In using the 90:10 methanol/water ratio for FOX assay, the absorbance of the ferric ion-xylenol orange (Fe-XO) complex was found to be relatively unstable, with more than 20% variation within a 2 h incubation (data not shown) which may have been due to the oxidation of lipids by oxygen. To improve the stability of the Fe-XO complex, the FOX reagent was degassed for periods ranging from 0 to 15 min. The experimental findings (data not shown) indicated that the stability of the FOX reagent increased with the degree of degassing. The color developed by the reagent, after being degassed for 10 min, was stable upon reaction at room temperature over a period of 2 h. However, this increase in stability was associated with a concomitant decrease in the sensitivity of the FOX assay by 20 and 29% corresponding to 2 and 15 min of degassing, respectively. This concomitant decrease in FOX sensitivity upon degassing may have been due to the removal of dissolved oxygen in the reagent solution which would otherwise have played a role in the chain radical oxidation complex of the xylenol reagent (Nourooz-Zadeh, 1999). Since a compromise between the two parallel phenomena, i.e., a decrease in sensitivity with a concomitant increase in stability, was necessary, the FOX reagent was degassed for 10 min prior to use throughout the study.

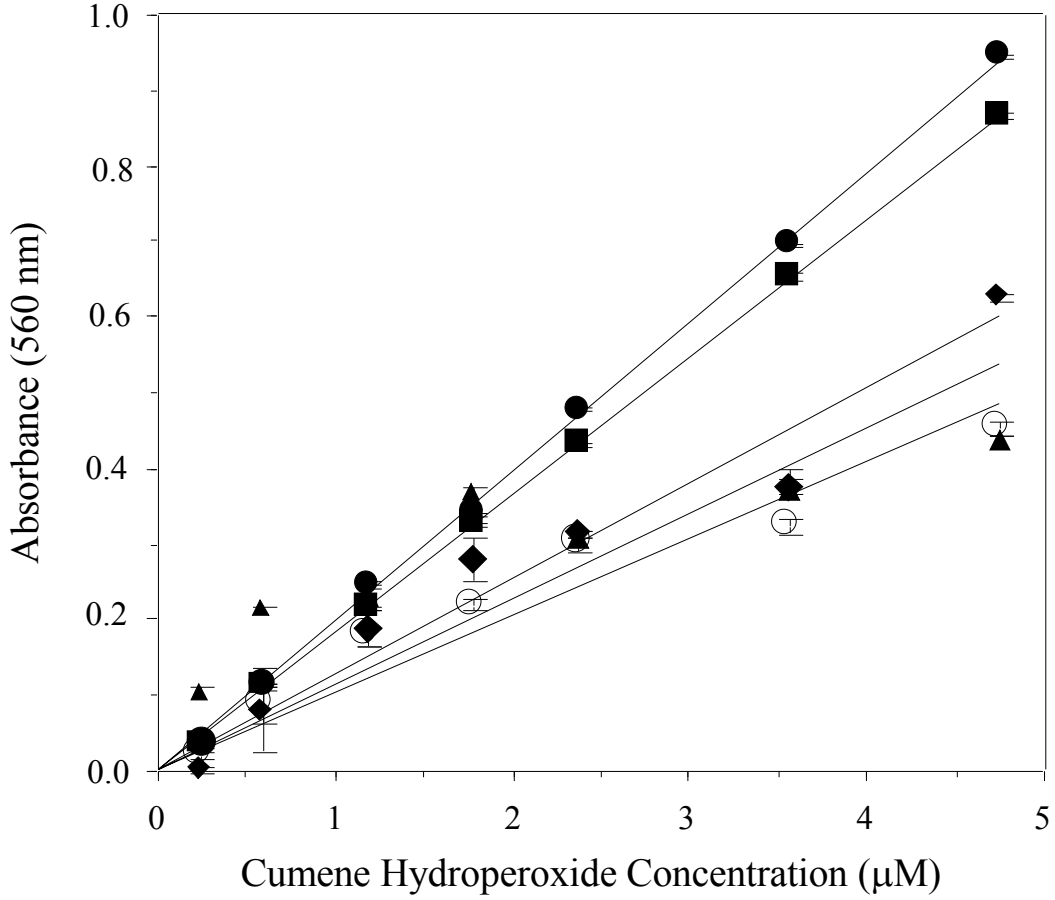


Figure 14. Effect of methanol content of the xylenol reagent on the sensitivity and reproducibility of the calibration curve for hydroperoxide quantification in hexane. Cumene hydroperoxide calibration curves were performed in xylenol orange (FOX) reagent composed of 100% deionized water (○), and in 45 (▲), 60 (◆), 75 (●), and 90% methanol (■) in deionized water. Error bars represent mean ± SD (n = 3).

5.4.2. Effect of BHT Addition on FOX Assay

Wolff (1994) reported that the addition of BHT, a chain breaking antioxidant, to the FOX reagent prevented the oxidation of lipids during analysis. However, the use of BHT in the FOX assay still remains ambiguous: some authors recommend the addition of BHT (Jiang *et al.*, 1991; Nourooz-Zadeh, 1999; Yin and Porter, 2003), whereas others discourage it (Hermes-Lima *et al.*, 1995; Grau *et al.*, 2000; Eymard and Genot, 2003). The effect of 4 mM BHT in the reagent on the FOX assay was investigated in the absence and presence of 14 mM linoleic acid.

Figure 15 shows that in the absence of linoleic acid, BHT has no effect on the ε_{560} value of cumene hydroperoxide. The results also show that in the presence of 14 mM linoleic acid, BHT produced a 32% decrease in the ε value of cumene hydroperoxide, whereas in the absence of BHT, a 50% increase in the ε value was observed. These findings suggest that in the absence of BHT, non-oxidized lipids, such as linoleic acid, can promote an oxidization chain reaction of the Fe^{2+} ions and their subsequent complexation with the xylenol orange compound thereby increasing the ε value of the FOX assay whereas in the presence of BHT, the opposite effect occurs, preventing the overall oxidation of Fe^{2+} ions by both linoleic acid and hydroperoxides.

Although it is difficult to propose a mechanism to explain the effect of linoleic acid on the formation of Fe^{3+} ions, the experimental findings (Fig. 15) are in agreement with those reported in the literature, where lower ε values were found in the presence of BHT, which could be attributed to limited oxidation of lipids and stabilization of the intermediate alkoxyl radicals of the FOX colorimetric reaction (Hermes-Lima *et al.*, 1995; Grau *et al.*, 2000). The overall results suggest that the presence of linoleic acid in the cumene hydroperoxide samples produced more accurate measurements for the construction of a calibration curve while the addition of BHT to the FOX reagent led to a decrease in the sensitivity of the assay in the presence of linoleic acid and was therefore omitted for all subsequent trials.

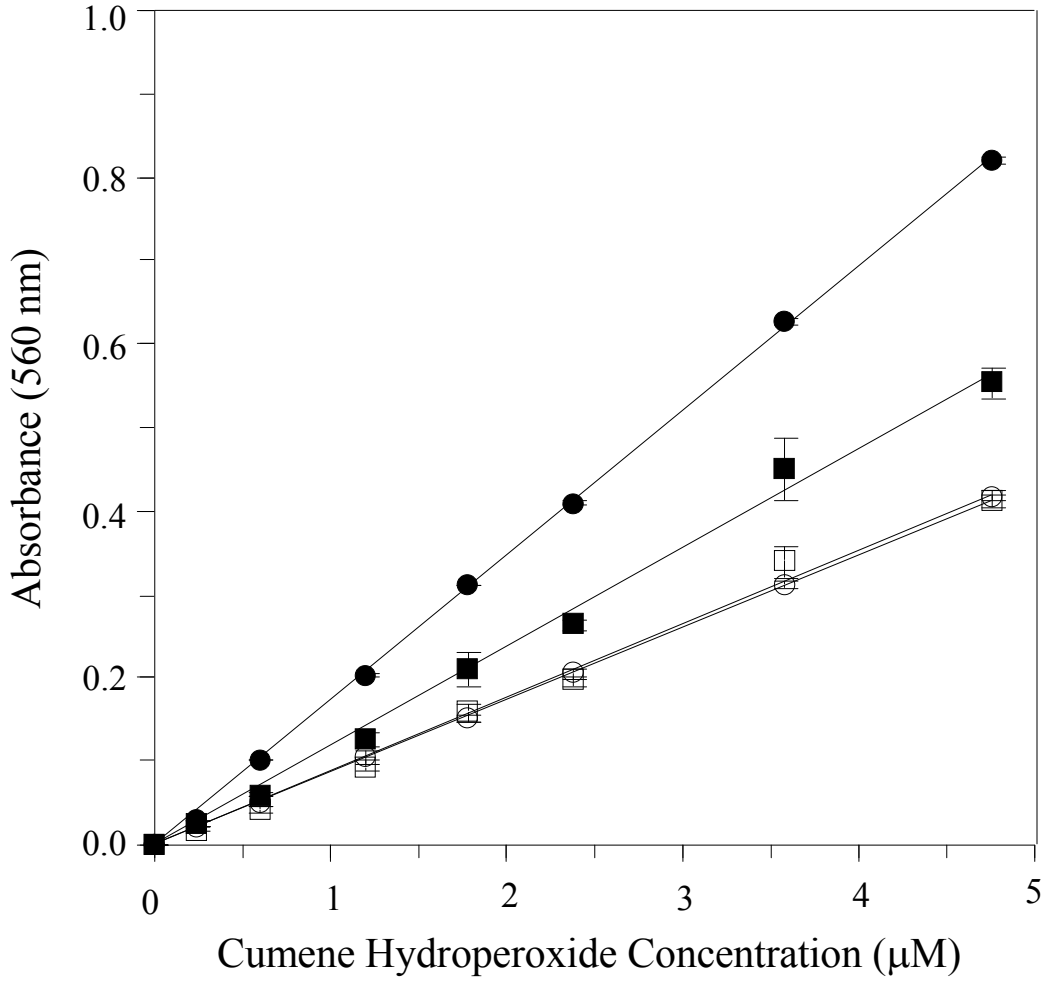


Figure 15. Effect of BHT and linoleic acid on the sensitivity and reproducibility of the calibration curve. Calibration curves were performed using the FOX reagent mixture in the presence of BHT (□ and ■) and absence of BHT (○ and ●). Linoleic acid was added to the hydroperoxide sample for the calibration curves with the filled symbols (● and ■). Error bars represent mean ± SD (n = 3).

5.4.3. Optimization of Reactants for FOX Assay

Several studies have indicated that the absorbance of the ferric ion-xylenol orange (Fe-XO) complex shows a strong dependence on the pH of the assay, with a maximal absorbance obtained in the pH range of 1.7-1.8 (Banerjee *et al.*, 2003). The effect of sulfuric acid and perchloric acid at a final concentration of 25 and 85 mM, respectively, on the FOX assay was investigated, using either ferrous sulfate or ammonium ferrous sulfate as a source of Fe^{2+} ions. The experimental results (data not shown) indicated that the overall absorbance profiles of the spectrophotometric scans of several different FOX colorimetric reaction mixtures consisted mainly of two peaks, the first peak with a maximum absorbance (λ_{max}) between 440 to 453 nm, corresponding to the xylenol orange reactant, and the second peak with a λ_{max} between 553 to 558 nm for the Fe-XO complex. The results also showed that when using ammonium ferrous sulfate as a source of Fe^{2+} ions, the λ_{max} of the Fe-XO complex was at 553 and 558 nm with sulfuric acid and perchloric acid, respectively, while with ferrous sulfate as a source of Fe^{2+} ions, the λ_{max} was at 556 nm with both acids.

Table 12 shows that, in the presence of perchloric acid, the molar absorption coefficients (ε_{560} values) of the Fe-XO complex were 105,110 and 110,380 M^{-1} cm^{-1} with ferrous sulfate and ammonium ferrous sulfate, respectively. However, in the presence of sulfuric acid, the ε_{560} value increased by a factor of 2.2 with ferrous sulfate compared with that obtained with ammonium ferrous sulfate. These results indicate that in the presence of perchloric acid, the source of the Fe^{2+} ions had no effect on the sensitivity of FOX assay whereas with sulfuric acid, the ε_{560} values were dramatically affected, suggesting that the low ε_{560} value obtained with ammonium ferrous sulfate could have been related to a lack of compatibility between these two compounds. The results also show that when the initial pH of the FOX reagent mixture was adjusted to 1.75 with sodium phosphate powder in the presence of ammonium ferrous sulfate, the ε_{560} values slightly increased by a factor of 1.1 and 1.2 with perchloric acid and sulfuric acid, respectively. However, since ammonium ferrous sulfate showed a certain degree of interference in the FOX assay, ferrous sulfate was used throughout the study.

Table 12. Molar absorption coefficients of the ferric ion-xylenol orange complex for cumene hydroperoxide using different xylenol orange reagents.

Reagent conditions[a]	ε_{560} (M^{-1} cm^{-1})[b]	pH Δ[c]	pH[d] Before	pH[d] After
Perchloric / fs	105,110 (±1,030)[e]	0.47	1.68	2.15
Perchloric / ammonium fs	110,380 (±890)[e]	0.29	1.6	1.89
Perchloric / adjusted ammonium fs	118,880 (±120)[e]	0.32	1.75	2.07
Perchloric / fs / LOX[f]	109,430 (±3,320)[e]	0.27	1.69	1.96
Sulfuric / fs	166,570 (±1,410)[e]	0.23	1.63	1.82
Sulfuric / ammonium fs	76,570 (±250)[e]	0.04	1.66	1.70
Sulfuric / adjusted ammonium fs	88,480 (±130)[e]	0.08	1.75	1.83
Sulfuric / fs / LOX[f]	101,150 (±370)[e]	0.61	1.73	2.34
Sulfuric / fs / TPP / with LA[g]	66,740 (±2,470)[e]	not applicable[h]		
Sulfuric / fs / TPP / without LA[g]	103,250 (±2,540)[e]	not applicable[h]		

[a]The FOX reagent was prepared using either perchloric or sulfuric acid as acid and ferrous sulfate (fs) or ammonium fs as a source of ferrous ions. The term 'adjusted' indicates that the pH of the reagent was adjusted before the assay.

[b]The molar absorption coefficient (ε) was calculated using cumene hydroperoxides. Linoleic acid was present in all samples at a 7 mM concentration. All curves were performed in triplicate.

[c]The pH variation was defined as the difference between the pH values of the reagent mixture before and after reaction.

[d]The pH was measured before the addition of the hydroperoxide samples and after the colorimetric assay.

[e]The standard error of the curve was calculated from the average of triplicate samples using SigmaPlot (SPSS, Chicago, IL).

[f]Lipoxygenase (LOX) denatured protein (3.2 μg) was added to the standard in order to evaluate the effect of the ferric ions of the enzyme.

[g]Linoleic acid (LA).

[h]The differences in the absorbance of the samples treated and non-treated with triphenylphosphine (TPP) were used for the construction of calibration curves and to determine the molar absorption coefficient.

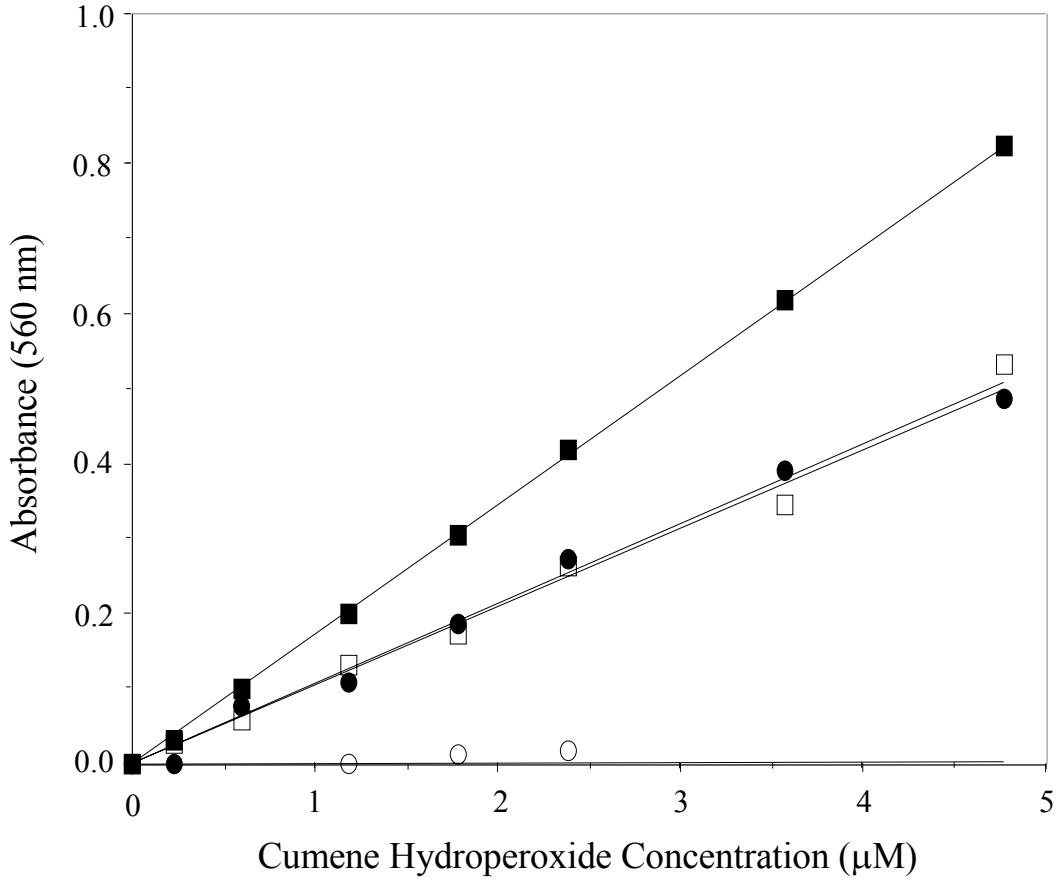


Figure 16. Effect of triphenylphosphine (TPP) on the specificity of the FOX assay. The absolute absorbance of the colorimetric reaction calibration curve was measured with samples treated with TPP (○ and ●) and without TPP (□ and ■). Linoleic acid (14 mM) was added to the hydroperoxide sample for the calibration curves with the full symbols (● and ■). All other parameters of the FOX reagent remained unchanged. Error bars represent mean ± SD (n = 3).

In the presence of denatured LOX possessing Fe^{2+}/Fe^{3+} ions at its active site, the ε_{560} value of the FOX assay slightly increased by 4% in the presence of perchloric acid and dramatically decreased by 40% with sulfuric acid compared to the ε_{560} values obtained in absence of the denaturated enzyme. The experimental results also show that in the presence of denatured LOX, variations in the pH of the reaction were more important with sulfuric acid than with perchloric acid. The high degree of interference of the LOX protein in the FOX assay with sulfuric acid may be explained by the lower capacity of the medium to adjust itself to the dramatic pH changes occurring during the reaction compared to perchloric acid (Gay and Gebicki, 2002).

In contrast to the literature (Gay and Gebicki, 2002; Banerjee *et al.*, 2003), the overall experimental findings (Table 12) indicate that there was no correlation between the sensitivity of the assay and the pH of the reagent mixture. Although variations in pH before and after the reaction were important with both perchloric acid and sulfuric acid, smaller changes in the ε_{560} values were observed in the presence of perchloric acid. Gay and Gebicki (Gay and Gebicki, 2002) also reported the use of perchloric acid for the FOX assay at lower pH optimum of 1.1 and with limited sensitivity to minor changes in pH. Perchloric acid was hence used in the optimized FOX assay.

5.4.4. Effect of TPP on the Specificity of the FOX Assay

Nourooz-Zadeh *et al.* (1994) reported that the use of TPP could increase the specificity of the FOX assay by reducing lipid hydroperoxides into alcohols so that only the potentially interfering substances, such as ferric ions or phenolic compounds, are measured (Nourooz-Zadeh, 1999; DeLong *et al.*, 2002). Differences in the absorbances, obtained using the FOX assay, between TPP-treated and untreated samples would therefore provide a specific quantification of lipid hydroperoxides (Banerjee *et al.*, 2003). Figure 16 shows the calibration curves of TPP-treated and untreated cumene hydroperoxide samples in the presence and absence of 14 mM linoleic acid. The results indicate that in the absence of linoleic acid, the TPP-treated cumene hydroperoxide samples did not absorb at 560 nm; however, in the presence of linoleic acid, a relative ε_{560} of 98,680 M^{-1} cm^{-1} as observed, suggesting that the presence of linoleic acid greatly

affected the reduction of cumene hydroperoxide by TPP. Nourooz-Zadeh *et al*. (1994) indicated that H_2O_2, used as a reference in the FOX assay, was not reduced by the TPP.

5.4.5. Selectivity of the FOX Assay

To investigate the specificity of the FOX assay, standard curves of different FA hydroperoxides were prepared using perchloric acid and ferrous sulfate in the FOX reagent. The results (Table 13) indicate a high dependence of the FOX assay on the nature of the hydroperoxides. The highest ε_{556} value, 172,540 M^{-1} cm^{-1}, was obtained for linoleic acid hydroperoxides while those of highly oxidizable lipids, including linolenic and arachidonic acids, showed lower ε_{556} values of 93,060 and 81,510 M^{-1} cm^{-1}, respectively. The ε_{556} values (Table 13) for cumene hydroperoxide were within the range of those reported by Gay and Gebicki (2002) and were much higher than those reported by Michaels and Hunt (1978) and Gay *et al*. (1999a). In addition, the overall ε values, estimated for the lipid hydroperoxides, were higher than those of 47,000 and 51,200 M^{-1} cm^{-1} previously reported in the literature by Jiang *et al*. (1991) and Gay and Gebicki (2002), respectively. The discrepancies between the ε values obtained in the present study and those reported in literature (Jiang *et al.*, 1991; Gay *et al.*, 1999a; Gay and Gebicki, 2002) could be related to the nature of the solvent, the reference source, the assay conditions as well as the presence of a linoleic acid chain-radical promoting compound.

The results (Table 13) suggest that an increase in the degree of unsaturation of the FA hydroperoxides affected the sensitivity of the FOX response. To interpret this phenomenon, the number of Fe^{3+} ions, generated by each –OOH group of the different hydroperoxides, was estimated using the ε_{560} of 20,100 $M^{-1}cm^{-1}$ obtained for the ferric ions of the Fe-XO complex reported by Gay *et al*. (1999b). Table 15 shows that linoleic acid hydroperoxides produced twice the amount of Fe^{3+} ions per molecule as the amounts produced by linolenic or arachidonic acid hydroperoxides. Overall, the ratios of Fe^{3+} ions per cumene and lipid hydroperoxides were found to be higher than those reported in literature (Jiang *et al.*, 1991; Gay *et al.*, 1999a and 1999b; Gay and Gebicki, 2002) which may be due to the different methods used for quantification of hydroperoxides. Using various hydroperoxide references, Gay *et al.* (1999b) attempted to explain the different

Table 13. Molar absorption coefficients of the xylenol orange assay[a] and the relative proportion of yielded Fe^{3+} ions.

Reference[b]	Molar Absorption coefficient (M^{-1} cm^{-1})	(Fe^{3+}:HP)[c] ratio
Cumene HP	111,800 (±7,590)[d]	5.6
HPOD	172,540 (±9,700)[d]	8.6
HPOT	93,060 (±2,680)[d]	4.6
HPETE	81,510 (±2,440)[d]	4.1

[a]The FOX assay conditions were described in the Material and Method section.

[b]The references, used for the construction of the calibration curve, were cumene hydroperoxide (cumene HP) and hydroperoxides of linoleic (HPODs), linolenic (HPOTs) and arachidonic (HPETEs) acids.

[c]The ratio of Fe^{3+}:HPs was defined as the number of Fe^{3+} ions generated by the reference and calculated as the extinction coefficient of the complex over that of the ferric ions, according to Gay *et al.* (1999b), in the same reagent.

[d]The standard error was calculated using Sigma plot software (SPSS, Chicago, IL).

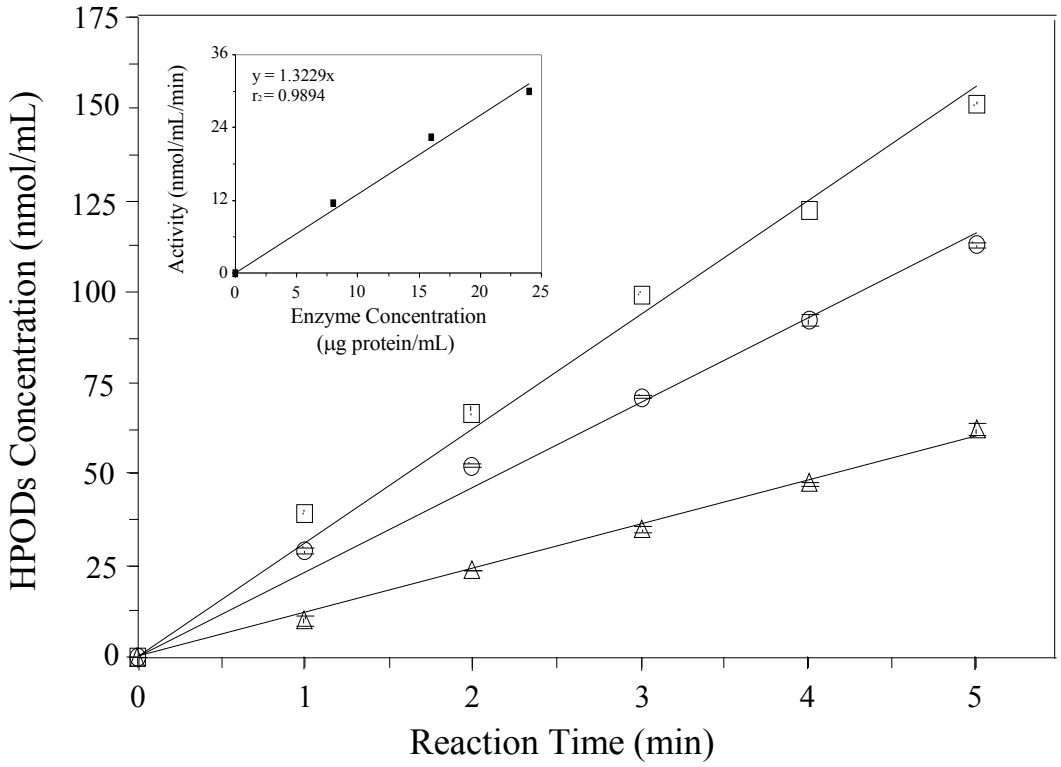


Figure 17. Kinetics of the formation of hydroperoxides of linoleic acid (HPODs) obtained by LOX activity, using 8 (△), 16 (○) and 24 (□) mg protein/mL reaction, determined using the FOX assay. v_i is the initial velocity of end product production, expressed in nmol of HPODs per mL reaction volume per min. Error bars represent mean ± SD (n = 3).

ratios of Fe^{3+} ions per hydroperoxide molecule on the basis of the mechanism for the production of radicals, but these authors did not provide a definite mechanism for the hydroperoxide references that produced higher amounts of Fe^{3+} ions, such as the cumene hydroperoxide. However, it has been reported (Gay *et al.*, 1999a; Nourooz-Zadeh, 1999; Gay and Gebicki, 2002) that alkoxyl radicals (equation 1) formed during the colorimetric assay, produce methyl free radicals by *β*-elimination, which could promote in the presence of oxygen, the formation of different radical species capable of oxidizing Fe^{2+} ions.

$$ROOH + Fe^{2+} \rightarrow Fe^{3+} + RO^{\cdot} + OH^{-} \quad (1)$$

5.4.6. Measurement of LOX Activity

LOX activity has been shown to be relatively higher in hexane media compared with other organic solvents (Kermasha *et al.*, 2001) which can be explained by its lower polarity (Laane *et al.*, 1987) that prevents it from stripping the essential water layer surrounding the enzyme molecule (Griebenow and Klibanov, 1996).

The kinetics of HPOD production by LOX were investigated, using a range of enzyme concentrations varying from 8 to 24 μg/mL, in the hexane reaction medium. The HPODs, produced at defined time intervals, were quantified by the modified FOX assay as described previously. The results (Fig. 17) show that a linear increase in HPOD concentration occurred with reaction time, demonstrating the high sensitivity of the assay even at the initial velocity stage. In addition, the initial velocity stage of the enzymatic reaction implied that the substrate was partially converted (<10%) into its respective end product; this condition being crucial as the presence of linoleic acid as substrate was shown to increase the ε_{556} of the FOX assay. Hence, a defined amount of linoleic acid (7 to 14 mM) was added to all samples used for the construction of calibration curve.

The initial velocity of HPOD production increased proportionally with enzyme concentration (insert of Fig. 17), indicating that the reaction was kinetically catalyzed by LOX and not by auto-oxidation. Although the determination of LOX activity in the non-polar organic solvent medium was reported previously (Piazza *et al.*, 1994; Kermasha *et al.*, 2001; Kermasha *et al.*, 2002a), the overall experimental results reported suggest that

the modified FOX assay may be a convenient, rapid and reproducible method for LOX assay in organic solvent media.

5.5. Conclusion

The experimental data from this study showed that the modified FOX assay could be used with acceptable precision and sensitivity to quantify LOX activity in hexane. However, a proper calibration curve, based on using the same components that were in the reaction medium, is mandatory since the presence of non-peroxidized lipids could dramatically influence the sensitivity of the assay.

CHAPTER VI

STATEMENT OF CHAPTER VI LINKAGE

Chapter VI describes the experimental results for the immobilization and biocatalysis of LOX, of the enriched enzymatic extract, obtained from *P. camemberti*. The optimization of the FOX assay, for its use in OSM was crucial for a rapid and accurate mean of determining LOX activity (Chapter V).

CHAPTER VI.

EFFECT OF ORGANIC SOLVENT REACTION SYSTEM ON AN IMMOBILIZED LIPOXYGENASE EXTRACT FROM P. CAMEMBERTI

6.1. Abstract

The immobilization of an enriched enzymatic extract from *Penicillium camemberti,* containing lipoxygenase (LOX, EC 1.13.11.12), and its biocatalysis in organic solvent media (OSM) were investigated. The highest immobilization efficiency (173.9%) was obtained with unmodified Eupergit®C250L. After 150 min of incubation at 50°C, the free LOX (LOX_f) extract retained only 7.6% of its enzymatic activity whereas the immobilized one (LOX_i) retained 40.3% of its initial activity at 55°C. The thermal inactivation of both LOX_f and LOX_i did not follow first order kinetic behavior. Moreover, the biocatalysis of LOX_i in OSM resulted in a 31.0% increase in its enzymatic activity. The specific activity of LOX_i was increased to a maximum of 7.73 nmol HPODs/mg immobilized protein/min, with the increase in tetradecane concentration up to 20% (v/v) in the ternary micellar system. Further increase in tetradecane content decreased the LOX activity. The LOX inactivation by tetradecane followed first order kinetic behavior. In addition, the enzyme affinity for the substrate was increased in the optimized reaction medium of iso-octane:tetradecane:potassium phosphate buffer (2:1:2; v/v/v). The kinetics results indicated that the enriched LOX extracts followed Michaelis-Menton behavior.

6.2. Introduction

Lipoxygenases (LOX, EC 1.13.11.12) are ubiquitous enzymes found in various animal tissues (Yamamoto *et al.*, 2004), plant cells (Gardner, 1996) and microorganisms (Perraud and Kermasha, 2000). LOX is an important class of non-heme iron enzymes that catalyze the specific di-oxidation of polyunsaturated fatty acids, containing a *cis,cis*-1,4-pentadiene moiety, into hydroperoxy fatty acids; these hydroperoxides are regarded as flavor precursors as they can be subsequently cleaved by hydroperoxide lyase and associated enzymes to flavor compounds, such as ketones, aldehydes and alcohols (Fauconnier and Marlier, 1997). The production of aroma compounds has been

recognized as one of the relevant applications of LOX (Gardner, 1996; Chikere *et al.*, 2001).

The biocatalysis of enzymes in non-conventional media, such as organic solvent media (OSM), may provide a better solvation of the hydrophobic substrate, an easier recovery of the products and of the insoluble biocatalyst, and a higher thermostability to the active enzyme. However, the outmost significance in this approach is the change in LOX specificity (Bell *et al.*, 1995) and hence the production of a wide range of flavor precursors. The LOX biocatalysis in various non-conventional media was investigated (Piazza *et al.*, 1994; Kermasha *et al.*, 2001; Vega *et al.*, 2005a); however, the efficiency of these systems was limited by the LOXs poor stability (Chikere *et al.*, 2001)

Enzyme immobilization has been proven to be a convenient mean of improving enzyme's performance and stability (Mateo *et al.*, 2002). Although the immobilization of soybean LOX and its biocatalysis in OSM have been reported for the purified form (Petrus Cuperus *et al.*, 1995; Pinto and Macias, 1996; Hsu *et al.*, 2000; Chikere *et al.*, 2001; Santano *et al.*, 2002; Vega *et al.*, 2005a), there is little information available regarding unpurified microbial enzymatic extracts.

The present work is part of ongoing research in our laboratory (Perraud and Kermasha, 2000; Kermasha *et al.*, 2001; Kermasha *et al.*, 2002a; Vega *et al.*, 2005a) aimed at the development of a biotechnological process for the use of microbial enzymatic extracts, containing LOX and associate enzymes, for the production of natural flavor precursors and flavors. The specific objectives were to investigate the use of Eupergit® type supports for the immobilization of the enriched enzymatic extract from *Penicillium camemberti* as well as the biocatalysis of the immobilized LOX in selected OSM, in terms of enzyme activity and stability.

6.3. Material and Methods

6.3.1. Enriched Enzymatic Extract Preparation

P. camemberti was grown in a modified Czapek medium and the culture harvested after 10 days following the method described by Perraud and Kermasha (2000),

where LOX was extracted from the fresh harvested biomass. After centrifugation, the supernatant was submitted to ultra-filtration at 4°C for 1.5 h (Millipore, Amicon, 30 kDa NMWCO, 40 psi). To this resulting ultra-filtrate, KCl salt (Fisher Scientific, Fair Lawn, NJ) was added in a ratio of 75 to 1 (KCl: protein, w/w), lyophilized and stored at -80°C. The lyophilized product was considered the enriched enzymatic extract containing LOX activity.

6.3.2. Materials

The modified Czapek medium was composed of glucose, $NaNO_3$, and $MgSO_4 \cdot 7$ H_2O which came from ACP Chemicals (Montreal, QC) and KCl and $FeSO_4 \cdot 7$ H_2O which were obtained from Fisher Scientific (Fair Lawn, NJ). Ammonium thiocyanate, NaCl, NaOH, Na_2CO_3 and $CuSO_4 \cdot 5$ H_2O were all of ACS grade and also purchased from Fisher Scientific. K-Na tartrate, Folin reagent (phospho-18-tungstic acid), xylenol orange salt (3,3'-bis(N,N-di(carboxymethyl)aminomethyl)-o-cresol-sulfonephthaleine sodium salt) and Tween 20 (polyoxyethylene sorbitan monolaurate) were purchased from Sigma Chemical Co. (St-Louis, MO), whereas meso-tetraphenyl-porphine (TPP) copper(II) and perchloric acid were obtained from Aldrich Chemical Co. (Milwaukee, WI). Dipotassium hydrogen phosphate (K_2HPO_4) and potassium dihydrogen phosphate (KH_2PO_4) were purchased from ACP Chemicals. Linoleic acid (*cis*-9, *cis*-12-octadecadienoic acid) was obtained from Nu-Chek Prep Inc. (Elysian, MN). All solvents used were of HPLC grade or more and were purchased from Fisher Scientific.

6.3.3. Protein Determination

Protein concentrations of the free and immobilized LOX_s were determined according to a modification of the Lowry method (Hartree, 1972). Bovine serum albumin (Sigma Chemical Co.) was used as a standard for the calibration curve.

6.3.4. LOX Immobilization

LOX immobilization was performed according to the method described by Vega *et al.* (2005a). Covalent immobilization of LOX was carried out by adding 1 mL of the enzyme suspension (5 mg protein) prepared in the potassium-phosphate buffer (0.1 M,

pH 6.5) to the wet support to obtain a final concentration of 10 mg protein/g wet support (1:100; w/w). Enzyme-support mixtures were stirred at 4°C for 16 h.

The immobilized protein yield (IPY) and the immobilized LOX_i yield (ILY) were calculated from the immobilized protein and LOX_i activity, respectively, divided by the total initial protein and total LOX activity before immobilization, respectively, multiplied by 100. The selectivity factor was defined as the specific activity of LOX_f over that of the supernatant, recovered after immobilization. The immobilization efficiency was calculated by dividing the specific activity of the LOX_i over that of LOX_f, multiplied by 100.

6.3.5. Preparation of the Modified Epoxy Support

Eupergit®C and *Eupergit®C250L* (oxirane acrylic beads) supports, provided kindly by Rohm Pharma (Darmstadt, Germany), were modified according to the method reported by Vega *et al.* (2005a). One gram of the wet support was suspended in 10 mL of 5% (v/v) ethylenediamine (EDA; Aldrich Chemical Co.) solution and subjected, at room temperature, to 15 min of continuous stirring.

6.3.6. LOX Enzymatic Assay

LOX biocatalysis in aqueous and OSM was carried out, in triplicate, according to a modification of the method described by Vega *et al.* (2005b). The aqueous phase of the enzymatic assay was composed of the potassium phosphate buffer. A stock solution of linoleic acid (8 mM) was freshly prepared in the buffer solution as described previously (Kermasha and Metche, 1986). The enzymatic assays were performed in a series of bioreactors under constant agitation. The reaction components were pre-incubated at 55°C. The ternary micellar reaction system (10 mL) contained 3.2 mM linoleic acid (4 mL of the substrate stock solution), 0.4% of Tween 20 (v/v), an adequate amount of the organic solvent and the buffer solution. The reaction was initiated by the addition of 0.8 and 1.0 mg of LOX_f and LOX_i, respectively. Reaction aliquots of 200 μL were recovered at every 4 min intervals for a total of 20 min. The enzymatic reaction was halted by subjecting the aliquot to the FOX colorimetric assay. Control reactions, without LOX,

were carried out in tandem with the enzymatic assays. The LOX activity was expressed in nmol linoleic acid hydroperoxides (HPODs) /mg protein/min.

6.3.7. Xylenol Orange (FOX) Colorimetric Reaction

FOX reagent was prepared following a modification of the method described by Vega *et al.* (2005b), but without methanol. FOX assays were initiated by the addition of 200 μL reaction aliquots to 2.0 mL of the FOX reagent. The mixture was stirred with a Vortex (Genie 2, Fisher Scientific) for 10 sec and repeated 3 times at 5 min interval. The mixture was centrifuged 30 sec (1,000x*g*) after 20 min and the supernatant's absorbance was measured at 556 nm against a blank assay, using a Beckman spectrophotometer (Model 650, Beckman Instruments, Inc., Fullerton, CA). In order to determine the effect of the enzymatic protein on the assay, inactivated extracts of LOX were also assayed. A calibration curve of HPOD standards, ranging from 0 to 62.5 μM, was used.

6.3.8. Preparation of Hydroperoxide Standards

The HPOD (hydroperoxide of octadecadienoic acid) isomers, used as standards in the FOX assay, were obtained by photooxidation of linoleic acid according to the procedure described by Schieberle *et al.* (1984). The separation of HPODs from the residual linoleic acid was performed following the method of Toschi *et al.* (1995), using LC-Si cartridges (Supelco, Bellefonte, PA). Photooxidized HPODs were quantified with the ferrous thiocyanate assay as described by Wurzenberger and Grosch (1984).

6.3.9. Thermal Stability of Free and Immobilized Extracts of LOX

The thermal stability of LOX was investigated by incubating the enzymatic extracts, at 55°C, in the ternary micellar system composed of 10% iso-octane and 0.4% Tween 20 (v/v) for a wide range of incubation periods (0 to 150 min). The residual LOX activity was measured and the inactivation constant (k_t) and the half-life (T_{50}) were determined from the semi-logarithmic plots of the inactivation kinetics, according to the following equation:

$$\ln (A/A_0) = -k_t T \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

where, *T* was the incubation time at 55°C; A and A_0 were the LOX activity with a defined incubation time *T* and at time 0, respectively.

6.3.10. Effect of Iso-Octane Concentration on LOX Activity

The effect of iso-octane concentration in the ternary micellar system on the LOX activity was investigated. The concentrations of iso-octane were 0 to 40% for the assay of LOX_f and 0 to 98% for LOX_i. The reaction temperature was 50°C for the assays involving LOX_f.

6.3.11. Effect of Solvent on the Activity of LOX

A wide range of OSM, including iso-octane:tetradecane (5:1, v/v), hexane, octane, heptane:hexane (1:1, v/v), iso-octane and heptane, with Log *P* values of 5.1, 3.5, 4.5, 3.8, 4.5 and 4.0, respectively, were investigated for their effect on the activity of LOX_i in the ternary micellar composed of 60% organic solvent (v/v), 40% potassium-phosphate buffer (v/v) and 0.4% Tween 20 (v/v).

6.3.12. Effect of Tetradecane Content on the Activity of LOX

The effect of tetradecane concentrations (0 to 50%, v/v) in the ternary micellar system on the activity of LOX_i was investigated. The system was otherwise composed of iso-octane (10 to 60%, v/v), 40% potassium-phosphate buffer (v/v) and 0.4% Tween 20 (v/v). A quantitative determination of LOX inactivation by tetradecane was obtained by calculating the first order inactivation constant (k_c) and the concentration of tetradecane required to decrease the LOX activity by 50% (C_{50}), using the following linear equation:

$$\ln (A/A_0) = -k_c C \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (2)$$

where, *C* was the concentration of tetradecane in the ternary system; A and A_0 were the LOX_i activity at concentration *C* of 50 and 20% tetradecane, respectively.

6.3.13. Effect of Immobilization on the Kinetics Parameters of LOX

The effect of substrate concentrations on the LOXi activity in ternary micellar media composed of either 10% iso-octane or iso-octane:tetradecane and potassium-phosphate buffer (2:1:2, v/v/v) was investigated, using a wide range of linoleic acid

concentrations (0 to 14 mM). The effects of immobilization on kinetic parameters, including Km and Vmax, were determined.

6.3.14. Effect of Temperature on LOX Activity

The effect of temperature of reaction (30 to 80°C) on LOX_f and LOX_i activity was investigated, using the optimized ternary micellar systems composed of 10% iso-octane and 60% iso-octane:tetradecane (3:2) mixture, respectively.

6.4. Results and Discussion

6.4.1. Immobilization of the Enriched LOX Extract from P. camemberti

The enriched enzymatic extract of LOX from *P. camemberti* was immobilized by covalent binding on different Eupergit®C supports, including Eupergit®C, Eupergit®C250L as well as modified Eupergit®C250L/EDA. The results (Table 14) show that the highest immobilized protein yield (IPY, 79.0%) and immobilized LOX_i yield (ILY, 99.2%) were obtained with the use of Eupergit®C250L/EDA; the IPY and ILY seamed dependent on the modification of the support by EDA. This modified support also resulted in the highest selectivity factor of 22.2 and hence showed its capacity to immobilize the LOX preferentially as compared to the other proteins present in the enriched enzymatic extract. These results are in agreement with those reported in the literature (Vega *et al.*, 2005a) where the selectivity of adsorption of purified soybean LOX was increased when the epoxy groups concentration on the support was low. The differences between IPY and ILY values may be due to differences in the adsorption selectivity onto the support between *P. camemberti* LOX and the other proteins (Vega *et al.*, 2005a). The selectivity factor can be used as a measure of the adsorption selectivity (Mateo *et al.*, 2001).

On the other hand, Eupergit®C250L and modified Eupergit®C250L/EDA showed a selectivity factor higher than 1.0 (Table 14), which confirmed the higher affinity of *P. camemberti* LOX for those supports as compared to the other proteins. As Eupergit®C250L has the same chemical structure as Eupergit®C, but with larger pores (Katchalski-Katzir and Kraemer, 2000), the differences in affinity and/or selectivity could

be related to the difference in support morphologies, such as the average pore diameter, which may affect that surface area of the supports (Clark, 1994).

The highest immobilization efficiency (173.9%) was obtained with the unmodified Eupergit®C250L (Table 14). These results could indicate that Eupergit®C250L support may favor an enzyme conformation that renders it more active; this could lower stearic hindrance at the active site as well as lower substrate diffusion limitations, probably by selectively adsorbing the active protein as compared to others present in the enriched enzymatic extract (Clark, 1994). The overall results suggest that the modified and unmodified Eupergit®C250L supports were the most appropriate supports for the immobilization of the LOX enriched enzymatic extract.

The enzyme activity was also compared. The highest activity per mg protein was found using the extract immobilized on Eupergit®250L (0.58 μmol/mg protein/min) whereas the one reported in U/g support was the extract immobilized on Eupergit®C250L/EDA (0.44 U/g support). It is worth mentioning that the large difference in the activity expressed in U/g support between the Eupergit®C250L/EDA support (0.44) and the Eupergit®C and Eupergit®C250L supports (0.05 and 0.06) is mainly due to the differences obtained in the IPY and ILY. As more LOX was immobilized on the modified support, it is most active in terms of U/g support on this support as compared to the unmodified ones.

A modified Eupergit®C250L support was also used for the immobilization of soybean LOX (Vega *et al.*, 2005a). The unmodified epoxy supports were used in other studies for the immobilization of soybean LOX (Chikere *et al.*, 2001; Santano *et al.*, 2002) and potato LOX (Pinto *et al.*, 1997). On the basis of the experimental findings (Table 14), Eupergit®C250L was used throughout this study for the immobilization of enriched enzymatic extract from *P. camemberti*.

6.4.2. Effect of Iso-Octane Concentration

LOX biocatalysis limitations in OSM may partly be attributed to the presence of insoluble hydrophobic fatty acids and to the more soluble hydrophilic HPODs in the

same reaction medium (Gardner, 1996; Carrea and Riva, 2000). In addition, the accumulation of HPODs in the microenvironment of the enzyme has an inhibitory effect on LOX activity (Siedow, 1991). To overcome the solubility restriction, the effect of iso-octane concentrations (0 to 40%) in the ternary micellar system was investigated on LOX_f activity at 50°C. The results (Fig. 18A) show that LOX activity in aqueous medium and in OSM containing 10% iso-octane was 35.66 and 15.70 nmol HPODs/mg protein/min, respectively. However, the increase in iso-octane concentration in the reaction medium inhibited LOX_f 2.3 fold.

The effect of iso-octane concentration on the LOX_i activity was also investigated using a ternary micellar system containing K-phosphate buffer, 0.4% Tween 20 (v/v) as well as iso-octane concentrations ranging from 0 to 98% (v/v). The results (Fig. 18B) show that at lower concentrations of iso-octane (20%), the LOX_i activity was at its minimum (2.31 nmol HPODs/mg protein/min). However, the increase in iso-octane content up to 80% resulted in an increase in LOX_i activity up to its maximum of 4.74 nmol HPODs/mg protein/min. Still, further increase in iso-octane concentration to 98% (v/v) reduced LOX_i activity by 3 fold.

The overall experimental findings (Fig. 18) show that the immobilization of the LOX enzymatic extract is an appropriate process for its biocatalysis in OSM, since it increased its tolerance to higher concentrations of organic solvent. LOX_i activity in the ternary micellar system, containing 80% iso-octane (v/v), is close to that in aqueous medium. Similarly, previous studies (Kermasha *et al.*, 2001) have shown a decrease in soybean LOX activity when iso-octane content in a ternary micellar system was increased beyond a certain threshold. However, Hsu *et al.* (2000) reported that soybean LOX activity was not affected by lower concentrations of iso-octane, pre-saturated with oxygen, in a biphasic system. Although the LOX_i activity was highest in 80% iso-octane, a concentration of 10% iso-octane was used in the subsequent thermal stability study since LOX_f was inactive at such increased levels of organic solvents. Furthermore, the stability of the ternary micellar system at 80% iso-octane was shown difficult to control with data bearing a relative standard deviation around 30%.

Table 14. Immobilization parameters for the lipoxygenase extract from *P. camemberti*, using selected modified Eupergit® supports.

Support	IPY (%)[a]	ILY (%)[b]	Selectivity factor[c]	Immobilization efficiency (%)[d]	Enzyme activity	
					μmol/mg protein/min	U/g support[e]
Eupergit®C	36.9	4.0	0.6	53.7	0.18	0.05
Eupergit®C250L	12.5	77.0	2.8	173.9	0.58	0.06
Eupergit®C250L/EDA	79.0	99.2	22.2	70.3	0.24	0.44

[a]The immobilized protein yield (IPY) was determined as the relative percentage of immobilized protein to that of the total initial protein. An enzyme to support ratio of 1:100 was used.

[b]The immobilized LOX yield (ILY) was calculated as the relative percentage of the total activity of the immobilized enzyme to that of the free enzyme treated under the same conditions.

[c]The selectivity factor was defined as the ratio of the specific activity of the free enzyme over that of the supernatant, obtained by filtration and centrifugation after immobilization.

[d]The immobilization efficiency was defined as the relative percent specific activity of the immobilized enzyme compared to that of the free one.

[e]The activity of the immobilized enzyme was also expressed as μmol/min/g support.

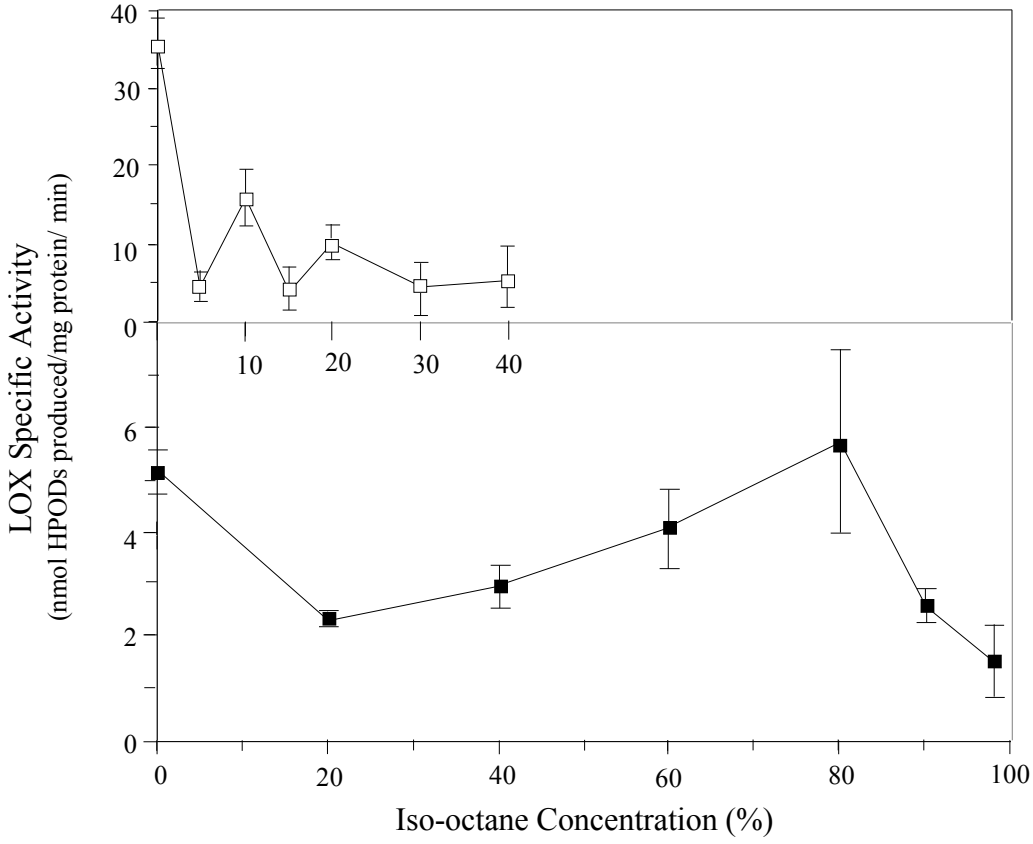


Figure 18. Effect of iso-octane concentration in the ternary micellar system on free LOX_f (□) and immobilized LOX_i (■) activities from *P. camemberti.*

6.4.3. Thermal Stability of LOX in OSM

The thermal stability of LOX in the ternary micellar system, composed of 10% iso-octane, was investigated. The enriched enzymatic extracts were incubated at 55°C for 0 to 150 min. The results (Fig. 19) indicate that after 150 min of incubation, LOX_f extract retained 7.6% of its activity, whereas the residual LOX_i activity was 40.3%, as compared to the initial one before incubation. The effect of temperature on the stability of the enriched enzymatic extract from *P. camemberti* had been studied previously (Perraud and Kermasha, 2000) in aqueous medium, where the residual LOX activity was 52% after 10 min of incubation at 80°C.

From the semi-logarithmic plots, the inactivation constant and half-life (T_{50}) of LOX_f and LOX_i were estimated (data not shown). From equation 1, the T_{50} of LOX_f and LOX_i were calculated at 44 and 165 min, respectively; the T_{50} hence increased by a factor of 3.75 upon immobilization on Eupergit®C250L. The results suggest that the immobilization increased the thermal stability of the enriched enzymatic extracts. Similarly, Mateo *et al.* (2002) have shown that immobilization, by epoxy supports, increased the thermal stability by multi-point covalent attachment, which stabilized the 3D structure enzymes.

The results (Fig. 19) also indicate that the thermal inactivation of both free and immobilized enzyme preparations did not follow first order kinetic behavior, as indicated by the poor linearity of those plots and the discrepancy between the calculated T_{50} and the remaining activity after 150 min for LOX_i. Chikere *et al*. (2001) and Vega *et al*. (2005a) reported that soybean LOX type I-B, immobilized on epoxy supports, displayed first order kinetics behavior. However, the kinetic inactivation of LOX from tomato (Anthon and Barrett, 2003), beans (Indrawati *et al.*, 1999) and potatoes (Park *et al.*, 1988) did not follow first order kinetics. Park *et al.* (1988) have demonstrated non first-order kinetics for potato LOX and hypothesized that this behaviour in a crude enzymatic preparation was due mainly to the presence of more than one LOX species with different thermal stabilities. This explanation could indicate that the enriched enzymatic extract from *P. camemberti,* used throughout this study, contained more than one LOX isozymes.

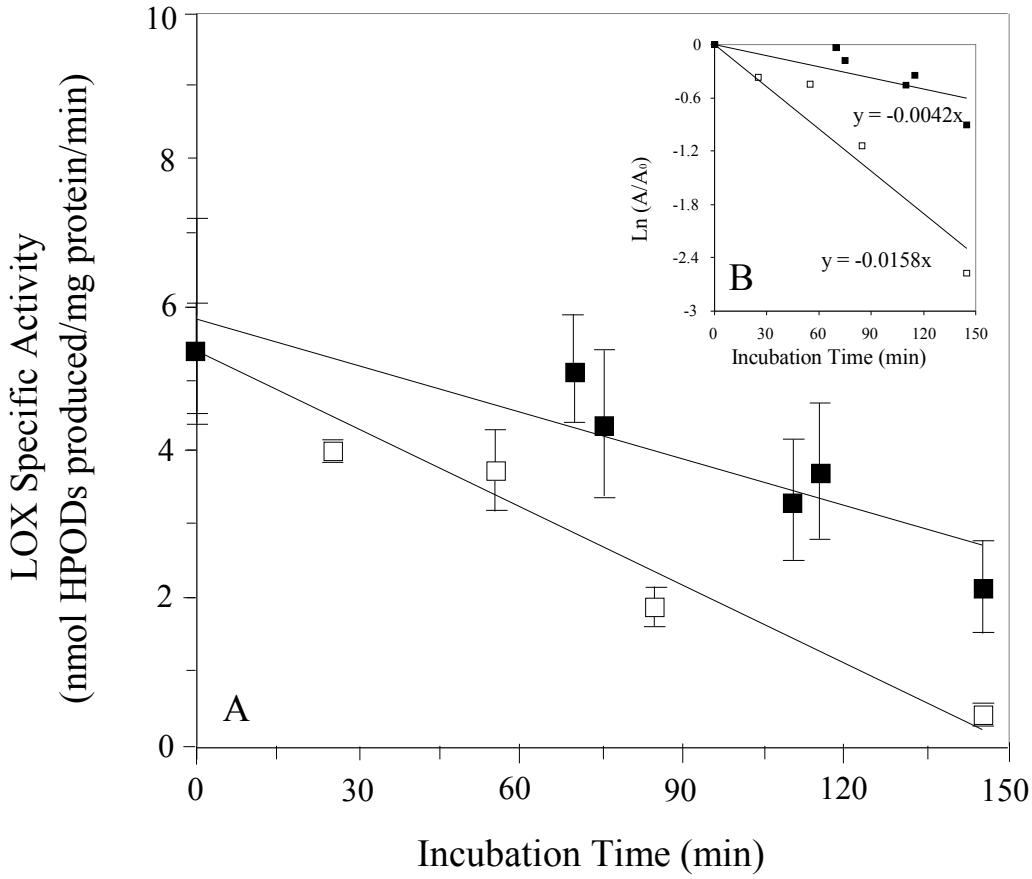


Figure 19. Effect of incubation period (0 – 150 min), at 55°C, on LOX activity (A) and stability (B) in a ternary micellar system composed of 10% iso-octane using LOX_f (□) and LOX_i (■) extracts from *P. camemberti*.

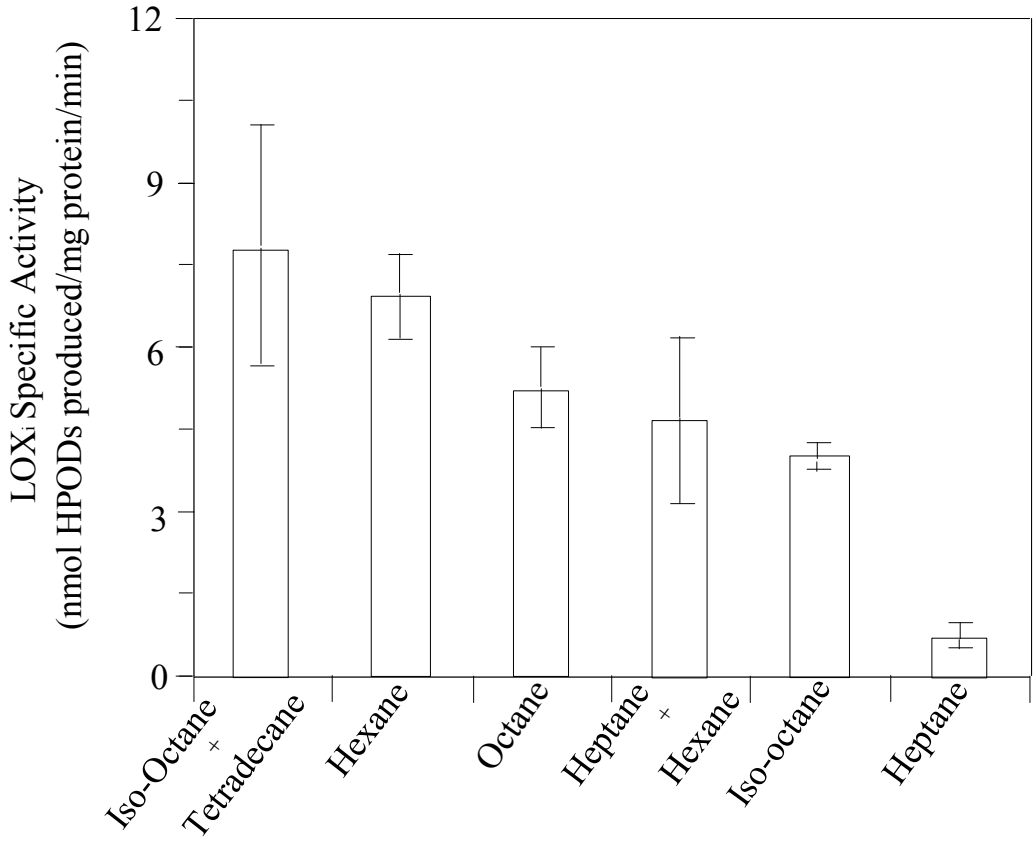


Figure 20. Specific activity of biocatalysis of immobilized LOX_i from *P. camemberti* in the ternary micellar systems composed of 60% solvent (v/v), 40% K-phosphate buffer (v/v) and 0.4 % Tween 20 (v/v).

6.4.4. Effect of Solvent on LOX Activity

The effect of a wide range of solvents or solvent mixtures on the LOX_i activity was investigated. The biocatalysis of LOX_i, immobilized on Eupergit®C250L, was performed in a ternary micellar system composed an organic solvent:potassium-phosphate buffer (3:2, v/v) and 0.4% Tween 20 (v/v). The results (Fig. 20) show that the specific activity of LOX_i in the ternary micellar system, with OSM composed of iso-octane:tetradecane (5:1; v/v), hexane, octane, heptane:hexane (1:1; v/v), iso-octane and heptane, was 7.87, 6.93, 5.25, 4.69, 4.09 and 0.79 nmol HPODs/mg immobilized protein/min, respectively. However, the specific activity for LOX_f in the same media displayed lower activity (data not shown). These findings suggest that the immobilization increased the tolerance of the enzyme for OSM, probably by preventing the denaturation of the enzyme (Clark, 1994). In addition, LOX_i activity increased in OSM as compared to that in aqueous medium. The increase in enzymatic activity could be attributed to a decreased end product partitioning effect by accelerating the removal rate of the HPODs from the micro-environment of the enzyme, which is a limiting factor in the conversion of linoleic acid into HPODs (Siedow, 1991).

The highest LOX activity was attained in the mixture with the highest calculated *Log P* value, and composed of iso-octane and tetradecane (5:1; v/v). Higher enzymatic activity was expected with the concomitant increase in solvent hydrophobicity; the increase in enzyme activity may be due to a decrease in the solvent ability to strip the water layer from the surface of the enzyme (Carrea and Riva, 2000). In addition, the molecules of non polar solvents are less likely to be present in the micro-environment of the enzyme thereby preventing denaturation (Griebenow and Klibanov, 1996). Bell *et al*. (1995) reported that in addition to the physical properties of solvents, such as their hydrophobicity, their molecular structure plays an important role in enzyme activity. Enzyme-solvent interaction may alter the conformation of the enzyme by a direct molecular binding of the solvent onto or near its active site, hence, resulting in a denaturating effect (Carrea and Riva, 2000).

6.4.5. Effect of Tetradecane Concentration on Immobilized LOX Activity

Since LOX_i was most active in the mixture containing iso-octane and tetradecane, the presence of tetradecane at variable concentrations (0 to 50%) was investigated. Figure 4 shows the effect of tetradecane concentration on LOX_i activity of the microbial enriched enzymatic extract. Using the ternary micellar system, with 60% organic solvent (v/v), the proportion of tetradecane as well as iso-octane was varied between 0 to 50% and 10 to 60% (v/v), respectively. The results (Fig. 21) indicate that the specific activity of LOX_i was increased to its maximum of 7.73 nmol HPODs/mg immobilized protein/min, with an increase in tetradecane concentration to 20% (v/v). The increase in LOX activity could be due to an increase in HPODs solvation, which may have lowered their micro-environmental effect and thereby their inhibition role (Siedow, 1991). However, further increase in tetradecane proportion to 50% resulted in a 21% decrease in the specific activity of LOX_i; this decrease in enzyme activity may be due to increased substrate diffusional limitations. The presence of specific interactions between the solvent and the enzyme may also account for the lower specific activity in the presence of higher proportions of tetradecane (Arroyo *et al.*, 2000).

The inserted diagram (Fig. 21) shows a straight line, with correlation coefficient of 0.99, indicating hence that the enzyme inactivation by tetradecane followed first order kinetic behavior. First order kinetics of enzyme inactivation by co-solvents in monophasic aqueous mixtures has also been reported for aminotransferase (Boross *et al.*, 1998) and acylase (Arroyo *et al.*, 2000) as well as in an organic solvent mixture of dioxane and hexane for soybean LOX (Vega *et al.*, 2005a).

A quantitative determination of LOX inactivation by tetradecane was obtained by calculating the inhibition factor (C_{50}). The theoretical C_{50} (equation 2) was estimated to be 187%; these findings suggest that tetradecane in a ternary micellar system, composed of iso-octane and the phosphate buffer, may not denature totally the immobilized enzyme. The low denaturating effect of tetradecane on LOX_i could be explained by the multipoint covalent attachment of the enzyme on the support, which may have led to less conformational changes (Mateo *et al.*, 2002), as well as by the hydrophobic character of tetradecane itself which prevents the denaturation of LOX.

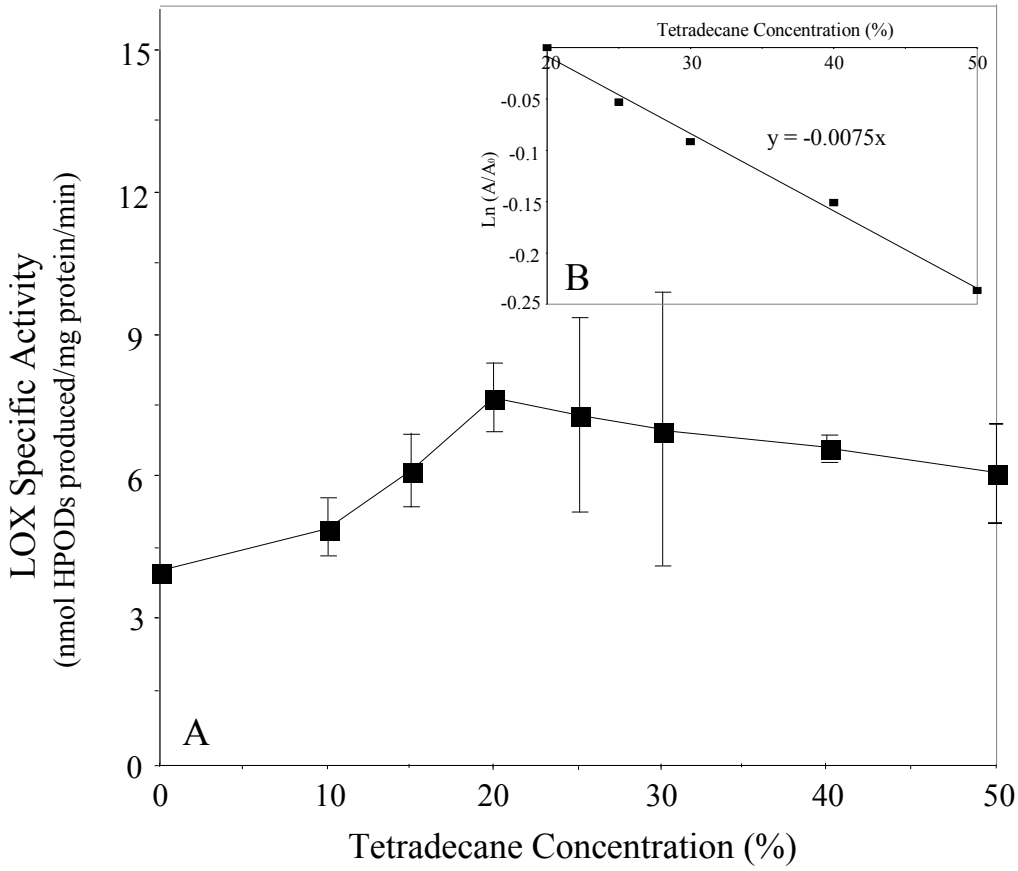


Figure 21. Effect of tetradecane content in the ternary micellar system composed of 3:2 organic solvent:K-phosphate buffer (v/v) on the specific activity of immobilized LOX_i from *P. camemberti*.

6.4.6. Kinetics parameters of LOX in OSM

Using linoleic acid, the effect of substrate concentrations on the specific activity of LOX_i in the ternary micellar media, composed of either 10% iso-octane or 60% iso-octane:tetradecane (2:1, v/v) was investigated. The kinetic parameters of LOX_f was also determined, but in the 10% iso-octane system. The Lineweaver-Burk plots of $1/v$ versus 1/[S] (data not shown) displayed a linear relationship with correlation coefficient of 0.99. 0.97 and 0.83, for the LOX_i in 10% iso-octane as well as in the optimized mixture containing tetradecane and for the LOX_f in 10% iso-octane, respectively.

The kinetic parameters, K_{m1}, K_{m2}, and K_{m3} as well as V_{max1}, V_{max2} and V_{max3}, for the LOX_f and LOX_i were estimated from the Lineweaver-Burk plots. The results (Table 15) indicate that K_{m1} and K_{m2} were determined as 1.55 and 0.03 mM for the LOX_f and LOX_i in 10% iso-octane, respectively; the K_{m3} for the LOX_i in the optimized tetradecane system was calculated as 0.84 mM. Using the reaction system with 10% iso-octane, the immobilization of LOX onto Eupergit®C250L decreased the K_m value by more than 50 fold. These findings suggest that the apparent affinity of the enzyme for the substrate increased upon immobilization. Although a decrease in enzyme affinity for the substrate could be expected with the immobilization process, which could be due to conformation changes, steric hindrance and substrate diffusion limitations (Clark, 1994; Vega *et al.*, 2005a), lower $K_m app.$ values have also been reported previously by Kermasha *et al.* (2002a) for the immobilized soybean LOX, with values as low as 0.06 mM in octane medium. Since the enzymatic extract used was crude and as the immobilization with Eupergit®C250L was relatively selective for LOX, the immobilization process could have had a concomitant effect by increasing affinity of the enzyme for the substrate.

From a different point of view, the $K_{m\ app.}$ value for LOX_i increased when the medium was changed to 60% iso-octane:tetradecane (3:2, v/v). Bindhu and Abraham (2003) has attributed the increase in K_m value for peroxidase to the increased hydrophobicity of the enzyme when placed in organic solvent medium as a result of the modification of the micro-environment of the enzyme and in turn its interaction with the substrate.

Table 15. Kinetics parameters of *P. camemberti* LOX in ternary micellar systems.

Kinetic parameters	Free LOX[a]	Immobilized LOX[b]	
		System 1[c]	*System 2*[c]
K_m[d]	1.55	0.03	0.84
V_{max}[e]	8.22	3.09	20.08
Enzyme efficiency[f]	5.30	123.60	23.90

[a]Free LOX kinetics parameters were determined in ternary micellar system 1.

[b]Enzyme was immobilized on Eupergit®C250L (Rohn Pharma). LOX_f was treated under the same conditions as LOX_i, without support.

[c]System 1 was the ternary micellar system composed of 10% iso-octane in K-phosphate buffer (0.1 M, pH 6.5) and 0.4% Tween 20 (v/v), whereas System 2 was composed of iso-octane: tetradecane: k-phosphate buffer (2:1:2, v/v/v) with 0.4% Tween 20.

[d]K_m expressed in mM linoleic acid.

[e]V_{max} expressed in nmol HPODs produced/mg protein/min.

[f]Catalytic efficiency was defined as the ratio of V_{max} over K_m.

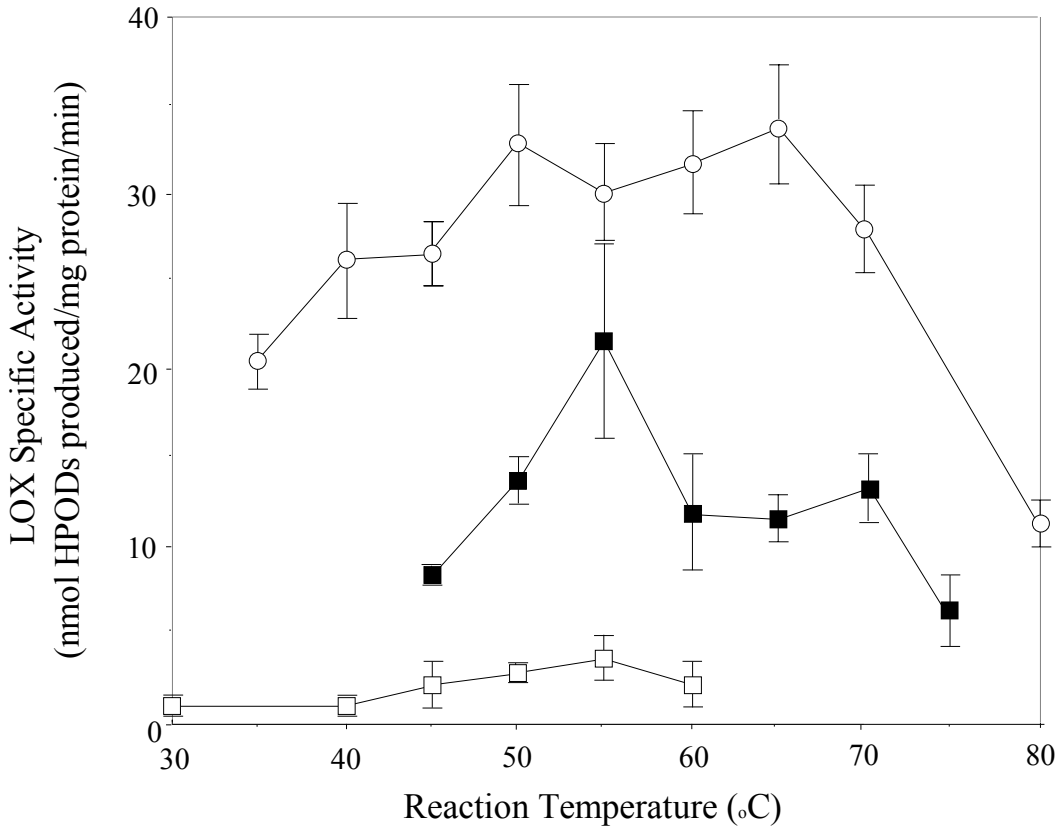


Figure 22. Effect of temperature (30 - 80°C), at a defined period of time, on free LOX_f activity from *P. camemberti* in the ternary micellar system composed of 10% iso-octane (□); LOX_f activity in aqueous medium (○) and immobilized LOX_i (■) activity in the ternary micellar system composed of iso-octane:teradecane and the buffer (2:1:2, v/v/v).

The results (Table 15) also show that the V_{max1} and V_{max2} values, for LOX_f and LOX_i in 10% iso-octane medium, were 8.22 and 3.09 nmol HPODs/mg protein/min, respectively. The V_{max3} value for LOX_i, in the optimized ternary micellar system composed of tetradecane, iso-octane and the buffer solution, was 20.08 nmol HPODs/mg protein/min. Using a partially purified extract from *P. camemberti* containing LOX activity in aqueous medium, Perraud and Kermasha (2000) reported a V_{max} value of 46.2 nmol HPODs/mg protein/min. Because of the poor stability of LOX in aqueous medium (Chikere *et al.*, 2001), its lower V_{max} value, as compared to the fresh one, could be due to a partial denaturation of the enzyme.

The experimental findings (Table 15) indicate that the apparent enzyme efficiency, defined as V_{max}/K_m, was 5.30 and 123.60 min^{-1} for LOX_f and LOX_i in 10% iso-octane, respectively, and 23.90 min^{-1} for the LOX_i in the medium with tetradecane; these values are higher than those reported in other studies. Using soybean LOX, Vega *et al.* (2005a) reported enzyme efficiency in the range of 0.14 to 0.41 in a mixture of hexane-dioxane, and Kermasha *et al.* (2001 and 2002) reported similar results in octane medium.

6.4.7. Effect of Temperature on LOX Activity

The effect of reaction temperature on LOX activity in the optimized ternary micellar systems was investigated, using 10% iso-octane for LOX_f and 60% iso-octane:tetradecane (3:2, v/v) for LOX_i. The results (Fig. 22) indicate that LOX_f in aqueous medium has maximum specific activity at 50 and 65°C, whereas LOX_f and LOX_i in OSM both exhibited maximum one at 55°C. These findings show that the addition of iso-octane (10 to 60%) to the reaction media increased slightly (by 5°C) the optimum temperature of enzymatic activity.

Optimum temperature for microbial LOX activity in aqueous medium was found in the range of 40°C (Perraud and Kermasha, 2000); however, Kermasha *et al.* (2001) have shown that soybean LOX-1 displayed an optimal activity at 20°C, above which the enzyme activity decreased. It has been suggested that the effect of temperature on the activity of enzymes could be due to the change in properties of the micelles (Atwood and

Florence, 1983). The solubility of non ionic surfactant has been shown highly dependant on the temperature which affected the stability of micelles (Overbeek *et al.*, 1984).

6.5. Conclusion

The experimental data obtained throughout this study showed that the immobilization of the enriched enzymatic extract from *P. camemberti,* containing LOX activity, increased its activity as well as its thermal stability as compared to the free one. EupergitC®250L was shown to be an appropriate immobilization support for the microbial LOX as it also increased the tolerance of the enzymatic extract to organic solvent. Finally, organic solvents with high *Log P* values, such as iso-octane and tetradecane, were shown valuable for the biocatalysis of linoleic acid by LOX activity.

CHAPTER VII

STATEMENT OF CHAPTER VII LINKAGE

Similarly to the characterization of LOX in OSM (Chapter VI), chapter VII reports on the results of the immobilization and biocatalysis of HPL of the enriched enzymatic extract, obtained from *P. camemberti*. Concomitantly with chapter VI, the following chapter's experimental findings provide an overall assessment for the potential bioconversion of PUFAs into flavor precursors and flavor compounds, using a biotechnological approach.

CHAPTER VII.

EFFECT OF ORGANIC SOLVENT REACTION SYSTEM ON AN IMMOBILIZED HYDROPEROXIDE LYASE EXTRACT FROM P. CAMEMBERTI

7.1. Abstract

The immobilization of an enriched enzymatic extract from *Penicillium camemberti* containing hydroperoxide lyase (HPL, EC 4.1.2.-) and its activity in organic solvent media (OSM) were investigated. High values of immobilization efficiency of 329.2 and 694.4% were obtained with Eupergit®C and Eupergit®C250L, respectively. The immobilization of the enzymatic extract with Eupergit®C250L resulted in the highest selectivity factor of 2.6., this immobilized enzyme (HPL_i) was used throughout this study. HPL_i retained 58% of its activity as compared to 37% for the free one (HPL_f), after incubation at 55°C for 150 min; the half-life of the activity of HPL increased by a factor of 2.5 upon immobilization. The results showed that the presence of low concentrations of iso-octane content (up to 30%, v/v) did not affect the HPL activity of both extracts. However, the use of 60% iso-octane (v/v) in the reaction medium increased the HPL_i activity by more than 5 fold as compared that in the aqueous medium. The addition of tetradecane in the reaction medium increased further the HPL_i activity by 17% as compared to that in 60% iso-octane medium without tetradecane. The K_m values were 27.14 and 1.88 μM for HPL_i in 10% iso-octane (v/v) and 60% iso:octane:tetradecane (2:1, v/v), respectively; whereas the V_{max} values were, respectively, 14.30 x 10^3 and 13.37 x 10^3 nmol HPOD/mg protein/min.

7.2. Introduction

Hydroperoxide lyase (HPL, EC 4.1.2.-) is a heme-enzyme that is found membrane-bound in higher plants and under soluble form in algae (Gardner *et al.*, 1991; Nuñez *et al.*, 1997). HPL catalyzes the specific cleavage of hydroperoxy fatty acids bearing a 1*Z*,4*Z*-pentadiene moiety, into non-volatile oxoacids and volatile flavor compounds such as alcohols (Gardner, 1991). The production of aroma has been recognized as one of the relevant application of the lipoxygenase (LOX) pathway, where HPL is associated. As HPL is the first enzyme of the LOX pathway to produce volatile

compounds, its characterization is fundamental (Salas *et al.*, 2000). The type of flavor compounds produced depends essentially on the specificities of both LOX and HPL.

The biocatalysis of enzymes in non-conventional media, such as organic solvent media (OSM), may provide a better solvation of the hydrophobic substrate, an easier recovery of both products and biocatalyst, a higher thermostability of the active enzyme and most importantly, it may change the specificities of LOX and HPL (Bell *et al.*, 1995). Although the optimization of LOX biocatalysis in non-conventional media was investigated (Piazza *et al.*, 1994; Kermasha *et al.*, 2001), there is little information reported on the stability of HPL and its biocatalysis in OSM.

HPL is commonly known as unstable, especially to its toxic substrate (Shibata *et al.*, 1995). Immobilization is considered a convenient mean of improving enzyme's performance and stability (Mateo *et al.*, 2000). Although HPL extracts from different plant sources have been immobilized (Gardner *et al.*, 1991; Rehbock and Berger, 1998; Simon *et al.*, 1998; Schade *et al.*, 2003), little information has been reported on those of microbial sources (Nuñez *et al.*, 1997).

The present work is part of ongoing research in our laboratory (Perraud and Kermasha, 2000; Kermasha *et al.*, 2001; Kermasha *et al.*, 2002b) aimed at the development of a biotechnological process for the use of microbial enzymatic extracts containing LOX and HPL activities for the production of flavor precursors and flavor compounds. The specific objectives of the present study were to investigate the use of oxirane acrylic bead supports for the immobilization of an enriched enzymatic extract from *Penicillium camemberti* as well as the biocatalysis of immobilized HPL in selected OSM, in terms of enzyme activity and stability.

7.3. Material and Methods

7.3.1. Enriched Enzymatic Extract Preparation

P. camemberti was grown in a modified Czapek medium and the culture harvested after 10 days following the method described by Perraud and Kermasha (2000), where HPL was extracted from the fresh harvested biomass. After centrifugation, the

supernatant was submitted to ultra-filtration at 4°C for 1.5 h (Millipore, Amicon, 30 kDa NMWCO, 40 psi). To this resulting ultra-filtrate, KCl salt (Fisher scientific, Fair Lawn, NJ) was added in a ratio of 75 to 1 (KCl: protein, w/w), lyophilized and stored at -80°C. The lyophilized product was considered the enriched enzymatic extract containing HPL activity.

7.3.2. Materials

The modified Czapek medium was composed of glucose, $NaNO_3$, and $MgSO_4 \cdot 7$ H_2O which came from ACP Chemicals (Montreal, QC) and KCl and $FeSO_4 \cdot 7$ H_2O which was obtained from Fisher Scientific (Fair Lawn, NJ). Ammonium thiocyanate, NaCl, NaOH, Na_2CO_3 and $CuSO_4 \cdot 5$ H_2O were all of ACS grade and also purchased from Fisher Scientific. K-Na tartrate, Folin reagent (phospho-18-tungstic acid), xylenol orange salt (3,3'-bis(N,N-di(carboxymethyl)aminomethyl)-o-cresol-sulfonephthaleine sodium salt) and Tween 80 (polyoxyethylene sorbitan monooleate) were purchased from Sigma Chemical Co. (St-Louis, MO), whereas meso-tetraphenyl-porphine (TPP) copper(II) and perchloric acid were obtained from Aldrich Chemical Co. (Milwaukee, WI). Dipotassium hydrogen phosphate (K_2HPO_4) and potassium dihydrogen phosphate (KH_2PO_4) were purchased from ACP Chemicals. Linoleic acid (*cis*-9, *cis*-12-octadecadienoic acid) was obtained from Nu-Chek Prep Inc. (Elysian, MN). All solvents used were of HPLC grade or more and were purchased from Fisher Scientific.

7.3.3. Protein Determination

Protein concentrations were determined according to a modification of the Lowry method (Hartree, 1972). Bovine serum albumin (Sigma Chemical Co.) was used as a standard for the calibration curve.

7.3.4. HPL Immobilization

HPL immobilization was performed as per the method described by Vega *et al.* (2005a). Covalent immobilization of HPL was carried out by adding 1 mL of the enzyme suspension prepared in K-phosphate buffer (0.1 M, pH 6.5) to the wet support to obtain a final concentration of 10 mg protein/g wet support (1:100; w/w). Enzyme-support

mixtures were stirred at 4°C for 16 h. The free and immobilized HPL were reported as HPL_f and HPL_i respectively in the text.

The immobilized protein yield (IPY) and the immobilized HPL_i yield (IHY) were calculated from the immobilized protein and immobilized HPL_i activity, respectively, divided by total initial protein and total HPL activity before immobilization, respectively, multiplied by 100. The selectivity factor was defined as the specific activity of HPL_f over that of the supernatant, recovered after immobilization. The immobilization efficiency was calculated by dividing the specific activity of HPL_i over that of HPL_f, multiplied by 100.

7.3.5. HPL Enzymatic Assay

HPL biocatalysis in aqueous and OSM was carried out, in triplicate, according to a modification of the method described by Kermasha *et al.* (2002b). The aqueous phase of the reaction system was composed of 0.1 M K-phosphate buffer at pH 6.5. The hydroperoxide isomer of linoleic acid (10-HPOD) substrate was prepared as described by Wurzenberger and Grosch (1984). The enzymatic assays were performed in tubes under constant agitation. The reaction components were pre-incubated at 55°C before the assays, unless otherwise indicated. The 50 μL reaction assays contained 6.3 to 25 μg HPL_f or 1 to 5 μg HPL_i and the appropriate amounts of the K-phosphate buffer and iso-octane solvents, depending on the biphasic system. The reaction was initiated by the addition of 0.4 mM 10-HPOD substrate. After 7.5 min reaction time, the FOX colorimetric reagent was added to the reaction tubes, which halted the enzymatic reaction. Control reactions without substrate were carried out in tandem with the assays. The HPL activity was calculated from the slope of the 10-HPOD consumed versus enzyme concentration and expressed in nmol linoleic acid hydroperoxide (HPOD)/mg protein/min.

7.3.6. Xylenol Orange (FOX) Colorimetric Reaction

FOX reagent was prepared following a modification of the method described by Vega *et al.* (2005b), but without methanol. The colorimetric reactions were initiated by the addition of 3 mL of FOX reagent to the 50 μL reaction volume. The mixture was

stirred with a Vortex (Genie 2, Fisher Scientific) for 10 sec and stirred again every 5 min until 15 min colorimetric reaction. After 30 sec centrifugation (1,000x*g*) of the mixture, the absorbance of the supernatant was measured at 20 min colorimetric reaction, at 556 nm against a blank assay, using a Beckman spectrophotometer (Model 650, Beckman Instruments, Inc., Fullerton, CA). Substrate was added independently to the colorimetric reaction to control its effect. A calibration curve of HPODs standards, ranging from 0 to 62.5 μM, was used.

7.3.7. Preparation of the 10-HPOD and Hydroperoxide Standards

Following the procedure described by Schieberle *et al.* (1984), photooxidation of linoleic acid yielded the HPOD (hydroperoxide of octadecadienoic acid) isomers, which were used as standards in the FOX assay. The 10-HPOD substrate was separated form the other isomers following the procedure described by Kermasha *et al.* (2002b). Photooxidized HPODs were quantified with the ferrous thiocyanate assay as described by Wurzenberger and Grosch (1984).

7.3.8. Effect of Iso-Octane Concentration on the HPL Activity

The effect of iso-octane concentration in the biphasic system on the HPL activity was investigated. The concentrations of iso-octane were 0 to 60% for the assay of HPL_f and 0 to 80% for HPL_i. The reaction temperature was set to 50°C for the assays involving HPL_f.

7.3.9. Thermal Stability of Free and Immobilized Extracts of HPL

The thermal stability was investigated by incubating HPL_f and HPL_i at 55°C in the biphasic OSM composed of 10% iso-octane (v/v) and traces of Tween 80 for a wide range of incubation periods (0 to 150 min and 0 to 310 min for HPL_f and HPL_i, respectively). The residual HPL activity was measured and the inactivation constant (k_t) and the half-life (T_{50}) were determined from the semi-logarithmic plots of the inactivation kinetics, according to the following equation :

$$\ln (A/A_0) = -k_t T \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

where, *T* was the incubation time at 55°C; A and A_0 were the HPL activity with a defined incubation time *T* and without incubation, respectively.

7.3.10. Effect of Tetradecane Content on the Activity of HPL in OSM

The effect of tetradecane concentration in the biphasic OSM on the activity of HPL_i was investigated by varying its proportion from 0 to 50% (v/v). The system was otherwise composed of iso-octane in concentration varying between 10 to 60% (v/v) as well as 40% of the K-phosphate buffer (v/v) and traces of Tween 80. A quantitative determination of HPL_i inactivation by tetradecane was obtained by calculating the first order inactivation constant (k_c) and the concentration of tetradecane required to decrease the HPL activity by 50% (C_{50}), using the following linear equation:

$$\ln (A/A_0) = -k_c C \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (2)$$

where, *C* was the concentration of tetradecane in the biphasic system; A and A_0 were the HPL_i activity at *C* concentration of 50 and 20% tetradecane, respectively.

7.3.11. Effect of Immobilization on the Kinetics Parameters of HPL

The effect of substrate concentrations on the enzymatic activity of HPL_i in biphasic OSM composed of either 10% iso-octane (v/v) or 60% iso-octane:tetradecane (2:1, v/v) in the K-phosphate buffer were investigated using a wide range of 10-HPOD concentrations (0.2-0.9 mM). The effects of immobilization on the kinetic parameters, including K_m *and* V_{max}, were determined.

7.3.12. Effect of Temperature on HPL Activity

The effect of temperature of reaction, from 35 to 70°C, was investigated for HPL_f and HPL_i in the optimized biphasic OSM composed of either 10% iso-octane (v/v) or 60% iso-octane:tetradecane (2:1, v/v) in the K-phosphate buffer, respectively.

7.3.13. Effect of Solvent on HPL Activity

A wide range of OSM, including iso-octane:tetradecane (5:1; v/v), hexane, octane, heptane:hexane (1:1; v/v), iso-octane and heptane, with Log *P* values of 5.1, 3.5,

4.5, 3.8, 4.5 and 4.0, respectively, were investigated for their effect on the activity of HPL_i in the biphasic system composed of 60% solvent (v/v) and 40% buffer (v/v).

7.4. Results and Discussion

7.4.1. Immobilization of the Enriched Enzymatic Extract Containing HPL

The enriched enzymatic extract of HPL from *P. camemberti* was immobilized by covalent binding on selected oxirane acrylic bead supports, including Eupergit®C and Eupergit®C250L. The results (Table 16) indicated that the highest immobilized protein yield (IPY) and immobilized HPL yield (IHY) were obtained with Eupergit®C, with values of 36.9 and 89.2%, respectively. However, the use of Eupergit®C250L resulted in the highest selectivity factor and immobilization efficiency. The results suggest that Eupergit®C250L immobilizes the HPL preferentially as compared to the other proteins present in the enzymatic preparation.

The selectivity factor can be used as a measure for the degree of adsorption of protein (Mateo *et al.*, 2000). The investigated supports displayed selectivity factors higher than 1.0 (Table 16), which suggest the high affinity of HPL from *P. camemberti* for epoxy supports. Since Eupergit®C250L has the same chemical structure as Eupergit®C but with larger pores (Katchalski-Katzir and Kraemer, 2000), the differences in affinity and/or selectivity must be related to the difference in support morphologies, such as the average pore diameter and the surface area of the supports (Clark, 1994). The results (Table 16) are in agreement with those of Vega *et al*. (2005a) who have reported that the selectivity of adsorption of soybean LOX was increased when the epoxy groups concentration on the carrier was lowered.

The high absolute values of immobilization efficiency, obtained with the oxirane acrylic bead supports (329.2 and 694.4% for Eupergit®C and Eupergit®C250L, respectively), may suggest that epoxy supports are carriers of choice for HPL immobilization. Previous results (Chapter VI) indicated high immobilization efficiency value (173.9 %) for the LOX extract from *P. camemberti,* using Eupergit®C250L; such results have been attributed to the capacity of the support to favor an enzyme conformation that rendered it more active as well as to its ability to lower stearic

Table 16. Immobilization parameters for the enzymatic extract of hydroperoxide lyase from *P. camemberti*, using selected Eupergit® supports.

Support	IPY (%)[a]	IHY (%)[b]	Selectivity factor[c]	Immobilization efficiency (%)[d]	Enzyme activity	
					nmol/mg protein/min	U/g support[e]
Eupergit®C	36.9	89.2	1.4	329.2	71.7	0.15
Eupergit®C250L	12.5	60.1	2.6	694.4	145.8	0.14

[a]The immobilized protein yield (IPY) was determined as the relative percentage of immobilized protein to that of the total initial protein. An enzyme to support ratio of 1:100 was used.

[b]The immobilized HPL yield (IHY) was calculated as the relative percentage of the total activity of the immobilized enzyme to that of the free enzyme treated under the same conditions.

[c]The selectivity factor was defined as the ratio of the specific activity of the free enzyme over that of the supernatant, obtained by filtration and centrifugation after immobilization.

[d]The immobilization efficiency was defined as the relative percent specific activity of the immobilized enzyme compared to that of the free one.

[e]The activity of the immobilized enzyme was also expressed as μmol/min/g support.

hindrance at the active sites and to increase substrate diffusion, probably by selectively adsorbing the active protein as compared to others present in the enriched enzymatic extract (Clark, 1994). The dramatic increase in the specific activity of immobilized HPL as compared to the free one confirmed the hypothesis that Eupergit® supports favored active enzyme conformation and selective immobilization. Although Eupergit®C250L modified with ethylenediamine was used in a previous study (Chapter 6) with the extract from *P. camemberti* containing LOX activity, it denatured the HPL enzyme and thus no activity could be detected in the present study; it was therefore discarded.

Overall, the experimental findings indicated that the two selected supports are appropriate carriers for the enzymatic extract, enriched with HPL activity, from *P. camemberti*. A modified Eupergit®C250L support was also used for the immobilization of soybean LOX (Vega *et al.*, 2005a). Although the literature indicated the use of epoxy supports for the immobilization of soybean LOX (Chikere *et al.*, 2001) and potato LOX (Pinto *et al.*, 1997), little information is available in regards to their use for the immobilization of HPL.

7.4.2. Effect of Iso-Octane Concentration

The effect of iso-octane concentration in a biphasic system, containing K-phosphate buffer, was investigated. At 50°C, the results (data not shown) indicated that the HPL activity for the free enriched enzymatic extract from *P. camemberti* (HPL_f) remained unchanged as the concentration of iso-octane increased from 0 to 30% (v/v), above which it decreased. The activity of HPL in 20% iso-octane (v/v) was 6.09 nmol HPOD/mg protein/min, close to that of 5.77 nmol/mg protein/min in aqueous medium. These findings suggest that HPL_f was not affected by low concentrations of iso-octane.

The effect of iso-octane concentration on the HPL_i activity of the immobilized enriched enzymatic extract from *P. camemberti* was also investigated in the biphasic system, with iso-octane concentrations ranging from 0 to 80% (v/v). The results (Fig. 23) show that, similar to HPL_f, low concentrations of iso-octane (0 to 30%, v/v) did not have any effect on the activity of HPL_i as compared to that in the aqueous medium. However, a further increase in iso-octane concentration to 60% (v/v) increased the HPL_i activity by

more than 5-fold. Additional increase up to 80% iso-octane (v/v) decreased the activity of HPL_i by 15%. Similarly, the activity of soybean LOX was not affected by lower concentrations of iso-octane (up to 30%), pre-saturated with oxygen, in a biphasic system of iso-octane and buffer (Hsu *et al.*, 2000).

The increase in HPL_i activity in OSM may be due to one or more effects, including the multi-point covalent attachment provided by the oxirane support which stabilized the active conformation of the enzyme (Mateo *et al.*, 2000), the effect of the immobilization process on the extract purity which may have decreased steric hindrance and increased the substrate diffusion to and away from the active site (Mateo *et al.*, 2000) as well as to the increased solubility of the HPOD substrate in OSM which could have prevented the accumulation of this substrate in the microenvironment of the enzyme and thus its irreversible inactivation effect on HPL (Shibata *et al.*, 1995). HPL has a unique characteristic in that it is irreversibly inhibited by its toxic substrate (Shibata *et al.*, 1995). At low concentrations of iso-octane, HPL_f activity probably benefited from the increased solubility of the HPOD substrate, however higher concentrations of iso-octane may have denatured its conformation.

On the basis of these findings, the optimized biphasic solvent used for further study was composed of 60% solvent and 40% K-phosphate buffer (v/v). Since HPL_f was inactivated by such a high level of solvent, a concentration of 10% iso-octane was used in the subsequent thermal stability study.

7.4.3. Thermal Stability of HPL in OSM

The thermal stability of HPL in a biphasic system composed of 10% iso-octane was investigated. The enriched enzymatic extracts were incubated at 55°C for periods varying between 0 and 300 min. The results (Fig. 24) indicated that after 150 min of incubation, the free and immobilized enzymatic extracts retained 37% and 58% of HPL activity, respectively, as compared to that of 5 min of incubation. From the semi-logarithmic plots, the inactivation constant and half-life (T_{50}) of HPL_f and HPL_i were estimated (data not shown). From equation (1) the T_{50} of HPL_f and HPL_i were calculated at 92 and 231 min, respectively; the T_{50} increased by a factor of 2.5 upon immobilization

onto Eupergit®C250L. These results suggest that the immobilization increased the thermal stability of the enriched enzymatic extract containing HPL activity.

Previous results (Chapter VI) indicated that the LOX activity from the same enriched enzymatic extract demonstrated that immobilization increased the thermal stability of *P. camemberti* LOX by a factor of 3.75. Mateo *et al.* (2000) have attributed the increased thermal stability of penicillin G acylase, immobilized by epoxy supports, to multi-point covalent attachment which stabilized the 3D structure of the enzyme. Because of the intrinsically high concentration of epoxy groups of Eupergit®C supports, which allows for them to be attach to a given protein at multiple locations, they could increase the stability of immobilized enzymes (Katchalski-Katzir and Kraemer, 2000).

Similar to this study, a study using an extract from *P. bispora* containing also showed that the HPL was more stable than the LOX activity. After storage at 0ºC for 5 h the residual activity of HPL was of 86% whereas that of LOX was 6%; the results suggested that *P. bispora* HPL was more stable than LOX (Wurzenberger and Grosch, 1984).

The results (insert of Fig. 24) indicate that the thermal inactivation of both HPL_f and HPL_i followed first order kinetic behavior, as indicated by the linearity of the semi-logarithmic plots, with correlation coefficients of 0.89 and 0.97, respectively. Vega *et al*. (2005a) reported similar linear results using soybean LOX type I-B, immobilized on epoxy supports, in a 98% hexane:dioxane (95:5, v/v) biphasic medium. However, non first order kinetic thermal inactivation data were also reported for LOX from *P. camemberti* (Chapter VI); the presence of more than one isozyme in the extract was thus hypothesized. Contrary to the LOX activity, the enriched enzymatic extract from *P. camemberti* most probably possessed only one isozyme form of the HPL activity. Anthon *et al.* (2003) have also demonstrated the first order kinetics behaviour for the HPL activity of tomato in aqueous medium.

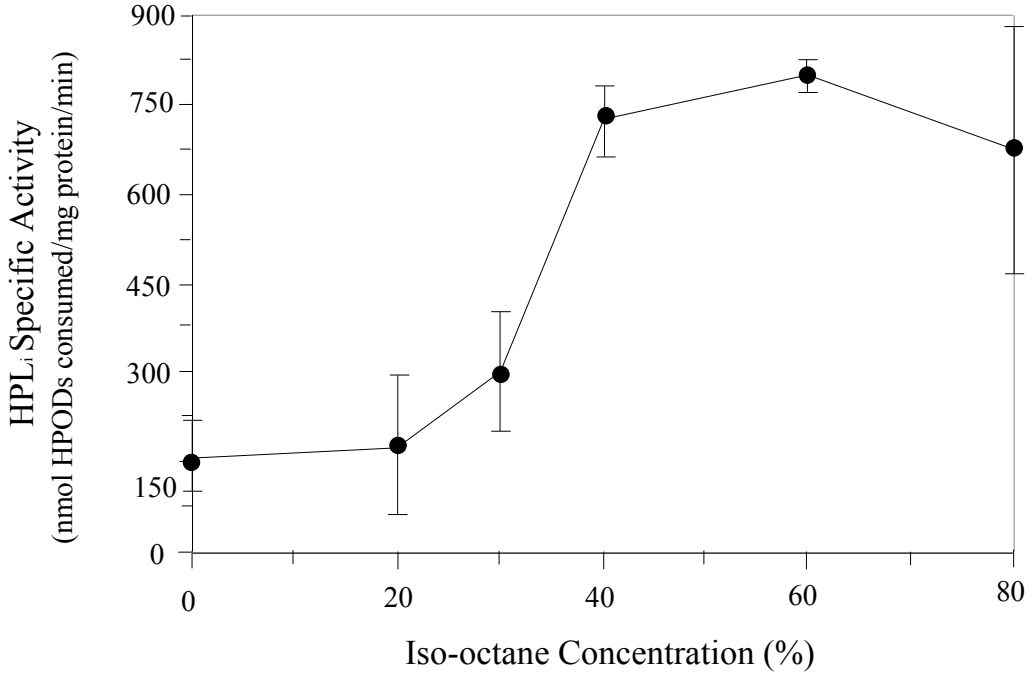


Figure 23. Effect of iso-octane concentration in biphasic organic solvent systems on the specific activity of immobilized HPL_i from *P. camemberti*.

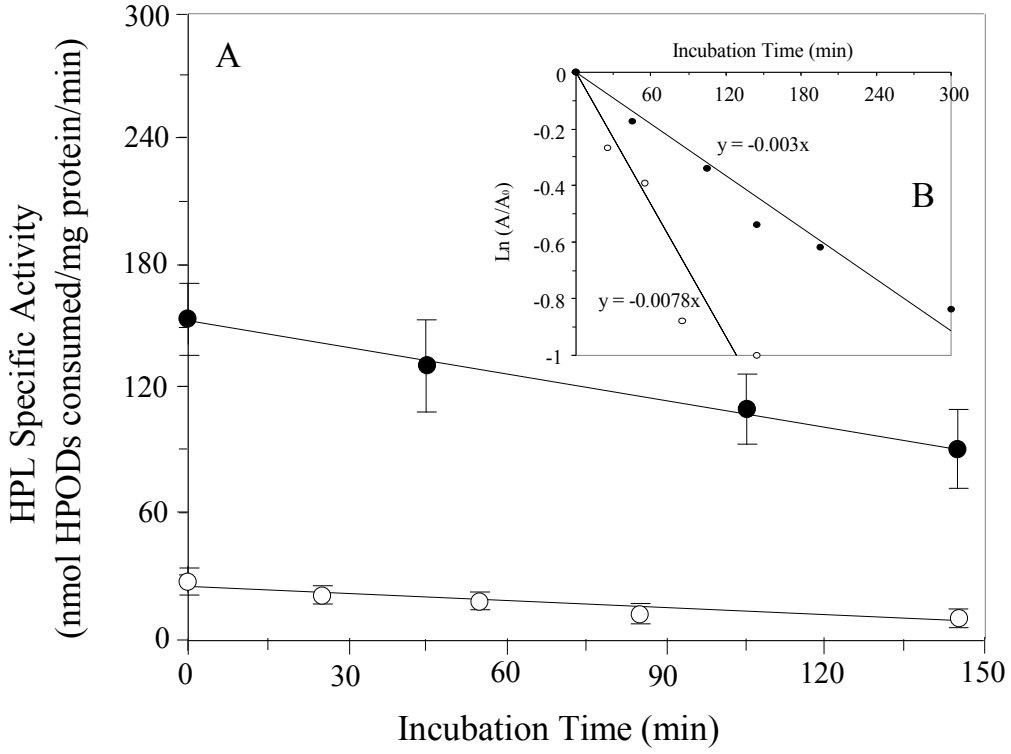


Figure 24. Effect of incubation period (0 – 240 min), at 55°C, on the specific activity (A) and stability (B) of the enzymatic extract of HPL from *P. camemberti* in a biphasic organic solvent system composed of 10% iso-octane using free (○) and immobilized (●) enzymatic extracts.

7.4.4. Effect of Tetradecane Concentration on Immobilized HPL Activity

The effect of mixtures of iso-octane:tetradecane, of variable ratios (from 6:0 to 1:5, v/v), was investigated on the activity of HPL_i in the biphasic system composed of 60% organic solvent (v/v) and 40% of the K-phosphate buffer (v/v). The results (Fig. 25) indicate that the specific activity of HPL_i was at its maximum of 2.31 x 10^3 nmol HPOD/mg immobilized protein/min, with a tetradecane concentration of 20% (v/v). The increase in HPL_i activity could be explained by an increase in HPODs solvation which may have decreased their tendency to irreversibly inhibit the enzyme (Siedow, 1991). However, further increase in tetradecane proportion to 50% resulted by a 69% decrease in the specific activity of HPL_i; this decrease in enzyme activity may be due to increased substrate diffusional limitations. The presence of specific interactions between the solvent and the enzyme may account for the lower specific activity in higher proportions of tetradecane (Arroyo *et al.*, 2000).

The inserted diagram (Fig. 25) shows a straight line with correlation coefficient of 0.88 indicating that the enzyme inactivation by tetradecane followed first order kinetic behavior. First order kinetics for enzyme inactivation by co-solvents in monophasic aqueous mixtures has been reported for aminotransferase (Moreno and Fagain, 1997) and acylase (Arroyo *et al.*, 2000) as well as in an organic solvent mixture of dioxane and hexane using soybean LOX (Vega *et al.*, 2005a). The tetradecane inactivation of LOX_i activity within the same enriched enzymatic extract from *P. camemberti* also followed first order kinetic behavior in similar medium (Chapter VI).

A quantitative determination of HPL inactivation by tetradecane was obtained by calculating the inhibition factor (C_{50}). The theoretical C_{50} (equation 2) was estimated to be 63% tetradecane (data not shown); these findings suggest that the presence of tetradecane in a biphasic system composed of 60% solvent may not denature totally the immobilized enzyme. The low denaturating effect of tetradecane on HPL_i may be explained by multipoint covalent attachment of the immobilized enzymes on the support, which may have led to less conformational changes (Mateo *et al.*, 2000) as well as by the hydrophobic character of tetradecane itself which prevents denaturation of proteins. Laane *et al.* (1987) have demonstrated that solvents with a Log *P* value higher than 4,

which is the case for tetradecane and all mixtures of tetradecane and iso-octane, favored an enzyme conformation that renders it maximal activity. Zaks and Klibanov (1988) have shown that hydrophobic solvents, such as the ones with a high Log *P* value, tend to protect the essential water layer of biocatalysts and thus favor their hydration. Furthermore, the immobilization support may also have protected the hydration level of the enzyme, which could explain the increase in the activity of the immobilized biocatalyst when used in reaction media containing tetradecane. The use of additional amount of tetradecane from 20 to 50% (v/v), however, could have increased mass transfer limitations due to stearic hindrance caused by the long carbon chain of the solvent (Arroyo *et al.*, 2000).

7.4.5. Effect of Temperature on HPL Activity

The effect of reaction temperature on HPL activity was also investigated, using the optimized biphasic media composed of 10% iso-octane for HPL_f and of 60% iso-octane:tetradecane (2:1, v/v) for HPL_i. The results (Fig. 26) indicate that HPL_f in aqueous medium has maximal specific activity at 55°C, whereas HPL_f and HPL_i exhibit maximal activity at 50°C in the optimized biphasic media. The addition of iso-octane to the systems slightly decreased the optimal temperature of reaction. However, the addition of iso-octane (Chapter VI) increased the LOX activity from the same enzymatic extract at its optimal temperature. Moreover, all HPL_i specific activities reported at the different investigated temperatures were higher than those shown by HPL_f.

7.4.6. Kinetics parameters of HPL in OSM

The effect of substrate concentration on the specific activity of HPL_i in biphasic media composed of either 10% iso-octane or 60% iso-octane:tetradecane (2:1, v/v) was investigated using a wide range of 10-HPOD concentrations. The kinetic parameters of HPL_f were determined in the 10% iso-octane (v/v) biphasic system only.

The Lineweaver-Burk plots of $1/v$ versus $1/[S]$ (data not shown) displayed linear relationship with correlation coefficients of 0.99, 0.99 and 0.96, for HPL_i in 10% iso-octane (v/v) and in the optimized mixture of 60% iso-octane:tetradecane (v/v) and for HPL_f in 10% iso-octane (v/v), respectively, which indicated Michaelis-Menten kinetic

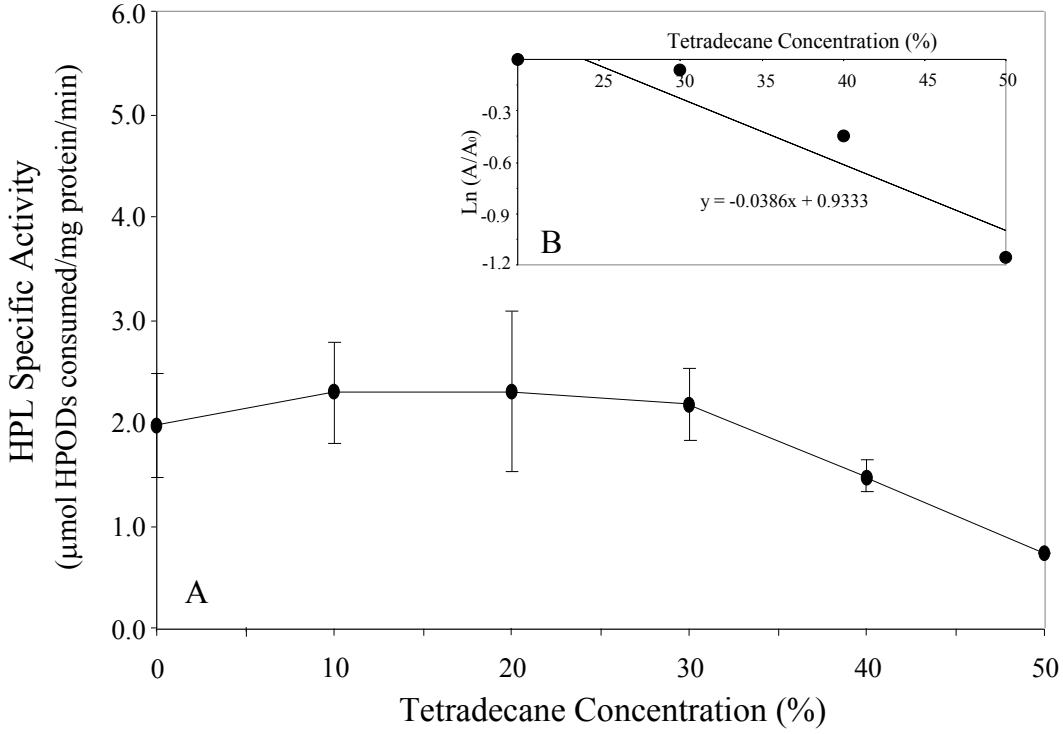


Figure 25. Effect of tetradecane content in the biphasic organic solvent system composed of 3:2 iso-octane mixture:buffer (v/v) on the activity of immobilized HPL_i from *P. camemberti*.

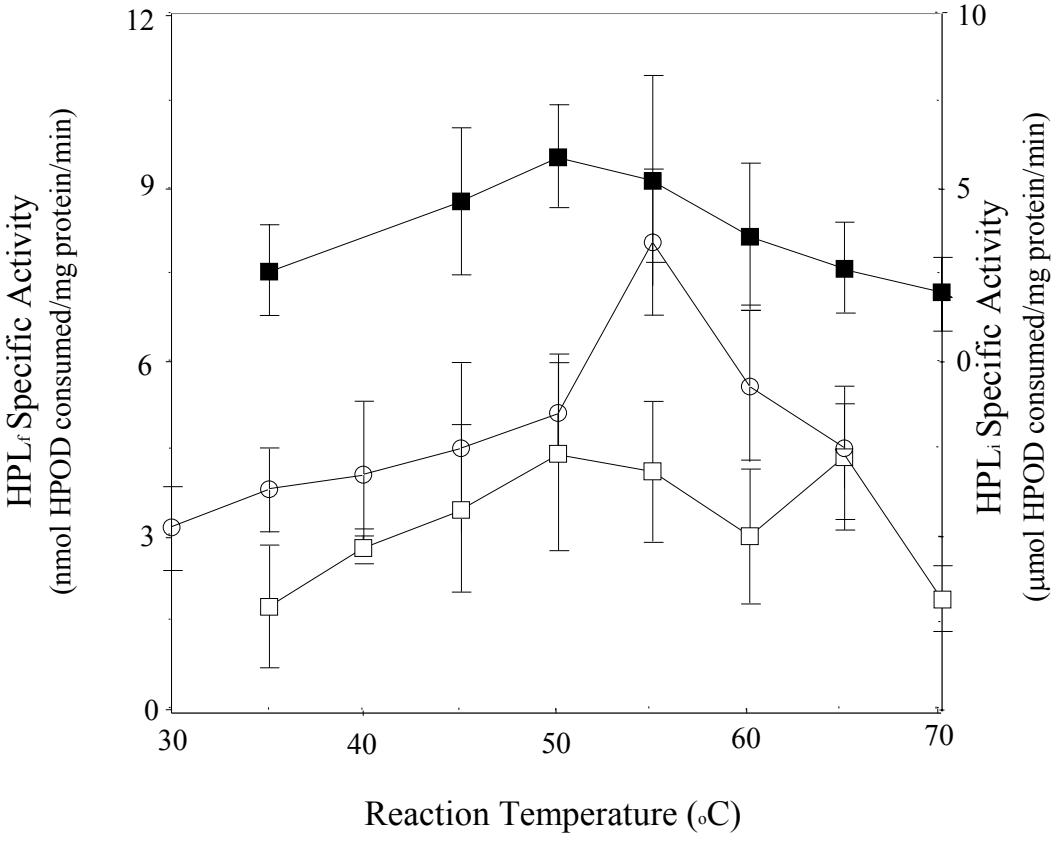


Figure 26. Effect of reaction temperature (30 - 80°C), at a defined time, on free HPL_f (□) activity from *P. camemberti* in a biphasic organic solvent system composed of 10% iso-octane (v/v); HPL_f activity in aqueous medium (○) and immobilized HPL_i (■) activity in a biphasic system composed of 60% iso-octane:teradecane (2:1, v/v) in the K-phosphate buffer.

behavior. Similarly, Michaelis-Menten type of behavior has been reported for tomato HPL (Suurmeijer *et al.*, 2000).

The kinetic parameters, for HPL_f and HPL_i were estimated from the Lineweaver-Burk plots. The results (Table 17) indicate that K_{m1} and K_{m2} were determined at 1.87 and 27.14 μM for HPL_f and HPL_i, respectively, in 10% iso-octane (v/v); the K_{m3} for HPL_i in the 60% iso-octane:tetradecane (2:1, v/v) was 1.88 μM. Both immobilization of HPL onto Eupergit®C250L and alteration in OSM affected the enzyme affinity for the substrate as seen by the two variations of K_m values by more than 14 fold. K_m values for HPL from fruits in aqueous medium, using 13-HPOD as substrate usually ranged from 10 to 30 μM (Shibata *et al.*, 1995; Salas *et al.*, 2000). The affinity of the enzyme for the substrate decreased upon immobilization whereas it increased with the use of the mixture containing iso-octane:tetradecane (2:1, v/v). A decrease in enzyme affinity for the substrate is expected with the immobilization process, which causes conformation changes, steric hindrance and substrate diffusion limitations; higher apparent K_m values have been reported previously by Vega *et al.* (2005a) for soybean LOX immobilized onto modified EupergitC250®L with values as high as 36 mM in hexane:dioxane:buffer (47:2:1, v/v/v) medium.

The increase in substrate affinity following the change of OSM system from 10% iso-octane (v/v) to the 60% iso-octane:tetradecane (v/v) in the K-phosphate buffer can be attributed to a possible increase in hydrophobicity of the enzyme upon transfer into another medium which modified its micro-environment (Bindhu and Abraham, 2003). Increase in K_m values upon modification of OSM has been reported previously for peroxidase (Bindhu and Abraham, 2003) and lipoxygenase (Chapter VI).

The results (Table 17) also show that the V_{max1} and V_{max2} values for HPL_f and HPL_i were, respectively, 120 and 14.30 x 10^3 nmol HPOD/mg protein/min in 10% iso-octane (v/v) reaction medium. The V_{max3} value for HPL_i in the optimized OSM composed of 60% iso-octane:tetradecane (2:1, v/v) was 13.37 x 10^3 nmol HPOD/mg protein/min. HPL_f activity in 10% iso-octane compares with others; an apparent V_{max} value 0.22 μmol·min^{-1} was reported for pea seeds HPL using 13-HPOD (Hornostaj and Robinson, 2000). The

immobilization increased the $V_{max\ app.}$ values of the enriched enzymatic extract as HPL_i showed similar results in both OSM; HPL_f displayed more than 100 fold smaller V_{max} value. The difference in the V_{max} values between the free and immobilized extract may be attributed to the purification effect that the immobilization process had on the extract as well as the multi-point covalent attachment of the support to the protein which stabilized the enzyme structure. In order to compare the kinetics data for both HPL_f and HPL_i, the present study treated HPL_f under the same conditions as for HPL_i, which implied continuous stirring for 16 h. Since HPL is poorly stable (Shibata *et al.*, 1995), the lower V_{max} value for HPL_f may be due to a partial denaturation of the enzyme.

The experimental findings (Table 17) indicate that the apparent enzyme efficiency, which is defined as V_{max}/K_m, was 0.06 and 0.53 min^{-1} for HPL_f and HPL_i in 10% iso-octane (v/v) medium, respectively, and 7.12 min^{-1} for HPL_i in the optimized medium of 60% iso-octane:tetradecane (2:1, v/v) in the K-phosphate buffer. The apparent enzyme efficiency in the optimized medium composed of 60% iso-octane:tetradecane was the highest as compared to the HPL_f and HPL_i in 10% iso-octane, by factors of 119 and 13, respectively. The V_{max}/K_m values are higher than those reported in other studies. In comparison, using pea seeds HPL in aqueous medium, Hornostaj and Robinson (2000) reported efficiencies of 0.02 and 0.03 using 9-HPOD and 13-HPOD, respectively.

7.4.7. Effect of Organic Solvent on HPL Activity

A wide range of organic solvents as well as their mixtures were investigated for their effect on the activity of HPL_i in biphasic OSM composed of 60% organic solvent (v/v) and 40% of the K-phosphate buffer (v/v). The organic phases were iso-octane:tetradecane (5:1; v/v), hexane, octane, heptane:hexane (1:1; v/v), iso-octane and heptane. The results (Fig. 27) show that the specific activity of HPL_i in the OSM was 0.87×10^3, 1.08×10^3, 1.22×10^3, 1.11×10^3, 0.96×10^3 and 1.07×10^3 nmol HPOD/mg immobilized protein/min, respectively, with relative standard deviation (RSD) smaller than 15%. These findings suggest that immobilization increased the tolerance of the enzyme for the OSM, probably by preventing the denaturation of the enzyme, since the HPL_i activity in OSM was drastically higher than that in aqueous medium. However, the results also imply that the type of solvent used had little influence on the biocatalysis of

Table 17. Kinetics parameters of *P. camemberti* HPL activity in organic solvent.

Kinetic parameters	Free HPL[a]	Immobilized HPL[b]	
		System 1[c]	*System 2*[c]
K_m	1.86	27.14	1.88
V_{max}	0.12	14.30	13.37
Enzyme efficiency[d]	0.06	0.53	7.12

[a]Free HPL kinetics parameters were determined in system 1.

[b]Enzyme was immobilized on Eupergit®C250L (Rohn Pharma). The HPL_f was treated under the same conditions as the immobilized HPL_i, without support.

[c]System 1 was the biphasic OSM composed of 10% iso-octane in k-phosphate buffer (0.1 M, pH 6.5) whereas System 2 was composed of iso-octane: tetradecane: k-phosphate buffer (2:1:2, v/v/v). Both systems had traces amounts of Tween 80.

[d]K_m expressed in mM 10-HPOD.

[e]V_{max} expressed in μmol 10-HPOD consumed/mg protein/min.

[f]Catalytic efficiency was defined as the ratio of V_{max} over K_m.

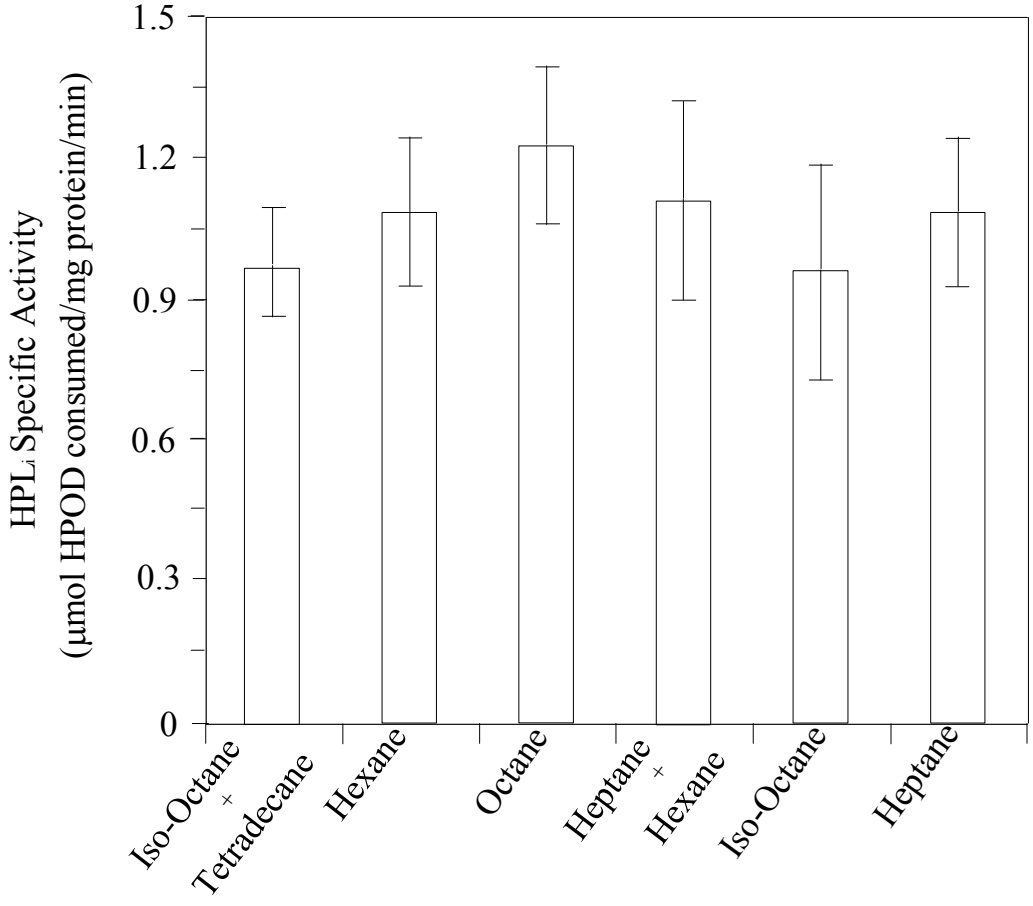


Figure 27. Specific activity of immobilized HPL_i of enzymatic extract from *P. camemberti* in biphasic organic solvent systems composed of 60% solvent (v/v) and minimal amounts of Tween 80.

HPL_i as the average specific activity in the selected solvents (1.05×10^3 nmol HPOD/mg immobilized protein/min) varied by a maximum of 11.4% as compared to that in other solvents; this limited variation could be attributed to increased solubility of the HPOD substrate in all solvents as compared to that in aqueous medium, which substrate is less likely to be present in the micro-environment of the enzyme thereby preventing denaturation.

7.5. Conclusion

The experimental data obtained throughout this study showed that the immobilization of the enriched enzymatic extract from *P. camemberti,* containing HPL, onto EupergitC®250L increased its enzymatic activity as well as its thermal and solvent stability. EupergitC®250L was shown to be an appropriate immobilization support for the microbial HPL as it also increased the tolerance of the enzymatic extract to organic solvent. Organic solvents with high *Log P* values, such as iso-octane and tetradecane, were concomitantly shown valuable for the biocatalysis of HPODs by HPL activity.

GENERAL CONCLUSION

The experimental data of the FTIR analysis indicated that changes in the secondary structure of purified soybean LOX in OSM affected the enzyme activity. The secondary structure of LOX showed that the conditions, in which LOX exhibited its highest enzyme activity, was predominantly ordered α-helix with minimal aggregate formation. However, the intermolecular β-sheet aggregation induced by the use of heat as well as by OSM resulted in a decrease in LOX activity. It was also shown that both LOX activity and the proportion of α-helical conformation in the overall secondary structure of LOX were increased by its pre-incubation in hexane solvent.

Immobilized soybean LOX activity and stability were increased in monophasic hexane OSM as compared to the use of the free one. In addition, the use of dioxane co-solvent in the reaction medium increased the enzyme activity. The relationship between LOX activity and both co-solvent concentration as well as thermal inactivation of the enzyme followed first-order kinetics. Immobilization provided with a pertinent tool for the stabilization of LOX in OSM.

Although the presence of nonperoxidized lipids could dramatically influence the sensitivity of the assay, the optimized FOX assay could be used, with acceptable precision and sensitivity for the determination of LOX in OSM. Appropriate calibration and manipulation of the colorimetric assay showed its validity for rapid, precise and accurate measurements of hydroperoxides of linoleic acid within organic solvent reaction media.

The immobilization of the enriched enzymatic extract, from *Penicillium camemberti,* showed an increase in LOX and HPL activities and thermal stability as compared to those of the free one. EupergitC®250L was shown to be an appropriate support for the immobilization of the microbial enzymatic extract enriched in LOX and HPL activities for enzymatic reactions in OSM. Optimized reaction systems for both LOX and HPL activities were determined to be 10% iso-octane (v/v) and 60% iso-

octane:tetradecane (2:1, v/v) in K-phosphate buffer, for the free and immobilized enzymatic extracts, respectively.

Epoxy type support was found suitable for the immobilization of both the plant and microbial LOX as well as of HPL; it increased the thermal stability of those activities and their tolerance to OSM. Concomitantly, biocatalysis in OSM increased activity of the immobilized extracts. It was also demonstrated that the support could also be used in a one-step purification and stabilization of crude enriched enzymatic extract of *P. camemberti* containing LOX and HPL activities.

This work has provided a better understanding of the enzymatic assay and of the behavior of microbial LOX and its associated enzyme HPL in OSM. The employment of the FOX method to quantify the activity of LOX and HPL in OSM showed time-efficient and accurate and the use of an enriched enzymatic extract immobilized on EupergitC®250L was shown appropriate for the biocatalysis of linoleic acid and its 10-isomers of hydroperoxide in OSM. The relative stability of this extract's activities in OSM as well as the amplified activity that LOX and HPL both exhibited could suggest potential in using such an approach in the application of these enzymes in the production of particular flavor precursors and flavor componds.

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