Characterization of Selected Microbial Lipoxygenase Extracts and Immobilization and Stabilization of an Enzymatic Preparation

by

Colin Eric Hall

A thesis submitted to McGill University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Department of Food Science & Agricultural Chemistry

McGill University

Montreal, Canada

January, 2007

Your file Votre référence
ISBN: 978-0-494-32190-4
Our file Notre référence
ISBN: 978-0-494-32190-4

Suggested Short Title

CHARACTERIZATION OF SELECTED MICROBIAL LIPOXYGENASE EXTRACTS

This thesis is dedicated to Aldwin Richards, may you rest in peace

ABSTRACT

Ph.D. Colin Eric Hall

Aspergillus niger and *Penicillium candidum* were grown and harvested on days 6 and 8, which corresponded to their maximal biomasses and lipoxygenase (LOX) activities. The extracts were enriched with ammonium sulfate precipitation at 30 to 70% and 20 to 60% of saturation, respectively. The LOX activity was assayed with linoleic, linolenic and arachidonic acids as substrates. Both enriched microbial LOXs demonstrated preferential substrate specificities towards free fatty acids, over acyl esters of linoleic acid. The LOXs had the highest catalytic efficiency values (ratio of V_{max} to K_m) for linolenic acid biocatalysis. Major and minor pH optima at 5.0 and 10.5 were observed for *A. niger*, whereas for *P. candidum* they were at 6.0 and 8.5. Normal phase high-performance liquid chromatography (NP-HPLC) and gas-liquid chromatography/ mass spectrometry (GC/MS) characterization of end products revealed that both LOXs produced the 10-hydroperoxide of linoleic acid (10-HPOD) at 15 to 16% of total isomers detected, respectively. Chiral studies of the *P. candidum* LOX catalyzed hydroperoxides revealed an excess in the production of (*S*) stereo-isomers resultant from linoleic, linolenic and arachidonic acids bioconversion. *Penicillium camemberti* was grown and harvested at its maximal biomass and LOX activity. The microbial extract was ultrafiltered (30 kDa NMWCO) and KCl (7.5 ppm) was added prior to lyophilization for the stabilization of enzyme activity. The LOX and hydroperoxide lyase (HPL) activities were assayed using linoleic acid and the 10-HPOD as substrates, respectively. The post-lyophilization residual activities were 93% and 223% for LOX and HPL, respectively. The long-term storage stability (-80°C) of the extract (KCl 7.5 ppm) was ~100% after 8 and 4 weeks for LOX and HPL, respectively. The investigated stabilizing chemical additives included glycine, mannitol, glycerol, sucrose and polyethylene glycol. The lowest $K_{inactivation}$ values were observed with glycine with 0.136 and 0.0296 for LOX and HPL, respectively. Thermostability studies indicated that 5 and 10% (w/v) mannitol and glycine effectively stabilized LOX and HPL, respectively. Immobilization of an enzymatic extract from *P. camemberti* containing LOX and HPL activities was performed on Eupergit®C and Eupergit®C250L-iminodiacetate (IDA), respectively. The free and immobilized extracts both possessed LOX activity with a pH optimum of 6.0,

whereas pH 6.0 and 4.0 were the optima of the HPL activity for free and immobilized extract, respectively. Optimal LOX reaction temperatures were 30 and 55°C for the free and immobilized extract, respectively, whereas 45 and 30°C were determined for the HPL activity of the free and immobilized extract, respectively. Long-term stability (–80°C) of the immobilized extract containing LOX and HPL activity showed residual activities of 82.6 and 93.8% after 4 and 8 weeks, respectively.

RÉSUMÉ

Ph.D. Colin Eric Hall

Aspergillus niger Penicillium candidum ont été cultivés et récoltés au jour 6 et 8 de la culture correspondant à leur sommet en biomasses ainsi qu'en activité lipoxigenase. Les extraits furent enrichis par précipitation au sulfate d'ammonium en utilisant respectivement 30 à 70% et 20 à 60% de saturation. L'activité lipoxigenase à été déterminée avec trois différents acides gras: linoleique, linoleique et arachinonique. Les extraits des deux souches microbiens ont démontré une plus grande spécificité pour les acides gras libres plutôt qu'en acyl ester. Parmi les différents substrats les lipoxidases démontrèrent également une plus grande efficacité catalytique (ratio de V_{max} sur K_m) pour la biocatalyse de l'acide linoléique. Les pH optimum observés pour *A.niger* ont été de 5 et 10.5 alors que des valeurs de 6.0 et 8.5 ont été observées pour *P. candidum.* L'étude des produits de la catalyse enzymatique à l'aide des techniques de chromatographie liquide à haute performance et la chromatographie liquide en phase gazeuse couplée à un spectre de masse pour la caractérisation révélèrent que les deux extraits produisent le 10-hydroperoxide d'acide linoléique en proportion de 15 à 16 % de tous les isomères détectés. Une étude chirale des HPOD produits par la catalyse de *P. candidum* à révelée que les (*S*) stereoisomères était produits en excès lors de la bioconversion des trois substrat cité plus haut. *P. camemberti* à été cultivé et récolté au maximum de sa biomasse, l'extrait microbien à été ultrafiltré (30 kDa NMWCO) puis on y a ajouté 7.5 ppm de KCl pour stabiliser les enzymes avant la lyophilisation. L'activité enzymatique post-lyophilisation résiduelle fût de 93% et 223% pour LOX et HPL respectivement. La stabilité de l'entreposage à long terme (-80 °C) de l'extrait (7.5ppm KCl) est demeuré au maximum (100%) après 8 et 4 semaines d'entreposage pour LOX et HPL, respectivement. Différent additifs pour la stabilisation ont été testés incluant la glycine, le mannitol, le glycérol, le sucrose et le polyéthylène glycol. La plus basse valeur d'inactivation à été observée avec la glycine 0.136 et 0.0296 pour LOX et HPL. L'étude de thermostabilité à démontré que 5 et 10 % (w/v) de mannitol et glycine stabilise LOX et HPL avec succès. L'activité d'un extrait enzymatique de *P camemberti* contenant LOX et HPL a été explorée avec les supports Eupergit®C and Eupergit®C250L-iminodiacetate (IDA). L'extrait enzymatique libre et immobilisé ont démontré différent pH optima de

6.0 pour LOX et 4.0 pour HPL. La température optimale pour la catalyse de l'enzyme libre à été déterminée à 30 °C et 55 °C pour l'activité LOX, alors qu'elle était de 45 °C et 30 °C pour l'activité HPL. La stabilité (entreposée à -80 °C) des extraits immobilisés LOX et HPL ont été évalué à 82.6 et 93.8% après 4 et 8 semaines, respectivement.

ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr. Selim Kermasha for his guidance and friendship throughout the course of my studies.

I would also like to thank Dr. Salwa Karboune for her invaluable advice and suggestions concerning my research work.

I would also like to thank all of the students with whom I worked in the laboraratory for their support and friendship. Special thanks to Dr. Barbara Bisakowski, Dr. Xavier Perraud, Dr. Abzal Hossain, Dr. Kebba Sabbally, Fredéric, Tammy and Jodie.

I would like to express my gratitude to Dr. Florence Husson for her collaboration during my experimental work.

I would also like to thank the staff at Macdonald Campus Housing especially Mrs. Ginette Legault, Mrs. Monique Verrette and Marie-Ève for making my stay in Québec so comfortable.

Lastly I would like to express my deep appreciation to my parents, sisters, my family and friends who were always encouraging, throughout my studies.

CLAIM OF ORIGINAL RESEARCH

1. This is the first study in which the lipoxygenase (LOX) extracts from *Aspergillus niger* and *Penicillium candidum* were isolated, enriched and characterized in terms of optimum pH, effect of inhibitors and kinetic parameters, using linoleic, linolenic and arachidonic acids as substrates.

2. This is the first study in which lipoxygenase (LOX) extracts from *Aspergillus niger* and *Penicillium candidum* were characterized in terms of the regio-specificities of their flavor-precursor end products, including the 10-hydroperoxides of linoleic and linolenic acids and the 8-, 9-, 12- and 15-hydroperoxides, using arachidonic acid, using linoleic, linolenic and arachidonic acids as substrates.

3. This is the first study in which an enzymatic extract preparation containing dual LOX / hydroperoxide lyase (HPL) activities from *Penicillium camemberti* was investigated in terms of their stability, using selected chemical additives.

4. This is the first study in which an enzymatic extract preparation containing dual LOX/HPL activities from *P. camemberti* was immobilized and characterized in terms of optimum pH, reaction temperature, stability and kinetic parameters, using linoleic acid and the 10-hydroperoxide of linoleic acid as substrates for LOX and HPL, respectively.

TABLE OF CONTENTS

LIST OF FIGURES

Figure Number **Page**

LIST OF TABLES

Table Number **Page**

LIST OF ABBREVIATIONS

LOX, lipoxygenase

HPL, hydroperoxide lyase

PUFA, polyunsaturated fatty acid

HPOD, hydroperoxide of octadecadienoic acid

HPOT, hydroperoxide of octadecatrienoic acid

HPETE, hydroperoxide of eicosatetrenoic acid

HOD, hydroxide of octadecadienoic acid

HOT, hydroxide of octadecatrienoic acid

HETE, hydroxide of eicosatetrenoic acid

HPLC, high-performance liquid chromatography

CP-HPLC, chiral phase HPLC

NP-HPLC, normal phase HPLC

RP-HPLC, reverse phase HPLC

DAD, diode array detector

ELSD, evaporative laser light scattering detector

MTMS, methyl trimethylsilyloxy

GC, gas-liquid chromatography

GC/MS, gas-liquid chromatography/mass spectrometry

FID, flame ionization detector

SPE, solid phase extraction

PEG, polyethylene glycol

PVP, polyvinyl polypyrrolidone

IDA, iminodiacetic acid

EDA, ethylene diamine

CHAPTER I

INTRODUCTION

Polyunsaturated fatty acids (PUFAs) may serve as subtrates for enzymes, which catalyze transformations into volatile chemicals, possessing particular organoleptic characteristics when sensed by the olfactory and taste receptors (Mosandl *et al.*, 1986). Often these volatiles have specific functionalities, including alcoholic, acidic, aldehydic or ketonic, which in combination with various aliphatic carbon chain lengths and points of unsaturation, result in a wide variety of flavors. The production and addition of such chemical flavorants is a lucrative industry and impacts the production of processed foods and beverages, cosmetic and household cleansers (Krings and Berger, 1998). Due to cost considerations most flavorants are produced chemically; however, such processes often produce racemic mixtures and their introduction into food products must be labelled "artificial" (Krings and Berger, 1998). The biotechonological production of flavors by enzymes has the potential to provide highly valued natural flavorants. A key challenge to this biocatalytic approach is that of enzymatic instability and inactivation. Using a variety of strategies, including stabilization with chemical additives/excipients or immobilization enzymatic activities may be preserved (Wang, 1999 and 2000; Kim *et al.*, 2006).

Lipoxygenase (LOX) is a dioxygenase that acts on 1(*Z*),4(*Z*)-pentadiene containing PUFAs, including linoleic ($C18:2^{10,12}$), linolenic ($C18:3^{10,12,15}$) and arachidonic ($C20:4^{5,8,11,13}$) acid substrates in the production of a variety of PUFA hydroperoxides. The PUFA hydroperoxide regio-isomers are often considered to be flavor precursors because they are substrates for hydroperoxide lyase (HPL), which is an enzyme that cleaves them into volatile flavor compounds and non-volatile moieties. Coupled LOX/HPL activities have been reported to be the principle pathway in the generation of flavor compounds in many higher plants, including tomato (Regdel *et al.*, 1994), cucumber (Hornostaj and Robinson, 1999) and alfalfa (Noordermeer *et al.*, 2000), where LOX converts linoleic acid to the 9-hydroperoxide of linoleic acid which is subsequently cleaved to hexanal and a C12 ω-oxoacid by HPL. LOX/HPL activities from microbial sources tend to demonstrate a different regio-selectivity, where C18 PUFAs are implicated with the production of C8 volatiles. Volatiles isolated in the headspace of

many species of molds and fungi have been reported to possess a variety of octanol and octanal isomers accounting for >85% of total volatiles isolated (Kaminski *et al.*, 1974; Mau *et al.*, 1994). Microbial LOX/HPL sources reported to possess these types of activities include, the molds *Penicillium camemberti* (Perraud and Kermasha, 2000; Kermasha *et al.*, 2002*b*), *Aspergillus niger* (Kaminski *et al.*, 1974) and the fungi *Pleurotus ostreatus* (Kuribayashi *et al.*, 2002) and *Psalliota bisporus* (Wurzenberger and Grosch, 1984). Structural studies of LOX and HPL have indicated that both enzymes are moderately thermolabile quite unstable (Gardner, 1991). Pea seed LOX and HPL were both reported to lose 75% of their activity after storage at 4°C for 30 days (Liagre *et al.*, 1996).

Enzymatic stabilization may be defined as a strategy aimed at the protection of enzymes from denaturation and/or maintenance of native conformation. (Wang, 1999). In the presence of a stabilizing excipient, a protein prefers to interact with water (preferential hydration) and the excipient is preferentially excluded from the protein domain (preferential exclusion), which aids in the maintainance of native enzyme conformation (Wang, 1999 and 2000). The most common chemical additives employed to stabilize LOX/HPL extracts are detergents (0.1-0.5%, w/v) such as Triton X-100 (Pérez-Gilabert *et al.*, 2001; Husson and Belin, 1995) and polyols (0.5 mM), including sucrose and glycerol (Götz-Schmidt *et al.*, 1986). Further, thiol compounds such as dithiothreitol (0.1-10 mM) have been used (Shibata *et al.*, 1995; Hornostaj and Robinson, 2000) to maintain the sensitive sulfhydryl residues of LOX/HPL in the reduced form. Some polymers (0.5-12% w/v), including polyvinyl polypyrrolidone have also been employed to stabilize LOX/HPL activity (Salas and Sánchez, 1999). Other compounds such as potassium chloride and amino acids, including glycine, have also been shown to impart greater stability to enzymes (Wang, 1999 and 2000). These compounds have been reported to reduce glass transition temperature (T_g), as well as increasing hydrophobic interaction, both of which led to a stabilized dry enzyme extract (Jensen *et al.*, 1996).

Another effective way of mitigating LOX/HPL enzyme denaturation is with immobilization. Enzyme immobilization refers to any technique in which an insoluble support material is added to an enzymatic extract to elicit an association between the

protein and the support, thereby improving the stability of the enzyme (Kim *et al.*, 2006) This process involves the coupling of an enzyme to an insoluble support via adsorptive/ionic, hydrophobic or covalent association (Bickerstaff, 1997). Ionic supports have been proven effective for some LOXs and HPLs, including immobilization on Dowex 50WX4-200 (Kermasha *et al.*, 2002*a*), on glass particles (Santano *et al.*, 2002) and talc (Liagre *et al.*, 1996). These supports have the advantage of very mild coupling conditions and little concomittant loss of enzymatic activity. Covalent immobilization supports include those which bond to amines, carboxyls or thiol functionalities (Nuñez *et al*, 1997; Rehbock and Berger, 1998). Several of these types of supports have been used to immobilize LOX/HPL, including modified and unmodified oxirane acrylic beads (Eupergit®) (Carmen Pinto *et al.*, 1997), Affi-Gels (Nuñez *et al*, 1997) and Ultralink Iodoacetyl resins (Rehbock and Berger, 1998). Immobilization often leads to decreases in the LOX/HPL enzymatic reaction rate and in the affinity of the enzyme for a substrate due to steric and electronic effects; however, this is often offset by significant gains in operational stability. Many studies of immobilized LOXs and HPLs have reported stable activities for several weeks/months in comparison to days for their free counterparts (Liagre *et al.*, 1996; Carmen Pinto *et al.*, 1997; Rehbock and Berger, 1998).

Volatile end products from either chemically stabilized or immobilized LOX/HPL extracts have widely been reported to be identical to their free counterparts. Characterization using chiral phase high-performance liquid chromatography (CP-HPLC) as well as gas chromatography with mass spectrometry (GC/MS) shows the production of the same relative ratios and types of flavor precursors and flavor compounds (Carmen Pinto *et al.*, 1997; Rehbock and Berger, 1998).

The specific objectives of this research work were:

(i) To produce biomasses and to prepare enzymatic extracts containing LOX activity from *Aspergillus niger* and *Penicillium candidum.*

(ii) To enrich and to characterize the LOX activities of the extracts from *Aspergillus niger* and *Penicillium candidum* in terms of pH, substrate specificity, enantio-

selectivity and kinetic parameters and to structurally characterize the flavor precursors using linoleic, linolenic and arachidonic acids as substrates.

(iii) To select the most appropriate enzymatic extract on the basis of experimental data in terms of activity and specificity, obtained throughout objective *(ii)*, as well those obtained previously in our laboratory on *Penicillium camemberti* and *Penicillium roqueforti*, for investigation in terms of stability and immobilization.

(iv) To determine the conditions for chemical stabilization of an enzymatic extract preparation containing LOX/HPL activities from *Penicillium camemberti* and its characterization in terms of inactivation rate, using linoleic acid and the 10-hydroperoxide of linoleic acid as substrates for LOX and HPL, respectively.

(v) To determine the conditions for immobilization of the stabilized LOX/HPL enzymatic extract from *Penicillium camemberti* and the characterization of these free and immobilized LOX/HPL activities in terms of pH, reaction temperature, stability of its enzymatic activity and kinetic parameters.

The current thesis contains six chapters. Chapter one is a brief introduction to the topics relevant to the research work, including the LOX and HPL catalyzed production of flavor compounds and strategies to stabilize these enzymes. Chapter two is a comprehensive literature review of the most recent and relevant research into the topics of LOX, HPL, chemical stabilization of enzymes and enzyme immobilization. Chapters three and four describe the growth, enrichment and characterization of LOX activities from *A. niger* and *P. candidum*, respectively, using linoleic, linolenic and arachidonic acid substrates. Chapter five deals with the preparation and stabilization of an enzymatic extract containing LOX/HPL activities from *P. camemberti*, with the use of selected chemical additives, as well as the characterization of the enzymatic activities in terms of long-term storage stability and thermostability, using linoleic acid and the 10-hydroperoxide of linoleic acid as substrates, respectively. Chapter six reports on the effects of the immobilization of a LOX/HPL extract from *P. camemberti*, as well as the

characterization of the enzymatic activities in terms of kinetics, long-term storage and thermostability.

CHAPTER II

LITERATURE REVIEW

2.1. Introduction

The overall dialogue on the biotechnological production of natural flavors from enzymes has led researchers to investigate those responsible for the bioconversion of polyunsaturated fatty acid (PUFAs) into volatile compounds (Kermasha *et al.*, 2002*b*). Figure 1. illustrates that lipoxygenase (LOX) oxidizes PUFAs producing various hydroperoxides, which are subsequently cleaved by hydroperoxide lyase (HPL) into moieties containing alcoholic, aldehydic and ketonic functionalities. Microbial LOX and HPL activities have been reported to produce many interesting and valuable flavor compounds. The main limitation to biotechnological approaches of enzymatic flavor production is the instability of the enzymes. Several approaches to the stabilization of LOX and HPL have been considered and the two most promising techniques are that of (1) chemical stabilization and (2) immobilization. These stabilization approaches may contribute to overall biocatalytic utility of an enzyme by counteracting forces that lead to denaturation and loss of activity (Wang, 1999 and 2000; Kim *et al.*, 2006).

2.2. Lipoxygenase (LOX)

2.2.1. Definition

Lipoxygenase (LOX) (linoleate: oxygen oxidoreductase; EC 1.13.11.12) catalyzes the dioxygenation of various PUFAs containing a 1(*Z*),4(*Z*)-pentadiene moiety into various regio-isomers of hydroperoxides of PUFAs (Gardner, 1996). Figure 2 illustrates the biocatalysis of linoleic acid hydroperoxides (octadecadieneoic acid hydroperoxides, HPODs).

2.2.2. Importance of LOX Activity to Flavor Production

The importance of LOX in flavor production was first described by Mukherjee (1951) who noted that microbial oxidation of butterfat caused the production of

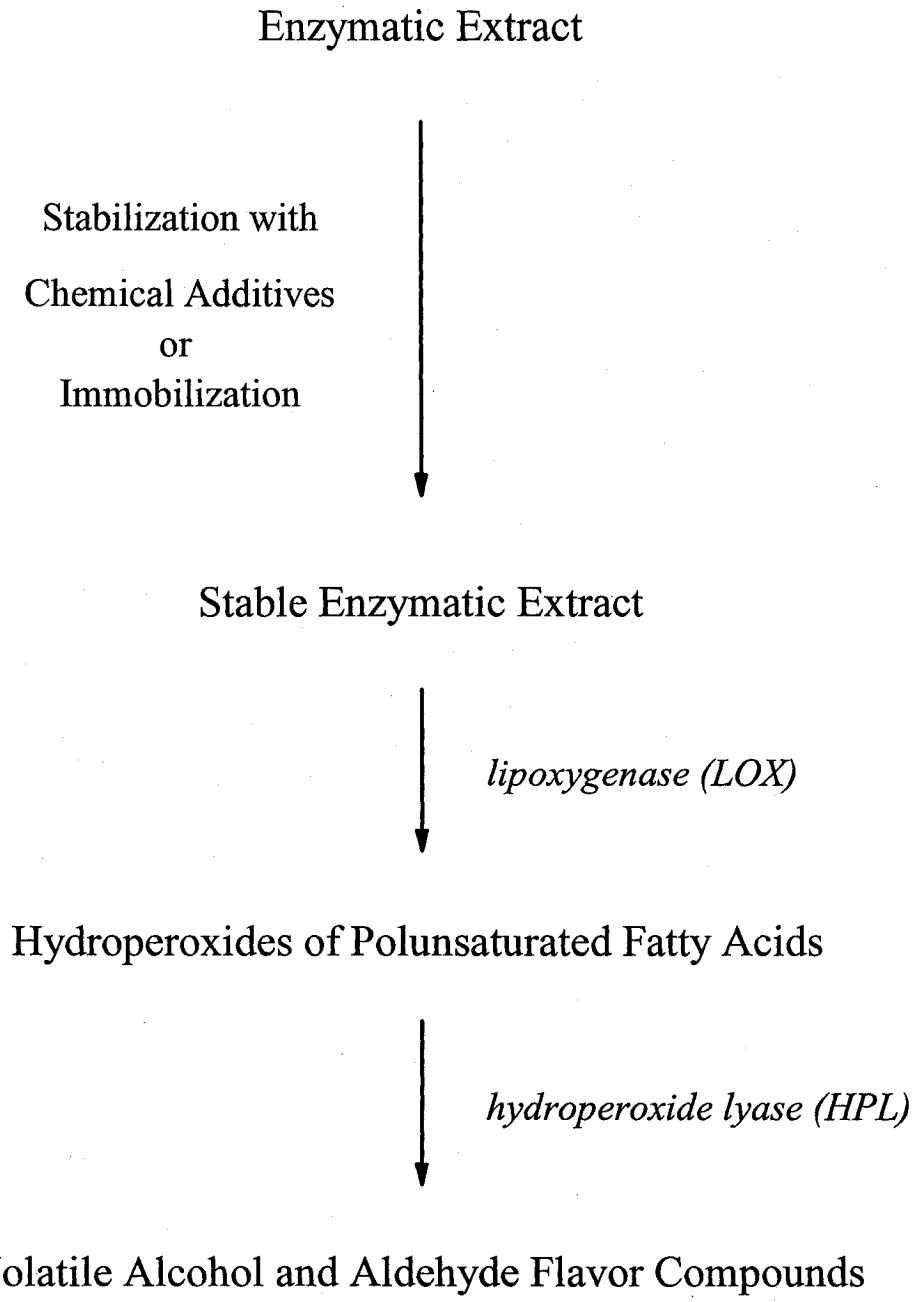

Figure 1. A general scheme for the production of natural flavor compounds using a microbial enzymatic extract from the lipoxygenase and hydroperoxide lyase activities, in the mold *Penicillium camemberti*.

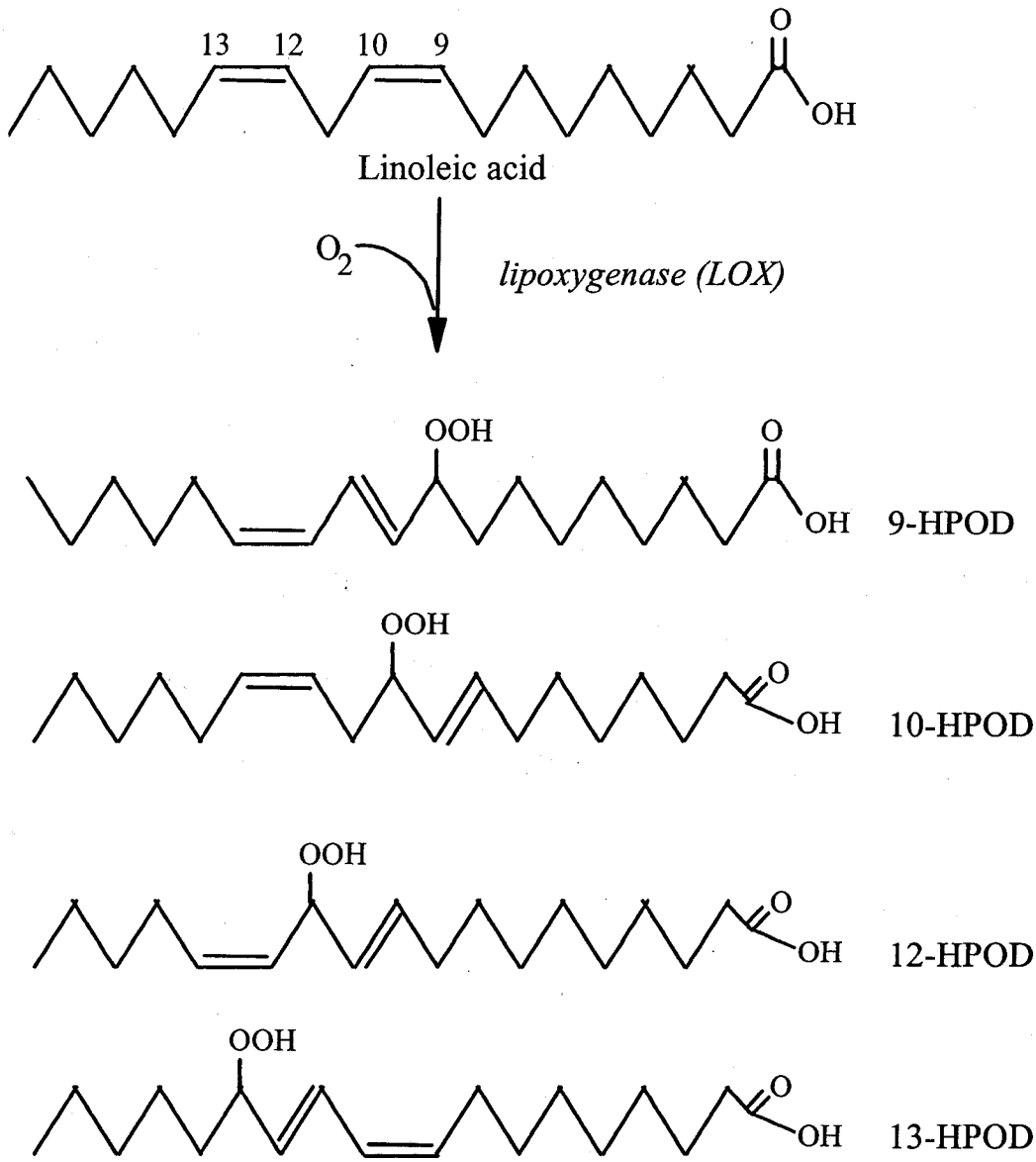

Figure 2. Production of 4 hydroperoxide regio-isomers of linoleic acid by lipoxygenase.

compounds that possessed a high spectral absorbance at 234 nm. Lipid peroxides produced by LOX alone contribute to off-flavors in products such as butter, milk, frozen vegetables and bread (Eskin *et al.*, 1977). The peroxides derived from LOX contribute significantly to rancid flavors in fermented products such as beer and some cheeses (Karahadian *et al.*, 1985*a*; Sovrano *et al.*, 2006). Lipid peroxides produced by LOX also mark the first product in a biocatalytic pathway that begins with lipid oxidation and ends with volatile flavor compounds which has been coined the lipoxygenase pathway (Gardner, 1991). The secondary enzyme in the LOX pathway is hydroperoxide lyase (HPL) which cleaves specific hydroperoxide isomers into a volatile and non-volatile moiety. The action of HPL will be elaborated on in Section 2.3. Further, transformation of the volatiles of the LOX pathway leads to flavor compounds with different organoleptic characteristics. These enzymes include dehydrogenases, *cis/trans* and enal isomerases (Gardner, 1991). These enzymes further transform lipid substrates into volatile compounds possessing particular flavor characteristics.

Research on flavor production from LOX appears to be divided into two distinct types. LOX is seen by some as a problematic enzyme that catalyzes the production of peroxides which impart foods and beverages with off flavors (Eskin *et al.*, 1977). The staling of beer has been, in part, linked to the production of peroxides from LOX in barley which acts on fatty acids present in malt or wort (Kuroda *et al.*, 2005). Other researchers see LOX as the primary enzyme in a pathway which biocatalyses the production of many flavors in foods stuffs as diverse as mushroom (Cronin and Ward, 1971), cheese (Hsieh, 1994) and shellfish (Kuo *et al.*, 1994).

2.2.3. Sources of LOX

LOX have been isolated and characterized from many sources, including vegetal (Lorenzi *et al.*, 2006; Kuo *et al.*, 2006), animal (Terao *et al.*, 1988; Mortimer *et al.*, 2006) and some microbial species (Schecter and Grossman, 1983; Su and Oliw, 1998). Overall, plants tend to possess the most active LOXs (Gardner, 1996). Soybean is the most common and active LOX source, from which commercial soybean LOX type-1B is produced, however tomato (Regdel *et al.*, 1994), cucumber (Feussner and Kühn, 1995) and banana (Kuo *et al.*, 2006) are also rich sources of LOX. Mammalian LOXs have been

under investigation for decades and have been localized from many tissues, including blood and skin cells (Terao *et al.*, 1988; Yamamoto, 1991).

A few studies have investigated LOX in microorganisms and included in this selection are some bacterial species (Iny *et al.*, 1993*a,b*), algae (Bisakowski *et al.*, 1995*c*; Kuo *et al.*, 1996) and yeasts, including *Saccharomyces* sp. (Shechter and Grossman, 1983; Bisakowski *et al.*, 1995*d*). A concensus amongst most researchers indicates that many important flavors derived from microbial metabolism of PUFA substrates involve LOX, particularly the generation of C8 volatiles, including 1-octen-3-ol and 1,5-octadiene-3-ol (Mau *et al.*, 1992). Assaf *et al.* (1995) reported the purified LOX from the fungus *Pleurotus pulmonarius* catalyzed the production of 1-octen-3-ol from incubation with linoleic acid substrate and suggested that the intermediate is the 13-HPOD regio-isomer of linoleic acid and similar results have been shown for *Agaricus bisporus* (Cronin and Ward, 1971) and *Lentinus edodes* (Chen *et al.*, 1984)

2.2.4. Substrates of LOX

Overall, the most commonly used substrates for LOX are the 1(*Z*),4(*Z*)-pentadiene containing PUFAs; linoleic ($C18^{9,12}$), linolenic ($C18^{9,12,15}$) and arachidonic ($C20^{5,8,11,13}$) acids (Gardner, 1996). Much lower substrate preferences are reported for mono-, di- and tri-substituted fatty acid acyl-glycerols esters. The C18 PUFAs are common substrates for plant and microbial LOXs, whereas the more substituted C20 PUFA ω-6 is seen most commonly as a substrate for animal LOXs. Plant LOXs initiate a pathway that ends in the biocatalysis of tissue wound response chemicals, including traumatin and jasmonic acid (Gardner *et al.*, 1991). The LOX of soybean (type-1B) has the greatest specificity towards linoleic acid, followed by linolenic and arachidonic acids (Hamberg and Samuelsson, 1967) and that of tomato follows an identical trend (Regdel *et al.*, 1994). An unusual LOX from cucumber cotyledon showed a preference for arachidonic acid (Feussner and Kühn, 1995).

Animal source LOX tend to display a preference for arachidonic acid above other free fatty acids or their acyl glycerols (Yamamoto, 1991). Generally mammalian LOXs are seen as the primary enzymes in the production of immune response chemicals,

including prostaglandins, leukotrienes and thromboxanes, which are all derived from biochemical pathways involving arachidonic acid (Lomnitski *et al.*, 1995).

Microbial LOXs are the producers of precursor molecules to volatile compounds such as alcohols and aldehydes, including as octanol and hexanal, that are exuded into the surrounding environment acting as bacteriostatic agents (Mau, 1992). The preferential usage of C18 PUFAs in the production of short chain aldehydes and alcohols could partly explain the preference over the larger C20 PUFA.

2.2.5. Enzymatic Mechanism of LOX

Figure 3 shows a proposed mechanism of action for soybean LOX type-1B. All LOXs structurally characterized to date contain one non-heme, non-sulfur iron atom at their core (Gardner, 1996; Knapp and Klinman, 2003). The mechanistic action of LOX proceeds via a radical intermediate, where the iron core of the enzyme cycles between oxidation states. The mechanism of LOX is initiated by the activation of the Fe atom from a resting ferrous (Fe^{3+}) form to an active ferric (Fe^{2+}) form by either molecular oxygen or the product hydroperoxide (Gardner, 1991). After activation, the ferric form (Fe^{2+}) binds the (*Z*,*Z*)-1,4-pentadiene moiety in the production of a pentadienyl radical which in turn reacts with molecular oxygen to yield a peroxyl radical. The peroxyl radical is ultimately reduced with ferrous, resulting in a peroxyl anion and a final reduction from water yields the end product PUFA hydroperoxide (Gardner, 1991).

2.2.6. Purification of LOX isozymes

The purification of LOX isozymes has been attempted using many different techniques. Briefly, the approaches usually apply the same general steps: (1) homogenization and centrifugation, (2) precipitation and concentration and finally (3) chromatographic separation.

Homogenization and centrifugation of LOX from *P. camemberti* was performed using a blender and glass bead homogenizer followed by centrigugation at 12,000×*g* for 15 min (Perraud and Kermasha, 2000). Centrifugation of *Gäunnanomyces graminnis* (Su and Oliw, 1998), *Oscillatoria* sp. (Beneytout *et al.*, 1989) and *Fusarium oxysporum*

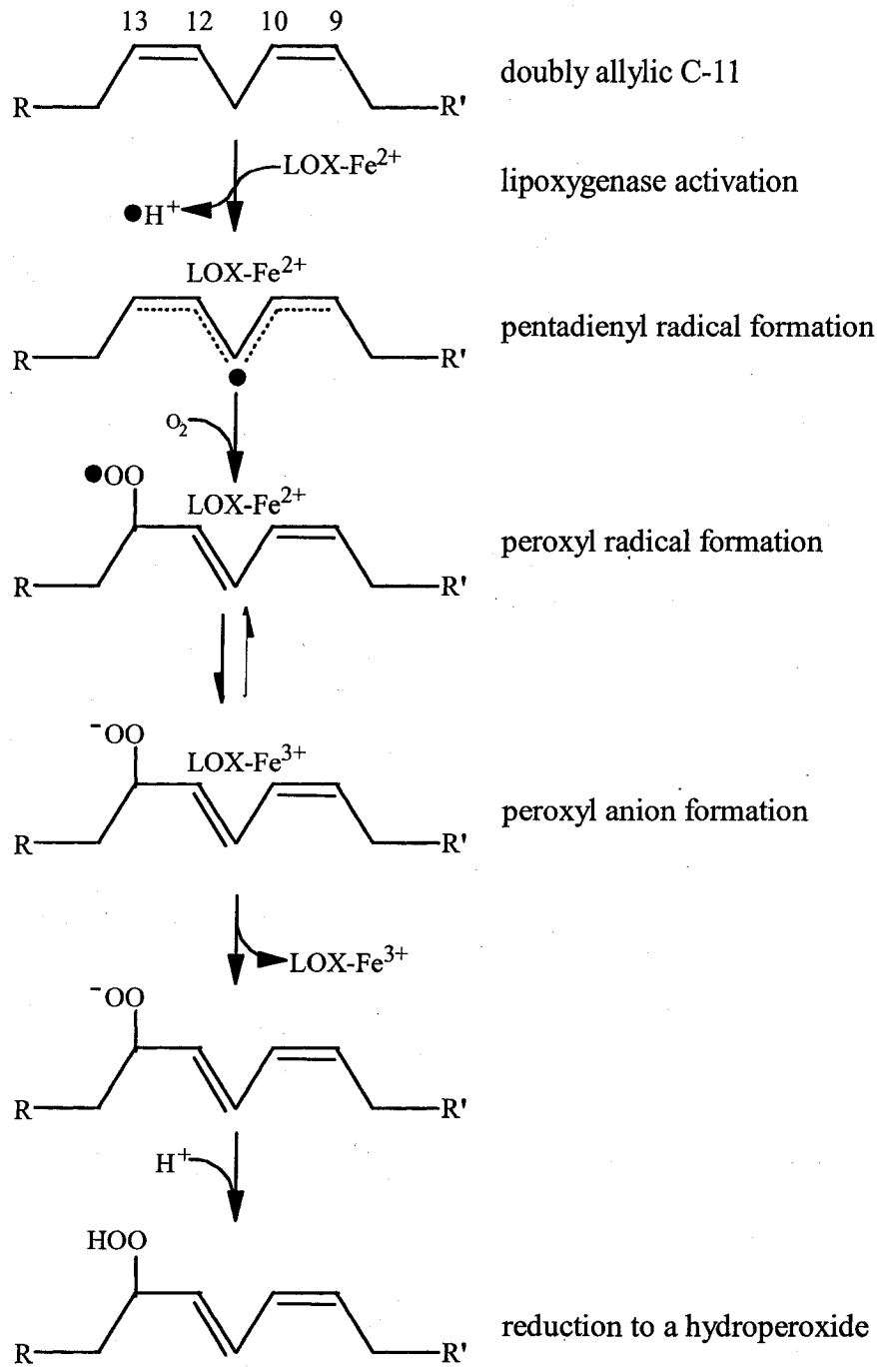

Figure 3. The proposed general mechanism of soybean lipoxygenase type-1B using linoleic acid as substrate in the production of the 13 regio-isomer hydroperoxide of linoleic acid where R = $CH_3(CH_2)_4$- and R' = -$(CH_2)_7COOH$ (modified from Gardner, 1991).

(Matsuda *et al*; 1978) were performed at 10,000×*g* for 30 min, 1,000×*g* for 45 min 12,000×*g* for 1 hour, respectively. For *Saccharomyces cerevisiae* (Schecter and Grossman, 1983) and *Thermoactinomyces vulgris* (Iny *et al.*, 1993*a,b*) the conditions were 100,000×*g* and 105,000 ×*g* for 1 hour, respectively.

Precipitation and concentration of LOX involved the addition of $(NH_2)_4SO_4$ (Matsuda *et al*; 1978) or polyethylene glycol 6,000 (Beneytout *et al.*, 1989). LOX from French bean seed (Kermasha and Metche, 1986) was purifed with $(NH_2)_4SO_4$ with a purification fold of 3.0. Using a similar strategy the LOX extracts from *P. camemberti* and *Penicillium roqueforti* yielded purification folds of 2.0 and 2.3, respectively (Perraud and Kermasha, 2000). Brash *et al.* (1996) used acetone to precipitate LOX from *Plexaura homomalla*, whereas ultrafiltration (NMWCO 100 kDa) was used to concentrate a LOX from *T. vulgaris* (Iny *et al.*, 1993*a,b*), as was the LOX of *Gersemia fruticosa* (Microcon, Amicon NMWCO 100 kDa) (Mortimer *et al.*, 2006).

Chromatographic separation of LOX isozymes involved several types of high-performance liquid chromatography. Hydrophobic interaction chromatography (HIC) on Phenyl sepharose was used to purify *G. gramininis* (Su and Oliw, 1998). Ion exchange chromatography (IEC) was used by Beneytout *et al.* (1989) when diethyl amino ethyl Tris Acryl was used to purify LOX from *Oscillatoria* sp. IEC on Mono-Q, Mono-S and carboxy methyl sepharose were used for the LOXs from *P. homomalla* (Brash *et al.* 1996), *F. oxysporum* (Matsuda *et al*; 1978) and *G. graminis* (Su and Oliw, 1998), respectively. Gel permeation chromatography (GPC) using Superdex 200 (Su and Oliw, 1998), Sephadex G-150 (Iny *et al.*, 1993*a,b*) and Sephadex G-100 (Matsuda *et al*; 1978) has also been used. LOX has also been separated by affinity chromatography (AC) on ConA-Sepharose (Su and Oliw, 1998), linoleyl AE Sepharose (Iny *et al.*, 1993*a,b*) and Sepharose 4B (Brash *et al.* 1996).

2.2.7. Characterization of LOX Activity

A review of relevant studies on LOXs from a variety of sources is provided in Table 1, key points of which are summarized in the current section.

Table 1. Properties of selected lipoxygenases (LOXs).

Type and Source	Regio-Specificity C18:2[9,12]	C18:3[9,12,15]	C20:4[5,8,11,14]	End-Product (% *S*)	Optimum pH	Inhibitors	V_{max} (nmol/mg/min)	K_m (mM)	Size (Kda)	References
Microbial										
Penicillium camemberti	9,10,12,13	n.d.[a]	n.d.[a]	53.0	6.5, 8.0	n.d.[a]	46	0.4	n.d.[a]	Perraud and Kermasha (2000)
Penicillium roqueforti	9,10,12,13	n.d.[a]	n.d.[a]	52.9	5.5, 8.0	n.d.[a]	84	0.3	n.d.[a]	Perraud and Kermasha (2000)
Geotrichum candidum	9,13	n.d.[a]	n.d.[a]	n.d.[a]	3.8, 8.0	HQ[b], BHT[c], KCN[d]	71	0.07	n.d.[a]	Perraud *et al.* (1999)
Gäunannomyces graminis	13	13,11	n.d.[a]	95	7.0	ETYA[e], *n*-3PCA[f]	8	0.03	73	Su and Oliw (1998)
Laminaria saccharina	13	13	15	n.d.[a]	8.5	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	Rorrer *et al.* (1997)
Polyneura lattissima	n.d.[a]	n.d.[a]	9	93	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	Jiang and Gerwick (1997)
Enteromorpha intestinalis	9,13	n.d.[a]	8,12,15	n.d.[a]	7.8	n.d.[a]	1180	n.d.[a]	n.d.[a]	Kuo *et al.* (1996)
Chlorella pyrenoidosa	n.d.[a]	n.d.[a]	8	n.d.[a]	4.5	KCN[d]	246	0.05	140	Bisakowski *et al.* (1995*c*)
Thermoactiomyces vulgaris	9,13	n.d.[a]	15	n.d.[a]	6,11	BHT[c], NDGA[g], *n*-PG[h]	840	1.0	160	Iny *et al.* (1993*a*,*b*)
Oscillatoria sp.	9,13	n.d.[a]	n.d.[a]	100	8.8	esculitin, NDGA[g]	51	0.25	124	Beneytout *et al.* (1989)
Saaccharomyces cerevisiae	9,13	n.d.[a]	n.d.[a]	n.d.[a]	6.5	KCN[d], *n*-PG[h]	n.d.[a]	0.27	n.d.[a]	Schecter and Grossman (1983)
Animal										
Gersemia fruticosa	n.d.[a]	n.d.[a]	11	100	8.0	n.d.[a]	n.d.[a]	n.d.[a]	77	Mortimer *et al.* (2006)
Rat dermis	13	n.d.[a]	12,15	99	5.5,8.6	*n*-PG[h]	51	0.25	176	Lomitski *et al.* (1995)
Trout gill	n.d.[a]	n.d.[a]	12	n.d.[a]	7.5	IA[j]	3	0.2	n.d.[a]	Hsieh *et al.* (1988)
Human blood plasma	n.d.[a]	n.d.[a]	15	99.4	8.6	*n*-PG[h]	52	0.2	166	Terao *et al.* (1988)
Vegetal										
Banana leaf	9	n.d.[a]	n.d.[a]	n.d.[a]	6.2	n.d.[a]	2400	0.15	85	Kuo *et al.* (2006)
Cucumber cotyledon	n.d.[a]	n.d.[a]	8,12,15	99	8.0	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	Feussner and Kühn (1995)
Tomato fruit	9	9	5,8,11	99	5.5,8.6	*n*-PG[h]	5500	0.21	150	Regdel *et al.* (1995)
Soybean type-1B	13	13	15	99	9.0	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	Hamberg and Samuelsson (1967)

[a] n.d. - not determined, [b]HQ – hydroquinone, [c]BHT – butylated hydroxytoluene, [d]KCN – potassium cyanide, [e]ETYA – 5,8,11,14-eicosatetryenoic acid, [f]*n*-3PCA – *N*-(3-phenoxycinnamyl) acetohydroxamic acid, [g]NDGA – nordihydroguaretic acid, [h]*n*-PG – *n*-propylgallate.

2.2.7.1. Kinetics of LOX Activity (K_m and V_{max})

Values for the the maximum velocity of an enzymatic reaction (V_{max}) and the substrate concentration at which said velocity is halfed (K_m) can give valuable information about an enzymes catalytic efficiency (Whitaker, 1994). The advantage of determining V_{max} for an enzyme is that it may a basis for comparisons between (eg. soybean LOX vs tomato LOX activities) or within various experimental treatments (eg. free extract LOX vs. immobilized extract LOX activities). The determination of K_m is essential because it is the single most important expression of the affinity of an enzyme for a substrate and in addition gives the researcher some insight into the concentration of a substrate *in vivo* (Whitaker, 1994).

Reports on the K_m values of most LOXs indicate that they are in the range of ~0.2 to 0.5 mM of PUFA subtrate. Some LOXs, including those extracted from *Chlorella pyrenoidosa* (Bisakowski, 1995*c*) and banana leaf (Kuo *et al.*, 2006) were reported to be lower than average at 0.05 and 0.15 mM, respectively. Elevated K_m values have also been reported as for LOX of the pathogenic fungus *T. vulgaris* at 1.0 mM (Iny *et al.*, 1993*b*).

Values for the maximum velocity (V_{max}) of the LOX reaction tend to vary more widely than K_m values. Generally, the lowest V_{max} values belong to animal and microbial LOXs which have been reported as being in the range of ~50 to 100 nanomol hydroperoxide produced/mg protein/min. V_{max} values for various microorganisms, including *G. candidum* (Perraud *et al*, 1999) and *P. camemberti* (Perraud and Kermasha, 2000) were reported to be 71 and 46, respectively. Greater velocities were determined from *T. vulgaris* (Iny *et al.*, 1993*b*) and *Enteromorpha intestinalis* (Kuo *et al.*, 1996) with 840 and 1180, respectively. That of banana leaf LOX was also slightly higher than most reported values at 2400 nanomol hydroperoxide produced /mg protein/min (Kuo *et al.*, 2006).

Catalytic efficiency values, defined as the ratio of V_{max} to K_m have been reported for several LOXs, including *P. camemberti* (2.6×10^{-4}). The appropriateness of the substrate for the enzyme is reflected by a large value; therefore large V_{max} values in combination with small K_m values indicate a "good" efficiency (Perraud and Kermasha,

2002). Knapp and Klinman (2003) investigated the kinetics of soybean LOX type-1B and reported that catalytic efficiency was independent of viscosity of the homogenate and determined that the O_2 diffusion effect is the main rate-limiting factor affecting velocity of the reaction. Further, the authors reported orientation of the LOX active site participated in the modulation of both stereo and regio-selectivity, whereas the Fe^{+2} atom primarily acts to generate an enzyme bound radical (Knapp and Klinman, 2003).

2.2.7.2. Effect of pH on LOX Activity and Stability

Enzymatic activities often have a narrow range of pH in which they are most efficient. LOXs tend to function best close to physiological pH. Most LOXs, despite their source have optima close to pH 7.0, including microbial (*G. graminis*, pH 7.0) (Su and Oliw, 1998), animal (trout gill, pH 7.5) (Hsieh *et al*, 1988) and vegetal (banana leaf, pH 6.2) (Kuo *et al.*, 2006). There was a basic pH optimum of 9.0 reported for soybean LOX type-1B (Hamberg and Sammuelson, 1967) and similar values reported for tomato fruit (Terao *et al.*, 1988), rat dermis (Lomintski *et al.*, 1995) and the alga *Oscillatoria* sp. (Beneytout *et al.*, 1989). Matsuda *et al.* (1976) reported on a single sharp optimum at pH 12 for the purified LOX of *F. oxysporum*, where the relative activities at the pH optimum was 2-fold that at either pH 11 or pH 13.

Dual optima for LOX have been reported by Iny *et al.* (1993*a*), for the bacterium *T. vulgaris*, at pHs of 6.0 and 11.0. Perraud and Kermasha (2000) also reported the presence of dual optima for the LOX of *P. camemberti*, at pHs of 6.5 and 8.0 and *P. roqueforti* at pHs of 5.5 and 8.0.

Varying pH values may affect not only LOX activity but stability as well. Tomato LOX was reported to have the highest stability against pressure induced denaturation at a pH value of 7 (Tangwongchai *et al.*, 2000). LOX type-1B was shown to be most stable at pH 7 and least stable at pH 9, at elevated temperature (55°C, 12 h) and pressure (2400 bar, 12 h) (Malvezzi-Campeggi *et al.*, 2001). The purified LOX of *P. ostreatus* was determined to be most stable over a pH range of 5.0 to 8.5, however, the major pH optima for the reaction using linoleic acid substrate was 8.0, with a minor optima at pH 4.5 (Kuribayashi *et al.*, 2002).

2.2.7.3. Effect of Temperature on LOX Activity and Stability

Temperature studies are often divided into two categories, (1) optimum reaction temperature (optimum temperature for catalytic functioning) and (2) thermostability (ability to catalyze after a discreet thermal treatment). Optimum reaction temperatures occur, in part, because low temperatures may contribute to decreased substrate/product diffusion rates while elevated temperatures initiate unfolding processes that damage the active site (Wang, 1999 and 2000). Thermostability, conversely, is imparted by an ability to maintain native enzyme conformation. Overall it appears that LOX optimum reaction temperatures are fairly broad and that it is moderately thermostable, due to high occurrence (+ 66%) of α-helices in the 3° structure of the enzyme (Gardner, 1991).

Matsuda *et al.* (1976) reported that the optimum temperature for the reaction of purified LOX from *F. oxysporum* with linoleic acid was 40°C. The optimal temperature for the LOX of *P. ostreatus* was reported to be 25°C (Kuribayashi *et al.*, 2002). Kuribayashi *et al.*, (2002) reported that the LOX of *P. ostreatus* was stable over a temperature range of 5 to 40°C (5 min treatment) but demonstrated sensitivity to thermal treatments above 40°C. The relative LOX activity at 10°C was 60% that of the optimum temperature and the authors believe this is evidentiary of the role of LOX initiation of fruiting in mushrooms which occurs at low temperatures (Kuribayashi *et al.*, 2002). Thermal inactivation of LOX from tomato fruits was reported by Anthon and Barrett (2003) to produce three different inactivation rate constants of 308, 336 and 349 kcal/mole, which corresponded to three structurally distinct LOX isozymes found in tomatoes.

2.2.7.4. Effect of Inhibitors on LOX Activity

Several authors have characterized the action of inhibitors on LOX. The classes of inhibitors deemed most effective at diminishing LOX activity are those which (1) quench free radicals (antioxidants), (2) chelate reactive metal species (chelators), or (3) act as substrate analogs (competitive inhibitors). Several antioxidants have been determined to inhibit LOX, including butylated hydroxytoluene (BHT) and *n*-propyl gallic acid (*n*-PG). Soluble cyanide salts, including KCN and NaCN also act to inhibit

LOX, by reducing the ability of the iron core to cycle between +II and +III oxidation states. EDTA acts as a chelator of metals and has actually been shown to sometimes activate LOX activity by reducing the amount of competing metal ions in solution. ETYA (eicosatetrayenoic acid) can act as a substrate analog of arachidonic acid (eicosatetraenoic acid) and this has been shown to be competitive inhibition. In this case the active site docking of the analog molecule is so similar to the substrate, as to remove free enzyme from the system through a reversible association that does not lead to products.

Matsuda *et al.* (1976) demonstrated that the addition of chelators (10 mM) of Fe^{2+}, including 8-hydroxyquinoline, thioglycollic acid and quinalizarin inhibited the specific activity by 46, 100 and 92%, respectively; whereas chelators of Fe^{3+}, including EDTA, *p*-chloromercurybenzoate caused no inhibition of the LOX of *F. oxysporum*. The addition of *p*-CMB (1 mM) caused 98.9% inhibition in the LOX activity of *P. ostreatus* (Kuribayashi *et al.*, 2002).

The addition of KCN (1 mM) did not inhibit the activity of the purified LOX from *S. cerivisiae*, using linoleic acid as substrate (Shecter and Grossman, 1983). Matsuda *et al.* (1976) reported that the addition of KCN (10 mM) inhibited *F. oxysporum* LOX by 40%. Kuribayashi *et al.* (2002) demonstrated the addition of KCN (10 mM) to the LOX of *P. ostreatus* caused only 5.8% inhibition compared to the control. Addition of urea (6 M, 37°C, 3 h) inhibited only 12% of the LOX activity in *F. oxysporum* (Matsuda *et al.*, 1976).

Several studies have also indicated that chemicals, such as detergents, may modify native enzyme conformation and be inhibitory to LOX. The requirement of PUFA substrate solubilization necessitates the addition of detergents, however excess amounts of Tween 20 (>0.25% w/v), have been shown to inhibit LOX activity by greater than 30% (Ben-Aziz *et al.*, 1970).

2.2.7.5. End product Specificity of LOX

The specificity of LOX is of two types, (1) Regio-specificity refers to the position of dioxygenation on the lipid chain of carbons and (2) Stereo-specificity refers to the (*R, S*) orientation of the chiral carbon in the lipid chain. Regio-specificity of LOXs varies widely depending on the source and substrate. Stereo-specificity of LOX end products are overwhelmingly (*S*) in orientation.

Regdel *et al.* (1994) reported that tomato LOX produces almost exclusively the (*S*)-enantiomer of the 9-HPOD and HPOT from linoleic and linolenic acids, respectively. Using arachidonic acid as substrate, tomato LOX was reported to produce the 5-, 8- and 11-HPETE regio-isomers, each with exclusively (*S*) configuration (Hawkins *et al.*, 1988; Regdel *et al.*, 1994). Soybean LOX type-1B was reported to produce the 13-HPOD and HPOT using linoleic and linolenic acids, respectively and enantiomeric excesses in favor of the (*S*) form, of greater than 97% have been obtained (Kühn *et al.*, 1987). Tomato (*Lycopersicon esculentum*), potato and corn have LOXs that oxygenate primarily at C-9. LOX regio-specificity can vary with LOX isozymes (Gardner, 1991). 80% of the 13-isomer was formed when linoleic acid served as substrate and 88% of this isomer was formed when linolenic acid was used as a substitute (Gardner, 1991).

The LOX from the coral *P. homomalla* is one of the few reported LOXs to produce *R*- enantiomers, yielding the 8-(*R*)-HPETE when incubated with arachidonic acid as substrate (Brash *et al.*, 1996). The authors suggest that the low substrate specificity of the LOX from *P. homomalla* may be due to an active site that allows the docking of arachidonic acid substrate in two orientations, resulting in either the 8-(*R*)- or the 12-(*S*)-HPETE end products (Brash *et al.*, 1996). Su and Oliw (1998) reported the enantiomeric excess of the 13-HPOD end product of *G. graminis* to be exclusively the 13(*S*)-HPOD. The purified LOX from *P. ostreatus* produced both the 13- and 9-HPODs using linoleic acid in a ratio of 9:1 (Kuribayashi *et al.*, 2002). The 13-HPOD was shown to be exclusively the (*cis, trans*)-13-HPOD stereoisomer, whereas both the *cis, trans* and *trans, trans* enantiomers were detected for the minor 9-HPOD product (Kuribayashi *et al.*, 2002).

2.2.8. Quantification of LOX Activity

Several techniques have been developed to detect and quantify lipid hydroperoxides. The main approaches adapted for the quantification of LOX activity include direct spectrophotometric monitoring, colorimetry, polarography and iodometry.

2.2.8.1. Direct Spectrophotometric Monitoring

Direct spectrophotometric monitoring at 234 nm of produced conjugated diene hydroperoxides is a common technique for the determination of LOX activity. Spectrophotometric monitoring at 234 nm for LOX activity was first reported for soybean LOX using linoleic acid as substrate, where the molar extinction coefficient (MEC) at 234 nm was estimated to be 25,000 M^{-1} cm^{-1} (Surrey, 1964). Matsuda *et al.* (1976) reported very close correlation in the results of direct spectrophotometric monitoring at 234 nm and polarography, for the quantification of LOX activity in *F. oxysporum*, concluding that the stoichiometry of the LOX reaction is 1 mole of PUFA substrate and O_2 converted for each 1 mole of HPOD end product generated.

2.2.8.2. Colorimetry/Xylenol Orange

Colorimetry refers to enzyme assays that develop a particular color, usually resulting from the oxidation/reduction of a dye in the presence of the LOX end product (Cheng, 1959). Ferrous oxidation (FOX) assays involve the hydroperoxide oxidation of ferrous (Fe^{2+}) ions to ferric (Fe^{3+}) form, which in turn oxidize a chromogenic salt. Several chromogenic salts may be employed for the detection of hydroperoxides, including thiocyanate (Wurzenberger and Grosch, 1984) and 3,3'-dimethoxybenzidine hydrochloride (deLumen and Kazeniac, 1976). MECs for lipids detected using these assays have been reported as ~ 26,000 M^{-1} cm^{-1} at 490 nm (Cheng, 1959; Wurzenberger and Grosch, 1984). The most common colorimetric salt for hydroperoxide detection in modern hydroperoxide analyses is xylenol orange.

Xylenol orange colorimetry is a robust technique used to detect hydroperoxides present in many materials, including animal tissues (Arab and Steghens, 2003; Södergren *et al.*, 1998), food stuffs (Navas *et al.*, 2004) and LOX enzymatic homogenates (Vega *et*

al., 2005 *a,b*; Cho *et al.*, 2006). Great care must be taken in determining the MEC for a particular hydroperoxide, due to wide variability in color formation at 560 nm. Most studies have indicated that detected hydroperoxides have molar extinction coefficients of approximately 26,000 M^{-1} cm^{-1} at 550-560 nm in aqueous environments (Gay *et al.*, 1999); whereas when in organic solvents are used, values range from 37,000 to 166,000 M^{-1} cm^{-1} (Södergren *et al.*, 1998; Vega *et al.*, 2005*a,b).*

2.2.8.3. Polarography

Polarography is a commonly used method of determining oxygen consumption in a LOX enzymatic homogenate, employing a metallic electrodrode that is sensitive to dissolved O_2 within the enzymatic homogenate (Pourplanche *et al.*, 1991). The technique has the advantage of being robust at low pH, where the solubility of PUFA substrates in aqueous systems may cause turbidity that renders direct spectrophotometric monitoring inefficient (Grossman and Zakut, 1979; Pourplanche *et al.*, 1991). Polarimetry was reported to yield results close to those obtained by chemical determination of dissolved oxygen (Pourplanche *et al.*, 1991).

Polarographic determination of purified LOX activity in *S. cerevisiae* was reported to be 8.35 μL O_2/mg protein/min (Shechter and Grossman, 1978). LOX from *F. oxysporum* was 19.4 μL O_2/mg protein/min (Matsuda *et al.*, 1976). The polarographic determination of O_2 consumption was performed for soybean LOX type-1B in environments containing various additives (1 M), including sucrose, sorbitol and glucose (Pourplanche *et al.*, 1991). Measurements performed in triplicate were closely clustered and suggested that despite the addition of excipients (up to 3 M) the LOX activity could be accurately estimated using polarography despite the viscosity present in the system (Pourplanche *et al.*, 1991).

2.2.8.4. Iodometry

The detection of LOX oxidation of lipid substrates by iodometry is based on the quantitative oxidation of Mn^{2+} to Mn^{3+} under alkaline conditions, followed by oxidation of I^- by Mn^{3+} under acidic conditions (Jiang *et al.*, 1991). The liberated iodine may then be titrated with thiosulfate to produce a colored chromophore at 500 nm. Pourplanche *et*

al. (1991) reported good agreement between this technique and that of polarographic oxygen determination for soybean LOX oxidation of linoleic acid, as did Jiang *et al.* (1991) for the detection of hydroperoxides in animal tissues.

2.2.9. Isolation of LOX End products

2.2.9.1. Thin-Layer Chromatography

Thin-layer chromatography (TLC) is an analytical and preparative technique whereby lipid hydroperoxides migrate through a polar support of bonded silica or alumina adherent to a plate that is positioned to allow a liquid mobile phase to progress via capillary action along its length (Pomeranz and Meloan, 1994). Shechter and Grossman (1983) used TLC to isolate the 9- and 13-HPOD end products of LOX from *S. cerevisiae* using linoleic acid as substrate, where silica plates where used with a mobile phase of petroleum ether/diethyl ether/acetic acid (60:40:1, v/v/v/). The 9- and 13-HPODs end products possessed retardation factors (R_fs) of 0.3 and 0.26, respectively (Shechter and Grossman, 1983). Matsuda *et al.* (1978) reported the use of silica gel TLC plates with a mobile phase of isooctane/diethyl ether/acetic acid (50:50:1, v/v/v) for the separation of the 9- and 13-HPOD end products of LOX from *F. oxysporum*.

Visualization of the end products may be performed using UV light and extracted from the silica with diethyl ether for further analyses (Shecter and Grossman, 1983). Matsuda *et al.* (1978) reported on TLC visualizations performed with either UV light, KI/starch or *N,N*-dimethyl-*p*-phenylenediamine for *F. oxysporum* LOX end products. The HPOD end products from *F. oxysporum* LOX were scraped from the silica TLC plates and extracted with chloroform/ethanol (2:1, v/v) (Matsuda *et al.*, 1978). Kuroda *et al.* (2005) reported that 9-HPOD produced by barley LOX was purified using TLC with a mobile phase consisting of hexane/ether (9:1).

2.2.9.2. High-Performance Liquid Chromatography

High-performance liquid chromatographic (HPLC) separation of LOX end products involves their application to columns containing either (1) a polar support such as bonded silica or alumina with non-polar mobile phases (designated normal phase or NP-HPLC) or (2) columns containing non-polar supports, such as C18 with polar mobile

phases (designated reverse phase or RP-HPLC). The use of either NP- or RP-HPLC offers rapid separation of lipid hydroperoxides regio-isomers and is widely used (Pomeranz and Meloan, 1994). A third type of HPLC designated chiral phase (CP) is sometimes applied to the analysis of (*R,S*) hydroperoxide stereo-isomersand is based on differential association of the enantiomers with a support that is itself chiral (Hawkins *et al.*, 1988).

The LOX of *F. oxysporum* using linoleic acid as substrate produced both the 9- and 13-HPOD in a ratio of 56:44 as determined by normal phase high-performance liquid chromatography (NP-HPLC) where the mobile phase was hexane:ethanol (995:5, v/v) and the analytical wavelength was 234 nm (Matsuda *et al.*, 1978). The 9-HPOD produced by banana leaf LOX was purified by NP-HPLC with isocratic elution of a mobile phase consisting of hexane/2-propanol/acetic acid (981:9:0.1), v/v/v) at a flow rate of 1.2 mL/min using UV/visible detection at 234nm (Kuo *et al.*, 2006).

The arachidonic hydroperoxide end products of a novel 11-(*R*)-lipoxygenase from the coral *Gersemia fruticosa* were purified using RP- HPLC (Rigel ODS 5μm, 250×4.6 mm) with a mobile phase consisting of methanol/0.017 M H_3PO_4 (82:18, v/v) using UV/visible detection at 234 nm (Mortimer *et al.*, 2006). A final purification was performed using a NP-HPLC column (Zorbax-Sil) with a mobile phase of hexane/2-propanol/water (98.3 : 1.2 : 0.012, v/v/v) at a flow rate of 1.0 mL/min (Mortimer *et al.*, 2006). CP-HPLC using a Chiralcel OD column confirmed the production of only the *R*-stereoisomer of 8-HPETE, produced from the LOX of *P. homomalla* (Brash *et al.*, 1996). Beneytout *et al.* (1989) used CP-HPLC (Pirkle, dinitrobenzoyl phenyl glycine) to resolve *R,S* enantiomers of *Oscillatoria* sp. LOX oxidation.

2.2.9.3. Gas-Liquid Chromatography/Mass Spectrometry

Modern gas-liquid chromography columns are thin, flexible fused silica columns made with polyimide and containing a liquid polymeric stationary phase which is adherent to the interior of the column (thickness from 0.1 μm to 10 μm). Older technologies involve shorter and thicker columns (1 to 3 m×4 mm i.d.) of steel or aluminum and packed with a support, usually a diatomaceous earth. Mass spectrometry is

an extremely powerful analytical tool, which can yield information on the molecular structure of an analyte. The process involves bombardment of an analyte molecule in a vacuum where the fragments of varying weights are accelerated towards a detector that produces a total ion chromatogram (Pomeranz and Meloan, 1994). Mass spectrometry is markedly more sensitive that flame ionization detection (FID) which does not yield information on the structure of the hydroperoxide derivative.

Analysis of hydroperoxide end products using GC/MS involves their conversion into more volatile methyl trimethyl silyloxy (MTMS) stearate/arachidate derivatives prior to injection. The injection solvent is usually a non-polar solvent such as dichloromethane (Bisakowski *et al.*, 1998; Perraud and Kermasha, 2000), or chloroform (Matsuda *et al.*, 1978). Oven temperatures are set at ~150°C and increase gradually to maximum temperatures of 300°C, whereas the flow of carrier gas varies on the type of column and nature of analyte but are in the range of 0.75 to 2.0 mL/min of helium (Schecter and Grossman, 1983).

The 11(*R*)-HPETE produced by coral LOX was MTMS derivatized and applied to a GC/MS column of fused silica (RSL, 30 m×0.12 mm) with MS detection (Hitachi M 80-B) where the temperature was initially 230 °C and was increased to 280°C at 7°C/min (Mortimer *et al.*, 2006). GC/MS analysis was also applied to linoleic acid hydroperoxides end products of the LOX from *F. oxysporum* (Matsuda *et al.*, 1978). The gas chromatographic separation of the 9- and 13-MTMS derivatives was performed using an initial column temperature of 165°C with a He carrier gas flow of 0.6 kg/cm^2 (Matsuda *et al.*, 1978). Characteristic mass fragmentations of 315 and 173 *m/z*s for the 13-MTMS and 259 and 279 *m/z*s for the 9-MTMS, respectively, were reported (Matsuda *et al.*, 1978).

2.3. Hydroperoxide Lyase (HPL)

2.3.1. Definition

Hydroperoxide lyase (HPL) is an enzyme capable of catalyzing the cleavage of a PUFA hydroperoxide in the generation of an alcohol or aldehyde and an ω-oxoacid (Matsui, 2006).

2.3.2. Importance of HPL Activity to Flavor Production

Figure 4 shows HPL cleavage of LOX catalyzed PUFA hydroperoxides produce a variety of flavor compounds that impart desirable tastes to foods and beverages. In food products, including breads (Hsieh, 1994), cheeses (Karahadian *et al.*, 1985*a*,*b*) and beers (Kuroda *et al.*, 2005) HPL contributes to the overall flavor profile. In plants, including pear (Kim and Grosch, 1981), apple (Schreier and Lorenz, 1981), mint (Gargouri *et al.*, 2004) and cucumber (Hornostaj and Robinson, 1999), HPL activity produces fresh, fruity and grassy notes (Hsieh, 1994). Animal HPLs, including those of some fish (Josephson, *et al.*, 1983; Hsieh *et al.*, 1988) shrimp (Kuo *et al.*, 1994) and lobsters (Whitfield *et al.*, 1982) produce savoury/metallic-tasting compounds. Microbial HPL activities in the molds *P. camemberti* (Kermasha *et al.*, 2002*b*), *A. niger* (Kaminski *et al.*, 1974), *F. oxysprum* (Bisakowski *et al.*, 1995*a*) and in the fungi *P. ostreatus* (Kuribayashi *et al.*, 2002) and *P. bisporus* (Wurzenberger and Grosch, 1984) have been implicated in the production of mushroomy/savoury flavors.

Flavors isolated from mushroom HPL activity include several C8 volatiles such as 1-octen-3-ol (mushroom alcohol), 1-octanol, 3-octanol, 3-octanone, 2-octen-1-ol and 1-octen-3-one and are widely considered to be the most important aroma components typical of most fungi (Mau *et al.*, 1994). These volatile components accounted +85% of all volatiles detected in headspace analysis, in more than 40 different mushroom species, including *Agaricus* sp. *Pleurotus* sp. and *Tricholoma* sp. (Mau *et al.*, 1994). HPL from *P. pulmonarius* was reported to produce 1-octen-3-ol as the major flavor volatile upon incubation with linoleic acid (Assaf *et al.*, 1995). The authors reported that only this PUFA substrate led to appreciable amounts of the mushroom alcohol. Wurzenberger and Grosch (1984 and 1986) made a similar observation in their investigation of *P. bisporus*, where 1-octen-3-ol originated from incubation with either linoleic acid, or its 10-HPOD, whereas 1,5-octadiene-3-ol was the end product of both linolenic acid biocatalysis and the 10-HPOT.

In the molds *Penicillium* sp., *Aspergillus* sp. and *Fusarium* sp. HPL has been demonstrated contribute to similar flavor compounds (Kaminski *et al.*, 1974). Relative

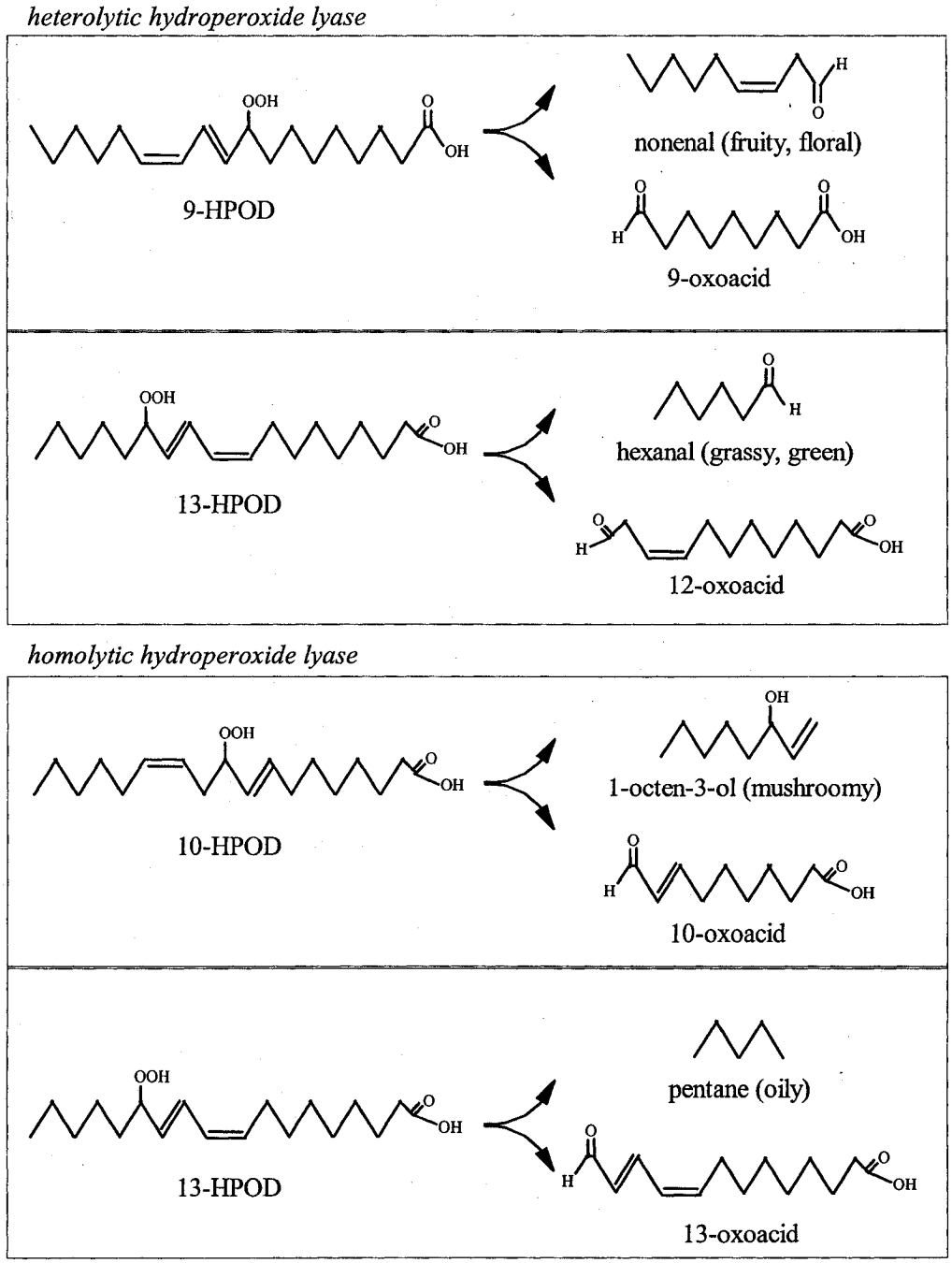

Figure 4. The importance of hetero- and homolytic hydroperoxide lyase activity to flavor production.

proportions of C8 alcohols, aldehydes, ketones and acids vary from varying species but it has been suggested that HPL could have industrial application in food flavor production because the concentration of 1-octen-3-ol isolated was comparable to boiled mushroom tissue, representing 0.9 μg volatile/L tissue homogenate (Kaminski *et al.*, 1974).

2.3.3. Sources of HPL

HPL has been isolated from the tissues microbial, plant and animals. HPL activities vary widely and this is explained with mechanistic distinctions (Matsui, 2006).

2.3.4. Enzymatic Mechanism of HPL

HPL catalyzed cleavage of PUFA hydroperoxides may be divided into two types depending on where chain cleavage occurs and is designated either homolytic or heterolytic (Gardner, 1996). Most microbial source HPLs possess homolytic features, while most vegetal and animal sources tend to be heterolytic in nature (Matsui, 2006). Figure 5 illustrates each HPL mechanism.

2.3.4.1. Homolytic HPL Activity

The enzymatic mechanism of homolytic HPL (Figure 5A) explains the cleavage of the 10-HPOD in mushrooms and the 13-HPOD in some algae and grasses (Matsui, 2006). The cleavage occurs on the carbon chain, between the hydroperoxide functionality and the carbon distal to the hydroperoxide functionality, yielding a volatile alcohol or aldehyde and a non-volatile ω-oxoacid (Gardner, 1991). The mechanism is reported to be similar to anaerobic LOX oxidation, where the formation of alkoxyl radicals and β-scission lead to similar fragments (Gardner, 1991). In isotopic studies it was reported that labelled $^{18}O_2$ was inserted into both volatile 1-octen-3-ol and non-volatile ω-oxoacid 10-oxo-8(*E*)-decenoic acid (Wurzenberger and Grosch, 1984; Adrianarison *et al.*, 1989; Vick and Zimmerman, 1976).

2.3.4.2. Heterolytic HPL Activity

The enzymatic mechanism of heterolytic HPL (Figure 5B) was elucidated for the cleavage of the 13-HPOT in soybean, tomato fruit and cucumber seedling and the 9-HPOD in pear fruit; the cleavage occurs on the carbon chain, between the hydroperoxide

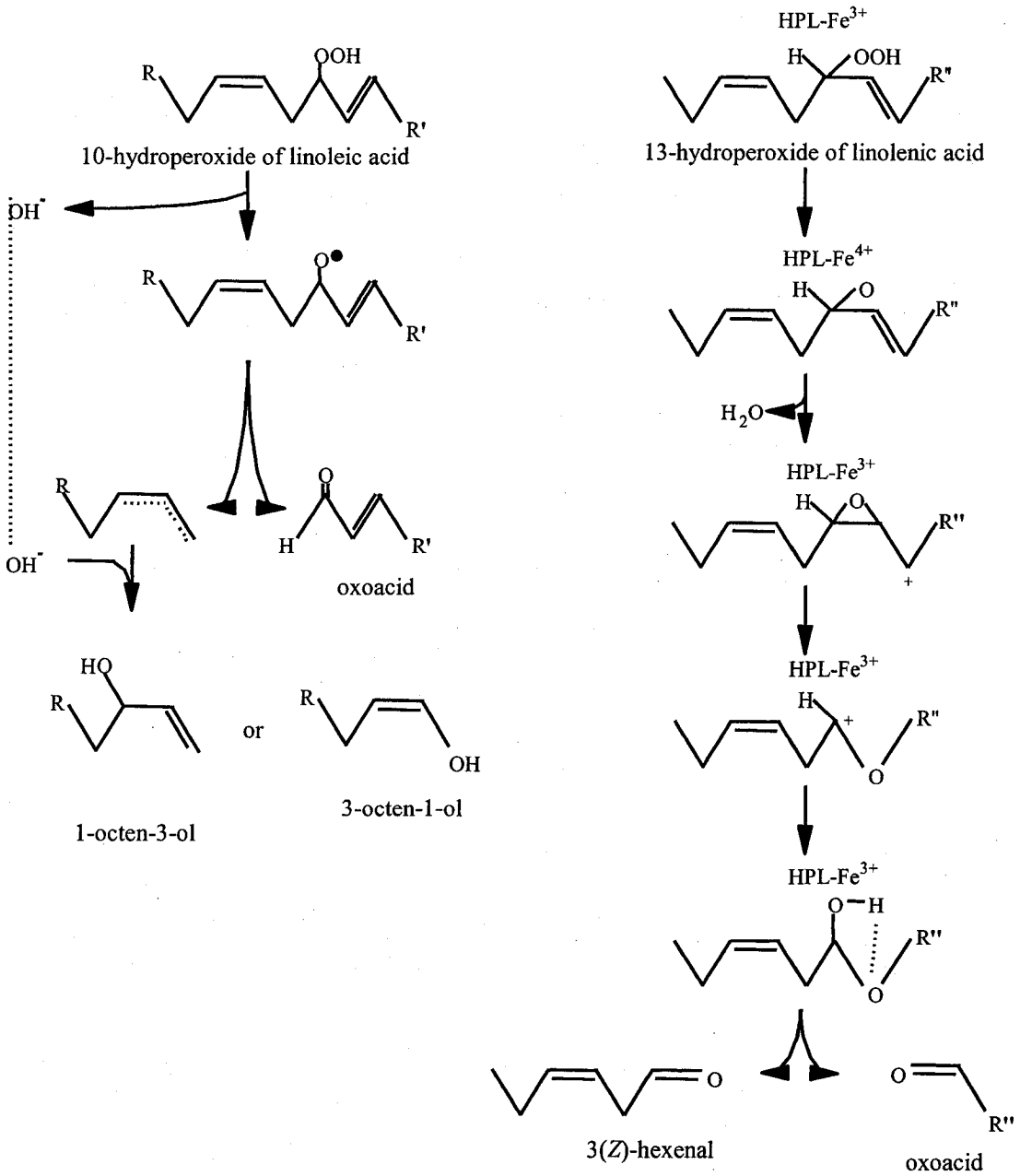

Figure 5. Proposed general mechanism for (A) homolytic hydroperoxide lyase (HPL) cleavage of the 10-hydroperoxide of linoleic acid and (B) heterolytic HPL cleavage of the 13-hydrperoxide of linolenic where R = $-(CH_3)_2$, R' = $-(CH_2)_6COOH$ and R'' = $-(CH_2)_2(CH_3)_7COOH$ (modified from Gardner, 1991).

functionality and the α-olefinic carbon, producing a volatile aldehyde and a non-volatile ω-oxoacid (Gardner, 1991).

2.3.5. Purification of HPL Isozymes

The purification of HPL isozymes has been sequentially performed using; (1) homogenization and centrifugation, (2) precipitation and concentration and finally (3) chromatographic separation.

Homogenization of an HPL extract from olive fruit was performed by manual grinding and centrifuged in two successive steps at 40,000×*g* for 20 min and 150,000×*g* for 80 min prior to further purification (Salas and Sánchez, 1999). HPL from cucumber and pea seed were both blended and centrifuged 25,000×*g* for 45 min (Hornostaj and Robinson, 1999 and 2000). Ultracentrifugation was used to obtain an active HPL from strawberry fruit (250,000×*g* then 350,000×*g*, for 120 min each). Some studies used lower speeds, as with sunflower hypocotyl (Itoh and Vick, 1999) and *P. camemberti* (Kermasha *et al.*, 2002*b*) at 12,000×*g* for 10 and 15 min, respectively.

Precipitation of HPL isozymes was peformed most commonly with polyethylene glycol (PEG) 6,000. Fauconnier *et al.* (1997) noted that PEG 6,000 precipitation (7% to 23%, w/v) was a simple and effective first step in the enrichment of HPL activity from tomato leaf, resulting in a fold purification of 3.7 and a recovery percentage of 47%. HPL activity from cucumber cotyledon was enriched using PEG 6,000 (22%, w/v) (Matsui *et al.*, 1989). The HPL from green bell pepper fruits was enriched using PEG 6,000 (30%, w/v) (Shibata *et al.*, 1995). Other compounds used for precipitation of HPL included ammonium sulfate (20-75% saturation) and polyvinyl polypyrrolidone (10%, w/v), for strawberry (Pérez *et al.*, 1999) and olive fruit (Salas and Sánchez, 1999), respectively.

Chromotographic separation of HPL isozymes was performed using many positively charged IEC supports, including DEAE-Toyopearl (Hornostaj and Robinson, 1999 and 2000), DEAE-cellulose (Itoh and Vick, 1999), DEAE-Sepharose (Fauconnier *et al.*, 1997). GPC chromatographic separation was performed with Sephacryl S-300 (Pérez *et al.*, 1999) and Superdex-200 (Fauconnier *et al.*, 1997). Other approaches included

hydroxylapatite chromatography on Biogel HT (Shibata *et al.*, 1995) and hydrophobic interaction chromatography using phenyl sepharose (Itoh and Vick, 1999).

2.3.5.1. Molecular Weight of HPL Isozymes

The molecular weight of HPL is ~ 150 to 200 kDa and usually consists of trimeric subunits of approximately equal weight. Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) of HPL from pea seed (Hornostaj and Robinson, 2000) and tomato leaf (Fauconnier *et al.*, 1997) indicated both were trimeric and contained monomers of 55 and 73 kDa, respectively. HPL from sunflower hypocotyls was an unusual tetramer consisting of 4×53 kDa subunits (Itoh and Vick, 1999).

2.3.6. Characterization of HPL Activity

A review of relevant studies on HPLs from a variety of sources is provided in Table 2, the key points of which are summarized in the current section.

2.3.6.1. Kinetics of HPL Activity (K_m and V_{max})

The K_m value for pepper fruit HPL was reported to be 20.8 μM of 13-HPOD (Shibata *et al.*, 1995) and similar values for plant source HPL were reported, 37 μM for soybean seed (Olías *et al.*, 1990) and 54 μM for alfalfa (Feussner and Kühn, 1995). That of olive fruit was slightly lower K_m at 6.8 μM (Salas and Sanchez, (1999).

The V_{max} values for the conversion of hydroperoxide substrate for HPL from cucumber fruit was reported to be 19 μmol/mg protein/min (Hornostaj and Robinson, 1999) while that of pea seed was 26 μmol/mg protein/min (Hornostaj and Robinson, 2000). The V_{max} for purified HPL from tomato fruit was approximated at 77 μmol/mg protein/min, which represented a 300-fold increase in HPL activity over the crude extract (Suurmeijer *et al.*, 2000).

2.3.6.2. Effect of pH on HPL Activity

The pH optima of most HPLs is 5.5 to 7. HPLs from green bell pepper fruit (Shibata *et al.*, 1995) and an edible button mushroom (Husson *et al.*, 2001) were reported as 5.5 and 6.5, respectively. HPLs from strawberry (Pérez *et al.*, 1999) and olive fruits (Salas and Sánchez, 1999) were both reported to possess a single pH optimum of 6.0.

Table 2. Properties of selected hydroperoxide lyases (HPLs).

Type and Source	Substrate hydroperoxide	End-Product volatile	Optimum pH	Optimum Rxn. (°C)	Inhibitors	V_{max} (μmol/mg/min)	K_m (mM)	References
Homolytic								
Penicillium camemberti	10-HPOD[a]	1-octen-3(*R*)-ol	6.5	25	13-HPOD[a], BHT[b]	n.d.[c]	n.d.[c]	Kermasha *et al.* (2002*b*)
Pear fruit	9-HPOD[a]	*cis*-3-nonenal	6.5	n.d.[c]	n.d.[c]	50	n.d.[c]	Kim and Grosch (1981)
Heterolytic								
Tomato fruit	13-HPOD[a] 13-HPOT[g]	hexanal *cis*-3-hexenal	6.5	n.d.[c]	NDGA[d], *n*-PG[e],BHA[f]	77	n.d.[c]	Suurmeijer *et al.* (2000)
Cucumber fruit	9-HPOD[a] 9-HPOT[g]	hexanal *cis*-3-hexenal	6.0	30	linoleic acid	19.3	6.8	Hornostaj and Robinson (1999)
Olive fruit	13-HPOD[a]	hexanal	6.0	n.d.[c]	n.d.[c]	9.6	5.8	Salas and Sánchez. (1999)
Strawberry fruit	13-HPOT[g]	*cis*-3-hexenal	6.0	n.d.[c]	n.d.[c]	n.d.[c]	n.d.[c]	Pérez *et al.* (1999)
Tomato leaf	13-HPOD[a]	hexanal	7.0	30	non-subtrate HPODs[a]	34	n.d.[c]	Fauconnier *et al.* (1997)
Alfalfa	13-HPOD[a] 13-HPOT[g]	hexanal *cis*-3-hexenal	6.5	n.d.[c]	n.d.[c]	71	54	Feussner and Kühn (1995)
Bell pepper fruit	13-HPOD[a]	hexanal	5.5	n.d.[c]	$HgCl_2$[h], α-tocopherol	n.d.[c]	21	Shibata *et al.* (1995)
Tea leaf chloroplast	13-HPOD[a]	hexanal	7.0	30	linoleic acid, 9-HPOD[a]	n.d.[c]	n.d.[c]	Matsui *et al.* (1991)
Soybean seed	13-HPOD[a]	hexanal	6.0-7.0	n.d.[c]	n.d.[c]	0.06	37	Olías *et al.* (1990)

[a]HPOD – hydroperoxide of octadecadieneoic acid, [b]BHT – butylated hydroxytoluene, [c]n.d. - not determined, [d]NDGA – nordihydroguaretic acid, [e]*n*-PG – *n*-propylgallate, [f]BHA – butylated hydroxy anisole, [g]HPOT – hydroperoxide of octadecatrieneoic acid, [h]$HgCl_2$ – mercuric chloride.

Matsui *et al.* (1989) reported the presence of two separate pH optima for HPL isozymes isolated from cucumber cotyledons at 6.5 and 8.0, corresponding to the 9- and 13-hydroperoxide cleaving HPL isozymes, respectively.

2.3.6.3. Effect of Temperature on HPL Activity and Stability

Temperature optima for most HPLs are 25 to 30°C, while its thermostability is often reported as being less than LOX (Anese and Sovrano, 2006). Olías *et al.* (1990) reported an 80% decrease in activity when the HPL from soybean was stored at 50°C for 5 min. Thermal treatment of an HPL from cucumber fruit showed no significant loss of specific activity up to 40°C for 2 min durations, however >50% of the activity was abolished at 45°C (Hornostaj and Robinson, 1999); these authors suggested that rapid loss in activity may be due to the presence of the detergent Triton X-100 (0.5%, w/v), which may have bound to the hydrophobic regions leaving them unable to associate with the aqueous medium thus preventing renaturation.

2.3.6.4. Effect of Inhibitors on HPL Activity

Antioxidants often used to inhibit LOX were effective in inhibiting HPL activity in tomato, including *n*-PG, butylated hydroxyanisole (BHA) and nordihydroguairetic acid (NDGA) (Suurmeijer *et al.*, 2000). Inhibition of HPL from green bell pepper fruits was accomplished using antioxidants at concentrations of 0.1 mM, including α-tocopherol and BHT (Shibata *et al.*, 1995); these authors proposed that the radical scavenging nature of these compounds slows the critical step of radical-formation in the HPL mechanism (Fig. 5B).

Inhibition by a non-substrate PUFA hydroperoxide regio-isomer was demonstrated by Fauconnier *et al.*, (1997) for tomato leaf HPL, which catalyzes the cleavage of the 13-hydroperoxide of linolenic acid. The authors reported an inhibition of >56% in HPL specific activity when 9-HPOT of linolenic acid (40 μM) was included in the enzymatic assay. Further, the authors determined a moderate inhibition of HPL activity using 5 other non-substrate PUFA hydroperoxides, including the 9-hydroperoxides of linoleic, linolenic, γ-linolenic acids and the 13-hydroperoxide of γ-linolenic acid (Fauconnier *et al.*, 1997).

2.3.6.5. Substrate Regio- and Stereo specificity of HPL

Regio-specicity of substrate usage by heterolytic HPL indicates a preference for the 13-regio-isomer of PUFA hydroperoxides. This preference is apparent with the cleavage of the13-HPOT of linolenic acid in tea leaf choloroplast HPL (Matsui *et al.*, 1991), or the 13-HPOD of linoleic acid in cucumber HPL (Feussner and Kühn, 1995). Regio-specificity of most homolytic HPLs is reported to prefer the 10-regio-isomer of PUFA hydroperoxides (Mau *et al.*, 1994; Kermasha *et al.*, 2002*b*).

Stereo-specificity of HPL substrate usage indicates a preference for the (*S*)-enatiomer (Gardner, 1991; Matsui, 2006). Only the 13-(*S*) and not the 13-(*R*) hydroperoxide of linoleic acid served as a substrate for HPL from soybean (Matoba *et al.*, 1985), or tea leaf chloroplasts (Kajiwara *et al.*, 1982). The HPL from mushroom converted only the 10-(*S*) regio-isomers of both linoleic and linolenic acids in the production of 1-octen-3-(*R*)-ol and 1,5-octadiene-3-(*R*)-ol, respectively (Wurzenberger and Grosch, 1984, 1986).

2.3.6.6. End product Specificity of HPL

The end product specificity of HPL is dependent on whether it is homolytic or heterolytic, as well as on substrate specificity. Table 2 and Figure 4 provide lists of some of the volatile end products flavor chemicals arising from various HPL biocatalyses.

2.3.7. Isolation of HPL End products

The isolation of volatile end products of HPL biocatalysis is commonly performed by either (1) extraction and/or (2) TLC. Pentane extraction was shown to be effective for the collection of C8 and C6 volatiles (Wurzenberger and Grosch, 1984; Olías *et al.,* 1990; Matsui *et al.* 1991; Shibata *et al.* 1995) whereas diethyl ether was effective for C5 volatile isolation (Adrianarison *et al.*, 1989). Non-volatile end products were mostly collected by extraction with chloroform or diethyl ether following acidification of the reaction medium (Wurzenberger and Grosch, 1984; Vick and Zimmerman, 1976). TLC was performed using a mobile phase consisting of diethyl ether/heptane/oxalic acid (60:40:1, v/v/v) (Wurzenberger and Grosch, 1984) or

hexane/dietyl ether/acetic acid (60:40:1, v/v/v) (Vick and Zimmerman, 1976) on fused silica plates, both of which were visualized by spraying phosphomolybdic acd (5%, w/v).

2.3.8. Characterization of HPL End products

2.3.8.1 High-Performance Liquid Chromatography

HPLC was used exclusively for the analysis of non-volatile oxoacids and was performed using Supersher Si60 (Kondo *et al.*, 1995) and Lichrosorb Diol (Nuñez *et al.*, 1997), each with a mobile phase of hexane/2-propanol (99:1, v/v). RP-HPLC on a Novapak C18 column with a mobile phase of methanol/water/triethylamine/acetic acid (80:20:0.05:0.1, v/v/v/v) was used to characterize decadienal produced by *V. sativa* HPL (Adrianarison *et al.*, 1991).

2.3.8.2. Gas-Liquid Chromatography/Mass Spectrometry

Gas chromatography was the most common technique used to characterize HPL volatiles. Non-polar stationary supports were most often chosen and included HP-5MS (Nuñez *et al.*, 1997; Kermasha *et al.*, 2002*b*), CP-Sil-5B (Wurzenberger and Grosch, 1984, 1986) and SE-30 (Assaf *et al.*, 1995). Kuroda *et al.* (2005) used a purge and trap cylinder fitted to a DB-1 column to detect 2(*E*)-nonenal from pear fruit HPL (Hewlett-Packard, HP 6890/MSD). Temperature ramping increased the initial temperature of 60°C by 5°C/min to 260°C, where the flow was maintained 1.0 mL/min He. A similar strategy was employed for the C-6 aldheydes produced by the HPL of mint (Gargouri *et al.*, 2004). The volatiles were introduced to a GC/MS instrument using a pressurized purge and trap autosampler (He 40 mL/mL for 10 sec) and were separated with a CP-Wax-57 fused silica column (Chrompack) with an initial oven temperature of 70°C, which was increased by 13°C/min to 220°C where it was maintained for 8 min (Gargouri *et al.*, 2004). Kim and Grosch (1981) applied the volatiles produced by pear HPL to a stainless steel column of 10% FFAP on Chromosorb W (3m×3.2 mm, 80-100 mesh), whereas Hornostaj and Robinson (1999) used a BPX-5 column (25m × 0.32 mm, 0.5 μm).

Kim and Grosch (1981) and Matsui *et al.* (1991) employed dinitrophenylhydrazine (DNPH) derivatization whereas other studies used methylation on the HPL end products (Adrianarison *et al.*, 1989). Most studies of the HPL volatiles used

no derivatization whatsoever, such as those produced by recombinant cucumber fruit (Hornostaj and Robinson, 1999) and bell pepper fruit source (Husson and Belin, 1995).

2.3.9. Quantification of HPL Activity

2.3.9.1. Direct Spectrophotometric Monitoring versus Colorimetry

Many studies used direct spectrophotometric monitoring of the enzymatic homogenate to quantify HPL activity (Vick, 1991; Matsui, 2006). A decrease in absorbance at 234 nm was observed for the HPL biocatalysis of pear fruit (Kim and Grosch, 1981), pepper fruit (Shibata *et al.*, 1995), recombinant alfalfa (Noordermeer *et al.*, 2000) and *Yarrowia lipolytica* (Husson *et al.*, 2006) as the PUFA hydroperoxide substrate is converted. This approach offers simplicity and rapid analysis, but is limited to those HPL activities that consume a diene-containing substrate. The activities of many homolytic HPLs, including those of *A. bisporus* (Wurzenberger and Grosch, 1984, 1986), *L. edodes* (Chen *et al.*, 1984) and *P. camemberti* (Kermasha *et al.*, 2002*b*) cannot be quantified by direct spectrophotometric monitoring. Therefore colorimetry using ferrous thiocyanate (MEC 26,000 M^{-1}, cm^{-1} at 490 nm) was used to quantify amounts of PUFA hydroperoxide substrate remaining after biocatalysis (Wurzenberger and Grosch, 1984 and 1986).

2.4. Chemical Stabilization of Enzymes

2.4.1. Definition of Enzymatic Stabilization

Enzymatic stabilization may be defined as any strategy aimed at the protection of enzymes from denaturation and/or maintenance of native conformation. Often chemicals are employed to mitigate destructive processes that cause decreases in enzymatic specific activity, due to their ability to aid the forces that impact native enzyme conformation (Wang, 1999).

2.4.2. Forces Influencing Native Enzyme Conformation

Many forces are involved in protein folding. These include hydrophobic interaction, electrostatic interactions (charge repulsion and ion pairing), hydrogen bonding, intrinsic propensities and van der Waals forces. Hydrophobic interactions are repulsive interactions between water and non-polar residues in proteins, leading to

minimal hydration of the hydrophobic core. These interactions are strongly disfavored and associated with a large increase in heat capacity. A hydrogen bond is the strong dipole-dipole attraction between covalently bonded hydrogen atoms and other strongly electronegative atoms such as oxygen and nitrogen. It is primarily a linear arrangement of donor, hydrogens and acceptor. Hydrogen bonds between amide hydrogen and carbonyl oxygen make up 68% of the total hydrogen bonds in globular proteins (Wang, 1999).

2.4.3. Aggregation of Enzymes

Protein aggregation is a major event of physical instability where the secondary, tertiary and quaternary structure of a protein may change and lead to protein unfolding. Protein aggregations, in many cases, result from intermolecular association of partially denatured protein chains (Carpenter *et al.*, 1990 and 1991).

Hydrophobic interaction, the reluctance of non-polar groups to be exposed to water, is considered to be the major driving force for both protein folding and aggregation (Wang, 2000). Protein aggregation may be induced by a variety of physical factors, such as temperature, ionic strength, vortexing and surface/interface adsorption. These factors can increase the hydrophobic surface area of proteins, causing aggregation (Carpenter *et al.*, 1997).

2.4.4. Preferential Exclusion

The most widely accepted mechanism of protein stabilization in aqueous solution is that of preferential interaction. Preferential interaction means that a protein prefers to interact with either water or an excipient (co-solute/co-solvent) (Carpenter *et al.*, 1990). In the presence of a stabilizing excipient, a protein prefers to interact with water (preferential hydration) and the excipient is preferentially excluded from the protein domain (preferential exclusion). In this case, proportionally more water molecules and fewer excipient molecules are found at the surface of the protein than in the bulk solution (Wang 1999 and 2000). The interaction of a protein with either water or an excipient is the consequence of a difference in affinity of a protein for water or an excipient. If there is a preference for an excipient, the measured interaction is positive and preferential

binding results. In the opposite case, the measured interaction is negative, preferential hydration is observed and the protein is stabilized (Wang, 1999).

2.4.5. Strategies of Stabilization

2.4.5.1. Definition of a Chemical Excipient

Excipients are defined as those chemicals included in enzymatic preparations to prevent denaturation resulting from freezing and drying stresses and are added prior to enzyme lyophilization.

2.4.5.2. Definition of a Chemical Additive

Additives are defined as those chemicals present in enzymatic preparations to resist protein denaturation resulting from physical stresses such as elevated temperature. These chemicals are added prior to the enzymatic assay.

2.4.5.2.1. Chemicals added to Stabilize LOX

Table 3 lists the chemicals added to enhance LOX solubility and/or stability and they include detergents, thiols and metal ions. Due to LOX's relatively high natural stability few studies have been undertaken to further increase its stability. Matsuda *et al.* (1976) reported that LOX of *F. oxysporum* is stabilized by the addition of Co^{2+} (0.25 mM) which was included in the form of $CoCl_2$ in the homogenization buffer. Removal of Co^{2+} by dialysis resulted in a 30% loss of the specific activity in comparison to LOX containing Co^{2+} (Matsuda *et al.*, 1976). Treatment at 30°C (10 min) abolished all activity in the LOX without Co^{2+}, whereas 100% activity remained in the Co^{2+} containing preparation (Matsuda *et al.*, 1976). Kuribayashi *et al.* (2002) demonstrated that the addition of Co^{2+} was not effective at stabilizing the LOX from *P. ostreatus*, reporting an inhibition of 77.1% activity in the extract containing $CoCl_2$ (1 mM). Several other metal ions (1 mM) were assayed for their effect on the LOX of *P. ostreatus*, including Ca^{2+}, Mn^{2+}, Hg^{2+} and Cu^{2+} (Kuribayashi *et al.*, 2002). All metals tested were shown to be inhibitory to LOX from *P. ostreatus*, except Cu^{2+}, which was slightly activitating resulting in 110.3% relative activity in comparison to the control (Kuribayashi *et al.*, 2002). Further, Kuribayashi *et al.* (2002) reported that the addition of thiol reducing

Table 3. Chemicals added to stabilize and/or solubilize lipoxygenase (LOX) from a variety of sources.

Source	Detergents	Thiols	Metal Ions	Others	References
Eggplant chloroplast	Triton X100[a] (0.5%, w/v)	dithiothreitol (10 mM)	-	3-PG[b] (4.5 mM) ATP[c] (2 mM) EDTA[d] (4 mM)	Pérez-Gilabert and García-Carmona (2001)
Penicillium camemberti	-	-	-	-	Perraud and Kermasha (2000)
Gaünamyces graminis	Tween 20[e] (0.04%, w/v) CHAPS[f] (0.5 mM)	-	-	EDTA[d] (1 mM) NaN_3[g] (2 mM)	Su and Oliw (1998)
Enteromorpha intestinalis	-	glutathione (1 mM)	-	-	Kuo *et al.* (1996)
Plexaura homomalla	Emulgen 911 (0.3%, w/v)	-	-	EGTA[h] (2 mM)	Brash *et al.* (1996)
Tomato fruit	Sodium cholate (0.2%, w/v)	-		EDTA[d] (1 mM) ascorbate (1 mM)	Regdel *et al.* (1994)
Thermoactinomyces vulgaris	Tween 20[e] (1%, w/v)	-	-	-	Iny *et al.* (1993 *a,b*)
Oscillatoria sp.	Brij 99[i] (0.1%, w/v)	-	-	-	Beneytout *et al.* (1989)
Trout gill	-	glutathione (1 mM)	-	-	Hsieh *et al.* (1988)
Saccharomyces cerevisiae	Tween 20[e] (1.0 %, w/v)	-	-	-	Shecter and Grossman (1983)
Watermelon seedling	-	dithiothreitol (0.1 mM)	-	EDTA[g] (0.3 mM)	Vick and Zimmerman (1976)
Fusarium oxysporum	Tween 40[j] (1.0 %, w/v)	-	Co^{2+}[k] (0.25 mM)	-	Matsuda *et al.* (1976)

[a]Triton X-100 - ([polyoxyethylene(10)isooctylphenyl ether]), [b]3-PG - (3-phosphoglycerate), [c]ATP - (adenosine triphosphate), [d]EDTA - (ethylenediaminetetraacetate), [e]Tween 20 - ([polyoxythelene sorbitan monolaurate], [f]CHAPS - (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate, [g]NaN_3 - (sodium azide), [h]EGTA - [(ethylene glycol-*bis*(2-aminoethylether)*N,N,N',N'*-tetraacetate)], [i]Brij 99 - (polyoxyethylene 20 oleyl ether), [j]Tween 40 - ([polyoxythelene sorbitan monopalmitate]; [k]Co^{2+} ($CoCl_2$).

agents such as dithiothreitol or β–mercaptoethanol in the preparation buffer was useful in stabilizing the essential LOX thiol group in the reduced form for catalysis.

2.4.5.2.2. Chemicals added to Stabilize HPL

Table 4 summarizes the chemicals added to solubilize and/or stabilize a variety of HPLs. Amongst the most common chemicals used for stabilization of HPLs are detergents. It has been suggested that they are necessary for the solubilization of the membrane bound HPL fraction, predominantly from plant sources (Gardner *et al.*, 1991). The few studies that have investigated HPL from fungal or microbial sources (Kermasha *et al.*, 2002; Wurzenberger and Grosch, 1984) omitted the inclusion of detergents from their enzyme preparations. The presence of nonionic detergents may reduce HPL surface adsorption and aggregation. Noordermeer *et al.* (2000) reported that the addition of Triton X-100 (0.2%, w/v) to alfalfa HPL led to 50% greater stability at 4°C and suggested that the detergent induced a change in the tertiary structure of the HPL enzyme. Vick and Zimmerman (1976) reported the inclusion of a detergent (Triton X-100 reduced, 0.1%, w/v) was essential for the reduction of aggregation in spinach leaf chloroplast HPL, during its purification.

Hornostaj and Robinson (1999) reported a decrease of 50% activity when purified HPL from cucumber fruit was subjected to storage at 50°C for 2 min. The authors suggested non-ionic detergents such as Triton X-100 may bind hydrophobic regions of the enzyme, exposed from heat denaturation at elevated temperature, thus preventing proper refolding of HPL in cucumbers, leading to a loss of activity (Hornostaj and Robinson, 1999). Olías *et al.* (1990) reported that detergents were necessary additives for the solubilization of HPL from soybean and that Triton X-100 (0.2% w/v) was the most effective and least denaturing of the detergents investigated.

Glycerol has been used to stabilize HPL from a variety of sources, including that of olive fruit (Salas and Sánchez, 1999) and sunflower (Itoh and Vick, 1999). The addition of PVP has also been proven effective in some stabilization protocols. Husson *et al.* (2001) reported that a 35% greater residual activity was obtained for mushroom LOX/HPL samples that contained PVP (0.5%, w/v), after 30 min of storage at 0°C.

Table 4. Chemicals added to stabilize and/or solubilize hydroperoxide lyase (HPL).

Source	Detergents	Sugars / Polyols	Thiols	Polymers	References
Green bell pepper fruit	Triton X-100[a] (0.5 %, w/v)	-	-	-	Husson and Belin (2002)
Button mushroom	-	-	-	PVP[b] (0.5 %, w/v)	Husson *et al.* (2001)
Tomato fruit	Triton X-100[a] (0.1 %, w/v)	-	Et-2-SH[c] (10 mM)	-	Suurmeijer *et al.* (2000)
Pea seed	Triton X-100[a] (0.5 %, w/v)	-	DTT[d] (4 mM)	PVP[b] (0.5 %, w/v)	Hornostaj and Robinson (2000)
Cucumber fruit	Triton X-100[a] (0.5 %, w/v)	-	DTT[d] (1 mM)	-	Hornostaj and Robinson (1999)
Sunflower hypocotyl	CHAPS[e] (0.3 %, w/v)	glycerol (30%, w/v)	DTT[d] (3 mM)	-	Itoh and Vick (1999)
Olive fruit	Triton X-100[a] (0.5%, w/v)	glycerol (10%, w/v)	Et-2-SH[c] (7 mM)	PVP[b] (2 %, w/v)	Salas and Sánchez (1999)
Green bell pepper fruit	Triton X-100[a] (0.5 %, w/v)	-	Et-2-SH[c] (100 mM)	-	Shibata *et al.* (1995)
Tea leaf	-	-	DTT[d] (5 mM)	-	Matsui *et al.* (1991)
Cucumber seedling	-	-	-	PVP[b] (4 %, w/v)	Matsui *et al.* (1989)
Soybean seedling	Triton X-100[a] (0.2 %, w/v)	-	-	-	Olías *et al.* (1990)
Strawberry fruit	Triton X-100[a] (0.1 %, w/v)	-	DTT[d] (3 mM)	PVP[b] (12.1 %, w/v)	Pérez *et al.* (1999)
Tobacco leaf	Tween 20[f] (0.5 %, w/v)	sucrose (400 mM)	-	-	Götz-Schmidt *et al.* (1986)
Pear fruit	Triton X-100[a] (0.2 %, w/v)	-	DTT[d] (4 mM)	-	Kim and Grosch (1981)

[a]Triton X-100 – polyoxyethylene-10-isooctylphenyl ether, [b]PVP - polyvinylpolypyrolidone 30 Kda, [c]Et-2-SH – 2-mercaptoethanol, [d]DTT – Dithiothreitol, [e]CHAPS - (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate, [f]Tween 20 - [polyoxythelene(20) sorbitan monolaurate].

Matsui *et al.* (1991) reported on the stabilizing effect of PVP on the HPL from tea leaf. The authors suggested the addition of PVP (4%, w/v) prevented the binding of HPL by polyphenols, oxidized by polyphenol oxidase, during the initial maceration of the leaves.

The use of thiol containing compounds is fairly common in the stabilization and/or solubilization of HPL fractions (Allison *et al.*, 1998; Pérez *et al.*, 1999; Suurmeijer *et al.*, 2000). The most common thiol containing reducing agent added is β–mercaptoethanol, however dithiothreitol (DTT) acts in a similar manner but is a more effective on a molar basis. Opinion on the mechanism of stabilization offered by thiols is contested, however, some researchers believe the addition of disulfide-reducing agents may aid in maintaining sensitive sulfhydryl groups within the active site of reduced HPL from a variety of sources (Matsui *et al.*, 1992; Rehbock and Berger, 1998). HPL from tea leaf chloroplasts was protected by ~25% above untreated trials with the addition of DTT (1.0 mM) (Matsui *et al.*, 1992). The HPL from mung bean also showed a greater maintenance of activity when DTT (20 mM) was included in the homogenate, a result not seen with the addition of cysteine (Rehbock and Berger, 1998). Suurmeijer *et al.* (2000) reported that tomato fruit HPL was very unstable, but the addition of Triton X-100 (0.1% w/v) and β–mercaptoethanol (0.01 M) yielded an active enzyme that could be stored for several months at –80°C. Fauconnier *et al.* (1997) reported that the exclusive use of detergents was insufficient to solubilize an HPL extract from tomato leaf but the addition of β–mercaptoethanol (0.1 mM) allowed for a 24-fold increase in specific activity upon subsequent purification. DTT is not universally effective at stabilizing HPL, as Husson *et al.* (2001) reported that inclusion of DTT (5 mM) to a LOX/HPL extract of *A. bisporus*, offered no significant stabilization and was in fact inhibitory.

2.5. Immobilization

2.5.1. Definition

Enzyme immobilization refers to any technique in which an insoluble support material is added to an enzymatic extract to elicit an association between the protein and the material, thereby improving the stability of the enzyme (Wang, 2000). Figure 6 illustrates various types of immobilizations commonly employed to stabilize enzymes.

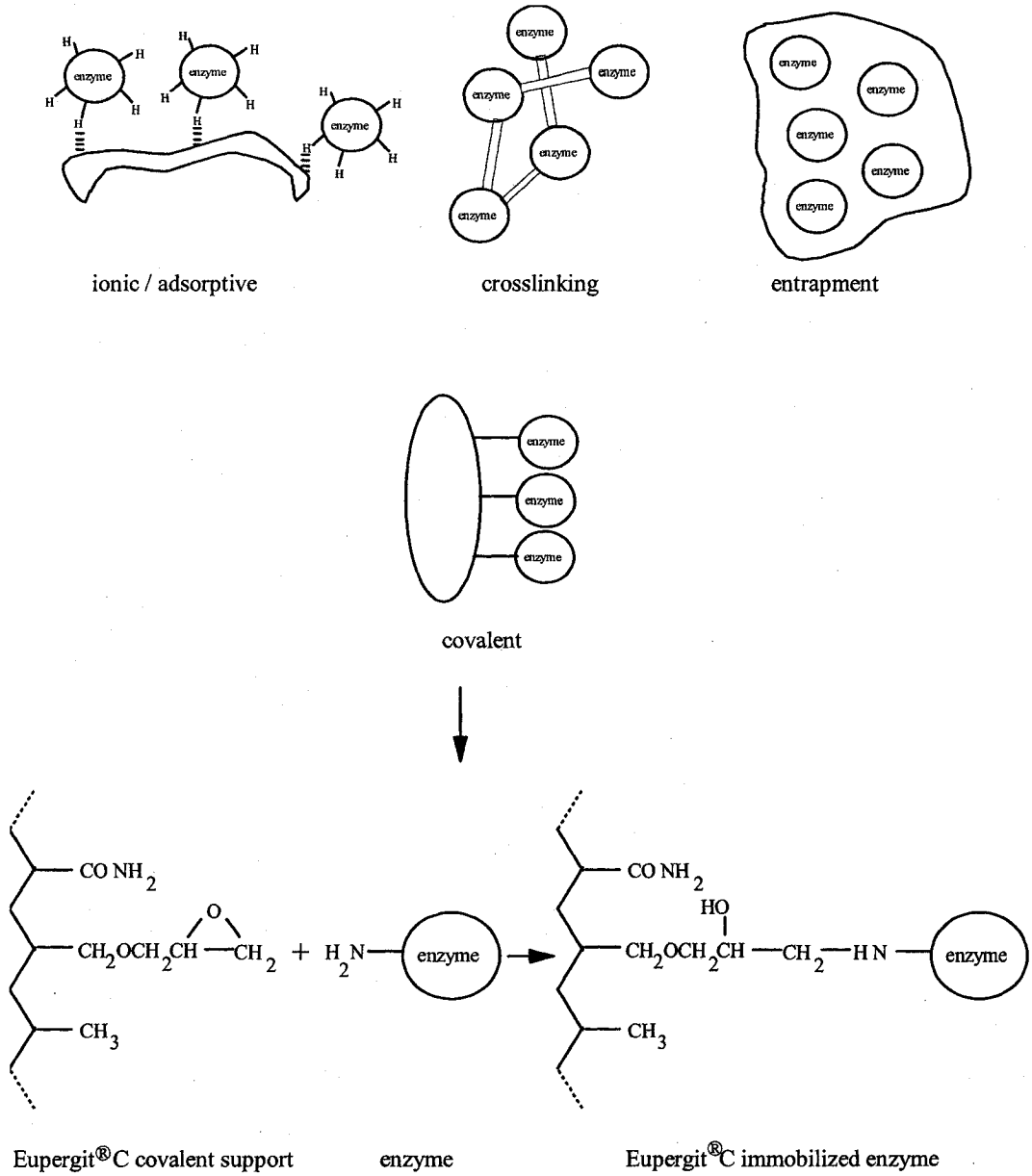

Figure 6. Various immobilization strategies including an expanded detail of Eupergit® C covalent support (modified from Kim *et al.*, 2006; Katchalski-Katzir and Kraemer, 2000).

2.5.2. Ionic/Adsorptive Supports

Ionic or adsorptive immobilization is a simple method of enzyme-support association involving weak interactions, including van der Waals forces, ionic binding, hydrogen bonding and hydrophobic interaction (Messing, 1976). Little damage results from this form of immobilization, due to the very low energy of the many weak bonds formed between enzyme and matrix. A drawback of this technique is that dissociation of enzyme from support (called leaching or leaking) (Bickerstaff, 1997). Also a limiting factor can be the inverse relationship between enzyme immobilization efficiency and enzyme concentration; this may serve to limit the applicability of a particular enzyme to a biocatalysis (Bickerstaff, 1997).

Supports of many types can be used for ionic immobilization of enzymes, however, those which offer microporous surfaces and high surface area to volume ratios are often the best choices. Inorganic silicates, alumina, active carbon or even biopolymers like chitosan are popular choices for immobilization as they offer good efficiencies for a broad cross section of enzymes and are relatively inexpensive (Messing, 1976). Parameters that are important in ionic immobilization include temperature of the coupling, enzyme/support ratio and agitation speed. Further, pH can impact on the efficience of coupling, as well as the stability of the immobilized enzyme.

2.5.3. Crosslinking Supports

Often times the use of cross-linking is applied to ionic matrices to improve immobilization efficiency. The use of glutaraldehyde has proven very successful for stabilizing enzymatic activity as it serves to covalently bind the enzyme to ionic matrices and is commonly used with dextran, talc and gelatin supports (Kim, 2006). Other crosslinking reagents may act on the protein alone. Sometimes called bi-functional reagents, they possess reactive groups at either ends of their molecular structure. Included in this family is dimethyl suberimidate, disuccinimidyl suberate and *bis*[2-(succinimidooxy-carbonyloxy)ethyl]sulfone, which react with lysine residues to crosslink proteins of the same type together (Kim *et al.*, 2006).

2.5.4. Entrapment

The immobilization of enzymes by entrapment entails the surrounding of the enzyme within the matrix and is commonly applied gels and to fibers. Gel entrapment involves securing the enzyme within the hydrated cavities of a water insoluble gel such as cellulose, gelatin or polyacrylamide (Wang, 2000). Alternatively, the use of fiber entrapment broadens the parameters of appropriate solvents, pH range and temperature that may cause gels to become unstable or depolymerize (Wang, 2000; Matsui, 2006).

2.5.5. Covalent Supports

Covalent immobilization involves the covalent bonding of an enzyme to the matrix. The bond formed occurs generally between an active group in the matrix and an (NH_2) amino group present in the side chain of lysine or the guanidino of arginine. Other matrices involve the covalent bonding of hydroxyl groups from serine, threonine or tyrosine, the carboxyl functionality of aspartic or glutamic acid, or the sulfhydryl group of cysteine. Included in this group of supports are Affi-Gels as well as Eupergit® C (Katchalski-Katzir and Kraemer, 2000). The Affi-Gels 10 and 15 are specific for amino groups, Affi-Gel 102 for carboxylate groups and Affi-Gel 501 for sulfhydryl groups.

2.5.6. Immobilization of Selected Lipoxygenases

2.5.6.1. Choice of support and Coupling conditions

The key aspects of several LOX immobilization studies are summarized in Table 5a.

2.5.6.2. Effect of Immobilization on LOX Biocatalysis

Kinetic parameters for immobilized LOX suggest a decrease in overall reaction velocity and an increase in K_m. Oxirane acrylic immobilized potato LOX activity had a higher K_m (0.312 mM) than that of the free (0.155 mM) (Carmen Pinto *et al.*, 1997). Polyacrylamide immobilized soybean LOX possessed a K_m of 0.145 and a V_{max} was 2.08 versus a K_m of 0.077 mM and a V_{max} of 6.25 μmol/mg/min for the free (Carmen Pinto *et al.*, 1996). The V_{max} for soybean LOX immobilized on phyllosillicates was 0.023 and the K_m was 1.7 mM, whereas those for the free were 56 nanomol/mg/min and 2.5 mM of linoleic substrate, respectively (Hsu *et al.*, 1998).

Table 5a. Parameters of several lipoxygenases (LOX) immobilizations onto various supports.

Source	Support	Activation Step	Enzyme/Support Ratio	Coupling Conditions	References
Soybean	Eupergit ® C250L/EDA[a]	-	100 mg / 1 g	4°C, pH 9.0, 16 h	Vega *et al.*, 2005*a*
Soybean	Dowex 50WX4-200	-	1 mg /3 mL	4°C, pH 9.0, 3 h	Kermasha *et al.*, 2002*a*
Soybean	gelatin	glutaraldehyde (2.5 %, w/v)	10 000 UI / 20 mg	38°C, pH 7.5, 1 h	Timur *et al.*, 2002
Soybean	glass particles	HNO_3 (5 %, w/v), 1 h	2.6 mg /1 mL	25°C, pH 9.0, 1 h	Santano *et al.*, 2002
Soybean	phyllosilicate clay	3× wash in NaCl (1M)	20 mg / 200 mg	25°C, pH 9.0, 12 h	Hsu *et al.*, 1999
Potato	oxirane acrylic beads	-	0.7 mg / 1 mL	25°C, pH 7.5, 24 h	Carmen Pinto *et al.*, 1997
Pea seed	talc	130°C, 2 h and cooled	28.2 mg / 250 mg	4°C, pH 6.4, 12 h	Liagre *et al.*, 1996

[a]Eupergit ® C250L/EDA was prepared using ethylenediamine tetracetate according to the procedure outlined by Mateo *et al.* (2001).

Table 5b. Parameters of several hydroperoxide lyase (HPL) immobilizations onto various supports.

Source	Support	Activation Step	Enzyme/Support Ratio	Coupling Conditions	References
Mung bean	Ultralink Iodoacetyl resin	-	1.98 mg / 2 mL	25°C, pH 7.0, 1.25 h	Rehbock and Berger, 1998
	Affi-Gel 15	-	1.98 mg / 2 mL	25°C, pH 7.0, 1.25 h	
Chlorella sp.	Affi-Gel 10	-	60 µg / 1 g	25°C, pH 7.0, 1.5 h	Nuñez *et al.*, 1997

The pH optimum for both free soybean LOX and that immobilized on gelatin was 9.0 (Timur *et al.*, 2002). Hsu *et al.* (1997) reported no change in pH optimum, as did Carmen Pinto and Macías (1996) for free versus polyacrylamide immobilized LOXs. Oxirane acrylic immobilized potato LOX had a pH optimum of 6.5, while that of the free was 6.0 (Carmen Pinto *et al.*, 1997). The effect of reaction temperature for LOX immobilized in gelatin showed an optimum at 30°C and that of LOX immobilized on phyllosillicates was 25°C, which are close to values reported for the free (Hsu *et al.*, 1998; Timur *et al.*, 2002). Oxirane acrylic immobilized potato LOX had an optimum temperature of 35°C, whereas it was 30°C for the free (Carmen Pinto *et al.*, 1997).

Recycling of the LOX immobilized in solgel indicated no decrease in activity after 5 cycles (Hsu *et al.*, 1997), wheras that of oxirane acrylic immobilized potato LOX (Carmen Pinto *et al.*, 1997) possessed 80% activity after 4 cycles of reuse and ~70% at the 9th cycle. Polyacrylamide immobilized soybean LOX was shown to retain all of its activity when recycled 20 times, provided the end products were removed from the medium (Carmen Pinto *et al.*, 1996). Immobilized potato LOX was less inhibited by the addition of NDGA (0.2 mM) than the free with 40% and 20% residual specific activities remaining, respectively (Carmen Pinto *et al.*, 1997).

Both free and immobilized potato LOX produced almost identical ratios of 9 and 13-regio-isomeric HPOD end products as the free (Carmen Pinto *et al.*, 1997). A similar result was reported for polyacrylamide immobilized soybean LOX (Carmen Pinto *et al.*, 1997). Substrate specificity of soybean LOX type-1B immobilized on phyllosillicates showed a preference for 1,3-dilinolein over free linoleic acid, which was different than the substrate preference reported for free LOX (Hsu *et al.*, 1998). The order of substrate preference was 1,3-dilinolein>linoleic acid>1-monolinolein>methyl oleate>trilinolein (Hsu *et al.*, 1998).

The LOX enzymatic assays used for immobilized extracts tend to be those which are less sensitive to potential interferences by the support. Xylenol orange colorimetry was frequently applied (Hsu *et al.*, 1997 and 1998; Vega *et al.*, 2005*a*,*b*), as was polarography (Carmen Pinto *et al.*, 1997).

2.5.6.3. Effect of Immobilization on LOX Stability

Soybean LOX type-1B immobilized on phyllosillicates was stable for up to 3 months when stored dry however when in aqueous conditions the immobilized LOX lost ~40% activity after 10 h (Hsu *et al.*, 1998). The authors postulated that decreased activity was related to protein leaching, particularly at neutral pH (Hsu *et al.*, 1998). Hsu *et al.* (1997) reported that soybean LOX immobilized in solgel displayed no decrease in activity for 25 days at 25°C, versus the free which possessed no activity after 24 h. Enhanced stability in oxirane acrylic immobilized potato LOX was demonstrated by a maintainance of ~90% activity after 30 days at 4°C, whereas the free LOX possessed no activity after an identical treatment (Carmen Pinto *et al.*, 1997). Polyacrylamide immobilized soybean LOX was also shown to be more stable than the free after storage at 4°C for 45 days, the immobilized LOX maintained 60% residual LOX activity versus 28% for the free (Carmen Pinto *et al.*, 1997).

2.5.7. Immobilization of Selected Hydroperoxide Lyases

2.5.7.1. Choice of support and Coupling conditions

The key aspects of several HPL immobilization studies are summarized in Table 5b.

2.5.7.2. Effect of Immobilization on HPL Biocatalysis

Few values for kinetic parameters of immobilized HPLs have been reported, however authors mention a relative decrease in V_{max} and an increase in K_m upon immobilization. Talc immobilized HPL was reported to be 20% less active than the free enzyme (Liagre *et al.*, 1996). The velocity of UltraLink immobilized HPL from mung bean was reported to be 1 μmol hexenal/mg protein, which is lower than values reported for the free HPL (Rehbock and Berger, 1998). The optimal pH values for free and talc immobilized *Pisum sativum* HPL were 7.0 and 6.4, respectively; those for both the free and Affi-Gel immobilized *C. pyrenoidosa* HPL were 6.4 (Nuñez *et al.*, 1997).

Inhibition of immobilized *P. sativum* HPL was conducted using putative LOX inhibitors, including NDGA and showed less sensitivity to inhibitors than did the free

HPL (Liagre *et al.*, 1996). The IC_{50} value indicated a ~9 fold greater activity in the immobilized trial compared to that of the free when NDGA (10 μM) was added to each extract. The authors cautioned that the effect of non-specific adsorption of the inhibitor to the talc could be responsible for some of the effect (Liagre *et al.*, 1996). Inhibition studies on the free and immobilized talc HPLs from *P. sativum* the order of inhibition was NDGA > 2-hydroxycinnamic acid > 4-hydroxycinnamic acid > 3-hydroxycinnmic acid>caffeic acid > 3,4-dihydroxycinnamic acid (Liagre *et al.*, 1996). For UltraLink immobilized HPL from mung bean inhibition by NDGA (0.5 μM) was the same as that for the free HPL (Rehbock and Berger, 1998).

Products of talc immobilized *P. sativum* HPL were identical to the free, including 2,4-decadienal. Hexenal end product from UltraLink immobilized HPL from mung bean was also identical to the free HPL (Rehbock and Berger, 1998).

The direct spectrophotometric monitoring of the production of 2,4-decadienal (MEC 22,000 M^{-1} cm^{-1}, 284 nm) was performed to determine the activity of talc immobilized HPL (Liagre *et al.*, 1996). For immobilized *C.. pyrenoidosa* HPL the conversion of 13-HPOD of linoleic acid was monitored by a decrease in absorbance at 234 nm (Nuñez *et al.*, 1997). The HPL activity of immobilized HPL from mung bean was assayed by extraction of the hexenal end product with pentane/diethyl ether (1:1.12, v/v) and was subjected to GC analysis (Rehbock and Berger, 1998).

Nuñez *et al.* (1997) reported recycling using Affi-Gel 501 HPL maintained 97% of the original activity after 5 cycles of reuse (Nuñez *et al.*, 1997). Rehbock and Berger (1998) reported on the the reusability of UltraLink Iodoacetyl immobilized HPL from mung bean decreased to 40, 13 and 5% at the 2^{nd}, 3^{rd} and 4^{th} cycles, respectively.

2.5.7.3. Effect of Immobilization on HPL Stability

Stability studies on talc immobilized HPL showed an increase in long-term stability at 4°C compared to the free, maintaining >60% of the activity after 30 days, whereas the free possessed 25% at the same interval. The Affi-Gel immobilized *C. pyrenoidosa* HPL was stable at 5°C for 4 months; whereas that of UltraLink immobilized

HPL from mung bean had a relative activity of 86% (versus 30% for the free) after 18 days at 4°C (Nuñez *et al.*, 1997; Rehbock and Berger, 1998).

CHAPTER III

STATEMENT OF CHAPTER III LINKAGE

Chapter III involves the characterization of LOX activity from the mold *Aspergillus niger,* in terms of substrate and end product specificities as well as kinetic parameters. The investigation of LOX from *A. niger* was part of ongoing work in our laboratory on a variety of microorganisms, where experimental work was previously conducted on enzymatic extracts from *Penicillium camemberti* and *Penicillium roqueforti.*

CHAPTER III

CHARACTERIZATION OF AN ENRICHED LIPOXYGENASE EXTRACT FROM *ASPERGILLUS NIGER* IN TERMS OF SPECIFICITY AND NATURE OF FLAVOR PRECURSORS PRODUCTION

3.1. Abstract

Aspergillus niger was grown for 6 days and harvested biomass was homogenized; the resultant supernatant, considered as the crude enzymatic extract, was enriched by ammonium sulfate pecipitation. The extract was assayed for its lipoxygenase (LOX) activity using a wide range of polyunsaturated fatty acids (PUFAs), including linoleic, linolenic and arachidonic acids as substrates. Two pH optima were determined at 5.0 and 10.5. The K_m and V_{max} values indicated that the microbial LOX displayed preferential substrate specificity towards linolenic acid at low pH. It was shown that the LOX activity of *A. niger* produced all monohydroperoxy regio-isomers of the PUFAs and there was a predominance of conjugated diene hydroperoxides. Significant production of the unconjugated 10-hydroperoxides of both linoleic and linolenic acids were obtained by the LOX activity. The amounts of 10-hydroperoxides ranged from 15 to 21% of the total produced isomers, for linolenic and linoleic acids, respectively. Values of 10-hydroperoxide ranged from 15-21%, for linolenic and linoleic acids, respectively. The greatest proportion of the 10-regio-isomer was attributed to the acidic optimum of activity at pH 5.0. Four major hydroperoxy-eicosatetraenoic acid (HPETE) regio-isomers were isolated from the bioconversion of arachidonic acid, including the 8-, 9-, 12- and 15-HPETE, which accounted for approximately 97% of total isomers.

3.2. Introduction

Lipoxygenase (LOX; EC 1.13.11.12) is distributed widely in nature and is responsible for the enzymatic conversion of 1(*Z*),4(*Z*)-pentadiene containing polyunsaturated fatty acids (PUFAs) into stereo-specific hydroperoxide isomers, by antarafacial insertion of molecular oxygen at the methylene carbon (Su and Oliw, 1998). Although plant LOXs have been studied widely, there has been a paucity of studies investigating the role of these dioxygenases in microorganisms (Feron *et al.*, 1996; Fuller *et al.*, 2001). However, selected microorganisms have been investigated, including bacterial species (Iny *et al.*, 1993*a*,*b*), algae (Beneytout *et al.*, 1989; Bisakowski *et al.*, 1995*c*, 1997; Kuo *et al.*, 1996) and industrially important yeasts such as *Saccharomyces cerevisiae* (Shechter and Grossman, 1983; Bisakowski *et al.*, 1995*d*).

LOX has been implicated in the biogeneration of both desirable and undesirable volatile flavor compounds in foods, stemming from an initial oxidation of PUFAs that resulted in the formation of PUFA hydroperoxides of linoleic (HPODs), linolenic (HPOTs) and arachidonic (HPETEs) (Kinderlerner, 1989). These hydroperoxides have long been considered flavor precursors and subsequent enzymatic activities such as hydroperoxide lyase (HPL) have been shown to convert them into flavor compounds (Kühn *et al.*, 1987; Hawkins *et al.*, 1988; Kinderlerner, 1989; Martini and Iacazio, 1997; Perraud and Kermasha, 2000, Kermasha *et al.*, 2002*b*).

The present work is part of ongoing research in our laboratory aimed at the biotechnological applications of microbial enzymes in the biogeneration of natural flavors from lipid substrates (Bisakowski *et al.*, 1995*a*,*b*,*c*,*d*, 1997; Perraud *et al.*, 1999; Perraud and Kermasha, 2000; Kermasha *et al.*, 2002*b*). The specific objectives of this study were to recover and to characterize LOX in the fungus *A. niger*, in terms of optimal pH, substrate specificity and other kinetic parameters as well as the structural characterization of end products, considered to be flavor precursors.

3.3. Materials and Methods

3.3.1. Culture Growth and Harvesting Conditions

The growing medium used for *A. niger* contained glucose (50.0 g/L), NH_4NO_3 (3.0 g/L), K_2HPO_4 (2.0 g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/L), $ZnSO_4 \cdot 7H_2O$ (16.0 mg/L), $FeSO_4 \cdot 7H_2O$ (12.0 mg/L), $CaCl_2 \cdot 4H_2O$ (10.0 mg/L), $MnCl_2 \cdot H_2O$ (5.0 mg/L) and $CuSO_4 \cdot 5H_2O$ (2.0 mg/L) according to the procedure outlined by Kermasha *et al.* (1993). *A. niger* was induced to sporulate and the resultant spore suspension was counted using a Neubauer Counting Chamber (Hausser Scientific, Horsham, PA), according to the procedure outlined by Perraud *et al.* (1999). The cultures of *A. niger* were incubated at 27°C using an orbital shaker at 100 rpm, the glucose conversion was followed up. After harvesting, the biomass was filtered, lyophilized and stored at –80°C.

3.3.2. Preparation and Enrichment of the Enzymatic Extract

The lyophilized biomass was homogenized using 0.45 to 0.50 mm diameter glass beads in an MSK cell homogenizer (Braun, Melsungen, Germany) for 2×2 min. The resultant suspension was centrifuged (12,000×*g*, 15 min) and the supernatant subjected to lyophilization using a FreezeZone® stoppering tray dryer (Labconco, Kansas City, MS). The lyophilized LOX was defatted and subjected to DNA removal as described by Bisakowski *et al.* (1995*a*); the resultant defatted microbial extract was considered as the crude enzymatic extract (FI). All subsequent steps were performed at 4°C, unless otherwise indicated.

The enrichment of the crude enzymatic extract was performed with ammonium sulfate precipitation at 0 to 30, 30 to 70 and 70 to 100% of saturation; the precipitated fractions, FIIa, FIIb and FIIc, respectively, were dialyzed and lyophilized as outlined by Bisakowski *et al.* (1995*a*).

3.3.2. Protein Measurement

The protein content of the enzymatic fractions was measured with a modified Lowry method (Hartree, 1972), using bovine serum albumin (Sigma Chemical Co., St. Louis, MO) as the standard for the calibration curve.

3.3.3. Substrate Preparation

The substrates used throughout this study, included linoleic acid (*cis*-9, *cis*-12-octadecadienoic acid), linolenic acid (*cis*-9, *cis*-12,*cis*-15-octadecatrienoic acid), arachidonic acid (*cis*-5,*cis*-8,*cis*-11,*cis*-14-eicosatetraenoic acid), monolinolein (1-mono[(*cis*,*cis*)-9,12-octadecadienoyl]-*rac*-glycerol), dilinolein (1,3-di[(*cis*,*cis*)-9,12-octadecadienoyl]-*rac*-glycerol) and trilinolein (1,2,3-tri[(*cis*,*cis*,*cis*)-9,12-octadecadienoyl]-*rac*-glycerol), were purchased from Nu-Chek-Prep Inc. (Elysian, MN) The substrate stock solutions were prepared at a concentration of 7.5×10^{-3} M in the appropriate buffer solutions (0.1M) according to the procedure outlined by Perraud *et al.* (1999).

3.3.4. Enzyme Assay

LOX activity was assayed spectrophotometrically using a Beckman DU-650 spectrophotometer (Beckman Instruments Inc., San Raman, CA) according to the procedure outlined by Perraud and Kermasha (2000). The specific activity was defined as nanomol of conjugated diene hydroperoxide produced per mg protein per min, using a molar extinction coefficient of 25,000 M^{-1} cm^{-1} (Surrey, 1964). LOX activity was also assayed polarographically using a Clark oxygen electrode (YSI, Yellow Springs, OH) and a Gilson 5/6 polarograph (Gilson Medical Electronics Inc., Middleton, WI) according to the procedure outlined by Grossman and Zakut (1979).

3.3.5. Effect of pH

The effect of pH on LOX activity was investigated using a wide range of pH buffer solutions (0.1 M), including citrate phosphate for the pH of 4.0 to 5.5; sodium phosphate for the pH of 6.0 to 8.0; glycine-NaOH for the pH of 11.0 to 11.5; and KCl-NaOH for the pH of 12.0 to 13.0.

3.3.6. Production of Hydroperoxide Standards

The different PUFA hydroperoxide standards were obtained using selected endogenous LOXs. The 9-HPOD and 9-HPOT were produced by tomato LOX according to the procedures outlined by Matthew *et al.* (1977). The 5-HPETE was produced by tomato LOX according to the procedure outlined by Regdel *et al.* (1994). The 13–

HPOD, 13-HPOT and 15-HPETE were produced by soya LOX type-1B, according to the procedure outlined by Hamberg and Samuelsson (1967).

3.3.7. Production and Recovery of Hydroperoxide End products

The enriched *A. niger* LOX extract FIIb (10 mg protein) was incubated with 30 mL of the substrate (7.5×10^{-3} M), prepared in buffer solutions (0.1 M) of citrate phosphate (pH 5.5) and glycine-NaOH (pH 10.5), according to the procedure outlined by Perraud and Kermasha (2000). Solid phase extraction (SPE) (Supelclean^{TM} LC-Si 6 mL, Supelco Inc., Bellefonte, PA) was used to purify the hydroperoxides prior to normal phase high-performance liquid chromatography (NP-HPLC) according to the procedure outlined by Toschi *et al.* (1995).

3.3.8. High-performance Liquid Chromatography of Hydroperoxide End products

The HPODs, HPOTs and HPETEs from the enzymatic oxidation of the selected PUFA substrates were reduced to HODs, HOTs and HETEs, respectively, using $NaBH_4$ according to the procedure described by Bisakowski *et al.* (1997). The hydroperoxide regio-isomers were subjected to HPLC using a Beckman Gold system (Beckman Instruments, Fullerton, CA) and an NP-HPLC alphabond silica column (300×3.9 mm i.d., 5 μm; Alltech Associates Inc., Deerfield, IL), using an ultra-violet diode array detector (UV-DAD) (Beckman, model 168 and an evaporative laser light scattering detector (ELSD) (Varex Corporation, Burtonsville, MD), according to the procedure outlined by Perraud and Kermasha (2000). The hydroperoxides were subjected to gas-liquid chromatography/mass spectrometry (GC/MS) analyses.

3.3.9. Gas-Liquid Chromatography/Mass Spectrometry of Hydroperoxide End products

The HODs and HOTs were derivatized into their corresponding methyl trimethylsilyloxystearate (MTMS stearate) and HETEs into their methyl trimethylsilyloxyarachidate (MTMS arachidate) derivatives according to the procedure described by Bisakowski *et al.* (1997). The derivatized hydroxides were analyzed on an HP 6890 Series GC System (Hewlett-Packard Co., Palo Alto, CA), with computerized integration and data handling and a 5973 Mass Selective Detector (Hewlett-Packard), as outlined by Perraud and Kermasha (2000). Manual tuning of the mass spectrometer was

accomplished with the use of perfluorotributylamine (PFTBA) calibrant, while selecting ion *m/z* ratios of 100, 264 and 414 for improved resolution.

3.4. Results and Discussion

3.4.1. Culture Growth of A. niger

The growth cycle of the *A. niger* was monitored in terms of changes in dry weight biomass, glucose, pH values and LOX activity. Figure 7 shows that the biomass of *A. niger* increased rapidly, 0.1 to 1.75 g dry weight/L, from 2 to 5 days, respectively, reaching a maximal mycelial mass (1.79 g dry weight/L) and LOX activity (37.7 nanomol diene/mg protein/min) after 6 days of growth. The findings are in agreement with those reported for *Penicillium camemberti* by Perraud and Kermasha (2000). Available glucose decreased from 50 to 40 g/L after 5 days of growth; however, the slow decline of glucose concentration is in contrast to that reported for *Geotrichum candidum* cultures, where complete utilization was observed after 5 days of incubation (Perraud *et al.*, 1999). A rapid drop in *A. niger*'s medium pH, from 4.5 to 2.2, resulting from the accumulation organic acids, was evident after 24 h of incubation, with a slight, steady decline over the following 13 days of the growth period to pH 1.4 (Tortora *et al.*, 1997).

3.4.2. Enrichment of LOX Enzymatic Extract

Table 6 summarizes the enrichment of the crude LOX enzymatic fraction (FI) from *A. niger*, obtained by ammonium sulfate precipitation at 0 to 30% (FIIa), 30 to 70% (FIIb) and 70 to 100% (FIIc). Fraction FIIb yielded the highest specific activity (84.2 nanomol diene/mg protein/min) and a purification fold of 2.2; this fraction was considered as the enriched LOX enzymatic extract and used for subsequent studies.

Enrichment of LOXs by ammonium sulfate fractionation of microbial extracts were also reported for *Fusarium oxysporum* (Bisakowski *et al.*, 1995*a*), *Chlorella pyrenoidosa* (Bisakowski *et al.*, 1995*c*) and *Saccharomyces cerivisiae* (Bisakowski *et al.*, 1995*d*), with purification folds of 13.3, 51.8 and 3.2, respectively. Purification folds of 2.0 and 2.7 for the LOX extracts from *P. camemberti* (Perraud and Kermasha, 2000) and *G. candidum* (Perraud *et al.*, 1999) are close to that (2.2) determined for *A. niger.*

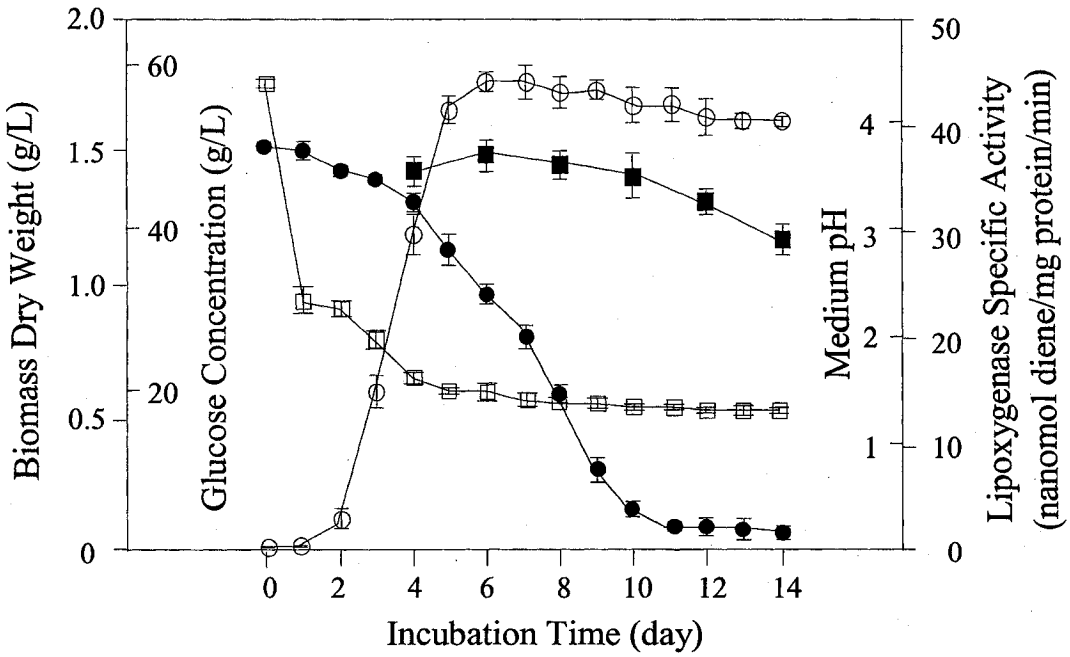

Figure 7. Changes in (●) glucose concentration, (□) media pH, (○) biomass dry weight and (■) lipoxygenase specific activity, during a 14-day incubation period for *Aspergillus niger*.

Table 6. Partial purification scheme for the lipoxygenase extract from *Aspergillus niger*.

Enzymatic fraction	Total protein (mg)[a]	Specific activity[b]	Total activity[c]	Recovery (%)[d]	Purification (fold)[e]
Crude (FI)	1125	38.0	42750	100	1.0
Ammonium sulfate precipitation (FII) at (%) of saturation					
0 - 30 % (FIIa)	25	11.0	275	1	0.3
30 - 70 % (FIIb)	560	84.2	4715	110	2.2
70 - 100 % (FIIc)	114	44.1	5027	11	1.1

[a]Protein concentration was determined as described by Hartree (1972).

[b]Specific activity was defined as nanomol conjugated diene hydroperoxides produced per mg protein per minute.

[c]Total activity was defined as nanomol conjugated diene hydroperoxides produced per min.

[d]Recovery percentage was defined as the ratio of the total activity of an enzymatic fraction to that of the crude, multiplied by 100.

[e]Purification fold was defined as the specific activity of an enzymatic fraction divided by the specific activity of the crude.

3.4.3. Effect of pH on enriched LOX activity

The effect of pH on the LOX activity was investigated using linoleic acid as substrate using both spectrophotometry and polarography, due to homogenate turbidity at acidic pH. The results (Fig. 8) demonstrate the presence of two optima for the LOX activity of the enriched extract from *A. niger*, at 5.0 and 10.5. However, the rate of enzymatic oxidation was determined to be greater when measured by the polarographic method as opposed to that measured spectrophotometrically. A similar trend was observed with the LOX of *G. candidum*, where both spectrophotometric and polarographic methods were employed (Perraud and Kermasha, 1999). The difference in relative higher enzyme activity shown by the polarographic method may be attributed to the nonselective consumption of oxygen by other enzymes present in the reaction homogenate, whereas the spectrophotometric monitoring at 234 nm yields information specific to the production of conjugated diene moieties (Grossman and Zakut, 1979). Kristie and Thomson (1989) reported that a variety of substances in the crude extract can interfere with spectrophotometric absorbance and that polarographic methods can be very effective for purified enzymes.

The presence of both acidic and basic pH optima for microbial LOXs was also reported in the literature. Perraud *et al.* (1999) determined that the LOX activity of *G. candidum* displayed two pH optima, at 3.75 and 8.0. The LOX of the alga *C. pyrenoidosa* (Bisakowski *et al.*, 1995*c*) was found to possess an optimum of activity at pH 4.5, whereas the bacterial LOX from *Thermoactinomyces vulgaris* possessed a single acidic optima at 6.5 (Iny *et al.*, 1993*b*).

3.4.4. Kinetic Studies of Enriched LOX with Selected PUFAs

K_m and V_{max} values for the enriched LOX extract from *A. niger*, using linoleic and linolenic acids as substrates, were calculated from Lineweaver-Burk plots (not shown). The assays at pH 5.0 were conducted spectrophotometrically using 7.5×10^{-4} M substrate solution, whereas those at pH 10.5 were conducted polarographically using 7.5×10^{-3} M according to the procedure outlined by Perraud *et al.* (1999).

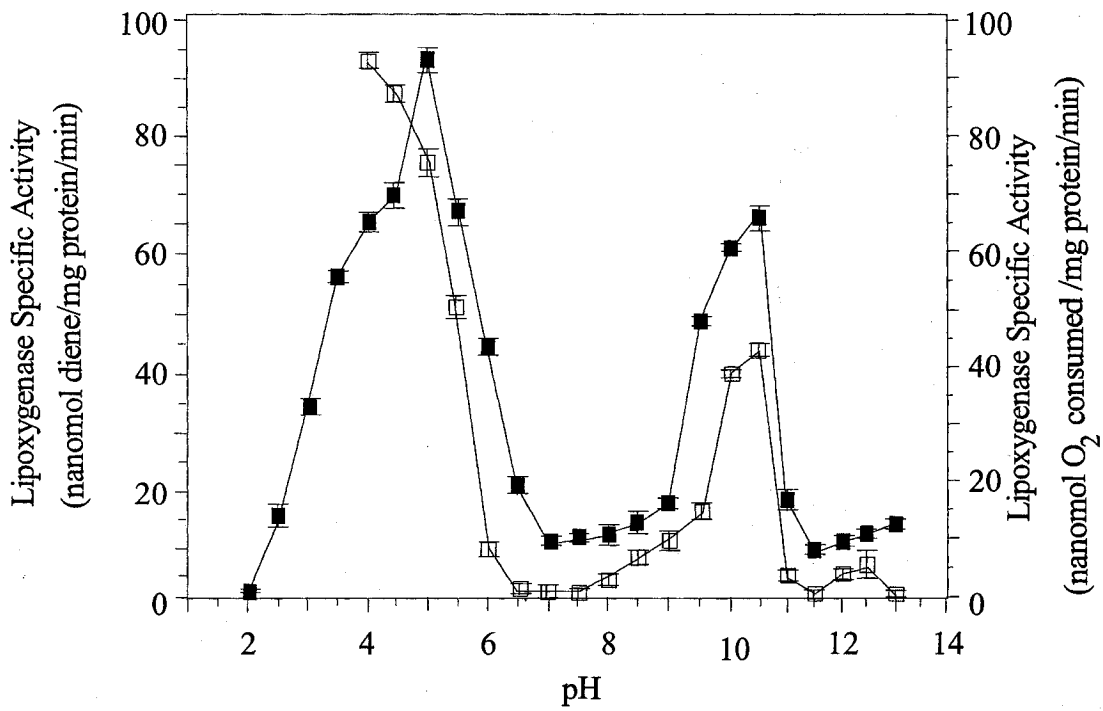

Figure 8. Effect of pH on the lipoxygenase specific activity of an enriched lipoxygenase extract from *Aspergillus niger* using linoleic acid substrate, determined (□) spectrophotometrically at 234 nm and (■) polarographically.

Using linoleic acid as substrate, the K_m values for the enriched LOX were determined to be 0.092 and 1.27 mM at pH values of 5.0 and 10.5, respectively, whereas the V_{max} values indicated a higher rate of oxidation for pH 5.0 than that for 10.5, with 78 and 67 nanomol diene/mg protein/min, respectively. The catalytic efficiency value, calculated as the ratio of V_{max} to K_m, at pH 5.0 (848), was 16-fold greater than at pH 10.5. Perraud *et al.* (1999) reported a 16-fold catalytic activity for the LOX of *G. candidum* at pH 3.75 (1051) compared to that at 8.0 (54).

Using linolenic acid as substrate, the K_m and V_{max} values for the enriched LOX at pH 5.0 were 0.095 mM and 84 nmol diene/ mg protein/ min, respectively. With a catalytic efficiency (8.84×10^{-4}) close to that obtained with linoleic acid. The K_m values reported for the LOXs of *C. pyrenoidosa* (Bisakowski *et al.*, 1995*b*) and *F. proliferatum* (Bisakowski *et al.*, 1995*c*) and *G. candidum* (Perraud *et al.* 1999) are close to that of *A. niger*. Values for the V_{max} of microbial LOXs vary widely, including 401 nanomol diene/mg protein/min for *C. pyrenoidosa* (Bisakowski *et al.*, 1995*b*) and 59 nanomol/mg protein/min for *T. vulgaris* (Iny *et al.*, 1993). The overall results indicate that the lower K_m and higher V_{max} values were obtained for the enriched LOX at pH 5.0.

3.4.5. Substrate Specificity of LOX using Selected PUFAs and Fatty Acyl Esters

Table 7 indicates that the enriched LOX at pH 5.0, demonstrated substrate specificity for linoleic, linolenic and arachidonic acids, of 84, 86 and 72 nanomol diene/mg/min, respectively, whereas that at pH 10.5 was 31, 26 and 28 nanomol diene/mg/min, repsectivley. Perraud *et al.* (1999) noted that the LOX of *G. candidum* displayed a substrate preference for linolenic acid at pH 3.75 and arachidonic acid at pH 8.0. However, most microbial LOXs showed preferential substrate specificity towards linoleic acid, including *F. oxysporum* (Bisakowski *et al.*, 1995*a*), *C. pyrenoidosa* (Bisakowski *et al.*, 1995*b*), *S. cerivisiae* (Bisakowski *et al.*, 1995*d*) and *P. camemberti* (Perraud and Kermasha, 2000).

Table 7 also demonstrates the enriched LOX form *A. niger* showed a preferential usage towards free fatty acids, including linoleic, linolenic and arachidonic acids with

Table 7. Substrate specificity of the lipoxygenase activity of the enriched extract from *Aspergillus niger* (FIIb), obtained by ammonium sulfate precipitation at 30 to 70% of saturation.

	pH 5.0		pH 10.5	
Substrate	Specific activity[a]	Relative Activity (%)[b]	Specific activity[a]	Relative activity (%)[b]
Linoleic acid	84.2	100 (4.9)[c]	31.1	100 (6.8)[c]
Linolenic acid	86.1	102 (4.4)[c]	26.0	84 (13.8)[c]
Arachidonic acid	71.9	85 (5.4)[c]	28.0	90 (11.8)[c]
Monolinolein	42.4	50 (3.3)[c]	0.6	2 (16.7)[c]
Dilinolein	37.5	45 (4.8)[c]	n.d[d]	-
Trilinolein	32.8	39 (4.6)[c]	n.d[d]	-

[a]Specific activity was defined as nanomol diene hydroperoxide produced per mg protein per min and was determined spectrophotometrically monitoring the increase in absorbance at 234 nm.

[b]Relative activity was expressed as a percentage with respect to the specific activity, determined for linoleic acid as substrate, at the relevant pH.

[c]Relative percentage standard deviation was defined as the standard deviation of triplicate samplings, divided by the mean, multiplied by 100.

[d]Not detected.

100%, 102% and 85% relative activity, in comparison to the fatty acid acyl-glycerols, including mono-, di- and trilinolein, with 50%, 45% and 39% relative activity, respectively. Bisakowski *et al.* (1995*c*) reported that LOX from *F. proliferatum* demonstrated low substrate specificity towards fatty acid acyl-glycerols, including mono-, di- and trilinolein, with 32.6, 37.8 and 40.7% relative activity, respectively, whereas Perraud and Kermasha (2000) indicated the same trend with LOX from *Penicillium roqueforti*, with 14, 17 and 15% relative activity, respectively. However, Feussner and Wasternack (1998) reported that LOX from cucumber cotyledon showed a higher substrate specificity towards acyl esters, including trilinolein; these authors suggested that the high substrate specificity might be due to the wide entry afforded by the enzyme's active site.

3.4.6. Effect of Selected Chemicals on LOX Activity

Various chemical antioxidants were investigated for their effect on LOX activity. The results (Fig. 9A) indicate that, at pH 5.0, hydroquinone (HQ; 0.25 mM), butylated hydroxy toluene (BHT; 0.1 mM) and *n*-propyl gallate (*n*-PG; 0.1 M) were slightly activating on the LOX activity of the enriched extract, 37.7, 16.4 and 19.7% increase in enzymatic activity respectively; the chemicals, however, are widely believed to be inhibitory to LOX activity (Kristie and Thompson, 1989; Perraud *et al.*, 1999) and displayed inhibition to LOX at pH 10.5. Antioxidants like *n*-PG and BHT reduce the redox iron in LOX from the catalytically active ferric to the inactive ferrous form (Bisakowski *et al.*, 1995*b*). Iron ligand inhibitors such as ethylenediaminetetraacetate (EDTA) and KCN chelate ferric and ferrous ions, inhibiting LOX activity (Kristie and Thompson, 1989). Eskin *et al.* (1977) reported that the use of HQ, BHT and *n*-PG at acidic pH decreases their effectiveness. An increase in activity of LOX from *G. candidum* was reported at pH 3.75 using selcted chemicals, including HQ, *n*-PG and BHT (Perraud *et al.*, 1999). However, at pH 10.5 the inhibition of the enriched LOX activity by HQ and BHT (Fig. 9B) was demonstrated, with a 67.8 and 29.0% decrease in enzyme activity, respectively. In addition, the effect of *n*-PG could not be investigated since *n*-PG has been shown to auto-oxidize at pH values greater than 9.0 (Kristie and Thompson, 1989). Perraud *et al.* (1999) reported that the LOX activity of *G. candidum* at pH 8.0 was

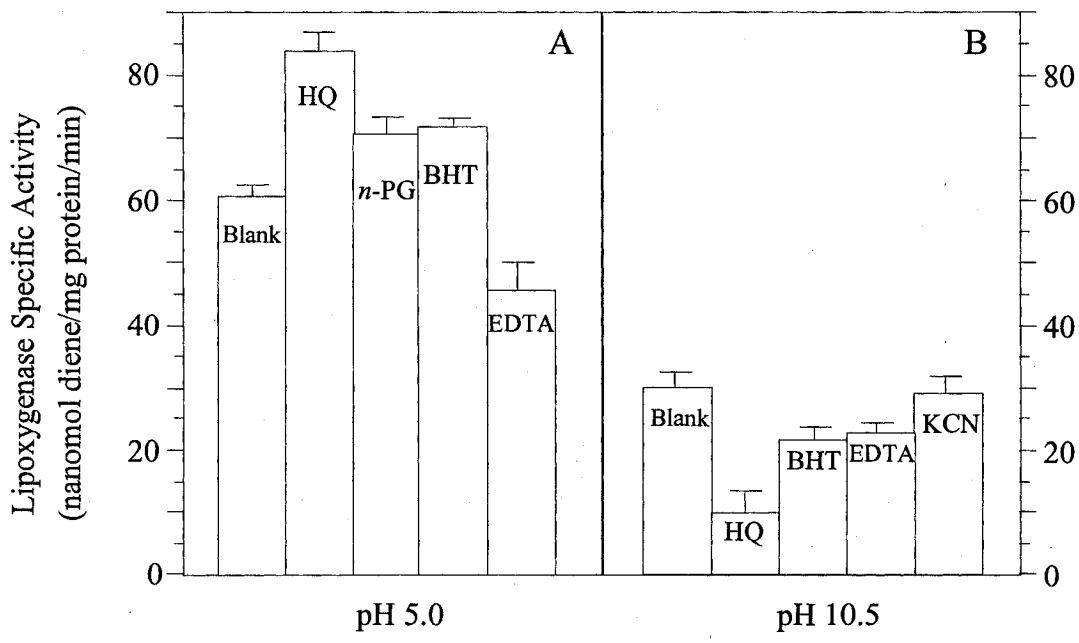

Figure 9. Effect of selected chemical substances on the specific activity of an enriched lipoxygenase enzymatic extract from *Aspergillus niger* at pH (A) 5.0 and (B) 10.5: (Blank) without inhibitor, (HQ) 0.25 mM hydroquinone, (*n*-PG) 0.1 mM *n*-propyl-gallate, (BHT) 0.1 mM butylated hydroxytoluene, (EDTA) 5 mM ethylenediaminetetracetate, and (KCN) 100 mM potassium cyanide.

inhibited by 72 and 30%, using HQ and *n*-PG, respectively. The most effective inhibitor for LOX activity of *A. niger* at pH 10.5 was HQ (Fig. 9B), which resulted in a decrease of 67.8% in the enzyme actitiy. The results (Figs. 9A and 9B) also indicate that addition of 5mM of EDTA inhibited LOX activity of the enriched extract by 26.2 and 25.8% at pH 5.0 and 10.5, respectively. Perraud *et al.* (1999) reported that using 5 mM of EDTA inhibited the LOX activity from *G. candidum* at pH 3.75 by 14.0%.

The experimental findings (Fig. 9B) indicate that the addition of 100 mM KCN inhibited the enzyme activity of LOX from *A. niger* at pH 10.5 by 9.7%. Perraud *et al.* (1999) reported that the addition of 100 mM KCN inhibited the LOX activity of *G. candidum* at pH 8.0 by 61.0%. Bisakowski *et al.* (1995*d*) indicated that the addition of 80 mM KCN inhibited the LOX activity of *F. proliferatum* by 90.0%.

3.4.7. Characterization of LOX End products

Figure 10 shows chromatograms of the NP-HPLC elution profiles of linoleic acid hydroperoxides (Figs. $10A_1$, A'_1, B_1, B'_1, C_1 and C'_1) obtained by the LOX activities of soybean type-1B (Fig. A_1), tomato (Fig. B_1) and of *A. niger* at pH 5.0 (Fig. C_1). Characterization of the separated hydroxides was performed with a UV/DAD detector at 234 nm (Figs. $10A_1$, B_1, C_1) and an ELSD detector (Figs. $10A'_1$, B'_1, and C'_1). The tentative chacterization of HOD, HOT and HETE regio-isomers, obtained from the enriched LOX of *A. niger* was based on the retention times and λ_{max} absorbances of the standards and with reference to the literature on tomato LOX (Matthew *et al.* 1977, Regdel *et al.*, 1993) and on soybean LOX type-1B (Kühn *et al.*, 1987; Hamberg and Samuelsson, 1967).Using linoleic acid substrate, peaks *a* and *a'* were characterized as 13-HOD *cis, trans* and 13-HOD *trans, trans* and coeluted with peaks 1, 13-HOD *cis ,trans* and peak 1', 13-HOD *trans, trans*. Peak *b* was identified as the 9-HOD (*cis, trans*) and co-eluted with peak 4 produced by *A. niger* (Fig. $10C_1$). Peaks 2 and 3 were determined to be the 12-HOD and the 10-HOD, respectively. The NP-HPLC of methyl-linoleate hydroxides (Thomas and Pryor, 1979) and linoleic acid hydroxides (Perraud and Kermasha, 2000) have been characterized; according to these authors, the order of NP-HPLC elution of regio-siomers was 13-, 12-, 10-, 9-regio-isomers, where only the 13- and 9-regio-isomers possess an absorbance at 234 nm.

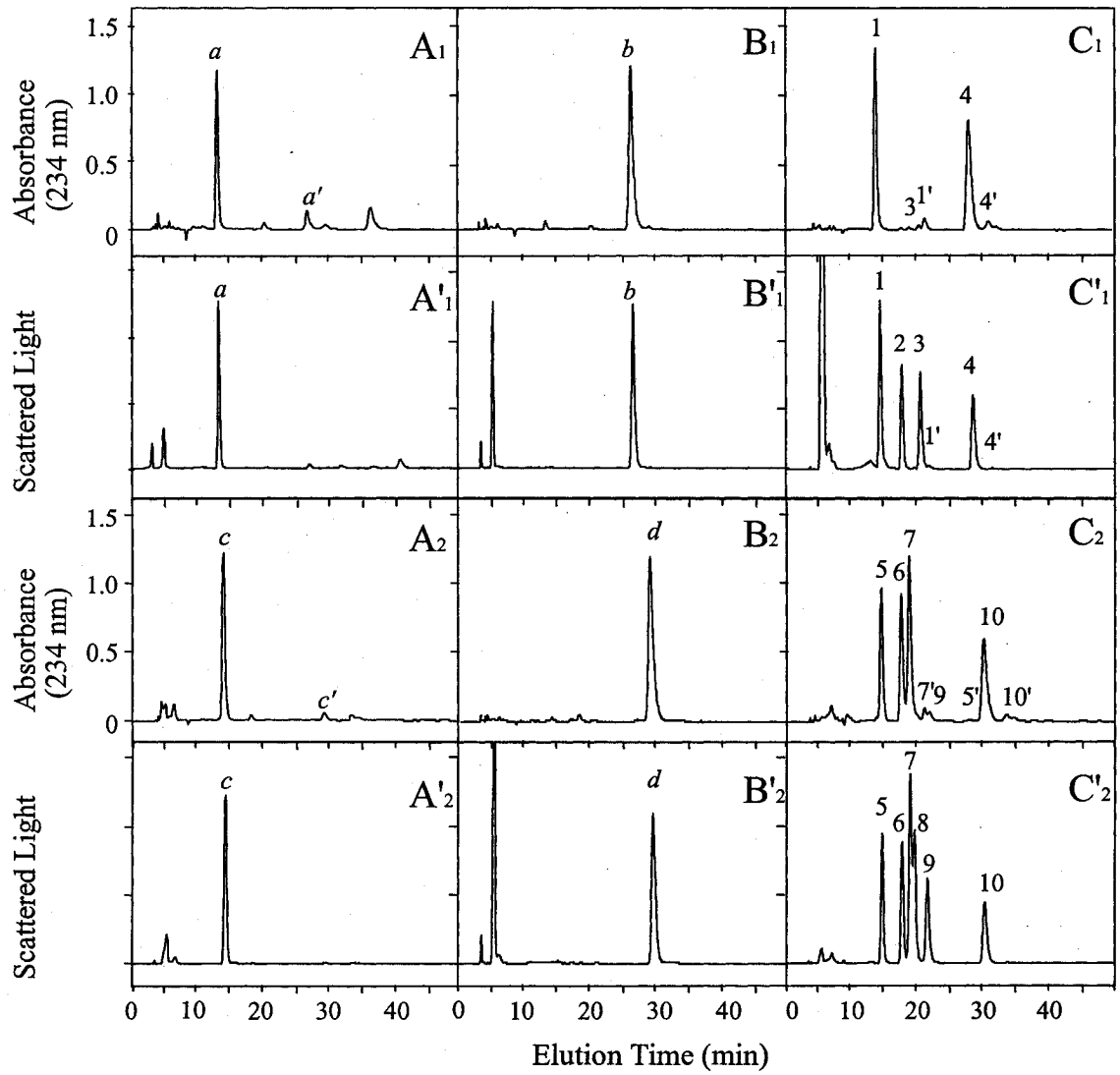

Figure 10. High performance liquid chromatograms of hydroperoxides of linoleic acid (HODs), using LOX from soybean type-1B (A_1 and A'_1), tomato (B_1 and B'_1) and *Aspergillus niger* (C_1 and C'_1), as well as hydroperoxides of linolenic acid (HOTs), using LOX from soybean type-1B (A_2 and A'_2), tomato (B_2 and B'_2) and *A. niger* (C_2 and C'_2), using ultraviolet diode-array detection at 234 nm (A, B and C) and evaporative laser light scattering detection (A', B' and C').

Using linolenic acid as substrate peaks c and c' were identified as 13-HOT *cis, trans* and 13-HOT *trans, trans* respectively (Fig. $10A_2$) and co-eluted with peaks 5 and 5', respectively, obtained by *A. niger* LOX (Fig. $10C_2$). Peak *d*, identified as the 9-HOT (*cis, trans*), was obtained by tomato LOX (Fig. $10B_2$) and co-eluted with peak 10, produced by *A. niger* LOX (Fig. $10C_2$). The HOTs obtained by *A. niger* LOX produced peaks 5 to 10' (Fig. $10C_2$). The use of ELSD characterized peaks 8 and 9, determined to be the unconjugated 10-HOT and 15-HOTs (Fig. $10C'_2$). Spectral scanning of the end products for both linoleic and linolenic acids (not shown), indicated that the double bonds in compounds 1', 4', 5', 7'and 10' are characterized as the 13-, 16- and 9-HOT, possessing *trans, trans* geometry and displaying λ_{max} values close to those of other end produts reported in the literature values (Perraud and Kermasha, 2000). The hydroxide isomers (Figs. $10C'_1$ and $10C'_2$) were hypsochromically shifted to a smaller maximal wavelengths, 232 nm, in comparison to the *cis, trans* isomers at 234 nm (Toshi, *et al.*, 1995).

Fragmentation patterns of GC/MS analyses (Fig. 11) of LOX end products were consistent with the presence of the 13-, 12-, 10- and 9- hydroperoxide regio-isomers of linoleic acid (Bisakowski *et al.*, 1997). Four major peaks each consisting of two intense ion abundances of predicted masses, were characterized for their MTMS derivative regio-isomers (I, II, III and IV).

The relative quantitative production of HOD, HOT and HETE regio-isomers were determined by integrating ion abundances during GC/MS analysis, the results of this integration are shown as percentages of total isomers detected for each of the investigated PUFAs (Table 8).

The major products of the enzymatic oxidation of linoleic acid by the LOX extract of *A. niger* were conjugated diene hydroxides with approximately 60% of 9- and 13-HOD regio-isomers, rising to almost 85% at pH 10.5. These results are in agreement those reported for the bacterial LOX from *T. vulgaris,* which was also noted to catalyze the dioxygenation of linoleic acid into the predominantly conjugated diene hydroperoxide

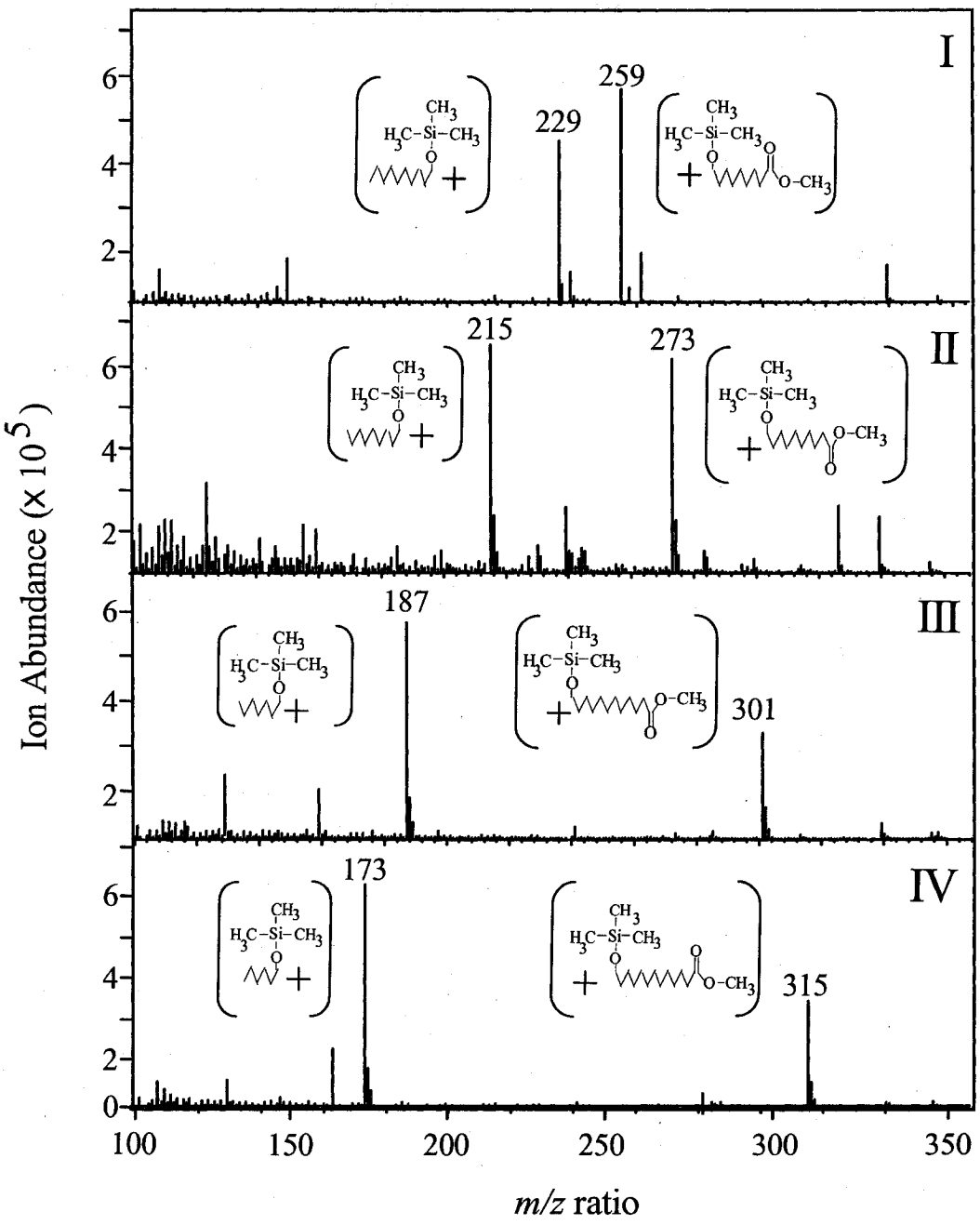

Figure 11. Mass spectra of the methyl trimethylsilyloxy (MTMS) stearate derivatives of hydroperoxides of linoleic acid produced by the lipoxygenase activity of the *Aspergillus niger* enriched enzyme extract: (I) 9-MTMS, (II) 10-MTMS, (III) 12-MTMS and the (IV) 13-MTMS stearate derivatives.

Table 8. Characterization of the relative percentages of hydroperoxide regio-isomers, obtained by the enzymatic activity of an enriched LOX extract from *Aspergillus niger* at two pH optima, using linoleic, linolenic and arachidonic acids as substrates.

Substrate	Hydroperoxide regio-isomer	Hydroperoxide Isomers[a] (%)	
		pH 5.0	pH 10.5
Linoleic acid	9-	30.1 (7.08)[b]	44.8 (3.58)[b]
	10-	21.4 (6.27)[b]	8.0 (8.89)[b]
	12-	18.1 (3.23)[b]	7.9 (5.05)[b]
	13-	30.4 (5.82)[b]	39.3 (2.79)[b]
Linolenic acid	9-	11.8 (8.47)[b]	14.3 (7.53)[b]
	10-	15.4 (5.35)[b]	10.4 (5.04)[b]
	12-	18.3 (3.97)[b]	18.9 (3.46)[b]
	13-	19.6 (4.20)[b]	25.7 (7.52)[b]
	15-	11.0 (5.08)[b]	5.5 (6.57)[b]
	16-	23.9 (3.09)[b]	25.2 (4.34)[b]
Arachidonic acid	8-	6.9 (4.64)[b]	n.d.[c]
	9-	38.7 (5.51)[b]	n.d.[c]
	12-	25.0 (2.80)[b]	n.d.[c]
	15-	29.4 (4.50)[b]	n.d.[c]

[a]The hydroperoxide regio-isomer was quantified by ion abundances, corresponding to the *m/z* ratios of its derivative, detected in GC/MS analyses, and expressed as a percentage relative to all hydroperoxides detected.

[b]Relative percentage standard deviation was defined as the the standard deviation of triplicate samplings divided by the mean, multipled by 100.

[c]Not determined.

species (Iny *et al.*, 1993*a,b*). An appreciable amount of 10-HPOD, 21.4% of total hydroxide characterized, was also obtained by the enriched LOX from *A. niger* at pH 5.0; this value is higher than that obtained by Perraud and Kermasha (2000) for *P. camemberti* at its acidic optima (8%). At higher pH the distribution of unconjugated regio-isomers of linoleic acid decreased to 8.0 and 7.9% for the 10 and 12 regio-isomers, respectively. These results suggest that the LOX of *A. niger* was capable of producing adequate unconjugated hydroperoxides to evolve appreciable amounts of volatile compounds when acted upon by HPL activity. The presence of 10-HPOD has been implicated in the production of volatile eight carbon compounds such as 1-octen-3(*R*)-ol (Wurzenberger and Grosch, 1984). Similarly, the preferential bioconversion of the 10-hydroperoxide of linolenic acid may be an important pathway in the production of compounds such as 1,5-octadiene-3(*R*)-ol, present in seafoods (Josephson *et al.*, 1983) and fungi (Wurzenberger and Grosch, 1986).

GC/MS analyses of end products resulted from the bioconversion of arachidonic acid by the LOX activity of *A. niger* were determined to be predominantly the 8-, 9-, 12- and 15-HPETE. Several studies have determined the oxidation products of bioconversion of arachidonic acid by LOX to be a mixture of HPETE regio-isomers. Kuo *et al.* (1996) determined the production of the 15-, 12- and possibly 8-HPETE from algal source LOX. Tomato source LOX has been shown to yield the 5-, 8- and the 11-HPETE, when incubated with arachidonic acid (Regdel *et al.*, 1994). The LOX of cucumber cotyledons was shown to produce a mixture of 15-, 12- and 8-HPETE (Feussner and Wasternack, 1998). Several authors suggested that these HPETE isomers are "flavor precursors", thought to be substrates for the production of a variety of melonlike fragrances in algae and fish (Hsieh *et al.*, 1988).

3.5. Conclusions

The enriched LOX extract from *A. niger* displayed a wide range of specificity towards free PUFAs, including linoleic, linolenic and arachidonic acids as well as acyl esters of linoleic acid. The enzyme showed two pH optima. Characterization of the end products, the hydroperoxide regio-isomers of PUFAs, confirmed the wide range of selectivity of the LOX from *A. niger*. The overall results suggested that the enriched

LOX of *A. niger* showed many of the characteristics of other microbial LOXs in terms of and nature of flavor precursor end products.

CHAPTER IV

STATEMENT OF CHAPTER IV LINKAGE

Chapter IV involves the characterization of LOX activity from another microorganism, *Penicillium candidum,* in terms of substrate specificities, kinetic parameters and nature of end products. The investigation of LOX from *P. candidum* continued ongoing work in our laboratory on a variety of microorganisms, where experimental work was previously conducted on enzymatic extracts from *Penicillium camemberti*, *Penicillium roqueforti* and *Aspergillus niger* (Chapter III).

CHAPTER IV

PRODUCTION OF FLAVOR PRECURSORS BY *PENICILLIUM CANDIDUM* USING SELECTED POLYUNSATURATED FATTY ACIDS

4.1. Abstract

Biomass of *Penicillium candidum* was harvested and homogenized prior to centrifugation. The resultant supernatant, considered as the crude enzymatic extract, was enriched by ammonium sulfate precipitation. The enriched enzymatic extract was assayed for its lipoxygenase (LOX) activity, using selected polyunsaturated fatty acids (PUFAs) as substrates. Two pH optima for LOX activity were determined at 6.0 and 8.5. The results indicated a wide range of LOX activity towards free PUFAs, including linoleic, linolenic and arachidonic acids, as well as preferential substrate specificity towards linoleic acid over its acyl esters. The catalytic efficiency values, defined as the ratio of V_{max} to K_m, were 2.14×10^{-4}, 1.63×10^{-4} and 1.60×10^{-4} for linoleic, linolenic and arachidonic acids, respectively. It was shown that the LOX activity produced many mono-hydroperoxy regio-isomer of the PUFAs and there was a predominance of conjugated diene hydroperoxides. Significant production (16%) of the unconjugated 10-hydroperoxides of octadecadienoic acid (HPOD) and octadecatrienoic acid (HPOT) was also obtained by the enriched LOX activity. Four major hydroperoxy-eicosatetraenoic acid (HPETE) regio-isomers, including the 8-, 9-, 12- and 15-HPETEs, were obtained from the bioconversion of arachidonic acid. Chiral studies of end products indicated varying degrees of enantio-selectivity, producing excesses in favor of the (*S*) stereo-isomer, for the hydroperoxide end products resulted from the bioconversion of linoleic, linolenic and arachidonic acids.

4.2. Introduction

Lipoxygenase (LOX, EC 1.13.11.12) is an enzyme responsible for the bioconversion of 1(*Z*),4(*Z*)-pentadiene containing polyunsaturated fatty acids (PUFAs) into stereo-specific hydroperoxide regio-isomers, by antarafacial insertion of molecular oxygen at the methylene carbon (Gardner, 1996). Mammalian and plant LOXs have been widely characterized but comparatively few studies have investigated the characteristics of this dioxygenase in microorganisms. Microorganisms that have been investigated include bacterial species (Iny *et al.*, 1993*a*), algae (Bisakowski *et al.*, 1995*c*, Kuo *et al*, 1996) and yeasts such as *Saccharomyces* sp. (Schecter and Grossman, 1983; Bisakowski *et al.*, 1995*d*).

LOX has been reported to contribute to the biogeneration of volatile flavor compounds from the initial oxidation of lipids (Tressl *et al.*, 1981). The regio-isomeric hydroperoxides can be derived from linoleic acid (octadecadienoic acid hydroperoxides, HPODs), linolenic acid (octadecatrienoic hydroperoxides, HPOTs) and arachidonic (eicosatetraenoic acid hydroperoxides, HPETEs), which have been considered flavor precursors, acting as substrates for subsequent enzymatic activities (Bisakowski *et al.*, 1997). One such activity is that of hydroperoxide lyase (HPL), reported to cleave the hydroperoxide, producing a volatile compound such as an alcohol (Kermasha *et al.*, 2002*b*). Selected molds reported to possess LOX activity include *Fusarium* sp. (Satoh *et al.*, 1976; Bisakowski *et al.*, 1995*a*; Bisakowski *et al.*, 1995*b*), *Geotrichum* sp. (Perraud *et al.*, 1999) and *Penicillium* sp. (Perraud and Kermasha, 2000).

The present work is part of ongoing research in our laboratory aimed at the biotechnological applications of selected microbial enzymes in the biogeneration of natural flavours from lipid substrates (Bisakowski *et al.*, 1997; Perraud *et al.*, 1999; Perraud and Kermasha, 2000). The specific objectives of this study were to recover and characterize LOX in the fungus *Penicillium candidum*, in terms of pH optima, substrate specificity and kinetic parameters, as well as the structural characterization of end products considered to be flavor precursors.

4.3. Materials and Methods

4.3.1. Culture Growth and Harvesting Conditions

P. candidum was induced to sporulate and the resultant spore suspension was counted using a Neubauer Counting Chamber (Hausser Scientific, Horsham, PA), according to the procedure outlined by Perraud *et al.* (1999). The cultures of *P. candidum* were incubated in a liquid medium at 20°C, using an orbital shaker at 100 rpm. Changes in pH, glucose concentration, biomass produced and lipoxygenase activity were monitored over a 16-day incubation period according to the procedure reported by Perraud and Kermasha (2000). After harvesting, the biomass was filtered, lyophilized and stored at –80°C.

4.3.2. Preparation and Enrichment of the Enzymatic Extract

The lyophilized biomass was homogenized using 0.45 to 0.50 mm diameter glass beads in an MSK cell homogenizer (Braun, Melsungen, Germany) for 2×2 min. The resultant suspension was centrifuged (12,000×*g*, 15 min) and the supernatant was subjected to lyophilization using a FreezeZone® stoppering tray dryer (Labconco, Kansas City, MS). The lyophilized LOX was defatted and subjected to DNA removal as described by Bisakowski *et al.* (1995*a*); the resultant defatted microbial extract was considered as the crude LOX enzymatic extract (FI). All subsequent steps were performed at 4°C, unless otherwise indicated.

The enrichment of the crude enzymatic extract was performed with ammonium sulfate fractionation at 0 to 20, 20 to 60 and 60 to 100% of saturation; the precipitated fractions, FIIa, FIIb and FIIc, respectively, were dialyzed and lyophilized as outlined by Bisakowski *et al.* (1995*a*).

4.3.3. Protein Measurement

The protein content of the enzymatic fractions was measured with a modified Lowry method (Hartree,1972), using bovine serum albumin (Sigma Chemical Co., St-Louis, MO) as a standard for the calibration curve.

4.3.4. Substrate Preparation

The substrates used throughout this study, included linoleic acid (*cis*-9,*cis*-12-octadecadienoic acid), linolenic acid (*cis*-9,*cis*-12,*cis*-15-octadecatrienoic acid), arachidonic acid (*cis*-5,*cis*-8,*cis*-11,*cis*-14-eicosatetraenoic acid), monolinolein (1-mono[(*cis*,*cis*)-9,12-octadecadienoyl]-*rac*-glycerol), dilinolein (1,3-di[(*cis*,*cis*)-9,12-octadecadienoyl]-*rac*-glycerol) and trilinolein (1,2,3-tri[(*cis*,*cis*,*cis*)-9,12-octadecadienoyl]-*rac*-glycerol) and were purchased from Nu-Chek-Prep Inc. (Elysian, MN). The substrate stock solutions were prepared at a concentration of 4.0×10^{-3} M in the appropriate buffer solutions (0.1 M) according to the procedure described by Perraud *et al.* (1999).

4.3.5. Enzyme Assay

LOX activity was assayed spectrophotometrically using a Beckman DU-650 spectrophotometer (Beckman Instruments Inc., San Raman, CA) and a reaction mixture containing 187 µL of a 4.0×10^{-3} M substrate solution and 33 µg of protein, according to the procedure outlined by Perraud and Kermasha (2000). The specific activity was defined as nanomol of conjugated diene hydroperoxide produced/mg protein/min, using a molar extinction coefficient of 25,000 M^{-1} cm^{-1} (Surrey, 1964).

4.3.6. Effect of pH

The effect of pH on LOX activity was investigated using a wide range of pH buffer solutions (0.1 M), including citrate phosphate for the pH of 4.0 to 5.5, sodium phosphate for the pH of 6.0 to 8.0, glycine-NaOH for the pH of 8.5 to 10.5, phosphate-NaOH for pH 11.0.

4.3.7. Production of Hydroperoxide Isomers

4.3.7.1. Photooxidation Hydroperoxides

The photooxidation hydroperoxide regio-isomers of linoleic and linolenic acids were produced according to the procedure described by Schieberle *et al.* (1984). The reaction medium was composed of 6 mL of the appropriate PUFA, 44 mL of benzene and 6.23 mg of meso-tetraphenyl porphine (Sigma Chemical Co.) as sensitizer. The photooxidation was performed at 15°C by bubbling O_2 into the reaction medium while

the stirred solution was irradiated with light from a 500 W halogen bulb, filtered through a 1 cm layer of deionized water to reduce IR radiation. Following photooxidation the benzene was evaporated using a SpeedVac System® model AES1010 (Savant Instruments, Holbrook, NY).

4.3.7.2 Hydroperoxide Production

The HPOD, HPOT and HPETEs were produced using selected LOXs and HPL activities. The 9(*S*)-HPOD, 9(*S*)-HPOT and 5(*S*)-HPETE were produced by tomato LOX with linoleic, linolenic and arachidonic acids as substrates, respectively, according to the procedure outlined by Matthew *et al.* (1977). Soybean LOX type-1B (Sigma Chemical Co.) biocatalysis using linoleic acid as substrate demonstrated 1 unit of enzyme caused an increase in absorbance at 234 nm of 0.001/min/unit at pH 9.0 at 25°C with 0.34 mg protein/ mg solid. The 13(*S*)–HPOD, 13(*S*)-HPOT and 15(*S*)-HPETE were produced by soybean LOX type-1B according to the procedure described by Hamberg and Samuelsson (1967). The 10(*R*)-HPOD and 10(*R*)-HPOT of linoleic and linolenic acids, respectively, were extracted from an enzymatic homogenate of the edible mushroom *Agaricus bisporus* containing HPL enzymatic activity, after incubation with racemic 10-HPOD and 10-HPOT, respectively, according to the procedure outlined by Wurzenberger and Grosch (1986).

4.3.7.3. Enzymatic Hydroperoxide End products

Hydroperoxide end products were produced by incubation of the enriched LOX extract from *P. candidum* (10 mg protein) with 30 mL of the substrate (4.0×10^{-3} M), prepared in sodium phosphate buffer solution (0.1 M, pH 6.0), according to the method outlined by Perraud and Kermasha (2000).

4.3.8. Purification of Hydroperoxides

Hydroperoxides produced by photooxidation, LOXs from tomato and soybean and the enriched extract of *P. candidum* were purified by solid phase extraction (SPE) (Supelclean™ LC-Si 6 mL, Supelco Inc., Bellefonte, PA) prior to normal phase high-performance liquid chromatography (NP-HPLC), according to the procedure described by Toschi *et al.* (1995).

4.3.9. High-Performance Liquid Chromatography of Hydroperoxide End products

The HPODs, HPOTs and HPETEs, obtained from the enzymatic oxidation of the selected PUFA substrates, were reduced to hydroxides of octadecenoic acid (HODs), octatrienoic acid (HOTs) and eicosatetraenoic acid (HETEs), respectively, using $NaBH_4$ according to the procedure outlined by Bisakowski *et al.* (1997). The hydroxide regio-isomers were separated by HPLC using a Beckman Gold system (Beckman Instruments, Fullerton, CA) and an alphabond silica column (300×3.9 mm i.d., 5 μm; Alltech Associates Inc., Deerfield, IL), using an ultra-violet diode array detector (UV-DAD) (Beckman, model 168) and an evaporative laser light scattering detector (ELSD) (Varex Corporation, Burtonsville, MD), according to the procedure reported by Perraud and Kermasha (2000). Portions of the hydroxides were collected and subjected to chiral phase HPLC (CP-HPLC) and to gas-liquid chromatography/mass spectrometry (GC/MS) analyses.

Hydroxide isomers were characterized using CP-HPLC according to the procedure outlined by Perraud and Kermasha (2000). The stereochemistry of the hydroxide isomers was analyzed with a Chiralcel OD column (Daicel Chemical Industries, Exton, PA), using a solvent mixture composed of hexanes/2-propanol/acetic acid (1000:30:1, v/v/v) at a flow rate of 1.0 mL/min. Detection was performed at 234 nm and with an ELSD detector for conjugated and unconjugated diene hydroxides, respectively.

4.3.10. Gas-Liquid Chromatography/Mass Spectrometry of Hydroperoxide End products

The HODs and HOTs were derivatized into their corresponding methyl trimethylsilyloxystearate (MTMS stearate) derivatives, whereas the HETEs were derivatized into their methyl trimethylsilyloxyarachidate (MTMS arachidate) derivatives, according to the method described by Bisakowski *et al.* (1997). The derivatized hydroxides were analyzed on an HP 6890 Series GC System (Hewlett-Packard Co., Palo Alto, CA), with computerized integration and data handling and a 5973 Mass Selective Detector (Hewlett-Packard), as outlined by Perraud and Kermasha (2000). Manual tuning of the mass spectrometer was accomplished with the use of perfluorotributylamine

(PFTBA) calibrant, while selecting ion *m/z* ratios of 100, 264 and 414 for enhanced resolution.

4.4. Results and Discussion

4.4.1. Culture Growth of P. candidum

Figure 12 summarizes the growth cycle of *P. candidum*, which was monitored in terms of changes in dry weight biomass, glucose conversion, pH values and LOX activity. The biomass for *P. candidum* increased slowly, from 0.0 to 0.06 g dry weight/L, from days 0 to 2. Growth was rapid during days 2 to 8 days, attaining a maximal mycelial biomass of 1.35 g dry weight/L and LOX activity of 14 nanomol diene/mg protein/min. These findings are in agreement with those reported for other *Penicillium* sp., including *P. roqueforti* and *P. camemberti*, which on day 10 attained maximal biomasses of 0.55 and 0.65 g dry weight/L, respectively and LOX activities of 14 and 11 nanomol diene/ mg protein/ min, respectively (Perraud and Kermasha, 2000). Available glucose in the medium decreased from 10.0 to 9.0 g/L after the first two days of culture incubation. The concentration of glucose in the medium decreased rapidly, from 9.0 to 0.02 g/L, from days 2 to 10, respectively. Perraud *et al.* (1999) reported the complete utilization of glucose from cultures of *Geotrichum candidum* after 5 days of incubation. A slow steady decrease in the pH of the *P. candidum* culture medium, from 6.0 to 2.9, was observed over the duration of the 16-day incubation. *P. camemberti* was also reported to display a slow steady decline in pH, however, the final value of 3.75 was less acidic than that obtained for *P. candidum* (Perraud and Kermasha, 2000). Overall, when culture growth changes for *P. candidum* are compared to those of *P. camemberti*, the trend appeared to be a faster growth curve, a more complete utilization of medium glucose and a larger production of biomass for *P. candidum*, however, the two *Penicillium* strains displayed similar crude LOX activities (Perraud and Kermasha, 2000).

4.4.2. Enrichment of LOX Enzymatic Extract

Table 9 summarizes the enrichment of the crude LOX enzymatic fraction (FI) from *P. candidum* which was performed by ammonium sulfate precipitation at 0 to 20 (FIIa), 20 to 60 (FIIb) and 60 to 100% (FIIc) of saturation, respectively. Fraction FIIb yielded the highest specific activity, with 26 nanomol diene/mg protein/min and a

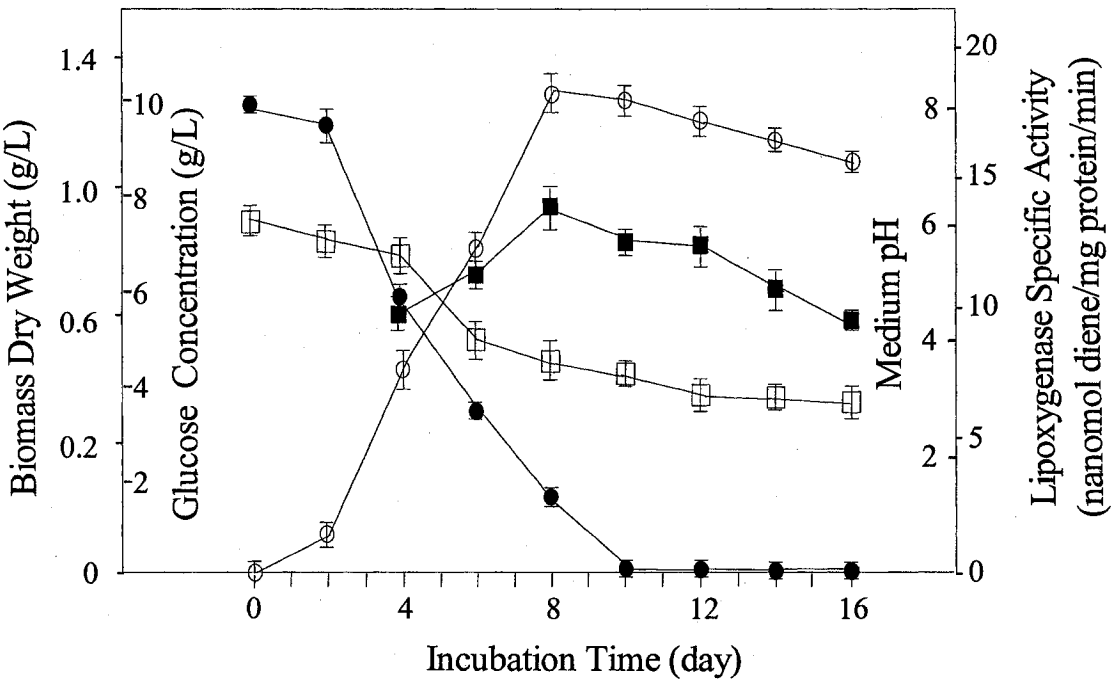

Figure 12. Changes in (○) biomass dry weight, (●) glucose concentration, (□) medium pH, and (■) lipoxygenase activity, during a 16-day incubation period for *Penicillium candidum*.

Table 9. Partial purification scheme for the lipoxygenase extract from *Penicillium candidum.*

Enzymatic fraction	Total protein (mg)[a]	Specific activity[b]	Total activity[c]	Recovery (%)[d]	Purification (fold)[e]
Crude (FI)	585	0.012	7.137	100	1.0
Ammonium sulfate precipitation (FII) at % of saturation					
0 - 20 (FIIa)	28	0.006	0.162	2	0.5
20 - 60 (FIIb)	361	0.026	9.458	132	2.1
60 - 100 (FIIc)	77	0.005	0.385	5	0.4

[a]Protein concentration was determined as described by Hartree (1972).

[b]Specific activity was defined as nanomol conjugated diene hydroperoxides produced per mg protein per minute.

[c]Total activity was defined as nanomol conjugated diene hydroperoxides produced per min.

[d]Recovery percentage was defined as the ratio of the total activity of an enzymatic fraction to that of the crude, multiplied by 100.

[e]Purification fold was defined as the specific activity of an enzymatic fraction divided by the specific activity of the crude.

purification of 2.1 fold; this fraction was considered as the enriched LOX enzymatic extract and used for subsequent studies.

Enrichment of LOXs by ammonium sulfate fractionation has been performed on plant source LOXs, including French bean seed (Kermasha and Metche, 1986) and canola seed sources (Khalyfa *et al.*, 1990) with purification factors of 3.0-fold and 4.4-fold, respectively. Microbial enzymatic extracts have been enriched using ammonium sulfate precipitation, including *Fusarium proliferatum* (Bisakowski *et al.*, 1995*b*) and *Saccharomyces cerivisiae* (Bisakowski *et al.*, 1995*d*), with purification factors of 2.66-fold and 3.2-fold, respectively. Purification factors of 2.0-fold and 2.3-fold for the LOX extracts from *P. camemberti* and *P. roqueforti* (Perraud and Kermasha, 2000), respectively, are close to that determined for *P. candidum*.

4.4.3. Effect of pH on Enriched LOX Activity

Figure 13 illustrates the specific activity of the LOX reaction over a broad range of pH, from 4.0 to 11.0, using linoleic and linolenic acids as substrates and shows two optima, a major one and minor one at pH 6.0 and 8.5, respectively. Galliard and Phillips (1971) reported that LOXs generally display optimal activity between pH 6.0 and 9.0. Axelrod *et al.* (1981) reported that three characterized isozymes of soybean LOX each demonstrated different pH optima using linoleic acid as substrate, with isozyme-1 most efficient at pH 9.0 to 9.5 and isozymes-2 and -3 most active at pH 6.0 to 7.0. Plant source LOXs, including those of canola seed (Khalyfa *et al.*, 1990) French bean seed Kermasha and Metche, 1986) and the green algae *Oscillatoria* sp. (Beneytout *et al.*, 1989) have been reported to possess single pH optima at 7.5, 7.3 and 8.8, respectively. However, the presence of dual optima for LOX has been reported by Satoh *et al.* (1976), for the mold *F. oxysporum*, at pH 6.0 and 10.0 and Iny *et al.* (1993), for bacterium *Thermoactinomyces vulgaris*, at pH 6.0 and 11.0. Perraud and Kermasha (2000) also reported the presence of dual optima for the LOX of *P. camemberti*, at pH 6.5 and 8.0 and *P. roqueforti* at pH 5.5 and 8.0. On the basis of the experimental findings, pH 6.0 was considered optimal for all characterization of LOX from *P. candidum*.

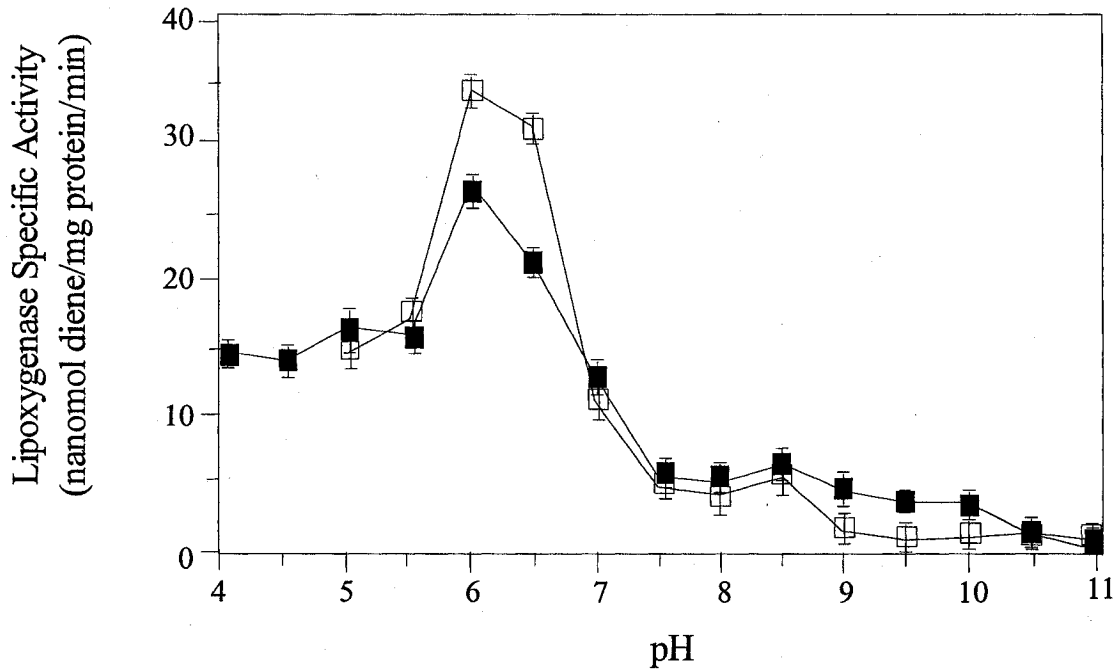

Figure 13. Effect of pH on the activity of an enriched lipoxygenase extract from *Penicillium candidum* determined spectrophotometrically at 234 nm using (■) linoleic acid and (□) linolenic acid as substrate.

4.4.4. Substrate Specificity of Enriched LOX

Table 10 indicates that *P. candidum* showed preferential substrate specificity for free linoleic acid versus its substituted acyl glycerols, including mono-, di- and trilinolein. The LOXs of canola seed (Khalyfa *et al.*, 1990) and French bean seed (Kermasha and Metche, 1986) displayed higher activity towards linoleic acid than for its acyl esters. The substrate preference for free fatty acids has been demonstrated for other fungal LOXs, including those of *G. candidum* (Perraud *et al.*, 1999) and *F. oxysporum* (Bisakowski *et al.*, 1995*a*).

The LOX activity of *P. candidum* showed a preferential substrate usage towards linolenic acid, with 126.8% relative activity compared to that of linoleic acid. Most LOXs characterized have demonstrated a substrate preference for linoleic acid over linolenic acid, including those of canola seed (Khalyfa *et al.*, 1990) and tomato fruit (Regdel *et al.*, 1994). This substrate preference was also demonstrated in the microbial LOXs from *P. camemberti*, *P. roqueforti* (Perraud and Kerasha, 2000), *F. oxysporum* (Bisakowski *et al.*, 1995*a*) and *F. proliferatum* (Bisakowski *et al.*, 1995*b*). LOX substrate specificity for *P. candidum* more closely resembled those of the fungi *G. candidum* (Perraud *et al.*, 1999) and *Gäumannomyces graminis* (Su and Oliw, 1998), which also demonstrated higher LOX activity towards linolenic acid substrate.

4.4.5. Kinetic Studies of the Enriched LOX

Using linoleic, linolenic and arachidonic acids as substrates, the K_m and V_{max} values, as well as the enzymatic catalytic efficiency (which was defined as the ratio V_{max} to K_m) for the enriched LOX extract from *P. candidum*, were calculated from Lineweaver-Burk plots (not shown). Using linoleic acid as substrate, the K_m value for the enriched LOX was 0.26 mM at pH 6.0, whereas the V_{max} value was 42.3 nanomol diene/mg protein/min. Using linolenic acid, the K_m value and V_{max} values were determined to be 0.26 mM and 55.2 nanomol diene/mg protein/min, respectively. Using arachidonic acid, the K_m and V_{max} values were determined to be 0.19 mM and 32.5 nanomol diene/mg protein/min, respectively. The K_m value for the LOX of *S. cerevisiae* was 0.26 mM (Schechter and Grossman, 1983), whereas that of *T. vulgaris* was 1.0 mM (Bisakowski *et al.*, 1995*c*), these results are close to those of *P. candidum*. Several

Table 10. Substrate specificity of the lipoxygenase activity of the enriched extract from *Penicillium candidum* (FIIb), obtained by ammonium sulfate precipitation at 20 to 60% of saturation.

Substrate	pH 6.0		pH 8.5	
	Specific Activity[a]	Relative Activity (%)[b]	Specific Activity[a]	Relative Activity (%)[b]
Linoleic acid	28.4	100.0 (10.2)[c]	6.8	100.0 (10.3)[c]
Linolenic acid	36.0	126.8 (7.8)[c]	6.0	88.2 (11.7)[c]
Arachidonic acid	21.3	75.0 (9.4)[c]	4.8	70.6 (8.3)[c]
Monolinolein	16.7	58.8 (3.6)[c]	3.9	57.3 (15.4)[c]
Dilinolein	12.2	43.0 (4.1)[c]	4.1	60.3 (9.8)[c]
Trilinolein	3.3	11.6 (4.6)[c]	3.4	50.0 (8.8)[c]

[a]Specific activity was defined as nanomol diene hydroperoxide produced per mg protein per min and was determined spectrophotometrically monitoring the increase in absorbance at 234 nm.

[b]Relative activity was expressed as a percentage with respect to the specific activity, determined for linoleic acid as substrate, at the relevant pH.

[c]Relative percentage standard deviation was defined as the standard deviation of triplicate samplings, divided by the mean, multiplied by 100.

microbial LOXs possess V_{max} values that resemble that of *P. candidum*, including *P. camemberti* with 31 nanomol diene/mg protein/min (Perraud and Kermasha, 2000) and *Geotrichum candidum* with 71 nanomol diene/mg protein/min (Perraud *et al.*, 1999), using linoleic acid as substrate.

With linolenic, linoleic and arachidonic acids as substrates, the overall results indicated that the catalytic efficiency values of LOX from *P. candidum* were 2.14×10^{-4}, 1.63×10^{-4} and 1.60×10^{-4}, respectively. Catalytic efficiency values for other *Penicillium* sp. are close to those obtained for *P. candidum* using linoleic acid as substrate, including *P. roqueforti* (2.67×10^{-4}) and *P. camemberti* (1.19×10^{-4}) (Perraud and Kermasha, 2000).

4.4.6. NP-HPLC and GC/MS of LOX End products

Figure 14 lists the NP-HPLC elution profiles of reduced hydroperoxides (hydroxides) produced by the enriched enzymatic extract of *P. candidum* at pH 6.0, tomato extract and soybean LOX type-1B, using linoleic, linolenic, arachidonic acids as substrates. Characterization of the hydroxides was performed with a UV/DAD detector at 234 nm and an ELSD detector. The tentative characterization of HOD, HOT and HETE regio-isomers, obtained by the enriched LOX of *P. candidum*, was based on the retention times and λ_{max} absorbances of tomato LOX (Matthew *et al.*, 1977; Regdel *et al.*, 1994) and soybean LOX type-1B (Hamberg and Samuelson, 1967) end products, respectively.

NP-HPLC characterization in terms of retention times and spectral scans were recorded for each hydroperoxide produced by the LOX activity of *P. candidum*; these results were combined with those of the GC/MS characterization, including *m/z* ratios and proportions of isomers produced. The combined results are reported for linoleic, linolenic and arachidonic acid substrates in Tables 11, 12 and 13, respectively; the statistical analysis of the data was performed using the formula outlined by Miller and Miller (1993).

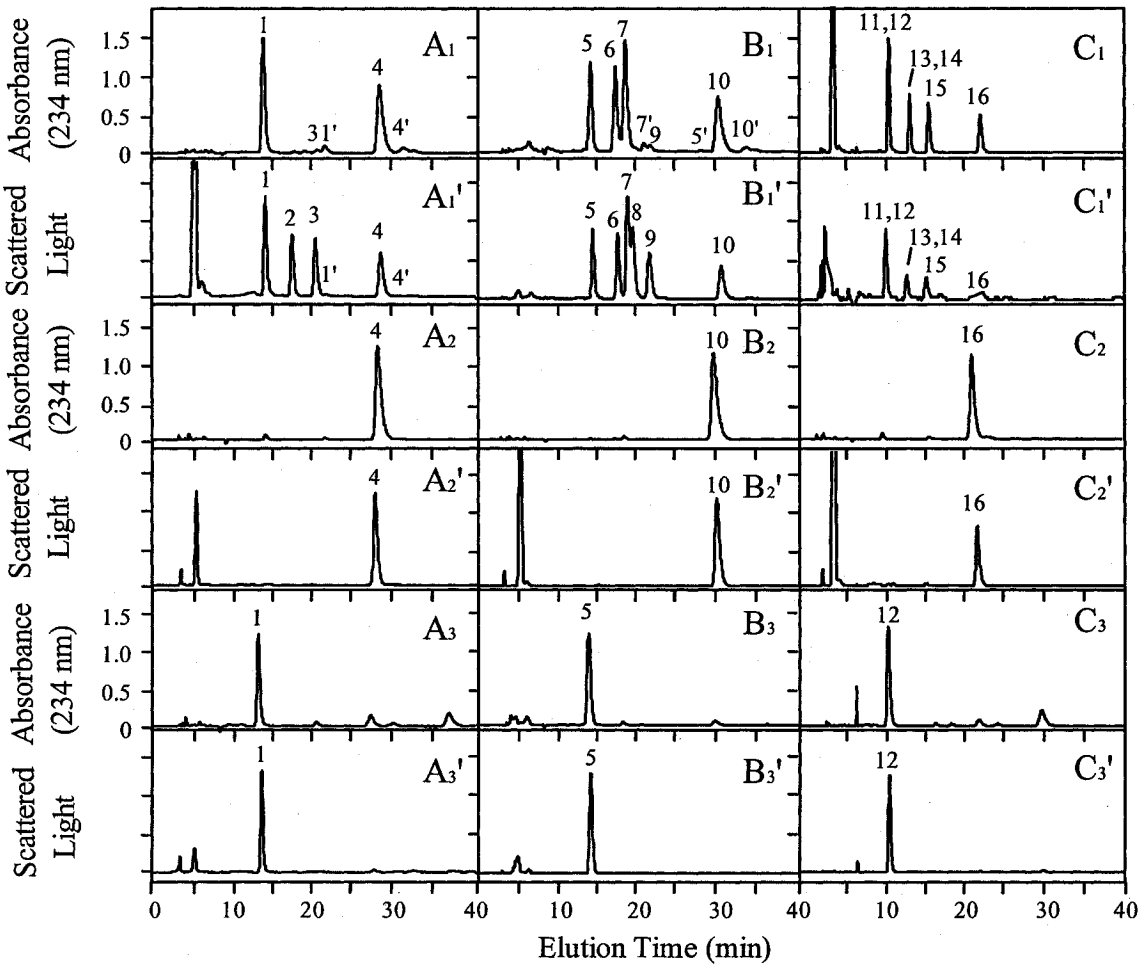

Figure 14. High-performance liquid chromatograms of hydroxides of linoleic acid (HODs), using LOX from *Penicillium candidum* (A_1 and A_1'), tomato (A_2 and A_2') and soybean type-1B (A_3 and A_3'), as well as hydroxides of linolenic acid (HOTs), using LOX from *P. candidum* (B_1 and B_1'), tomato (B_2 and B_2') and soybean type-1B (B_3 and B_3'), and hydroxides of eicosatetraenoic acid (HETEs), using LOX from *P. candidum* (C_1 and C_1'), tomato (C_2 and C_2') and soybean type-1B (C_3 and C_3'), using ultraviolet diode-array detection at 234 nm (A, B and C) and evaporative laser light scattering detection (A', B' and C').

4.4.6.1. Linoleic Acid Substrate

Figure 14 shows the NP-HPLC elution profiles of reduced hydroperoxides produced by the LOX activity of *P. candidum* (Figs. $14A_1$ and $14A_1$'), tomato LOX (Figs. $14A_2$ and $14A_2$') and soybean LOX (Figs. $14A_3$ and $14A_3$'), using linoleic acid as substrate. The predominant peaks 1, 1', 4 and 4' were detected at 234 nm and were identified as the conjugated diene isomers 13-*cis, trans*, 13-*trans, trans*, 9-*cis, trans* and 9-*trans, trans* HODs, respectively. Elution profiles of the 9-*cis, trans* HODs (Fig. $14A_2$ and $14A_2$') produced with tomato LOX and 13-*cis, trans* HODs (Figs. $14A_3$ and $14A_3$') with soybean LOX displayed similar retention times to peaks 4 and 1, respectively. Peaks 2 and 3 were detected with the ELSD and did not share similar elution times to those HODs produced by either the tomato or soybean LOXs and were determined to be the 12- and 10-HODs respectively. Figure 14 demonstrates the elution order of the HOD regio- isomers, using NP-HPLC, is 13-, 12-, 10- and 9-HOD and is identical to that reported in the literature for the oxidation products of linoleic acid by Perraud and Kermasha (2000) and Wurzenberger and Grosch (1986).

Spectral scans of HODs peaks 1 and 4 (Table 11) indicated a maximal wavelength of absorbance at 234 nm, which is consistent with *cis, trans* geometry, whereas HODs 1' and 4' displayed a maximal absorbance of 231 nm, which is consistent with *trans, trans* geometry, as demonstrated by a hypsochromic shift to a lower wavelength (Toschi *et al.*, 1995). Spectral scanning of peaks 2 (12-HOD) and 3 (10-HOD) demonstrated maximal absorbance of 203 nm, which is in agreement with absorbances reported by Terao *et al.* (1988). The fragmentation patterns obtained using GC/MS analyses (Table 11) are consistent with the presence of several mono-hydroperoxy stearate derivatives, including the 9-, 10-, 12- and 13-HOD. The *m/z* ratios of the 9- (*m/z*s 229 and 259), 10- (*m/z*s 215 and 273), 12- (*m/z*s187 and 301) and 13-MTMS (*m/z*s 173 and 315), were consistent with the fragmentation of the 4 regio-isomeric HODs (Bisakowski *et al.*, 1997; Perraud and Kermasha, 2000).

The LOX from *P. candidum* was determined to produce mostly unconjugated 9- and 13-HPODs, with 66% of total isomers produced. Other microbial LOX activity was determined to produce exclusively the 13- and 9-HPOD when linoleic acid was used as

Table 11. Characterization of reduced hydroxy-octadecadienoic acid isomers (HODs), and their methyl trimethylsilyloxy (MTMS) stearate derivatives obtained by the enzymatic activity of an enriched lipoxygenase extract from *Penicillium candidum*, using linoleic acid as substrate at pH 6.0; determined by normal phase high-performance liquid chromatography (NP-HPLC) and gas-liquid chromatography/ mass spectrometry (GC/MS).

NP-HPLC analysis of linoleic HODs			GC/MS analysis of MTMS derivatives					
Peak Number	Retention time (min)	Maximal λ_{max} (nm)	Peak number	Retention time (min)	MS fragments (*m/z*)	Derivative identification[b]	Hydroperoxide identification[c,d]	Percentage of total isomers detected[e]
1	13.4	234	1	22.3	173 (100)[a], 315 (45)[a]	13-MTMS	13 *cis, trans*[c,d]	35.2 (3.25)[f]
2	16.8	203	2	22.1	187 (100)[a], 301 (52)[a]	12-MTMS	12[c]	13.7 (9.84)[f]
3	20.0	203	3	22.0	215 (94)[a], 273 (100)[a]	10-MTMS	10[c,d]	16.3 (5.64)[f]
1'	20.6	231	1'	22.2	173 (100)[a], 315 (35)[a]	13-MTMS	13 *trans, trans*[c]	1.8 (3.88)[f]
4	28.3	234	4	22.0	229 (85)[a], 259 (100)[a]	9-MTMS	9 *cis, trans*[c,d]	30.5 (4.71)[f]
4	30.7	234	4	22.0	229 (86)[a], 259 (100)[a]	9-MTMS	9 *trans, trans*[c]	2.5 (4.73)[f]
							Total[g] =100.0 (14.10)[h]	

[a]Relative percentage of intensity of the defined fragment to that of the fragment with the highest intensity.
[b]The compound was identified by its mass spectrum.
[c]The compound was identified by its retention times (HPLC and GC), maximal absorbance (λ_{max}), and mass spectrum.
[d]The compound was identified by its retention times, (λ_{max}), and mass spectrum, and the values compared to those of a standard compound. Enzymes used for production of standards were soybean LOX type-1B, and tomato LOX.
[e]The hydroperoxide isomer was quantified by ion abundances, corresponding to its *m/z* ratio.
[f]The relative percent standard deviation of triplicate samplings.
[g]The sum of all isomer percentages detected, expressed as a percentage.
[h]The standard deviation of the total of all detected isomers was calculated according to the equation $[(RSD1)^2 + (RSD2)^2 + (RSD3)^2]^{1/2}$, where RSD1 was the relative percent standard deviation of a single isomer (Miller and Miller, 1993).

substrate, including those of *T. vulgaris* (Iny *et al.*, 1993*a*) and *G. candidum* (Perraud *et al.*, 1999). The amount of 10-HPOD produced was determined to be greater than 16% of all product hydroperoxides. Perraud and Kermasha (2000) reported that the 10-HPOD accounted for 8 and 9% of total end products, using the LOX of *P. camemberti* and *P. roqueforti*, respectively.

4.4.6.2. Linolenic Acid Substrate

Figure 14 shows the NP-HPLC elution profiles of reduced hydroperoxides produced by the LOX activity of *P. candidum* at pH 6.0 (Fig. $14B_1$ and $14B_1$'), tomato LOX (Fig. $14B_2$ and $14B_2$') and soybean LOX (Fig. $14B_3$ and $14B_3$'), using linolenic acid as substrate. The peaks 5, 5', 10 and 10' were detected at 234 nm and were identified as the 13-*cis, trans*, 13-*trans, trans*, 9-*cis, trans* and 9-*trans, trans* HOTs, respectively. Elution profiles of the 9-(*cis, trans*)HOT (Figs. $14B_2$ and $14B_2$') produced using the tomato LOX and 13-(*cis, trans*) HOT (Figs. $14C_3$ and $14C_3$') produced by soybean LOX produced identical retention times to peaks #10 and #5, respectively. Peaks 6, 7, 8 and 9 were determined to be the 12-, 16-, 15- and 10-HOTs, respectively. The elution order of the linolenic HOTs was identical to that reported by Wurzenberger and Grosch (1986).

Table 12 summarizes the results of spectral scanning obtained from NP-HPLC of HOTs peaks 5-10'. Maximal absorbances of 234 and 231 nm were used to distinguish between *cis, trans* and *trans, trans* geometry, respectively (Toschi *et al.*, 1995). Spectral scanning of peaks 8 (15-HOT) and 9 (10-HOT) demonstrated maximal absorbances of 204 nm and were determined to be unconjugated regio-isomers (Terao *et al.*, 1988).

The mass spectra of the MTMS derivatives (Fig. 15), produced from the enriched LOX of *P. candidum* using linolenic acid at pH 6.0, are mostly unconjugated HPOTs, with 66% of total isomers produced. Results from the 9- and 13-MTMS of linolenic acid, produced by tomato and soybean LOXs, are close to those determined for the enriched enzymatic extract from *P. candidum*, with respect to their *m/z* ratios and retention times (not shown). The profile of enzymatically produced hydroperoxides was different than that produced by photooxidation of linolenic acid, where conjugated regio-isomers accounted for 77% of all hydroperoxides produced (Khalyfa *et al.*, 1990) Peaks 5, 6, 9

Table 12. Characterization of reduced hydroxy-octadecatrienoic acid isomers (HOTs), and their methyl trimethylsilyloxy (MTMS) stearate derivatives obtained by the enzymatic activity of an enriched lipoxygenase extract from *Penicillium candidum*, using linolenic acid as substrate at pH 6.0; determined by normal phase high-performance liquid chromatography (NP-HPLC) and gas-liquid chromatography/ mass spectrometry (GC/MS).

NP-HPLC analysis of linolenic HOTs			GC/MS analysis of MTMS derivatives					
Peak Number	Retention time (min)	Maximal λ_{max} (nm)	Peak number	Retention time (min)	MS fragments (*m/z*)	Derivative identification[b]	Hydroperoxide identification[c,d]	Percentage of total isomers detected[e]
5	13.7	235	5	22.3	173 (100)[a], 315 (39)[a]	13-MTMS	13 *cis, trans*[c,d]	17.9 (5.20)[f]
6	16.5	234	6	22.1	187, 301	12-MTMS	12[c]	14.0 (3.86)[f]
7	17.6	236	7	23.7	131 (100)[a], 357 (21)[a]	16-MTMS	16 *cis, trans*[c]	18.6 (11.02)[f]
8	19.5	204	8	23.1	145, 343	15-MTMS	15[c]	18.0 (4.71)[f]
7'	20.8	232	7'	23.7	131 (100)[a], 357 (9)[a]	16-MTMS	16 *trans, trans*	1.4 (7.08)[f]
9	21.6	204	9	22.1	215, 273	10-MTMS	10[c,d]	16.0 (5.07)[f]
5'	26.6	234	5'	22.3	173 (100)[a], 315 (38)[a]	13-MTMS	13 *trans, trans*[c]	1.1 (5.61)[f]
10	31.6	236	10	22.0	229 (85)[a], 259 (100)[a]	9-MTMS	9 *cis, trans*[c,d]	11.8 (5.78)[f]
10'	34.8	233	10'	22.0	229 (84)[a], 259 (100)[a]	9-MTMS	9 *trans, trans*[c]	1.2 (5.36)[f]
							Total[g] =100.0 (23.44)[h]	

[a]Relative percentage of intensity of the defined fragment to that of the fragment with the highest intensity.
[b]The compound was identified by its mass spectrum.
[c]The compound was identified by its retention times (HPLC and GC), maximal absorbance (λ_{max}), and mass spectrum.
[d]The compound was identified by its retention times, (λ_{max}), and mass spectrum, and the values compared to those of a standard compound. Enzymes used for production of standards were soybean LOX type-1B, and tomato LOX
[e]The hydroperoxide isomer was quantified by ion abundances, corresponding to its *m/z* ratio.
[f]The relative percent standard deviation of triplicate samplings.
[g]The sum of all isomer percentages detected, expressed as a percentage.
[h]The standard deviation of the total of all detected isomers was calculated according to the equation $[(RSD1)^2 + (RSD2)^2 + (RSD3)^2]^{1/2}$, where RSD1 was the relative percent standard deviation of a single isomer (Miller and Miller, 1993).

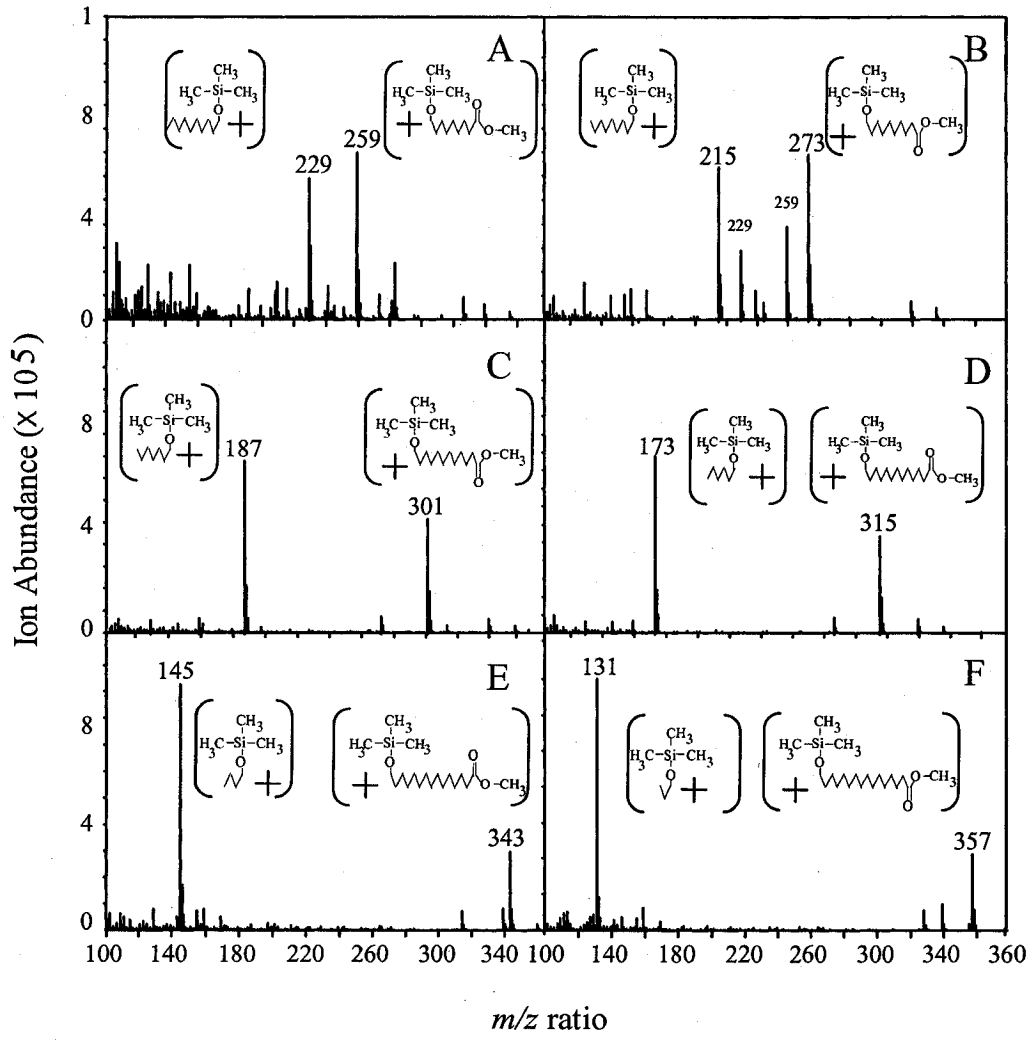

Figure 15. Mass spectra of the methyl trimethylsilyloxy (MTMS) stearate derivatives of hydroperoxides of linolenic acid (HPOTs) produced by the lipoxygenase activity of the *Penicillium candidum* enriched enzyme extract: (A) 9-MTMS, (B) 10-MTMS, (C) 12-MTMS, (D) 13-MTMS, (E) 15-MTMS, and the (F) 16-MTMS stearate derivatives.

and 10, characterized as the 13-, 12-, 10- and 9-MTMS of linolenic acid, respectively, shared identical *m/z* ratios as those produced by the LOX of *P. camemberti*, using linoleic acid (Perraud and Kermasha, 2000); the similarity in *m/z* ratios between these linoleic and linolenic MTMS regio-isomeric hydroperoxides could be the result of the reduction of all double bonds, prior to further derivatization.

The literature reported little information on the end products of LOX biocatalysis using linolenic acid; however, *G. graminis* was reported to produce mainly the 13-HPOT and a minor 11-HPOT product (Su and Oliw, 1998). Wurzenberger and Grosch (1986) suggested that LOX production of the 10-HPOT in the mushroom *A. bisporus* may be the first step in the bio-generation of 8-carbon alcohols, such as 1,5-octadiene-3-ol. An analogous pathway was proposed for the production of 1,5-octadiene-3-ol in *Penicillium caseicolum* (Karahadian *et al.*, 1985*a*). Characterization of the LOX end products of *Penicillium candidum*, using linolenic acid substrate, suggests the production of many hydroperoxide end products, including the 10-HPOT.

4.4.6.3. Arachidonic Acid Substrate

Figure 14 shows the NP-HPLC elution profiles of reduced hydroperoxides produced by the LOX activity of *P. candidum* (Figs. $14C_1$ and $14C_1$'), tomato LOX (Figs. $14C_2$ and $14C_2$') and soybean LOX (Figs. $14C_3$ and $14C_3$'), using arachidonic acid as substrate. The peaks 11, 12, 13, 14, 15 and 16 were detected at 234 nm and were characterized as possessing conjugated diene chromophores. Elution profiles of the 5-HETE, (Figs. $14C_2$ and $14C_2$') produced by the tomato LOX and 15-HETE (Figs. $14C_3$ and $14C_3$') generated by soybean LOX, displayed similar retention times to those of peaks 16 and 12, respectively. Peaks 13, 14 and 15 possessed elution times different from those HETEs produced by tomato and soybean LOX and were determined to be the 11-, 9- and 8-HETEs, respectively. The elution order of the arachidonic regio-isomers, using NP-HPLC, was 12-, 15-, 11-, 9-, 8- and 5-HETE, respectively and this result is in agreement with that reported by Kühn *et al.* (1987) and Pace-Asciak and Asotra (1989).

The mass spectra of the MTMS derivatives (Fig. 16), produced by the enriched LOX of *P. candidum* using arachidonic acid at pH 6.0, suggests a broad regio-selectivity

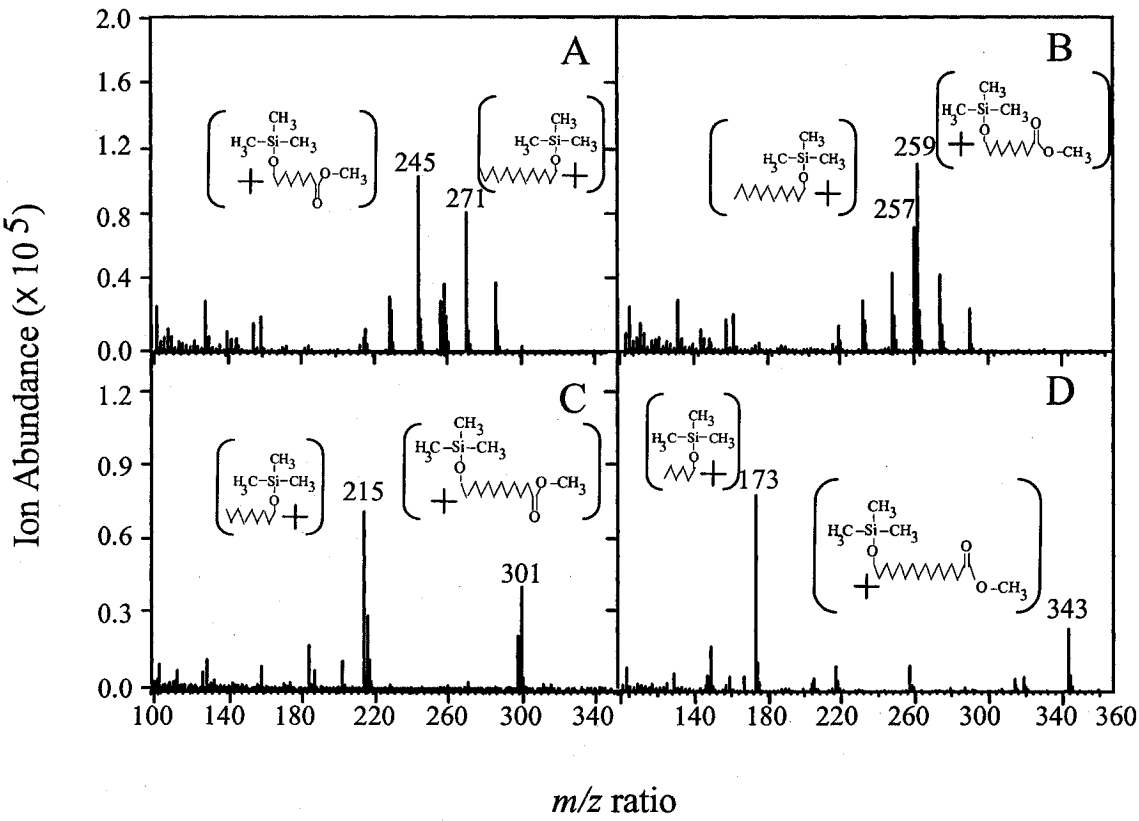

Figure 16. Mass spectra of the methyl trimethylsilyloxy (MTMS) arachidate derivatives of the major hydroperoxides of arachidonic acid (HPETEs) produced by the lipoxygenase activity of the *Penicillium candidum* extract: (A) 8-MTMS, (B) 9-MTMS, (C) 12-MTMS, and the (D) 15-MTMS arachidate derivatives.

of the LOX reaction. Results from the 5- and 15-MTMS, produced by tomato and soybean LOX, respectively are close to those determined for *P. candidum* with respect to their *m/z* ratios and GC retention times (not shown). Regdel *et al.* (1994) reported identical *m/z* ratios in the characterization of HPETEs produced by purified tomato LOX, including *m/z* ratios of 203 and 313 for the 5-MTMS, 245 and 271 for the 8-MTMS and 229 and 287 for the 11-MTMS HPETE derivatives.

Table 13 lists the main HPETE products, including the 15-, 12-, 9- and 8-HPETE at 38.4, 20.7, 21.0 and 16.6% of the total HPETEs produced, respectively. Characterization of HPETEs obtained by other LOXs has been reported to produce mixtures of regio-isomers. LOX from cucumber seedling was shown to produce the 15-, 12- and 8-HPETEs using arachidonic acid substrate at 76, 4 and 20% of total HPETEs produced, respectively (Feussner and Kühn, 1995). The LOX-I of pea seed was reported to produce a mixture of hydroperoxide end products, including the 15-, 12-, 11-, 9-, 8- and 5-HPETE regio-isomers, at 24, 4, 27, 18, 1 and 26%, respectively (Kühn *et al.*, 1987). The enriched LOX of *P. candidum* produced appreciable amounts of 15-HPETE and 12-HPETE, 20.7, 38.4%, respectively. Herman and Hamberg (1986) reported that the 15-HPETE to be the major product of LOX in the fungus *Saprolegnia parasitica*, 12-HPETE in the alga *Gracilariopsis lemaneiformis* (Moghaddam and Gerwick, 1990) and the 15-, 12- and possibly 8-HPETE in the alga *Enteromorpha intestinalis* (Kuo *et al.*, 1996). Tomato LOX has been demonstrated to produce a mixture of hydroperoxide products when incubated with arachidonic acid, including the 5-, 8- and 11-HPETE (Regdel *et al.*, 1994); these authors suggested that the multiple positional specificity of the HPETE end products may be due to the arachidonic acid substrate being bound in the LOX active site in such a position that the three allylic methylenes at C-7, C-10- and C-13 are located a comparable distance from the enzyme's iron, which is implicated in hydrogen abstraction (Regdel *et al.*, 1994). These HPETE regio-isomer "flavor precursors" are thought to be substrates for the production of green, seafood, cucumberlike, or melonlike fragrances in algae and fish (Hsieh *et al.*, 1988).

Table 13. Characterization of reduced hydroxy-eicosatetraenoic isomers (HETEs), and their methyl trimethylsilyloxy (MTMS) arachidic derivatives obtained by the enzymatic activity of an enriched lipoxygenase extract from *Penicillium candidum*, using arachidonic acid as substrate at pH 6.0; determined by normal phase high-performance liquid chromatography (NP-HPLC) and gas-liquid chromatography/ mass spectrometry (GC/MS).

NP-HPLC analysis of arachidonic HETEs			GC/MS analysis of MTMS derivatives					
Peak Number	Retention time (min)	Maximal λ_{max} (nm)	Peak number	Retention time (min)	MS fragments (*m/z*)	Derivative identification[b]	Hydroperoxide identification[c,d]	Percentage of total isomers detected[e]
11	11.3	235	11	27.1	215 (100)[a], 301 (76)[a]	12-MTMS	12[c]	20.7 (6.05)[f]
12	11.5	234	12	27.6	173 (100)[a], 343 (31)[a]	15-MTMS	15[c,d]	38.4 (6.71)[f]
13	12.5	235	13	27.1	229 (100)[a], 287 (92)[a]	11-MTMS	11[c]	1.1 (5.67)[f]
14	12.7	235	14	27.0	257 (71)[a], 259 (100)[a]	9-MTMS	9[c]	21.0 (9.09)[f]
15	16.4	236	15	27.0	245 (100)[a], 271 (100)[a]	8-MTMS	8[c]	16.6 (5.66)[f]
16	23.6	234	16	27.3	203 (100)[a], 313 (23)[a]	5-MTMS	5[c,d]	2.2 (8.72)[f]
							Total[g] =	100.0 (17.45)[h]

[a]Relative percentage of intensity of the defined fragment to that of the fragment with the highest intensity.
[b]The compound was identified by its mass spectrum.
[c]The compound was identified by its retention times (HPLC and GC), maximal absorbance (λ_{max}), and mass spectrum.
[d]The compound was identified by its retention times, (λ_{max}), and mass spectrum, and the values compared to those of a standard compound. Lipoxygenases used for production of standards were soybean type-1B, and tomato source.
[e]The hydroperoxide isomer was quantified by ion abundances, corresponding to its *m/z* ratio.
[f]The relative percent standard deviation of triplicate samplings.
[g]The sum of all isomer percentages detected, expressed as a percentage.
[h]The standard deviation of the total of all detected isomers was calculated according to the equation $[(RSD1)^2 + (RSD2)^2 + (RSD3)^2]^{1/2}$, where RSD1 was the relative percent standard deviation of a single isomer (Miller and Miller, 1993).

4.4.7. Chiral HPLC Analysis of LOX End products

Using linoleic acid substrate the stereo-selectivity of the LOX from *P. candidum* was compared to those of the tomato and soybean LOXs. The 9(*S*)-HPOD produced by tomato LOX, whereas the 13(*S*)-HPOD was obtained by soybean LOX type-1B. The 10(*R*)-HPOD was recovered from mushroom enzyme extract containing HPL activity, following incubation with racemic 10-HPOD. The three HPOD regio-isomers (not shown) displayed a higher enantiomeric purity than that of the end products of *P. candidum* LOX.

As for linolenic acid as substrate, the experiental findings (not shown) demonstrated that the end products, HPOTs, of LOX from *P. candidum* have lower optical purity than those produced by tomato and soybean LOX type-1B. However, the 9(*S*)- and 13(*S*)-HPOTs produced by tomato and soybean LOX type-1B, respectively, were close to being optically pure. The 10(*R*)-HPOT, obtained from mushroom enzyme extract containing HPL activity and after its reaction with racemic 10-HPOT displayed high optical purity.

For the use of arachidonic acid as substrate, the data indicated (not shown) that the enantiomeric hydroperoxide end products obtained by LOX of tomato and soybean LOX type-1B were almost exclusively 5(*S*)- and 15(*S*)-HPETE, respectively. The HPETE end products of LOX from *P. candidum* were lower in their optical purity than those produced by tomato and soybean type-1B LOXs.

The results relating to the characterization of the hydroperoxides produced by tomato and soybean LOXs and converted by the HPL activity of mushroom enzymatic extract, are in agreement with studies of their regio- and enantio-selectivities. Hawkins *et al.* (1988) reported that tomato LOX produces almost exclusively the (*S*)-enantiomer of the 9-HPOD and HPOT from linoleic and linolenic acids, respectively. Using arachidonic acid as substrate, tomato LOX was reported to produce the 5-, 8- and 11-HPETE regio-isomers, each with exclusively (*S*) configuration (Regdel *et al.*, 1994). Soybean LOX type-1B was reported to produce the 13-HPOD and HPOT using linoleic and linolenic acids as substrates, respectively and enantiomeric excesses in favor of the (*S*) form, of

greater than 97% have been obtained (Gardner, 1996; Kühn *et al.*, 1987). Hawkins *et al.* (1988) reported obtaining a 99.7% optical purity in the production of the 15(*S*)-HPETE from soybean LOX type-1B. Wurzenberger and Grosch (1986) reported that incubation of HPL from mushroom *A. bisporus* with racemic 10-HPOD and 10-HPOT (both produced by photooxidation), displayed exclusively stereo-selective cleavage of the (*S*) enantiomers, in the production of 1-octen-3-(*R*)ol and 1,5-octadiene-3-(*R*)ol, respectively. A similar HPL activity has been reported in *P. camemberti* and *P. roqueforti*, demonstrating selective cleavage of the 10-HPOD regio-isomer, in the production of 1-octen-3-ol (Kermasha *et al.*, 2002*b*). Josephson *et al.* (1983) suggested that the 10(*S*)-HPOT may be a substrate in the production of 1,5-octadiene-3(*R*)-ol in some seafoods such as whitefish and shrimp.

Table 14 reports the relative percentages of (*R*) and (*S*) enantiomers, produced by the LOX of *P. candidum* using various PUFA substrates at two pH optima. At more alkaline pH values, *P. candidum* showed less stereo-selectivity; the production of the 10(*S*)-HPOD enantiomer decreased from 66.2 to 53.7%, relative to the 10(*R*)-HPOD enantiomer. A similar trend is demonstrated concerning the production of the 10-HPOT, decreasing from 66.8 to 53.7%. CP-HPLC analysis of the HETEs produced from arachidonic acid demonstrated the highest ratios of (*S*):(*R*), including that of the 8-HETE, displaying 73.7% (*S*)- configuration, at pH 6.0. van Os *et al.* (1979) reported that the proportion of 13(*S*)- to 13(*R*)-HPOD produced by soybean LOX type-1B changed from 94 to 77% (*S*) enantiomer when it was incubated at pH 10.5 and 7.0, respectively. Gardner (1996) indicated that the two main factors influencing enantiomeric purity in favor of the (*S*) form for end products of soybean LOX type-1B using linoleic and linolenic acid substrates, were incubation at a pH above 10 and the presence of available oxygen. Higher proportions of (*S*) enantiomers were obtained for soybean LOX type-1B at alkanline pH in this study (not shown). However, the general lack of regio- and enantio-selectivity for various LOXs has been reported in the literature. Kühn *et al.* (1987) reported the production of the 5-, 9-, 11- and 15-HPETE by pea seed LOX II, with (*S*):(*R*) isomer ratios of 67:33, 50:50, 48:52 and 91:9, respectively. Perraud and Kermasha (2000) indicated that, using linoleic acid as substrate, the LOXs of *Penicillium* sp.

Table 14. Chiral phase high-performance liquid chromatography (CP-HPLC) analysis of a selection of reduced hydroperoxide isomers, obtained by the enzymatic activity of a lipoxygenase extract from *Penicillium candidum*, using linoleic, linolenic and arachidonic acids as substrates.

		Relative Peak Area (%)[a]			
	Hydroperoxide	pH 6.0		pH 8.5	
Substrate	regio-isomer	(*R*)	(*S*)	(*R*)	(*S*)
Linoleic Acid	HOD[d]				
	9-[b]	44.6	53.4	49.1	50.9
	10-[c]	33.8	66.2	46.3	53.7
	13-[b]	39.0	61.0	48.2	51.8
Linolenic Acid	HOT[e]				
	9-[b]	39.7	60.3	49.3	50.7
	10-[c]	33.2	66.8	46.3	53.7
	13-[b]	43.3	56.7	47.3	52.7
	15-[c]	44.1	55.9	47.5	52.5
Arachidonic Acid	HETE[f]				
	8-[b]	26.3	73.7	33.5	66.5
	9-[b]	29.1	70.9	34.9	65.1
	12-[b]	29.9	70.1	36.0	64.0
	15-[b]	33.0	67.0	38.4	61.6

[a]Relative percent of peak area, determined by CP-HPLC analysis, of the defined hydroperoxide enantiomer, (*R*) or (*S*), to that of the total of both enantiomers.

[b]The detection was performed using diode array detection at 234 nm.

[c]The detection was performed using a evaporative laser light-scattering detector (ELSD).

[d]Hydroxy-octadecadienoic acid (HOD).

[e]Hydroxy-octadecatrienoic acid (HOT).

[f]Hydroxy-eicosatetraenoic acid (HETE).

produced several hydroperoxide end products, including the 9- and 13-HPOD with (*S*):(*R*) isomer ratios of 52.2:48.3 and 52.8:47.2 for *P. camemberti* and 52.9:47.1 and 55.3:44.7 for *P. roqueforti*, respectively. Overall, the experimental findings (Table 14) suggested a higher degree of stereo-selectivity in the LOX of *P. candidum* than that of other *Penicillium* sp.(Perraud and Kermasha, 2000).

4.5. Conclusion

The enriched LOX extract from *P. candidum* displayed a wide range of specificity towards free PUFAs, including linoleic, linolenic and arachidonic acids as well as acyl esters of linoleic acid. The enzyme showed two pH optima. Characterization of the end products, the hydroperoxide regio-isomers of PUFAs, confirmed the wide range of regio-selectivity of the investigated LOX. The LOX displayed stereo-selectivity towards production of (*S*) hydroperoxide enantiomers. The overall results suggested that the enriched LOX from *P. candidum* showed many of the characteristics of other microbial LOXs in terms of enzyme specificity and nature of flavor precursor end products.

CHAPTER V

STATEMENT OF CHAPTER V LINKAGE

As a result of the research work conducted previously in our laboratory on *Penicillium camemberti* and *Penicillium roqueforti* as well as that in the current thesis on *Aspergillus niger* (Chapter III) and *Penicillium candidum* (Chapter IV), the most appropriate microorganism with which to conduct further investigations into enzymatic stabilization was determined to be *P. camemberti*. The selection of this microorganism was based on experimental data gathered on its lipoxygenase and hydroperoxide lyase activities, with respect to substrate specificity, kinetic parameters and nature of end products.

CHAPTER V.

STABILITY OF AN ENZYMATIC EXTRACT PREPARATION CONTAINING LIPOXYGENASE AND HYDROPEROXIDE LYASE ACTIVITIES FROM *PENICILLIUM CAMEMBERTI* USING SELECTED CHEMICAL ADDITIVES

5.1. Abstract

The stability of an enzymatic extract containing lipoxygenase (LOX) and hydroperoxide lyase (HPL) activities, obtained from the mycelia of *Penicillium camemberti*, was investigated using selected additives. The addition of KCl had little impact on lyophilized LOX activity (93%); however, it was activating towards HPL activity (223%), whereas the addition of dextran led to LOX/HPL inactivation. The storage stability of the solid lyophilized LOX/HPL enzymatic extract was greater in extracts containing KCl (7.5 ppm) than those without salt, with ~100% residual activity after 8 and 4 weeks, for LOX and HPL, respectively. The rate constants of inactivation ($K_{inactivation}$) and the $C_{1/2}$ (concentration at ½ enzymatic activity) values showed glycine to be the most appropriate additive for LOX and HPL. All other additives investigated, including glycerol, polyethylene glycol, mannitol and sucrose were more inactivating. Thermostability (25-80°C, 1h) was conferred best by mannitol 5% (w/v) for LOX and by glycine 10% (w/v) for HPL. All additives investigated provided some enhancement to the thermostability for LOX activity, whereas all additives, except mannitol, led to a higher HPL thermostability.

5.2. Introduction

Lipoxygenase (LOX) (linoleate: oxygen oxidoreductase; EC 1.13.11.12) catalyzes the dioxygenation of various polyunsaturated fatty acids (PUFAs) containing a 1(Z),4(Z)-pentadiene moiety into various regio-isomers of hydroperoxides of PUFAs (Gardner, 1996). Lipoxygenases have been isolated and characterized from many sources, including vegetal (Kuo *et al.*, 2006), animal (Mortimer *et al.*, 2006), bacterial species (Iny *et al.*, 1993) and in *Penicillium camemberti* (Perraud and Kermasha, 2000). In addition to increased importance of LOX in medical applications (Adamjee *et al.*, 2006; Shim *et al.*, 2006; Hayashi *et al.*, 2006) its end products, PUFA hydroperoxides are often considered to be flavor precursors, which are cleaved by hydroperoxide lyase (HPL) into flavor compounds such as ketones, aldehydes and alcohols. Microbial HPL is associated with the conversion of the 10-hydroperoxide of linoleic acid, which is believed to be the principle substrate in many molds and fungi, including *P. camemberti* (Kermasha *et al.*, 2002*b*). The production of aroma chemicals has been recognized as a relevant application of the biocatalytic process involving LOX/HPL. However, the enzymes are widely regarded as fairly unstable and thermolabile and must be stabilized if they are to have biotechnological value (Beneytout *et al.*, 1989).

Enhanced stability may be achieved by counteracting the forces that lead to denaturation and loss of activity. A practical approach to enzymatic stabilization is with the addition of chemicals, which may include a variety of salts (Kohn *et al.*, 1997), sugars/polyols (Götz-Schmidt *et al.*, 1986) and polymers (Hatley and Franks, 1991; Hornostaj and Robinson, 1999). Several LOXs have been shown to be stabilized with detergents (Beneytout, 1989), thiol-containing compounds including dithiothreitol (DTT) (Vick and Zimmerman, 1976; Kuo *et al.*, 1996), metal ions such as cobalt (Matsuda *et al.*, 1976) and as well as other chemicals including ethylene diamine tetraacetate (EDTA) (Pérez-Gilabert *et al.*, 2001) and ethylene glycol tetraacetate (EGTA) (Brash *et al.*, 1996). Chemical compounds used to stabilize HPLs include detergents (Olías *et al.*, 1990; Pérez *et al.*, 1999), glycerol (Salas and Sanchez, 1999), sucrose (Götz-Schmidt *et al.*, 1986), DTT (Matsui *et al.*, 1991; Hornostaj and Robinson, 1999) and polyvinylpolypyrrolidone (PVP) (Matsui *et al.*, 1989). The use of chemical additives could improve the operational

stability of LOX/HPL from *P. camemberti*, making it more suitable to be used as a stable enzymatic preparation for the biocatalytic transformation of lipid substrates into flavor compounds.

The overall objective of this study was to obtain a stabilized LOX/HPL enzymatic extract preparation from *P. camemberti*, by addition of selected chemical additives in the lyophilized (solid) and reconstituted (liquid) states. The specific objectives were to determine the potential lyo-protective effects of potassium chloride (KCl) and dextran on the enzymatic extract as well as to determine the long-term storage stability of LOX/HPL activities when the extract was stored in the lyophilized state. In addition, the study was aimed at the investigation of the potential efficacy of selected chemicals, including glycine, sucrose, mannitol, polyethylene glycol (PEG) and glycerol to enhance the thermostability of the LOX/HPL extract in the liquid state.

5.3. Materials and Methods

5.3.1. Chemicals

The additives used in this study included KCl, mannitol and glycine which were purchased from BDH Inc. (Poole, U.K.) and dextran 72,200 which was purchased from Sigma Chemical Co. (St-Louis, MO). Glycerol and Carbowax® polyethylene glycol 20,000 were both purchased from Fisher Scientific (Nepean, ON) and sucrose was purchased from ACP (Montreal, QC). Other chemicals used to prepare buffer were mono and dibasic potassium phosphate, purchased from Fisher Scientific (Fair Lawn, NJ).

5.3.2. Culture Growth and Harvesting Conditions

P. camemberti was induced to sporulate and the resultant spore suspension was counted using a Neubauer Counting Chamber (Hausser Scientific, Horsham, PA) and used to inoculate liquid medium according to the procedure outlined by Perraud and Kermasha (2000). After harvesting on day 10, the biomass was filtered through cheesecloth and the mycelia were washed (2×50 mL) with cold water (4°C), then potassium phosphate buffer (pH 6.5, 0.1 M). The mycelia were then drained, patted dry with paper towel and were subjected to enzymatic extraction. All subsequent steps were performed at 4°C, unless otherwise stated.

5.3.3. Preparation of Enzymatic Extract

The lyophilized biomass was blended (5 mL phosphate buffer per 1 g biomass) and homogenized using 0.45 to 0.50 mm diameter glass beads in an MSK cell homogenizer (Braun, Melsungen, Germany) for 2×2 min. The resultant suspension was centrifuged (12,000×*g*, 15 min) and the supernatant was subjected to ultrafiltration (Amicon, 30 kDa NMWCO, 40 psi) until a 2-fold concentration was attained. The retentate thus obtained was considered to be the fresh LOX/HPL enzymatic extract. The ultrafiltrate and retentate were aliquotted for enzymatic activity and protein determination. The bulk of the retentate was set aside for lyophilization.

5.3.4. Protein Measurement

The protein content of the enzymatic extract was measured with a modified Lowry method (Hartree, 1972), using bovine serum albumin (Sigma Chemical Co.) as a standard for the calibration curve.

5.3.5. Addition of Additives to LOX/HPL Enzymatic Extract

Prior to lyophilization KCl or dextran was added to the fresh enzymatic extract. The additives were added to the lyophilized enzymatic extract following reconstitution with potassium phosphate buffer (pH 6.5, 0.1 M). All crystalline chemicals were ground manually in a ceramic mortar and pestle prior to being added stepwise to prevent precipitation.

5.3.6. Substrate Preparation

LOX studies used linoleic acid (*cis*-9,*cis*-12-octadecadienoic acid purchased from Nu-Chek-Prep Inc. (Elysian, MN). The substrate stock solutions were prepared at a concentration of 4.0×10^{-3} M in the appropriate buffer solutions (0.1 M) according to the procedure described by Perraud *et al.* (1999).

HPL studies used the 10-hydroperoxide of octadecadienoic acid (10-HPOD) produced by photooxidation of linoleic acid and purified by solid phase extraction (SPE) and normal phase high-performance liquid chromatography (NP-HPLC). Briefly, the photooxidation of linoleic was performed according to the procedure described by

Schieberle *et al.* (1984). The reaction medium was composed of 6 mL of linoleic acid, 44 mL of benzene and 6.23 mg of meso-tetraphenyl porphine (Sigma Chemical Co.) as sensitizer. The photooxidation was performed at 15°C by bubbling O_2 into the reaction medium while the stirred solution was irradiated with light from a 500 W halogen bulb, filtered through a 1 cm layer of deionized water to reduce IR radiation. Following photooxidation the benzene was evaporated using a SpeedVac System® model AES1010 (Savant Instruments, Holbrook, NY). The dried hydroperoxides were purifed by SPE (Supelclean™ LC-Si 6 mL, Supelco Inc., Bellefonte, PA) according to the procedure outlined by Toschi *et al.* (1995). Separation of the 10-HPOD from other HPOD regio-isomers was accomplished using NP-HPLC, according to the procedure described by Perraud *et al.* (2000). Quantification of the 10-HPOD was performed using ferrous thiocyanate colorimetry (25,000 M^{-1} cm^{-1}, 490 nm) according to the procedure outlined by Wurzenberger and Grosch (1986) and dried substrate was stored in tubes (0.5 μmol/tube) at –80°C.

5.3.7. Enzyme Assays

The LOX/HPL reactions were conducted under the same standard conditions (pH 6.5, 0.1M, 25°C). LOX activity was assayed spectrophotometrically using a Beckman DU-650 spectrophotometer (Beckman Instruments Inc., San Raman, CA) and a reaction mixture containing 187 μL of a 4.0×10^{-3} M substrate solution and 33 μg of protein, according to the procedure outlined by Perraud and Kermasha (2000). LOX specific activity was defined as nanomol of conjugated diene hydroperoxide produced/ mg protein/ min (MEC 25,000 M^{-1} cm^{-1} at 234 nm) (Surrey, 1964). All LOX assays were performed in duplicate series and run in tandem with blanks containing all components of the enzymatic assay except protein.

HPL activity was assayed using xylenol orange [(3,3'-*bis*(*N*,*N*-di(carboxymethyl)aminomethyl)-*o*-cresol)] colorimetry according to a modified procedure outlined by Jiang *et al.* (1991). The xylenol orange reagent solution was prepared as a mixture of deionized/degassed water, ferrous sulfate (0.25 mM), perchloric acid (85 mM) and xylenol orange salt (0.1 mM). Fresh xylenol orange reagent was prepared each 6 hours and stored away from light. The 10-HPOD substrate (10 nanomol)

containing Tween 80 (0.3 µL per 0.9 µmol) was placed into 5 mL pyrex tubes and 10 µL of deionized water was added. The mixture was sonicated for 3 min according to the procedure outlined by Kermasha *et al.* (2002). The reaction was initiated by the addition of 15 µL of HPL enzyme extract (0.1 - 1 mg protein/mL) and allowed to react for 5 min. The reaction was stopped by the addition of xylenol orange reagent (2 mL) to the reaction homogenate and read after 20 min of color development (10-HPOD; MEC 18,765 M^{-1} cm^{-1}, 560 nm). HPL specific activity was defined as nanomol of 10-HPOD converted/ mg protein/ min, (MEC 18,765 M^{-1} cm^{-1} at 560 nm). All HPL trials were run in triplicate in tandem with a blank, containing all components of the test except the presence of active HPL extract. The blank enzyme assay was performed by adding active HPL enzyme directly to the xylenol orange reagent containing the 10-HPOD substrate and allowing for color development as previously described.

5.3.8. Long-Term Stability

Lyophilized LOX/HPL enzyme extracts were stored in 15 mL polypropylene tubes (10 mg protein) contained in desiccators containing Dry-Rite®. The desiccators were stored at –80 and 4°C for a period of 9 weeks and assayed for LOX/HPL activities weekly, using enzymatic extract from the same tube, according to the procedures previously described.

5.3.9. Effect of Additives

The effect of additive concentration on the LOX/HPL activity was investigated by varying the chemical concentration from 0 to 10 % (w/v) in reaction homogenate. The rates of inactivation ($K_{inactivation}$) for LOX/HPL activity caused by chemical additives were investigated by calculating the first-order inactivation constant on semilogarithm plots, using standard LOX/HPL reaction conditions. The concentration of an additive required to abolish LOX/HPL activity by 50% denoted $C_{(1/2)}$, was calculated using the linear equation:

$$ln\ (A/A_0) = -K_{inactivation}C$$

where C is the concentration of the chemical additive and A and A_0 are the LOX/HPL activities at C concentration with chemical additive and without chemical additive, respectively (Rodrigo *et al.*, 2006).

5.3.10. Thermostability Study

The effect of temperature on LOX/HPL thermostability was determined by pre-incubating reconstituted lyophilized extracts to a wide range of temperatures (25 to 80°C, 1 h) using a Precision reciprocal shaking bath model 25 (Precision Scientific, Chicago, IL), followed by cooling (4°C, 0.5 h). The extracts were gently mixed to resuspend the protein and the residual LOX/HPL activity was measured using the standard assay conditions.

5.4. Results and Discussion

5.4.1. Effect of Additive Concentration on Lyophilized LOX/HPL Activities

The addition of KCl and dextran were investigated for their potential efficacy as lyoprotective additives, in the maintenance of high LOX and HPL activities post-lyophilization. The effect of the KCl and dextran additives and their concentrations on the LOX and HPL activities in the lyophilized enzymatic extracts are summarized in Table 15. The LOX activity of the lyophilized extract was determined to be 1.34-fold greater than that of the fresh one. The presence of dextran inactivated the LOX activity, where the inactivation was increased proportionally to the increases in the additive concentration in the lyophilized enzymatic extract. The addition of KCl (7.5 ppm) (182.7 mg retentate solid/ g solid preparation) resulted in the lowest decrease in LOX activity after lyophilization, corresponding to 92.9% that of the no-additive trial. At KCl concentrations higher (10.0 ppm) and lower (5.0 ppm) than this optimum the LOX residual activity were 82.7 and 78.4 %, respectively. Results (Table15) of additive effect on HPL biocatalysis after lyophilization indicate that KCl resulted in an activation of HPL, whereas the addition of dextran led to HPL inactivation.

Several studies have indicated that dextran additive was an effective lyoprotectant for the protection of enzymatic activity (Gavalas *et al.*, 2000; Gloger *et al.*, 2003). Gavalas *et al.* (2000) noted that the addition of dextran 10 kDa (2.0% w/v) to glucose oxidase, prior to lyophilization, resulted in a 50% increase in residual specific activity in comparison to the control. Gloger *et al.* (2003) noted that the addition of dextran 1 kDa (8%, w/v) aided in the maintenance of 100% residual activity in plant source aviscumine.

The authors also noted that freeze drying the enzyme with higher molecular weights dextrans (1 to 1,000 kDa) led to a decrease of 20% in post-lyophilization residual specific activity (Gloger *et al.*, 2003). Gloger *et al.* (2003) suggested the decreased enzymatic activity in the presence of higher molecular weight dextrans was due to steric hindrance of the enzyme onto large dextran polymers. In the current study a large dextran polymer (72 kDa) was investigated for its lyoprotective effect and the low stabilization observed may be due to post-lyophilization steric/conformational strains on the LOX and HPL. For all concentrations of dextran, added prior to lyophilization, a relatively higher decrease in HPL activity was observed.

Many studies have reported KCl to be an effective lyoprotectant of enzymatic activity including for microbial lipase (Yu *et al*, 2005), penicillin amidase (Lindsay *et al.*, 2002) and various proteases (Ru *et al.*, 2000; Würges *et al.*, 2005). Würges *et al.* (2005) reported a greater than 100% residual specific activity when subtilisin/protease was lyophilized in the presence of KCl (98.5%, w/w). Lindsay *et al.* (2002) noted that the addition of KCl (98%, w/w) to penicillin amidase prior to lyophilization led to a 10-fold higher activity in the salt containing trial compared to the control; these authors suggested that this effect may be due to an ordering of water molecules around the enzyme (kosmotropicity). Carpenter *et al.* (1997) reported that effective lyoprotectants function by affecting the glass transition temperature (T_g) of the enzymatic preparation. T_g is defined as the temperature, where a freezing enzymatic extract becomes maximally concentrated with respect to all of its solutes and a transition occurs from a viscous liquid (20 to 50% water) to a brittle glass. With respect to additives, salts such KCl, possess low T_gs (~ 0 °C) as do small chemicals like mannitol (T_g of ~13 °C) and sucrose (T_g of ~31 °C) (Wang, 2000). Large molecules, such as dextran or PVP tend to have higher T_gs, >100 °C and are deemed not as effective at stabilizing enzyme extracts (Hatley and Franks, 1991). It may be noted that several studies used dextran polymers in combination with sucrose at 5% (w/v) to effectively retain enzyme activity and long-term storage stability, including actin (Allison *et al.*, 1998) and microbial lipase (Kreilgaard *et al*, 1999).

Table 15. Percentage of residual specific activities of lipoxygenase (LOX) and hydroperoxide lyase (HPL), remaining in the enzyme extract from *Penicillium camemberti*, after lyophilization with either potassium chloride or dextran additive, using linoleic acid and the 10-hydroperoxide of octadecadienoic acid (10-HPOD) as substrates, respectively.

Additive[a]	Concentration of Extract Ultrafiltrate Solid/ Solid Prep. (mg/g)[b]	Additive (%, w/v)[c]	Lyophilization	Residual Specific Activity (%)[d] LOX	HPL
None		0	-[e]	74.6 (17.5)[g]	48.1 (6.5)[g]
None		0	+[f]	100.0 (28.4)[g]	100.0 (3.8)[g]
Potassium chloride	304.9	2.5	+[f]	86.7 (21.8)[g]	110.4 (7.1)[g]
	276.8	5.0	+[f]	82.7 (23.7)[g]	165.2 (21.4)[g]
	182.7	7.5	+[f]	92.9 (16.8)[g]	223.2 (12.3)[g]
	98.5	10.0	+[f]	78.4 (29.3)[g]	114.2 (1.9)[g]
Dextran	304.9	2.5	+[f]	55.0 (29.9)[g]	29.7 (64.5)[g]
	276.8	5.0	+[f]	22.5 (46.8)[g]	4.5 (10.8)[g]
	182.7	7.5	+[f]	9.7 (76.0)[g]	n.d.[h]
	98.5	10.0	+[f]	13.4 (51.7)[g]	n.d.[h]

[a]Dextran or potassium chloride additive was included in the enzymatic extract prior to lyophilization.
[b]The concentration of the enzymatic extracts (ultrafiltered, 30,000 NMWCO) expressed in mg ultrafiltrate solid/g solid preparation, was prepared according to selected amounts of additives, included prior to lyophilization.
[c]The concentration of dextran or potassium chloride additive expressed as a weight/volume percentage of the enzymatic extract.
[d]Residual specific activity percentage was defined as the specific activity of the lyophilized trial in comparison to that containing no additive, for LOX and HPL, respectively.
[e]- denotes that lyophilization was not conducted.
[f]+ denotes that lyophilization was conducted.
[g]Relative percentage standard deviation was defined as the standard deviation of LOX duplicate samplings and HPL triplicate samplings, divided by their respective means, multiplied by 100.
[h]Not detected.

In the current study the optimum KCl concentration of 7.5 ppm led to an increase (223.2%) in the recovered HPL activity. On the basis of the experimental data (Table 15) the addition of KCl 7.5 ppm was chosen for future work.

5.4.2. Effect of KCl Additive on LOX/HPL Long-Term Stability

Table 16 summarizes the long-term storage stability of the LOX/HPL preparation (–80°C, 9 weeks) for trials with and without KCl addition. LOX extracts containing no KCl demonstrated a decrease in activity from week 0 to week 3, from 100 to 78.4%, respectively. However, the LOX activity was determined to be approximately the same as was determined for week 0 as at week 4 to 6, after which a second decrease was observed. After 9 weeks of storage trial lacking KCl had lost 28.5% of its activity. In comparison the KCl containing trial maintained a much more constant LOX activity, retaining approximately 100% of its activity up until week 8. At week 9 the KCl containing trial lost slightly which was less (16.5%) than the loss in the control. Overall the LOX activity appeared to be stabilized by the addition of KCl (7.5 ppm) additive effectively up to week 8. HPL activity, for trials containing no additive, was determined to be ~100% for the first 2 weeks of storage, after which a steady decrease to 44.6% was observed at week 9. The stored KCl containing enzyme extract possessed a residual HPL activity of ~124% at weeks 1 to 4, after which a dramatic constant decrease was observed. Long-term storage stability studies tend to suggest that the decrease in the enzyme stability past 4 weeks may be due to the protein aggregation, resultant from excessive moisture in the enzymatic extracts (Wang, 2000). Chemical degradation processes in proteins, including deamidation and hydrolysis, were reported to be initiated by small quantitities of water (1 to 5% w/w), present in lyophilized enzyme extracts (Wang, 2000). The LOX/HPL stability of the enzyme extract is in agreement with studies that have suggested that the higher the T_g of the preparation the more stability will be displayed in the long-term; a protein preparation with a T_g of 40°C will be stable at 25°C (Carpenter *et al.*, 1997; Wang, 2000). The presence of a KCl may stabilize the LOX/HPL activity by increasing the ionic strength of the homogenate which can lead to increase in hydrophobic interactions and the accumulation of water molecules around the protein (Volkin and Klibanov, 1989).

Table 16. Long-term stability of lipoxygenase (LOX) and hydroperoxide lyase (HPL) activities of a lyophilized enzyme extract from *Penicillium camemberti*, with and without potassium chloride additive (7.5 ppm), stored at –80°C, using linoleic acid and the 10-hydroperoxide of octadecadienoic acid (10-HPOD) as substrate, respectively.

	Residual Specific Activity of LOX (%)[a]		Residual Specific Activity of HPL (%)[b]	
Storage (Weeks)	KCl[c]	No KCl	KCl[c]	No KCl
0	100.0 (8.7)[d]	100.0 (20.9)[d]	100.0 (18.3)[d]	100.0 (5.9)[d]
1	79.6 (8.5)[d]	93.3 (4.2)[d]	124.9 (20.8)[d]	102.4 (9.7)[d]
2	106.2 (2.9)[d]	65.3 (14.8)[d]	115.2 (6.9)[d]	85.6 (3.7)[d]
3	128.6 (8.9)[d]	78.4 (8.9)[d]	131.1 (12.9)[d]	63.2 (21.2)[d]
4	103.7 (4.9)[d]	112.6 (14.8)[d]	124.6 (11.3)[d]	58.1 (35.2)[d]
5	97.8 (0.1)[d]	115.4 (9.6)[d]	77.0 (12.8)[d]	62.7 (20.6)[d]
6	109.2 (8.2)[d]	99.9 (4.3)[d]	61.0 (20.6)[d]	49.1 (17.9)[d]
7	103.3 (17.2)[d]	70.6 (10.7)[d]	18.4 (24.0)[d]	49.0 (14.4)[d]
8	107.2 (9.3)[d]	65.5 (16.1)[d]	18.9 (26.3)[d]	43.4 (25.5)[d]
9	83.5 (10.4)[d]	71.5 (13.1)[d]	34.8 (32.8)[d]	44.6 (15.5)[d]

[a] Residual specific activity of LOX percentage was calculated by dividing the specific activity (nanomol diene hydroperoxide produced/mg protein/min) of a specified trial by the specific activity of the trial at week 0, multiplied by 100. LOX specific activity was determined spectrophotometrically by monitoring the increase in absorbance at 234 nm, using linoleic acid as substrate. All LOX trials were performed in duplicate series run in tandem with a blank, at pH 6.5, 25°C.

[b] Residual specific activity of HPL percentage was calculated by dividing the specific activity (nanomol 10-hydroperoxide of linoleic acid converted/mg protein/min) of a given trial by the specific activity of the trial at week 0, multiplied by 100. HPL specific activity was determined spectrophotometrically by monitoring the increase in absorbance at 560 nm, using 10-hyfroperoxide of linoleic acid as substrate. All HPL trials were performed in triplicate series run in tandem with a blank, at pH 6.5, 25°C.

[c] Potassium chloride (KCl) was added at a concentration of 7.5 ppm to the LOX/HPL enzymatic extract (ultrafiltered, 30,000 NMWCO), prior to lyophilization.

[d] Relative percentage standard deviation was defined as the standard deviation of LOX duplicate samplings and HPL triplicate samplings, divided by their respective means, multiplied by 100.

5.4.3. Effect of Additive Concentration on LOX/HPL Activities ($K_{inactivation}$) and $C_{(1/2)}$

Table 17 illustrates that the additive with the least damaging effect overall on LOX was glycine, which was determined to have a $K_{inactivation}$ of 0.0136 and a $C_{(1/2)}$ of 50.96% (w/v). Large $C_{(1/2)}$ values and depressed $K_{inactivation}$ values for a given chemical additive were taken to be representative of suitability of an additive for the LOX/HPL preparation; on this basis, an optimal concentration for each additive (expressed in %, w/v), was chosen for LOX/HPL thermostability studies. The order of $C_{(1/2)}$ for the investigated additives was glycine > mannitol > PEG followed by glycerol and sucrose, which were not significantly different from each other, with $C_{(1/2)}$ values of 2.23 and 1.86 % (w/v), respectively. All polyhydroxyl containing additives possessed dramatically higher $K_{inactivation}$ than did glycine. Mannitol was the second least inactivating additive, however, glycine could be added at 10-fold the concentration before an equivalent drop in activity was observed. All the additives investigated resulted in a decrease in the LOX and HPL activity; this decrease may be attributable to the stripping effect of the additive on the water layer surrounding the enzyme, which is essential for the enzyme activity (Wang, 1999). The decrease in the LOX/HPL activity may also be due to the direct effect of the molecular structure of the additive on the enzyme itself, by binding either in or near its active site, resulting in conformational changes (Chang *et al.*, 1988; Lopez and Burgos, 1995).

HPL activity followed almost the same trend as was seen in LOX where glycine was the most appropriate investigated additive, possessing a small $K_{inactivation}$ of 0.0296 and a $C_{(1/2)}$ of 10.02 % (w/v). The order of appropriateness for the investigated additives for HPL was glycine > sucrose > mannitol > PEG > glycerol, which differed from LOX only in the position of sucrose and not mannitol being the second most appropriate additive investigated. Glycine could be added at ~2-fold the concentration of sucrose before 50% of the HPL activity was lost. The most inappropriate additive investigated was glycerol, which possessed a $K_{inactivation}$ of 0.2604 and a $C_{(1/2)}$ of 2.66 % (w/v). It may be noted that $K_{inactivation}$ and $C_{(1/2)}$ values were also calculated for KCl additive but no loss in LOX/HPL activity could be determined up to the limit of solubility for the salt

Table 17. The effect of selected additives on the lipoxygenase (LOX) and hydroperoxide lyase (HPL) specific activities of an enzymatic extract from *Penicillium camemberti*.

Additive[c]	LOX[a]		HPL[b]	
	$K_{inactivation}$[d]	$C_{(1/2)}$[e]	$K_{inactivation}$[d]	$C_{(1/2)}$[e]
Glycerol	0.3104	2.23	0.2604	2.66
Polyethylene glycol (20K)	0.2214	3.13	0.2246	3.09
Mannitol	0.1236	5.61	0.1765	3.93
Sucrose	0.3720	1.86	0.1475	4.70
Glycine	0.0136	50.96	0.0296	10.02

[a]Lipoxygenase specific activity was defined as nanomol diene hydroperoxide produced/mg protein/min and was determined spectrophotometrically by monitoring the increase in absorbance at 234 nm, using linoleic acid as substrate. All trials were performed in 3 duplicate series, run in tandem with blanks.

[b]Hydroperoxide lyase specific activity was defined as nanomol 10-hydroperoxide of linoleic acid converted/mg protein/min and was determined from the plot of the residual hydroperoxide versus enzyme concentration an molar exctinction coefficient of 18 765 M^{-1} cm^{-1} absorbance at 560 nm in the xylenol orange assay. All trials were performed in triplicate, run in tandem with blanks.

[c]Additives were defined as chemicals included in the ultrafiltered, lyophilized LOX/HPL enzyme extract (ultrafiltered 30 000 NMWCO).

[d]The rate constant of inactivation ($K_{inactivation}$) expressed as 1/% w/v $^{-1}$ was calculated by plotting the ln percentage residual specific activity versus the percentage of additive containing trial for LOX and HPL, respectively.

[e]The $C_{(1/2)}$ expressed in % w/v, was defined as the additive concentration at which 50% of the initial activity was obtained.

(data not shown). On the basis of the experimental results (Table 17) the thermostability studies were conducted using glycine at 10% (w/v), mannitol and glycerol at 5% (w/v), respectively, whereas for sucrose and PEG, 2.5 % (w/v) was chosen.

The most common type additive included in LOX and HPL extracts are non-ionic detergents, including a variety of Tritons® (polyoxyethylene octyl alkyl ethers) or Tweens® (polyoxyethylene sorbitol esters) which are added at concentrations ranging from 0.1 to 1% (w/v) with no reported decrease in LOX or HPL activity (Su and Oliw, 1998; Pérez-Gilabert and García-Carmona, 2001). Non-ionic detergents are, however, widely regarded as inactivating to both LOX and HPL activities at concentrations greater than 1% (w/v) (Busto *et al.*, 1999). Hughes *et al.* (2006) reported that the specific activity of plant hydroperoxide lyase could be modified with the addition of non-ionic detergents including Emulphogene® (polyoxyethylene 6-tridecyl ether). The authors reported that the addition of the detergent at concentrations up to 80 mM resulted in an 8-fold higher catalytic efficiency (K_{cat}) versus the non-detergent containing HPL trial. The authors suggested that the effect may be due to coordination of the detergent with the non-heme iron, as well as conformational changes in the HPL molecule (Hughes *et al.*, 2006). The addition of hydrophilic polyols and non-reducing sugars including glycerol and sucrose, have been reported for LOX (Leone *et al.*, 2006) and HPL (Götz-Schmidt *et al.*, 1986) extracts. Typically, these stabilizing additives are included at concentrations of 10 to 30% (w/v) and are not reported to decrease enzymatic activities relative to the control.

Reducing sugars, including glucose, have been reported to decrease LOX activity at concentrations greater than 20% (w/v) (Busto *et al.*, 1999). Other chemical additives have been included in various LOX extracts including metal chelators such as ethylene diamine tetraacetate (EDTA) (García-Carmona, 2001) and ethylene glycol tetraacetate (EGTA) (Brash *et al.*, 1996) at concentrations rangings from 0.5 to 4.0 mM. Thiol containing additives at concentrations ranging from 1 to 7 mM of are commonly included in HPL extracts, including dithiothreitol (DTT) (Hornostaj and Robinson, 1999) and 2-ethanethiol (Suurmeijer *et al.*, 2000).

5.4.4. Effect of Additives on LOX Thermostability

Figure 17 illustrates the thermostability profiles of LOX and reveals that all additives investigated conferred some level of stabilization at elevated temperature. The enzyme activity of the control LOX assay, with 7.5 ppm KCl, decreased with values of 100.0, 75.6, 37.2 and 8.2% at 4, 25, 40 and 50°C, respectively. A treatment at 60°C completely inactivated the LOX activity. Assays containing mannitol 5% (w/v) appeared to be the most stabilized of those investigated and showed a decrease in LOX activity from 100 to 81.2, 86.4 and 74.9% at 4, 25, 40 and 50°C, respectively. For mannitol at 60 and 70°C, the enzyme activity was 48.5 and 27.0%, respectively. When glycine 10% (w/v) was added, the LOX decreased from 100 to 79.1, 50.9 and 63.0% residual activity at 4, 25, 40 and 50°C, respectively. At 60°C glycine preserved 37.1% residual LOX activity. PEG 2.5% (w/v) was effective in maintaining 100% LOX activity up to 40°C treatment, after which a drop in residual activity was observed. Sucrose was ineffective for the maintenance of LOX activity and showed a decrease to 65.4% after 25°C treatment, which was lower than any additive tested. Glycerol showed the highest residual activity after 25°C temperature treatment, after which a drop to 72.7 and 37% was seen at 40 and 50°C, respectively. In all trials, LOX activity was completely inactivated at 80°C.

The $K_{inactivation}$ of *P. camemberti* LOX is in agreement with those reported for other LOXs, where inactivation occurred in the range of 45-65°C (Rodrigo *et al.*, 2006). The thermostabilizing effect of sucrose and glycerol was reported (Lopez and Burgos, 1995) for soybean LOX type- 1B (72.5°C, 2 min), as being due to increased hydrophobic interaction due to the of exclusion of the additive; these authors suggested a solvophobic effect where glycerol fits well into the water lattice and forms hydrogen bonds that reinforce water interactions, thus making contacts between hydrophobic residues and water more favourable. The enhancement in LOX thermostability (70°C, 8 min) in a pea seed extract containing KCl (1 M) and sucrose (2 M) was reported to be 1.5 and 6.0-fold greater than that of the control, respectively (Busto *et al.*, 1999). The pea seed LOX thermostability (70°C, 8 min) using mannitol, glycerol and sorbitol additives (2M) was 2.3, 1.7 and 1.2-fold above that of the control, respectively (Busto *et al.*, 1999). The LOX

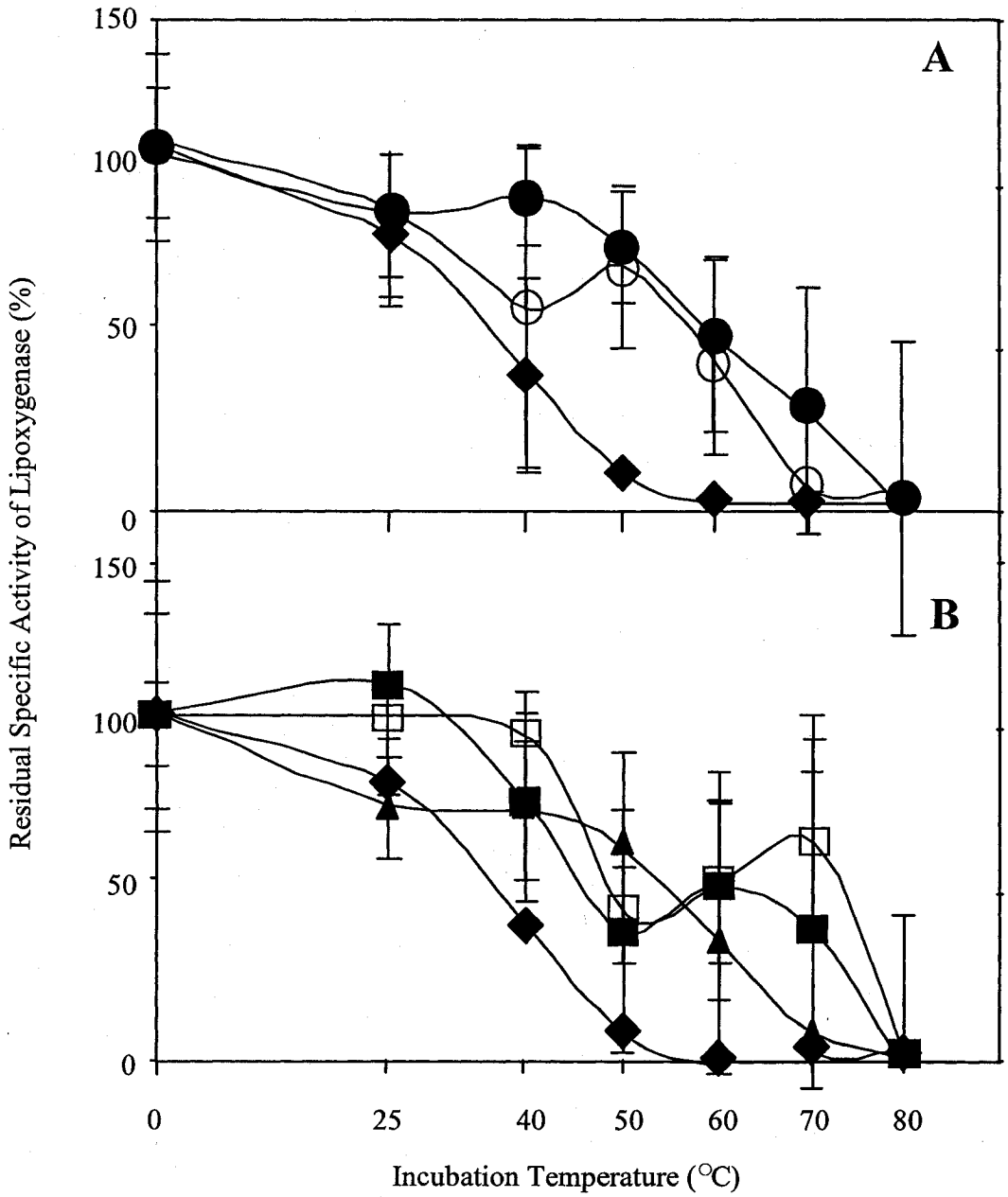

Figure 17. Thermostability profiles of lipoxygenase (LOX) activity in enzymatic extracts from *Penicillium camemberti*, after temperature treatments of 1 h, where the (%) residual specific activity of LOX was determined for trials containing: (A) (◆) KCl 7.5 ppm, (○) glycine 10% w/v, (●) mannitol 5% w/v and (B) (□) polyethelyne glycol 2.5% w/v, (▲) sucrose 2.5% w/v, and (■) glycerol 5% w/v, additive in the extract, respectively.

of *Pleurotus ostreatus* (Kuribayashi *et al.*, 2002) was inactivated after a thermal treatment at 40°C for 5 min, wheras that of fish gill (Hsieh *et al.*, 1988) lost 95% activity at 50°C for 10 min. Iny *et al.* (1993) reported that *Thermoactinomyces vulgaris* LOX maintained 80% residual activity after treatment at 70°C (20 min), but activity was completely inactivated after treatment at 85°C for 20 min, whereas *Fusarium oxysporum* LOX (Matsuda *et al*, 1976) was active after a thermal treatment of 70°C (15 min) and lost all activity after treatment at 100°C for 60 min. Paprika LOX was reported to be inactivated at 80°C for 10 min; however, peroxidase activity was unaffected (Schweiggert *et al.*, 2005). The decrease in *P. camemberti* LOX activity (Fig. 17) may be explained by the sequential inactivation of several different isozymes present in the LOX/HPL extract. Anthon and Barret (2003) reported that thermal inactivation of tomato LOX yielded 3 separate $K_{inactivation}$ constants corresponding to 3 distinct LOX isozymes. A similar result was reported for three isozymes of LOX isolated from soybean, thermally treated at 70°C for 10 min (Matoba *et al.*, 1985). Perraud and Kermasha (2000) postulated that *P. camemberti* may also contain several LOX isozymes, owing to the multiple regiosomeric hydroperoxides isolated from biocatalysis with linoleic acid substrate.

5.4.5. Effect of Additives on HPL Thermostability

Figure 18 illustrates HPL thermostability in the presence and absence of additives. It may be seen that HPL activity was more stable than that of LOX over a broader range of temperatures. The enzyme activity of the control HPL assay, with 7.5 ppm KCl, showed residual HPL activity of 89.7, 93.0, 94.2, 86.3 and 40% at 25, 40, 50, 60 and 70°C, respectively. At 80°C, the HPL activity was completely inactivated in the control (KCl, 7.5 ppm) assay. By contrast, the HPL assay containing glycine 10% (w/v), possessed stable residual activity at temperatures from 25 to 70°C and possessed 51.5% residual HPL activity at 80°C. The least appropriate additive investigated for HPL thermostability was mannitol 5% (w/v), which appeared to inactivate HPL activity at all temperatures investigated. Assays containing sucrose 2.5% (w/v) possessed HPL residual activity of 95% up to 60°C, after which a rapid decrease in HPL activity was observed. The HPL thermostability profile of assays containing PEG 2.5% (w/v) was a slow decline in HPL residual activity, from 100 to 53, 34 and 0% at 25, 50, 60 and 70°C, respectively.

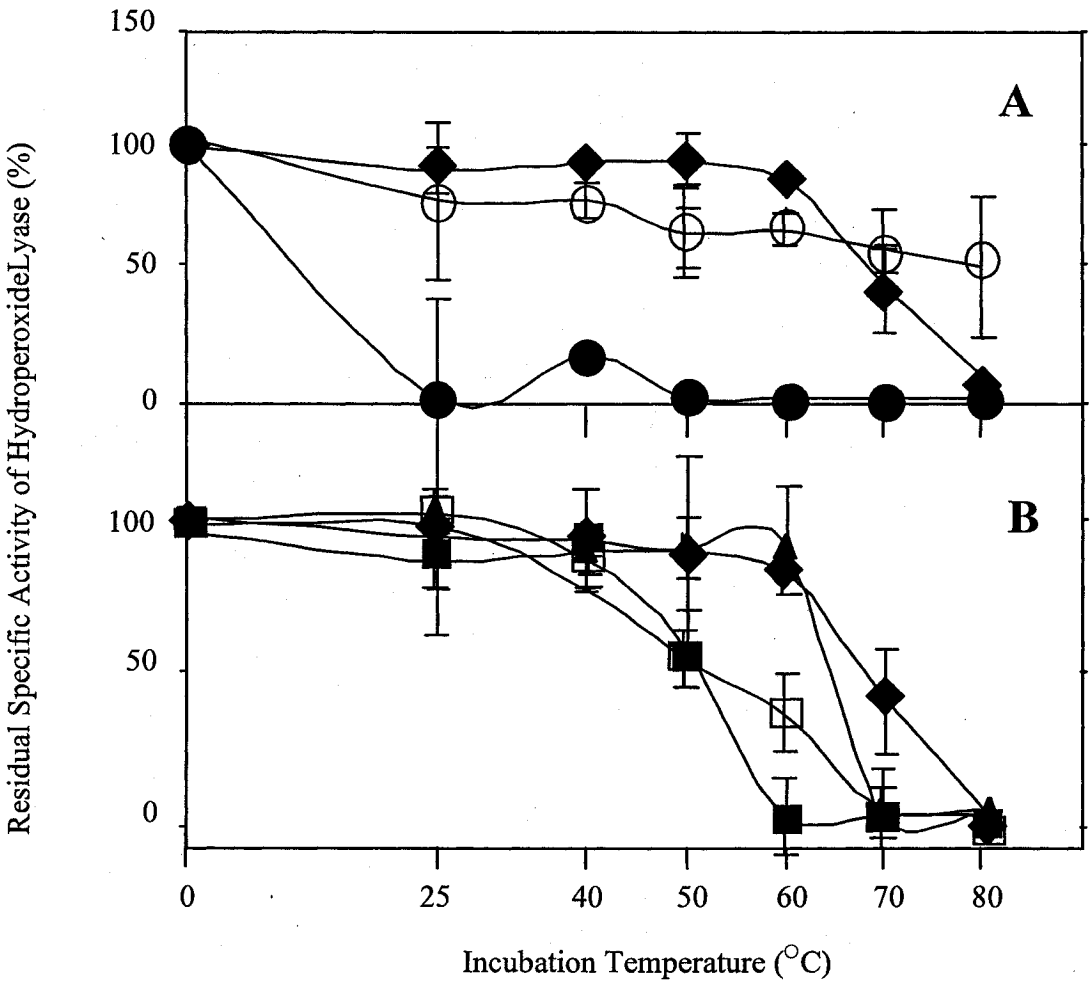

Figure 18. Thermostability profiles of hydroperoxide lyase (HPL) activity in enzymatic extracts from *Penicillium camemberti*, after temperature treatments of 1 h, where the (%) residual specific activity of HPL was determined for trials containing: (A) (◆) KCl 7.5 ppm, (○) glycine 10% w/v, (●) mannitol 5% w/v and (B) (□) polyethelyne glycol 2.5% w/v, (▲) sucrose 2.5% w/v, and (■) glycerol 5% w/v, additive in the extract, respectively.

Assays containing glycerol 5% (w/v) possessed HPL residual activity of 50% at the temperature of 50°C.

A comparison of the thermostability of the LOX and HPL controls (KCl, 7.5 ppm) indicate that LOX was less thermostable than HPL, and displayed a greater decrease in residual specific activity at all thermal treatment temperatures investigated (25 to 80°C) (Figs. 17 and 18). This result is consistent with the LOX thermostability (50°C, 0.5 h) of carrots, green beans and green peas extracts which maintained 0, 30 and 2% residual activity, respectively (Akyol *et al.*, 2006). This result is also consistent with the thermal inactivation of barley LOX (Hirota *et al.*, 2006) and broccoli LOX (Morales-Blancas *et al.*, 20002). In the current study LOX and HPL controls displayed a linear decrease in thermal inactivation, but were predominantly non-linear with respect to trials containing additives (Figs. 17 and 18). Morales-Blancas *et al.* (2002) noted that thermal inactivation profiles of crude enzyme extracts possessing multiple enzymatic activities, demonstrated a linear decrease for enzymes with large rate constants of inactivation ($K_{inactivation}$), whereas heat resistant enzymes caused deviation from linearity. Our results suggest that the addition of chemical additives did improve LOX and HPL thermostability and may be responsible for the non-linearity of the thermal inactivation in additive containing trials.

Results (Fig. 18) of the thermostability of HPL are in agreement with values for the thermal inactivation of HPL activity reported from other sources, including soybean which maintained 20% residual activity after thermal treatment of 50°C for 5 min (Olías *et al.* 1990). Cucumber fruit HPL showed a 50% decrease in activity after treatment at 45°C for 5 min (Hornostaj and Robinson, 1999), whereas Kim and Grosch (1981) reported that pear fruit HPL lost 30 and 100% activity after treatment at 50°C for 10 and 60 min, respectively. Studies (Suurmeijer *et al.*, 2000; Anese and Sovrano, 2006) suggested that the thermostability of HPL is not as great as that of LOX from the same source. *Pleurotus pulmonarius* HPL activity was completely inactivated at 70°C for 5 min, whereas the LOX residual activity remained at 100% (Assaf *et al.*, 1995). The experimental results (Fig. 18) indicate that the presence of glycine, PEG, sucrose and

glycerol in the enzyme extract, led to an increase in HPL thermostability, suggesting that the HPL activity was stabilized from inactivation by increased hydrophobic interaction due to exclusion of the additive from the protein surface (Gekko and Timasheff, 1981; Timasheff, 1993; Jenson *et al.*, 1996; Remmele *et al.*, 1998; Timasheff, 2002).

5.5. Conclusion

The LOX and HPL activities of the extract from *P. camemberti* were stabilized by the addition of chemical additives. KCl (7.5 ppm) additive was effective at minimizing lyophilization inactivation in the LOX/HPL extract, as well as it was determined to be activating toward HPL activity. Dextran, at all concentrations investigated, increased lyophilization inactivation in the LOX/HPL extract. The long-term stability of the lyophilized extract containg KCl (7.5 ppm) was 2 months for LOX and 1 month for HPL. The chemical inactivation rate constants ($K_{inactivation}$) for glycine, mannitol, sucrose, glycerol and PEG indicated that glycine was the most appropriate for LOX/HPL acitivity. Thermostability studies suggested that the addition of mannitol to the enzyme extract was most appropriate for the stabilization of LOX activity, whereas, the addition of glycine was most appropriate for the stabilization of HPL activity. Overall, the stability of the flavor-producing enzymes, LOX/HPL, was stabilized by the addition of selected chemicals.

CHAPTER VI

STATEMENT OF CHAPTER VI LINKAGE

As a result of the research work conducted previously on the chemical stabilization of lipoxygenase and hydroperoxide lyase activities in extracts of *Penicillium camemberti* (Chapter V) investigation into the immobilization of the enzymatic extract from same microorganism was conducted. The further investigation of stability in the enzyme extract from *P. camemberti* offers an opportunity to compare the efficacy of two different stabilization strategies.

CHAPTER VI

IMMOBILIZATION OF AN ENZYMATIC EXTRACT FROM *PENICILLIUM CAMEMBERTI* CONTAINING LIPOXYGENASE AND HYDROPEROXIDE LYASE ACTIVITIES

6.1. Abstract

An enzymatic extract from *Penicillium camemberti*, containing lipoxygenase (LOX) and hydroperoxide lyase (HPL) activities was immobilized on (oxirane acrylic beads) Eupergit®C and Eupergit®C250L-iminodiacetate (IDA). The optimum pH for LOX activity was determined to be 6.0 and 4.0 for the free and 6.0 for the immobilized enzyme preparation, whereas pH 6.0 was the optimum for the HPL activity for both free and immobilized one. Optimal reaction temperatures for LOX activity was 30 and 55°C for the free and immobilized enzyme preparation, respectively, whereas the HPL activity showed its optima at 45 and 30°C, for the free and immobilized one, respectively. The immobilization of the enzyme preparation dramatically enhanced the thermostability of LOX and HPL activity. Long-term stability of the lyophilized immobilized preparation showed that LOX activity at -80°C was more stable compared to that at 4°C, whereas the HPL activity was equally stable for both free and immobilized extracts at both temperatures. The results indicated a decrease and an increase in enzyme efficiency for LOX and HPL activity, respectively.

6.2. Introduction

The sequential action of lipoxygenase (LOX) (linoleate: oxygen oxidoreductase; EC 1.13.11.12) and hydroperoxide lyase (HPL) results in the bioconversion of polyunsaturated fatty acids into a wide variety of flavor compounds (Gardner, 1996). Although there has been an increasing interest in the LOX/HPL biocatalyzed production of aroma compounds (Chen *et al.*, 1984; Kermasha *et al.*, 2002*a*), the limited stability of the enzymes has restricted their biotechnological utility (Hsu *et al.*, 1999). Production of natural flavors from lipid sources requires enzymes with high thermal stability. Immobilization offers substantial enzymatic stabilization and is simple, as well as it offers the possibility for reuseability of the biocatalyst in continuous operation packed-bed reactors (Hsu *et al.*, 1999). Further benefits of immobilization include the ease of separation of the enzyme from its end products, which minimizes downstream processing costs (Kim *et al.*, 2006). The coupling of an enzyme to a support is contingent on chemical bond formation between the functional groups of the immobilization support and those of the enzyme. Several types of immobilizations exist, including adsorptive, entrapment and crosslinking (Bickerstaff, 1997; Kim *et al.*, 2006). Covalent supports form bonds at a variety of attachment points on the enzyme, including $-NH_3$, $-COO^-$, or -SH groups (Nuñez *et al.*, 1997; Rehbock and Berger, 1998). Oxirane acrylic resins or beads possess many oxirane groups that bind primary amines in the formation of covalently bound enzymes (Katchalski-Katzir and Kraemer, 2000). Eupergit® supports are among the most popular types of covalent supports and have been used to stabilize dozens of different types of enzymes, often by multi-point attachment of enzyme and support (Katchalski-Katzir and Kraemer, 2000). Several studies have been conducted immobilized extracts containing LOX (Carmen Pinto *et al.*, 1996; Vega *et al.*, 2005*a*) and HPL activities (Nuñez *et al.*, 1997; Rehbock and Berger, 1998).

The overall objective of this study was to investigate the effect of immobilization on a LOX/HPL enzymatic extract from *P. camemberti* using a variety of supports. The specific objectives were to characterize the LOX/HPL activity in free and immobilized extracts in terms of optimum pH, reaction temperature, thermostability, long-term stability and other kinetic parameters.

6.3. Materials and Methods

6.3.1. Culture Growth and Harvesting Conditions

The culture growth and harvesting of *P. camemberti* was conducted according to the procedure outlined by Perraud and Kermasha (2000), as described in Section 5.3.1.

6.3.2. Preparation of Enzymatic Extract

The preparation of the enzymatic extract was conducted according to the procedure as described in Section 5.3.2.

6.3.3. Protein Measurement

The protein content of the LOX/HPL enzymatic extract was measured with a modified Lowry method (Hartree, 1972), using bovine serum albumin (Sigma Chemical Co.; St-Louis, MO) as a standard for the calibration curve.

6.3.4. Immobilization Supports

The supports investigated in this study included the covalent oxirane acrylic supports Eupergit® C and Eupergit® C250L (Röhm Pharma Polymers, Darmstadt, Germany) which were both used unmodified and modified with ethylene diamine (EDA) and iminodiacetate (IDA), according to a procedure outlined by Mateo *et al.* (2001). The modifications involved suspension of Eupergit® C or Eupergit® C250L (1 g wet weight) in 10 mL of EDA (5%, w/v) or 5 mL of IDA (1.8 M), under gentle stirring at 25°C for 15 min and 5 h, respectively. The modified EDA and IDA supports were then washed with deionized water. The silica gel support was purchased from Silicycle (Quebec City, Qc) and the anion exchange resin Dowex® 50WX4-200 was purchased from Aldrich (Milwaukee, WI).

6.3.5. Immobilization of Enzyme Preparation

The immobilization of the enzyme extract, containing LOX/HPL activity, was conducted at 4°C, using 40 mg protein/g wet support. Potassium phosphate buffer (pH 6.5, 0.1 M) was used for all steps unless otherwise indicated. Unmodified supports were coupled using 1.0 M buffer, whereas EDA and IDA modified supports were coupled using 0.1 M buffer, in conical 5 mL screw-cap tubes under mild agitation. After 18 h, the

agitation was halted and any free enzyme was removed by pipetting and aliquotted for protein determination. The immobilization support was washed 1×15 mL of deionized water and 2×15 mL of buffer and each wash was aliquotted for later protein determination. The support containing the immobilized enzyme extract was then re-suspended in buffer (0.1 g wet support/mL) and set aside for LOX/HPL assays.

Blank trials for the free and immobilized enzyme preparations were carried out with thermally inactivated (95°C, 1 h) LOX/HPL activities, using 15 mL screw-cap tubes, which was followed by cooling (4°C, 0.5 h). Prior to the enzymatic assay, the enzyme extract was allowed to warm to 25°C and was gently mixed to re-suspend the protein.

The enzyme activity per g support was defined as the specific activity of either LOX or HPL per g of wet support expressed as nanomol of hydroperoxide produced or converted/mg protein/min, for LOX and HPL, respectively. Protein immobilization yield (%) was defined as the ratio of protein, immobilized onto a support (mg), divided by the initial protein content (mg) multiplied by 100. The retention of enzyme activity (%) was defined as the specific activity of LOX or HPL of the immobilized enzyme extract, divided by the specific activity of LOX or HPL of the free extract and multiplied by 100.

6.3.6. Substrate Preparation

LOX studies used linoleic acid (*cis*-9,*cis*-12-octadecadienoic acid) purchased from Nu-Chek-Prep Inc. (Elysian, MN). The substrate stock solutions were prepared according to the procedure outlined by Perraud *et al.* (1999) as described in Section 5.3.6.

HPL studies used the 10-hydroperoxide of octadecadienoic acid (10-HPOD), which was prepared according to the procedure outlined by Kermasha *et al.* (2002*b*) as described in Section 5.3.6.

6.3.7. Enzyme Assays

LOX/HPL activities were assayed spectrophotometrically with xylenol orange [(3,3'-*bis*(*N*,*N*-di(carboxymethyl)aminomethyl)-*o*-cresol)] (Sigma Chemical Co.), using a Beckman DU-650 spectrophotometer (Beckman Instruments Inc.; San Raman, CA),

according to a modified procedure outlined by Jiang *et al.* (1991) as described in Section 5.3.7.

6.3.7.1. Free and Immobilized LOX Assays

The free LOX assay was initiated by the addition of 180 μL of enzyme extract (1.5 mg protein/mL) to 0.6 mL of substrate solution (4.0 mM), with sufficient buffer to adjust the final volume to 1.5 mL. The LOX assay for the immobilized extract was initiated by the addition of 0.6 mL of immobilized enzyme extract (0.1 g support/mL) to 1 mL of substrate (4.0 mM), with sufficient buffer to obtain a total volume of 2.6 mL. The LOX assays for the free and immobilized enzyme extracts were carried out at 25°C, under mild stirring, for 12 and 35 min, respectively. Aliquots of the reaction homogenate (0.1 mL) were taken at selected time intervals and were immediately added to 1 mL of xylenol orange reagent. Any small quantity of immobilization support present in the aliquot was allowed to settle out of xylenol orange solution prior to taking a second aliquot (0.6 mL) for spectrophotometric measurement. LOX specific activity was defined as nanomol of conjugated diene linoleic acid hydroperoxide produced/mg protein/min. All LOX assays were performed in duplicate series and run in tandem with a blank that contained all components of the enzymatic assay; however thermally inactivated LOX extract (95°C, 1 h) was substituted for the active LOX extract.

6.3.7.2. Free and Immobilized HPL Assays

HPL assays were conducted using the 10-HPOD substrate (10 nanomol) containing Tween 80 (0.3 μL per 0.9 μmol) placed into 5 mL thermostated tubes with 10 μL of the appropriate buffer solution (0.1 M). The mixture was sonicated for 3 min according to the procedure outlined by Kermasha *et al.* (2002*a*). The HPL reaction was initiated by the addition of 15 μL of the free enzyme extract (1.5 mg protein/mL) or the immobilized enzyme extract (0.1 g support/mL) and allowed to react for 5 min, under gentle agitation. The HPL reaction was halted by the addition of xylenol orange reagent (2 mL) to the reaction homogenate. As with LOX, any immobilization support was allowed to settle out of the xylenol orange solution prior to taking a 0.6 mL aliquot for spectrophotometric measurement. HPL specific activity was defined as nanomol of 10-HPOD converted/mg protein/min and was determined from several activity curves

constructed using serial dilutions of the HPL extract. All HPL trials were run in duplicate in tandem with a blank, containing all components of the assay; however, thermally inactivated HPL (95°C, 1 h) was substituted for the active HPL extract.

6.3.8. Effect of pH

The effect of pH on free and immobilized extract containing LOX/HPL activities, was investigated using a wide range of buffers (0.1 M), including citrate phosphate buffer solution for the pH range of 3.0 to 5.5; potassium phosphate buffer solution for the pH range of 6.0 to 8.0 and glycine-NaOH buffer for the pH values of 8.5 and 9.0.

6.3.9. Effect of Reaction Temperature

The effect of reaction temperature on the LOX/HPL activity of the free and immobilized enzyme extracts was conducted over a wide range of temperatures (5 to 75°C), as previously described in Sections 6.3.7.1. and 6.3.7.2., respectively.

6.3.10. Effect of Thermal Treatment

Free and immobilized enzymatic extracts were subjected to an extended thermal treatment at 25°C (0 to 84 h), using a Precision reciprocal shaking bath Model 25 (Precision Scientific, Chicago, IL) and the residual LOX/HPL activities were measured at regular time intervals of (0, 2, 4, 8, 12, 24, 36, 48, 60, 72 and 84 h) and (0, 1, 2, 3, 4, 5 and 6 h) for LOX and HPL, respectively. The concentration of the free enzymatic extract was 1.50 mg protein/mL, whereas that of the immobilized preparation was 0.15 to 0.50 mg protein/mL.

6.3.11. Long-Term Stability

Lyophilized immobilized enzyme extracts were stored in 15 mL polypropylene tubes (1.5 mg protein) contained in desiccators containing Dry-Rite® desiccant. The tubes were stored at 4°C and at −80°C for a period of 9 weeks and assayed for LOX/HPL activities weekly, using immobilized enzymatic extract from the same sample, according to the procedures previously described in Sections 6.3.7.1. and 6.3.7.2., respectively.

6.3.12. Kinetic Parameters of Free and Immobilized LOX and HPL

The effect of linoleic acid substrate concentration on the specific activity of the free and immobilized LOXs was investigated using concentrations of 12 to 1.5×10^3 μM and 77 to 1.5×10^3 μM, respectively. The effect of 10-HPOD substrate concentration on the specific activity of the free and immobilized HPLs was investigated using substrate concentrations of 25 to 0.8×10^3 μM and 100 to 0.7×10^3 μM, respectively.

6.4. Results and Discussion

6.4.1. Selection of Immobilization Supports

Table 18 summarizes the experimental data for the immobilization of the enzymatic extract from *P. camemberti*, containing LOX/HPL activities, using selected supports. The results indicate that for LOX activity, wide range of protein immobilization yield % (10.4-32.9%) were obtained with the use of Eupergit® C-EDA, Eupergit® C-IDA, Eupergit® C250L and Eupergit® C250L-EDA. The protein immobilization yield % was higher for Eupergit® C (28.4%) than that for Eupergit® C250L-IDA (10.1%) and retention of LOX activity was 366.6 and 422.5%, respectively. These results suggest that the greater number of reactive epoxides in Eupergit® C (~600 μmol oxirane functionalities /g dry weight support) were effective at covalently binding amino, hydroxyl and thiol residues in LOX (Mateo *et al.*, 2000*b*). It may be noted (Table 18) that for LOX, many of the investigated supports, including silica and the epoxy supports Eupergit® C-EDA, Eupergit® C-IDA, Eupergit® C250L and Eupergit® C250L-EDA, did not contain detectable enzyme activity. The decrease in enzyme activity may be the result of a combination of conformational changes in the protein during immobilization, steric hindrance at the active site and substrate diffusion limitations (Mateo *et al.*, 2000*a*,*b*), as well as the formation of color arising from the support itself (Katchalski-Katzir and Kraemer, 2000). Vega *et al.* (2005*a*) reported the value for protein immobilization yield % obtained for soybean LOX type-1B on Eupergit® C250L-IDA as 29.3%, which is greater than that determined (10.13%) for the present LOX from *P. camemberti*. These results suggest that Eupergit® supports can have differential immobilization yields depending on the source and concentration of the enzyme (Katchalski-Katzir and Kraemer, 2000). The tendency of oxirane acrylic beads to cause interfering oxidation of

Table 18. Summary of the immobilization parameters of the enzyme extract from *Penicillium camemberti* containing lipoxygenase (LOX) and hydroperoxide lyase (HPL) activities, using linoleic acid and the 10-hydroperoxide regio-isomer of octadecadienoic acid (10-HPOD) as substrate, respectively.

Support	Activity per Gram Support[b] (nanomol HPOD/ g support/ min)	Protein Immobilization yield[c] (%)	Retention of Activity[d] (%)
LOX[a]			
Eupergit C	33.1	28.4	366.6
Eupergit C-EDA[e]	nd[f]	15.9	0
Eupergit C-IDA[g]	nd[f]	10.4	0
Eupergit C250L	nd[f]	16.3	0
Eupergit C250L-EDA[e]	nd[f]	32.9	0
Eupergit C250L-IDA[g]	16.5	10.1	422.5
Silica	nd[f]	20.5	0
Dowex 50WX4-200	14.9	26.8	281.2
HPL[h]			
Eupergit C	50.1	18.1	145.1
Eupergit C250L	213.6	12.7	472.5
Eupergit C250L-EDA[e]	284.6	36.7	197.5
Eupergit C250L-IDA[g]	151.5	12.5	356.0
Silica	nd[f]	24.2	0
Dowex 50WX4-200	nd[f]	30.3	0

[a]Lipoxygenase (LOX) activity was defined as nanomol hydroperoxides produced per mg protein per min and was determined using a molar exctinction coefficient of 18 765 M^{-1} cm^{-1} absorbance at 560 nm in the xylenol orange assay, using linoleic acid as substrate.

[b]Activity per gram support was defined as nanomol hydroperoxide produced (LOX) or converted (HPL) per gram wet support per min.

[c]Protein immobilization yield percentage was defined as ratio of immobilized protein relative to the initial amount of free protein, expressed as a percentage.

[d] Retention of activity percentage was defined as the specific activity of either immobilized LOX or HPL divided by the specific activity of free LOX or HPL and multiplied by 100.

[e,g]The Eupergit® supports were modified with ethylene diamine (EDA)[e] and iminodiacetate (IDA)[g], according to the procedure outlined by Mateo *et al.* (2001).

[f]not detected.

[h]Hydroperoxide lyase (HPL) activity was defined as nanomol hydroperoxides converted per mg protein per min and was determined using a molar exctinction coefficient of 18 765 M^{-1} cm^{-1} absorbance at 560 nm in the xylenol orange assay, using the 10-hydroperoxide of linoleic acid as substrate.

the substrate was reported by Carmen Pinto *et al.* (1997); this interference was addressed by the reduction of excess/unreacted oxirane groups in the immobilization support, where the oxirane acrylic support, used for the immobilization of potato LOX, was incubated overnight in 2-mercaptoethanol (5%, w/v), for use in the blank assays. The retention of enzyme activity for the immobilized *Pisum sativum* LOX was 290% (Liagre *et al.*, 1996). Results for the immobilization of the enzymatic extract of *P. camemberti* (Table 18) suggest that 18 h is the optimal coupling time for the Eupergit® supports, however, shorter coupling times for ionic or adsorptive supports of 1 to 3 h have been reported (Kermasha *et al.*, 2002*a*; Santano *et al.*, 2002; Timur *et al.*, 2002). Most covalent supports require coupling times from 12 to 24 h (Liagre *et al.*, 1996; Hsu *et al.*, 1999; Vega *et al.*, 2005*a*). The optimum ratio of enzyme to support for the immobilization yield % for the enzyme extract from *P. camemberti*, was 40 mg protein/g wet support (data not shown). Vega *et al.* (2005*a*) used 100 mg protein/mL wet support for Eupergit® supports for the immobilization of soybean LOX type-1B. Carmen Pinto *et al.* (1997) reported that 0.7 mg protein/mL wet support was used for the immobilization of potato LOX on Eupergit®. On the basis of experimental findings (Table 18), Eupergit® C was selected for future work with LOX activity of *P. camemberti*.

The immobilization of enzymatic extract from *P. camemberti* for HPL activity on various supports (Table 18) shows a protein immobilization yield % ranging from (12.5 to 36.7 %), which is close to that for LOX; whereas HPL activity per gram support (50.1 to 284.6 nanomol HPOD/g support/min) was much greater than values obtained for LOX (14.9 to 33.1 nanomol HPOD/g support/min). The parameter of protein immobilization yield % for Eupergit® C250L and Eupergit® C250L-IDA were 12.70 and 12.45%, respectively. The HPL activity per g support for Eupergit® C was the lowest ratio and hence this support was discarded. However, Silica, Dowex® 50WX4-200, Eupergit® C250L and Eupergit® C250L-EDA all caused large interferences with the xylenol orange assay; this interference may be due to structural denaturation at or near the active site of HPL. Based on the overall results for HPL activity (Table 18), the immobilization of the enzyme extract from *P. camemberti* on Eupergit® C250L-IDA was considered for future work; this selection is due the low interference this support with the xylenol orange assay

and the acceptable enzyme activity/g support value of 151.49 nanomol of 10-HPOD converted/g support/min. The retention of enzyme activity %, determined for HPL activity from *P. camemberti*, ranged from 145 to 472%; these results suggest that the immobilization enhanced the HPL activity. These experimental findings are in agreement with those reported for the immobilized HPL from *P. sativum*, which was reported to possess 226% retention of activity (Liagre *et al.*, 1996). Decrease in immobilized HPL activity for the immobilized extract as compared to the free one was also reported for that from *Chlorella* sp., which was covalently bound on a variety of supports, including Affi-Gel 10 and Affi-Gel 501, with 55.3 and 67.1% residual specific activity, respectively (Nuñez *et al.*, 1997). HPL from mung bean, immobilized on Ultralink Iodoacetyl, possessed 47% of residual activity after immobilization (Rehbock and Berger, 1998). The overall results (Table 18) for protein immobilization yield % were quite low for the extract from *P. camemberti*, ranging from 12.5 to 36.7%. These results are close those reported for HPL from potato (18.9%) (Liagre *et al.*, 1996) and from mung bean (26%) (Rehbock and Berger, 1998).

6.4.2. Effect of pH

LOX activity of the free enzyme extract and that immobilized on Eupergit® C were both determined to possess an optimum of activity at pH 6.0 (Fig. 19A). These results are close to the pH optimum of 6.5 reported by Perraud and Kermasha (2000) for *P. camemberti* LOX; however, no basic optimum at pH 8.0 is determined in the current study; instead a minor optimum at pH 4.0 was observed. The pH optimum for the HPL activity of the free enzyme extract from *P. camemberti* was determined to be 6.0, whereas that of the enzyme extract immobilized on Eupergit® C250-IDA was most active at pH 4.0 with a minor optimum at 8.0 (Fig. 19B). Kermasha *et al.* (2002*a*) reported that the free HPL from *P. camemberti* had relatively high activity at pH 6.5; however, no pH profile was provided. The pH shifts associated with immobilization on Eupergit® C250-IDA may be attributable to the polycationic nature of the support, which attracts more OH^- ions around the immobilized enzyme, thus making the pH of the enzyme's micro-environment higher than the bulk solution (Katchalski-Katzir and Kraemer, 2000). The immobilized enzyme therefore requires a lower pH for optimal activity than the free

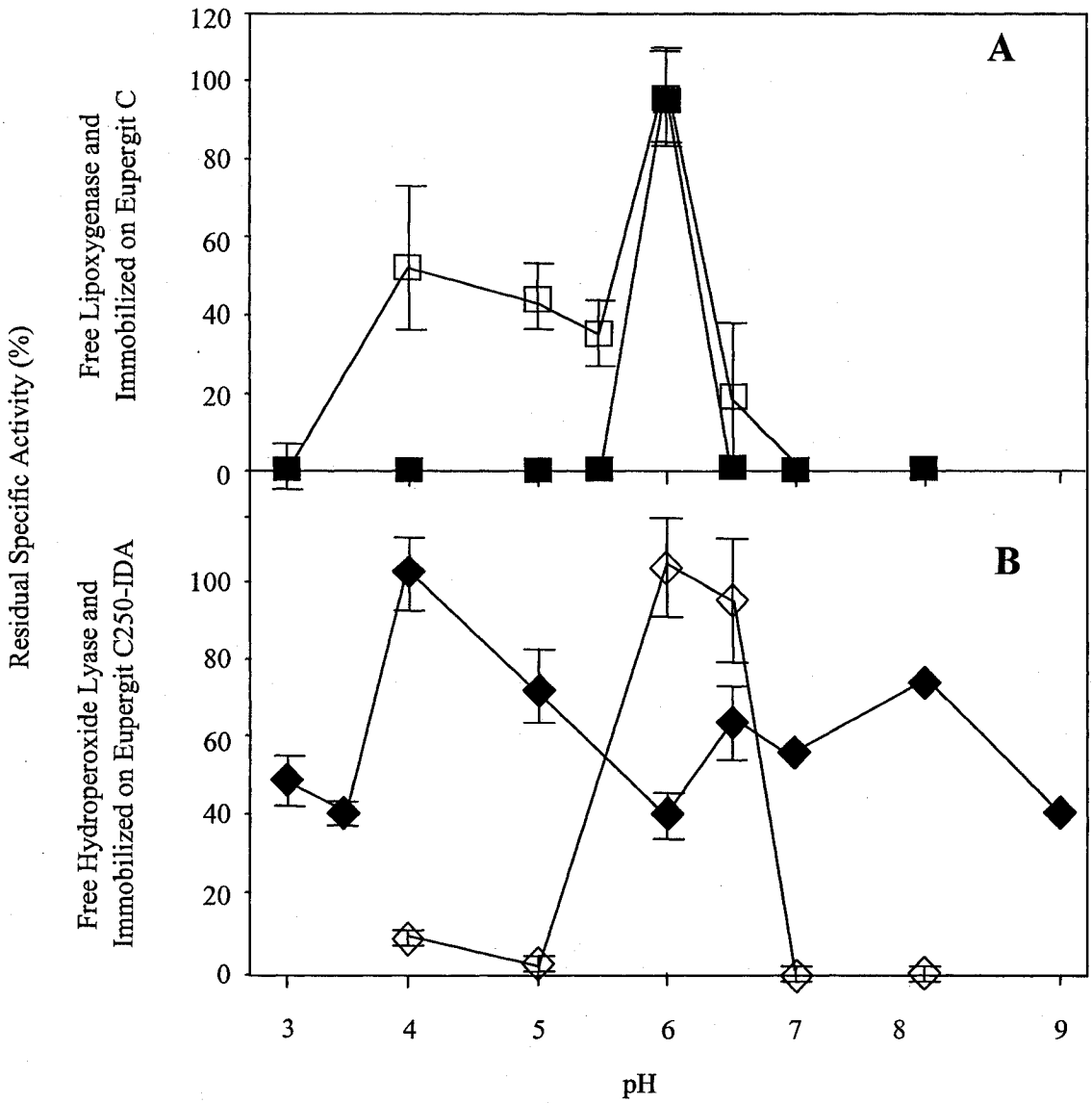

Figure 19. The pH profiles of lipoxygenase (LOX) and hydroperoxide lyase (HPL) activities in enzymatic extracts from *Penicillium camemberti*, where the (%) residual specific activity of LOX was: (A) (□) free, and (■) immobilized and that of HPL was (B) (◇) free and (◆) immobilized, respectively.

enzyme. The optima for potato LOX were 6.0 and 6.5 for the free and immobilized preparations, respectively (Carmen Pinto, 1997). Soybean LOX type-1B was reported to possess a pH optimum at 9.0 for both the free and solgel immobilized preparation (Hsu *et al.*, 1997), whereas free and immobilized HPL from *Chlorella* sp. were reported to possess the same pH optimum at 6.4 (Nuñez *et al.*, 1997). The free LOX and HPL from *P. sativum* were reported to share a pH optimum at 7.0, whereas after immobilization on talc an optimum of 6.4 was reported (Liagre *et al.*, 1996). The pH optimum of Eupergit® C250-IDA immobilized HPL from *P. camemberti* was quite low (4.0); however other HPLs have been reported to possess acidic pH optima, including bell pepper (Shibata *et al*, 1995), cucumber (Hornostaj and Robinson, 1999) and olive fruits (Salas and Sánchez, 1999).

6.4.3. Effect of Reaction Temperature

LOX activity of free enzyme extract and that immobilized on Eupergit®C is shown over a wide range of reaction temperatures from 10 to 65°C (Fig. 20A), whereas the HPL activity of free enzyme extract and that immobilized on Eupergit®C250L-IDA was investigated at 5 to 75°C (Fig. 20B). The results show that the optimum temperature for LOX activity of the free extract was 30°C, whereas that of the immobilized one was 55°C. The optimal temperature for HPL activity of the free enzyme was 45°C, whereas that of the immobilized one was relatively constant from 5 to 30°C, after which a sharp decrease in HPL activity was observed. The results (Figs. 20A and 20B) suggest an increase in the stability of LOX activity upon immobilization on Eupergit C (higher optimal reaction temperature), whereas the opposite is seen for the HPL activity for the immobilized extract on Eupergit® C250-IDA (lower optimal reaction temperature) in comparison to their respective free counterparts; this may be may be due to conformational changes in the HPL enzyme structure upon immobilization. Many free LOXs characterized in terms of reaction temperature have optima in the range of 40 to 60°C, including banana leaf (40°C) (Kuo *et al.*, 2006), *Thermoactinomyces vulgaris* (50°C) (Iny *et al*, 1993) and *Gaümannomyces graminis* (60°C) (Su and Oliw, 1998). Several LOXs possessed reaction temperature optima close to those determined in this study, including *Gersemia fruticosa* (15°C) (Mortimer *et al.*, 2006) and *Pleurotus*

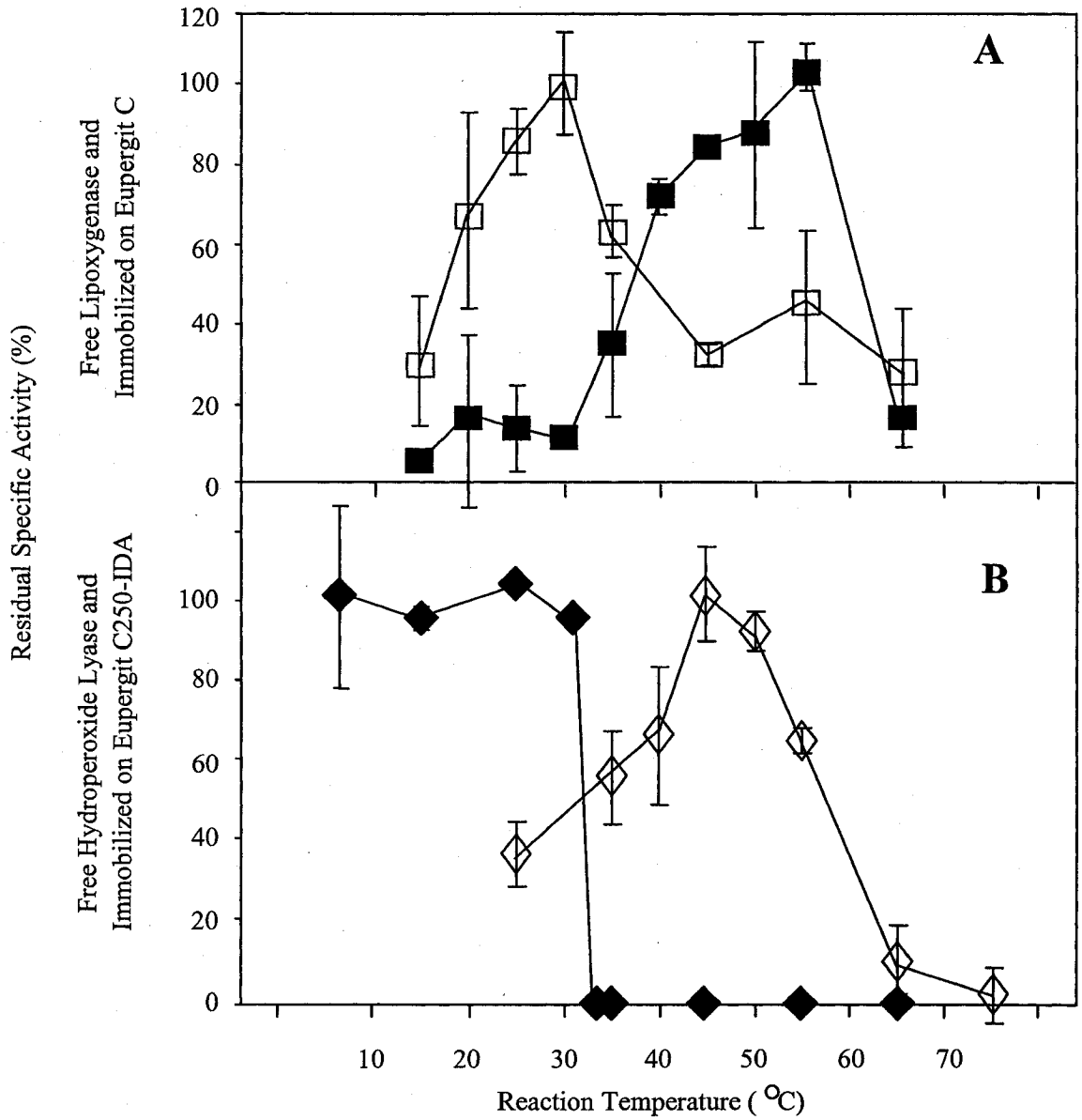

Figure 20. The reaction temperature profiles of lipoxygenase (LOX) and hydroperoxide lyase (HPL) activities in enzymatic extracts from *Penicillium camemberti*, where the (%) residual specific activity of LOX was: (A) (□) free and (■) immobilized and that of HPL was (B) (◇) free and (◆) immobilized, respectively.

pulmonarius (25°C) (Kuribayashi *et al.*, 2002). Carmen Pinto and Macias (1996) reported that soybean LOX immobilized on polyacrylamide gel, possessed an optimum reaction temperature of 35°C, whereas its free counterpart was 30°C. The authors hypothesized that due to a decrease in the energy of activation for the immobilized LOX (19 kJ/mol) in comparison to the free LOX (23 kJ/mol), linoleic acid substrate diffusion effects were minimized (Carmen Pinto and Macias, 1996). A similar profile seen for the LOX activity of the free enzyme extract and the immobilized one, in the current study (Fig. 20A) and is suggestive of an enhanced thermostability in the immobilized extract. Several free HPLs have been reported to have reaction temperature optima of 30°C, including *Oscillatoria* sp. (Adrianarison *et al.*, 1989) tomato leaf (Fauconnier *et al.*, 1997) and cucumber fruit (Hornostaj and Robinson, 1999). Few studies have endeavored to investigate the optimal temperature for HPL activity, instead opting to investigate dual LOX/HPL activities at the LOX temperature optimum (Liagre *et al.*, 1996; Kuroda *et al.*, 2005). Despite this fact, high immobilized HPL activities have been reported at 20°C for *P. sativum* (Liagre *et al.*, 1996) and at 25°C for mung bean (Rehbock and Berger, 1998) and *Chlorella* sp., respectively (Nuñez *et al.*, 1997).

6.4.4. Thermostability of LOX/HPL

Thermostability studies (25°C, 0 to 84 h) of the enzymatic extract LOX activity of the free and immobilized extracts were performed and the results are presented in Figures 21A and 21B, respectively, whereas the thermostability of (25°C, 0 to 36 h) for the free enzyme extract and the immobilized one are displayed in Figures 22A and 22B, respectively. Thermal inactivation of LOX/HPL activities of the free and immobilized enzymatic extracts followed first-order kinetics (data not shown). The free enzymatic extract (1.5 mg/mL) displayed very rapid decrease in LOX activity upon thermal treatment (25°C), with residual specific activity (%) of 95.5, 58.8, 27.2 and 0% after 4, 12, 24 and 36 h, respectively (Fig. 21A). The enzymatic extract immobilized on Eupergit® C (0.15-0.50 mg protein/mL) possessed LOX residual specific activities (%) of 95.3, 88.4, 82.2 and 72.4%, over the same period of time. After h of storage (25°C), greater than 50% of the LOX residual activity of the immobilized enzyme extract remained (Fig. 21B). The HPL activity of the free extract (1.5 mg protein/mL) displayed

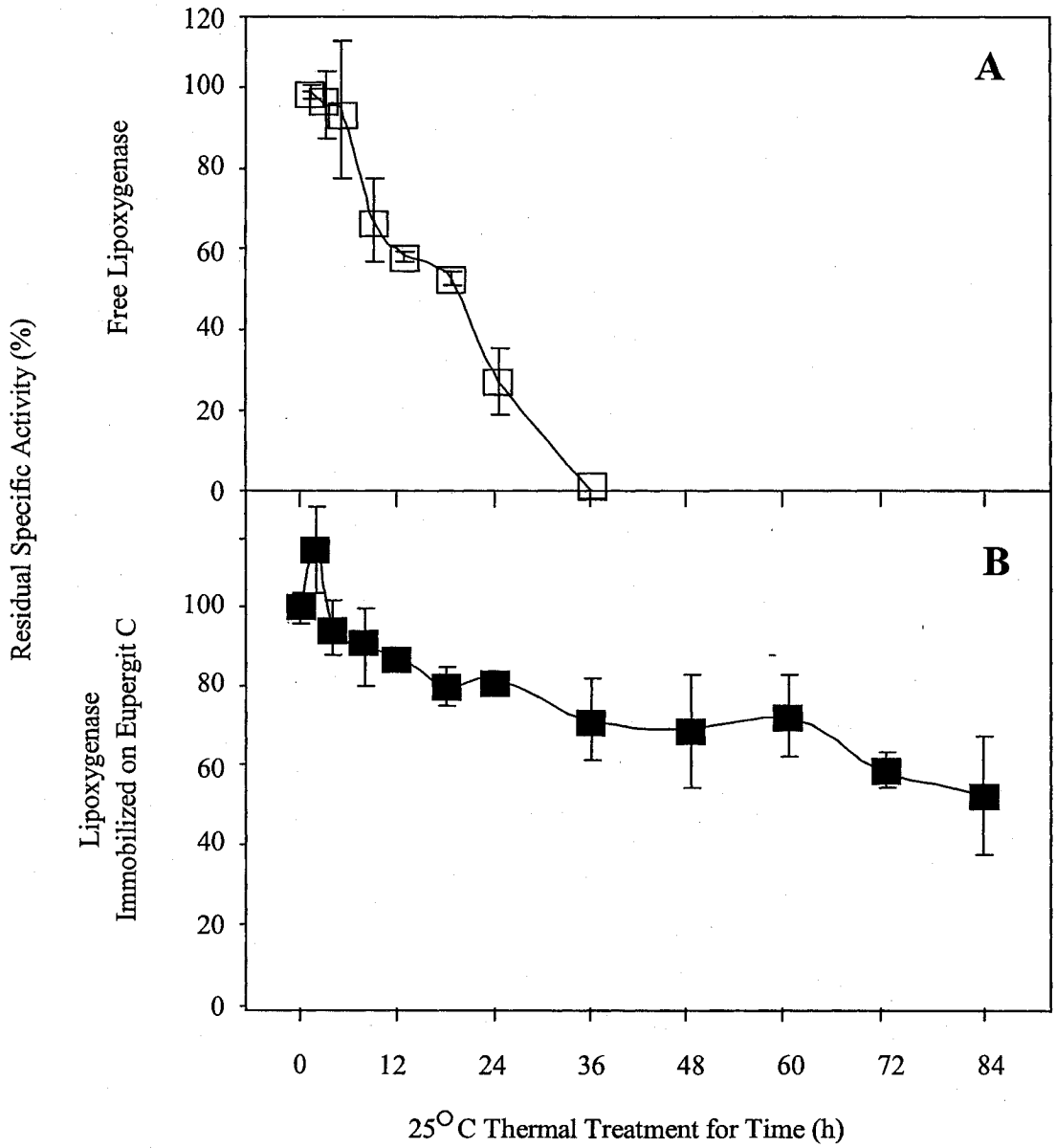

Figure 21. The thermostability profiles of lipoxygenase activity in enzymatic extracts from *Penicillium camemberti* for the: (A) (□) free and (B) (■) immobilized preparations, respectively.

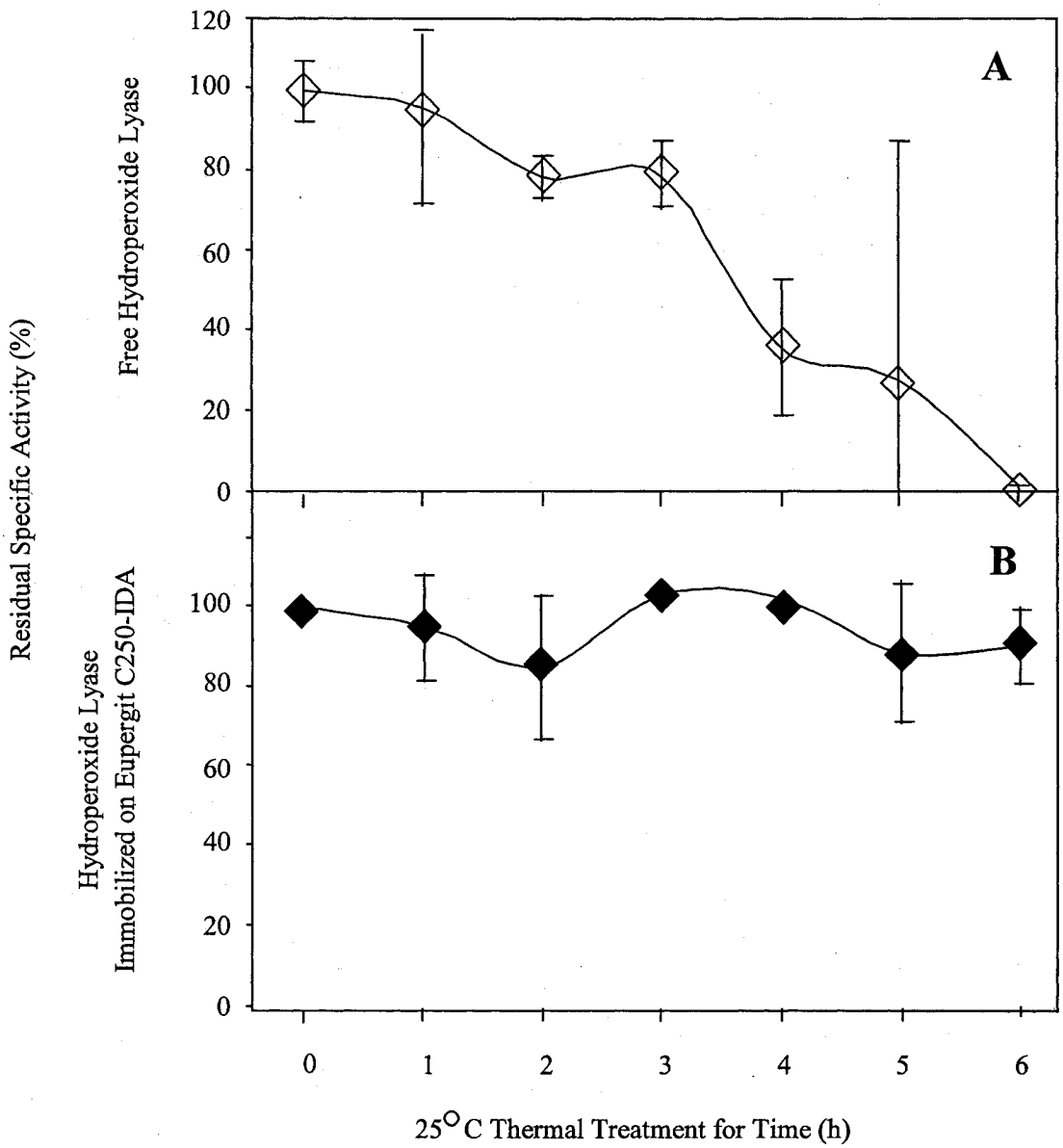

Figure 22. The thermostability profiles of hydroperoxide lyase activity in enzymatic extracts from *Penicillium camemberti* for the: (A) (◇) free, and (B) (◆) immobilized preparations, respectively.

a steady decrease in HPL residual specific activity (%) throughout the thermal treatment (25°C), with values of 96.3, 78.1, 80.1, 35.1 and 27.3% after 1, 2, 3, 4 and 5 h, respectively (Fig. 22A). The HPL activity of the enzymatic extract immobilized on Eupergit®C250-IDA (0.15-0.50 mg protein/mL) had residual specific activity (%) of ~100% over the duration of the thermal treatment (25°C) (Fig. 22B).

Both free LOX and HPL activities were relatively unstable with respect to thermal treatment, however, the free LOX demonstrated a greater thermostability than did the free HPL (Figs. 21A and 22A). The immobilization of the extracts onto their respective supports yielded greater thermostability for both LOX and HPL activities (Figs. 21B and 22B). These results may indicate a higher resistance to thermal denaturation present in the immobilized enzme trials versus the free ones, due to the the multipoint covalent attachment between the enzyme and the support (Mateo *et al.*, 2000*a*; Cho *et al.*, 2006). Knezevic *et al.* (2006) investigated the enhanced thermostability (75°C, 10 h) of Eupergit®C immobilized lipase and determined that the free enzyme possessed a linear inactivation profile, whereas that of the immobilized enzyme was linear at temperatures ranging from 37 to 75°C and biphasic above 75°C. A similar result was reported for the thermal inactivation profile of Eupergit®C immobilized penicillin acyclase (Torres-Bacete *et al.*, 2001). The authors suggested that this may be due to a variety of subpopulations of multipoint immobilized enzyme molecules onto the Eupergit® support (Knezevic *et al.*, 2006). In the current study both free and immobilized LOX and HPL possessed linear inactivation rate constants (data not shown), however the thermal inactivation temperature of 25°C was below that associated with biphasic inactivation profiles in other studies (Torres-Bacete *et al.*, 2001; Knezevic *et al.*, 2006).

Hamberg (1986) reported that 6 h thermal treatments of free enzymatic extract from *Saprolegnia parasitica* (1 mg protein/mL) resulted in 24 and 0% residual LOX activity at 22 and 37°C, respectively. Beneytout *et al.* (1989) reported that a free enzyme extract from *Oscillatoria* sp. (1 mg protein/mL) subjected to 45°C for 5 min resulted in a 50% decrease in LOX activity and complete inactivation at 80°C. Trout gill LOX (1 mg protein/mL) retained only 10% activity after 10 min at 50°C (Hsieh *et al.*, 1988). HPL investigations on thermostability generally reported appreciable decreases in HPL

activity after short (~10 min) thermal treatments in the range of 40-50°C. Hornostaj and Robinson (1999) reported that free cucumber HPL (0.03 mg protein/mL) possessed 50% activity after 10 min at 50°C and was completely inactivated at 80°C. Free pear HPL (0.9 mg protein/mL) retained 70% activity after 10 min at 50°C (Kim and Grosch, 1981). Results of the present study indicate that greater instability towards thermal treatments (25°C) is exhibited in the HPL activity of the enzyme extract from *P. camemberti* in comparison HPL activity in extracts from other sources; however enhanced thermostability of HPL activity is afforded by the immobilization of the extract on Eupergit®C250-IDA.

6.4.5. Long-Term Stability of LOX/HPL

The effect of long-term stability at –80 and 4°C on the LOX activity of the lyophilized Eupergit®C immobilized enzyme extract (Fig. 23A) and HPL activity of the lyophilized Eupergit® C250L-IDA immobilized enzyme extract (Fig 23B) indicated that, for LOX, the rate of inactivation was less at –80°C than that at 4°C, whereas HPL appeared to be relatively stable regardless of the storage temperature. Figure 23A illustrates that throughout the first 8 weeks of storage at –80°C, the residual LOX activity (%) of the immobilized enzyme extract decreased to 90.8, 82.6, 73.2 and 59.8% at 2, 4, 6 and 8 weeks, respectively, whereas those at 4°C possessed residual LOX activity (%) of 77.2, 69.1, 53.5 and 12.6 %, at the same time intervals. Further, the results (Fig. 23A) indicate at week 9, close to 50% residual LOX activity of the immobilized extract stored at –80°C, whereas no activity was detected in the immobilized preparation stored at 4°C. These results (Fig. 23A) suggest that the enzymatic extract immobilized on Eupergit®C possessed sufficient LOX stability to be utilized for a period of up to a month without substantial loss of enzymatic activity. Further, the results (Figs. 23A and 23B) indicate that, with regards to the enzyme extract immobilized on Eupergit®C, there may be a greater sensitivity to moisture induced degradation processes, including chemical hydrolysis and deamidation, leading to increased LOX inactivation, whereas the enzyme extract immobilized on Eupergit® C250L-IDA possessed stable HPL activity and seemed more resistant to these chemical degradations (Wang, 1999). Results indicate (Fig. 23B) that when the enzyme extract immobilized on Eupergit® C250L-IDA was stored at

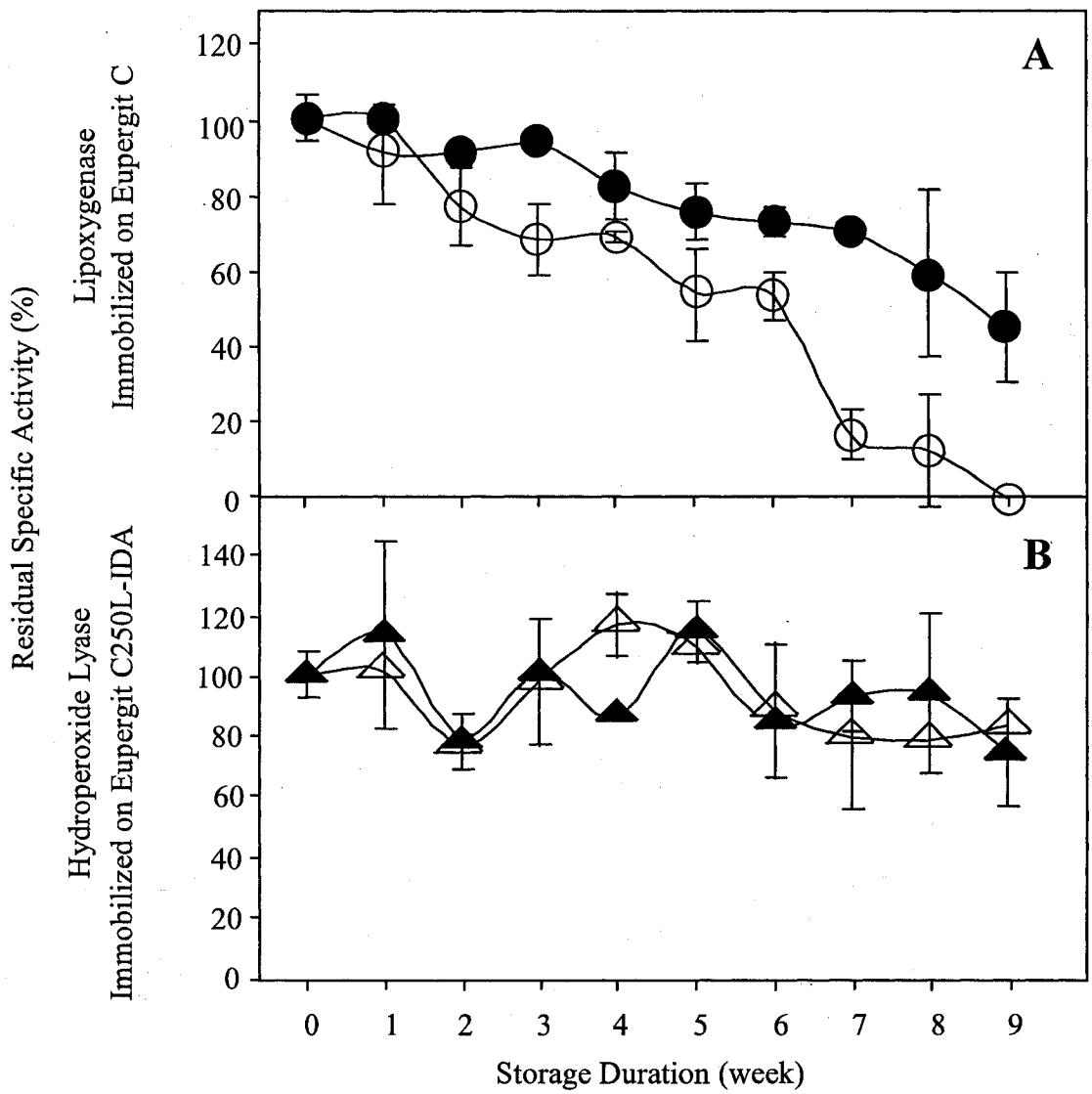

Figure 23. The long-term stability profiles of lipoxygenase (LOX) and hydroperocxidelyase (HPL) activities in lyophilized immobilized enzymatic extracts from *Penicillium camemberti*, where the (%) residual specific activity of the preparation for (A) LOX was stored at (●) -80°C and at (○) 4°C and that of (B) HPL was stored at (▲) -80°C and at (△) 4°C, respectively.

-80°C, no appreciable decrease in HPL residual activity (%) occurred during weeks 0 to 7, after which time the values of 93.8 and 73.9% residual immobilized HPL activity, were obtained at weeks 8 and 9, respectively. HPL residual activity (%) were ~100% for the the enzyme extract immobilized on Eupergit® C250L-IDA during weeks 0 to 5 and ~80-90% during weeks 6 to 9. The results (Fig. 23B) suggest that enzyme extract immobilized on Eupergit®C250L-IDA possessed relatively stable HPL activity for a period of up to 8 weeks.

Most LOX long-term stability studies have focused on liquid enzyme extracts at ~4°C, perhaps owing to their potential application as biocatalysts (Liagre *et al.*, 1996). When free enzyme extract from *P. sativum* (0.07 M, pH 6.4) was stored at 4°C for 30 days, it maintained 25% residual LOX activity, whereas the extract immobilized on talc maintained ~50% residual LOX activity (Liagre *et al.*, 1996). Free and polyacrylamide immobilized soybean LOX (0.05 M, pH 9.0) were subjected to storage at 4°C for 45 days and the results demonstrated maintenance of 28% and 60% activities, respectively (Carmen Pinto and Macías, 1996). Hsu *et al.* (1997) reported that storage of sol-gel immobilized soybean LOX type-1B (0.2 M, pH 9.0) lost ~50% activity after 168h of storage at 25°C. The results of the current study (Fig. 23A) indicate that storage at 4°C of the enzyme exctract immobilized on Eupergit® C containing LOX activity may be associated with the initiation of chemically initiated hydrolytic and/or deamidation reactions leading to denaturation or aggregation of the active enzyme forms in the lyophilized preparation (Wang, 1999 and 2000).

HPL storage stability studies usually investigate enzymatic activity losses in the liquid state and immobilization of HPL has been show to contribute to maintaining high activity for several weeks (Liagre *et al.*, 1996). Free enzyme extract containing HPL activity from *P. sativum* was stored at 4°C for 30 days (0.07 M, pH 6.4) and resulted in ~25% residual HPL specific activity, whereas upon immobilization of the extract on talc the HPL residual specific activity was ~75% (Liagre *et al.*, 1996). AffiGel 10 immobilized enzymatic extract from *Chlorella* sp. containing HPL activity (0.05 M, pH 7.0) (Nuñez *et al.*, 1997) was stable for 4 months at 4°C and an extract from mung bean immobilized on UltraLink Iodoacetal containg HPL activity (0.1 M, pH 6.5) was stable

after 18 days at 4°C (Rehbock and Berger, 1998). The results of the current study (Fig. 23B) are similar to values for long-term stability of HPL activities from other sources, suggesting that the immobilization of the enzymatic extract on Eupergit®C250L-IDA possesses an appropriately long shelflife for use in biocatalytic protocols.

6.4.6. Determination of Kinetic Parameters

Table 19 summarizes the kinetic parameters of V_{max} and K_m for the LOX activity of both the free enzymatic extract and that immobilized on Eupergit®C, as well as the HPL activity of both the free enzymatic extract and that immobilized on Eupergit®C250L-IDA. The kinetic parameters were calculated from Lineweaver-Burk plots of $1/v$ versus $1/[S]^2$, using linoleic acid substrate concentrations of 77 to 1.5×10^3 μM and $1/v$ versus $1/[S]$, using the 10-HPOD of linoleic acid at concentrations of 25 to 0.8×10^3 μM, for LOX and HPL, respectively. The results show the LOX activity of the enzymatic extract immobilized on Eupergit®C possessed a higher V_{max} value than the free one with 46.95 and 12.17 nmol HPOD produced/mg protein/min, respectively. In addition the results showed that the K_m value for LOX activity of the free enzymatic extract (57 μM) is much lower than for the enzymatic extract immobilized on Eupergit®C (58.47 mM), suggesting a greater substrate affinity for the free extract, possibly due to substrate partitioning and/or conformational changes in enzyme structure (Halling, 1994). The LOX enzymatic catalytic efficiency value for the free enzymatic extract (2.13×10^{-4}) is much greater than that of the the extract immobilized on Eupergit®C (8.00×10^{-7}). The results (Table 19) show V_{max} value for HPL activity of the enzymatic extract immobilized on Eupergit®C250L-IDA immobilized was higher than that of the free extract, with 1383.07 and 54.17 nmol 10-HPOD converted/mg protein/min, respectively. Further, the results (Table 19) indicate that the K_m values for the HPL activity for the free enzymatic extract (0.16 mM) is lower than that of the enzymatic extract immobilized on Eupergit®C250L-IDA (0.25 mM), which suggests a greater substrate affinity for the free extract. The HPL enzymatic catalytic efficiency value for the extract immobilized on Eupergit®C250L-IDA immobilized (5.55×10^{-3}) is larger than for the free extract (3.31×10^{-4}). The LOX enzymatic catalytic efficiency value for the free extract was close to that reported for a partially purified LOX fraction from *P. camemberti* (1.19×10^{-4})

Table 19. Kinetic studies of the lipoxygenase (LOX) activity in an enzyme extract from *Penicillium camemberti* in the free preparation and that immobilized on Eupergit C, using linoleic acid substrate and hydroperoxide lyase (HPL) activity in the free preparation and that immobilized on Eupergit C250L-IDA, using the 10-hydroperoxide of linoleic acid as substrate.

	LOX[a]			HPL[b]		
	K_m[c]	V_{max}[d]	Enzymatic Catalytic Efficiency[e]	K_m[c]	V_{max}[d]	Enzymatic Catalytic Efficiency[e]
Free	0.057	12.17	2.13×10^{-4}	0.16	54.17	3.31×10^{-4}
Immobilized	58.49	46.95	8.00×10^{-7}	0.25	1383.07	5.55×10^{-3}

[a]Lipoxygenase specific activity was defined as nanomol hydroperoxides produced/mg protein/min and was determined from the plot of the residual hydroperoxide produced versus enzyme concentration using the xylenol orange assay (molar exctinction coefficient 18,765 M^{-1} cm^{-1}, 560 nm). All LOX trials were performed in duplicate series run in tandem with a blank, at pH 6.0, at 30 and 55°C, for the free extract and the immobilized one, respectively.

[b]Hydroperoxide lyase specific activity of was defined as nanomol 10-hydroperoxide of linoleic acid converted/mg protein/min and was determined from the plot of the residual 10-hydroperoxide substrate versus enzyme concentration using xylenol orange assay (molar exctinction coefficient 18,765 M^{-1} cm^{-1}, 560 nm). All HPL trials were performed in duplicate series run in tandem with a blank, at pH 6.0, 45°C and pH 4.0, 30°C, for the free extract and the immobilized one, respectively.

[c]The K_m values were defined as mM of substrate.

[d]The *V*max values were defined as substrate produced or converted/mg protein/min, for LOX and HPL, respectively.

[e]The enzymatic catalytic efficiency was defined as the ratio of V_{max} to K_m.

(Perraud and Kermasha, 2000). The effect of immobilization on kinetic parameters usually result in a decrease in V_{max} and an increase in K_m. Oxirane acrylic immobilization of potato extract containing LOX activity resulted in a K_m value of 0.312 mM versus 0.155 mM for the free extract (Carmen Pinto *et al.*, 1997). The results (Table 19) for *P. camemberti* LOX activity suggest less enzymatic catalytic efficiency and a lower substrate affinity for the immobilized extract in relation to the free extract, due to a diffusion limitation of the substrate into the microenvironment of the immobilized preparation (Liagre *et al.*, 1997; Kermasha *et al.*, 2002*b*). Several studies have indicated a relative increase in K_m and decrease in V_{max} upon immobilization of extract containing HPL activity (Liagre *et al.*, 1996; Rehbock and Berger, 1998), however, an enhanced velocity with a concomitant increase in K_m for the enzymatic extract immobilized on Eupergit®C250L-IDA was observed (Table 19). These results suggest a high selectivity for HPL coupling of the Eupergit®C250L-IDA immobilization support for enzymatic extract of *P. camemberti*.

6.5. Conclusion

The findings show that the enzyme extract from *P. camemberti* was stabilized most effectively by immobilization on Eupergit®C for LOX and Eupergit®C250L-IDA for HPL activities. The immobilized extracts were more thermostable than their free counterparts, with respect to LOX and HPL and possessed high residual activity after being stored for many weeks. Parameters of optimum pH, reaction temperature and kinetic parameters indicated that the LOX and HPL activities share many similarities to LOXs and HPLs from other sources. The enhanced stability of the immobilized enzymatic extracts makes them more potentially useful as biocatalysts for the conversion of lipid substrates to volatile flavor compounds.

GENERAL CONCLUSION

The research into the characterization of *Aspergillus niger* and *Penicillium candidum* lipoxygenases (LOXs) has confirmed several long-held assertions concerning the nature of the biocatalysis of PUFAs into natural flavors. Both microbial LOX extracts displayed a substrate specificity towards free fatty acids over the acyl esters of linoleic acid. This result suggests that predominantly free fatty acid hydroperoxides are produced and that they are potential substrates for subsequent hydroperoxide lyase (HPL) activity. This is in strong agreement with mechanistic studies of other LOXs, which also prefer free fatty acids. Further, the overall LOX reaction velocity indicated the production of an equivalent amount of flavor-precursor hydroperoxides as volatile flavor compounds reported in classical headspace studies of microbial homogenates. This strongly suggests that these LOXs are capable of catalyzing organoleptically significant amounts of flavor-precursor compounds. Further, both LOXs possessed activities that produced appreciable ratios of the 10-hydroperoxide of linoleic (10-HPOD) and linolenic acids (10-HPOT) at 15-21% of total isomers detected, respectively. This result suggests the endogenous production of the appropriate flavor-precursors, which are implicated in the biogeneration of characteristic C8 volatile flavor compounds, commonly isolated in molds and fungi. Both LOXs displayed the production of only mono-hydroperoxides, which is suggestive of a discreet enzymatic insertion of molecular oxygen into the substrate PUFA.

For both *A. niger* and *P. candidum*, varying the pH of the LOX reaction modified the relative ratios of hydroperoxide regio-isomers, which is a result similar to that in other vegetal LOXs. These changing ratios are strongly associated with changes in ionization state of the residues near the active site of the LOX, further confirming an enzymatic catalysis. Further, chiral studies at both pH optima for the LOX of *P. candidum* revealed an enantio-selective reaction where the hydroperoxide production favored the (*S*)- configuration, resultant from either linoleic, linolenic or arachidonic acids bioconversion, which is in agreement with several other microbial source LOXs. This type of enantio-selectivity has been shown to yield downstream flavor compounds of predominantly (*R*)- configuration, including 1-octen-3(*R*)-ol and 1,5-octadiene-3(*R*)-ol.

Overall, both microbial LOXs displayed appropriate substrate specificities, pH optima and kinetic parameters, to be of potential utility in biocatalytic applications. Further, both LOXs were determined to produce appreciable amounts of the 10-HPOD and 10–HPOT flavor-precursors, which are considered unusual in comparison to other LOXs characterized.

Investigations into LOX and HPL activities in *Penicillium camemberti* suggested that both could be effectively stabilized by the addition of KCl (7.5 ppm) prior to lyophilization. The lyophilized enzymatic extract had residual activities of 93 and 223% for LOX and HPL, respectively. The addition of 7.5 ppm KCl to the enzymatic extract maintained LOX and HPL activities for 8 and 4 weeks storage at -80°C, respectively. These results suggest that the *P. camemberti* extract possessed sufficient stability and activity to be potentially useful as a biocatalyst. With regards to enzymatic thermostability, the results showed that the addition of glycine and mannitol could be useful as a means of preserving LOX and HPL activities. The least damaging investigated stabilizing additive for LOX/HPL extract was glycine, which had the lowest $K_{inactivation}$ values and highest $C_{1/2}$ values of any additive tested. These results confirm that the addition of chemicals is an appropriate method to enhance the stability of a LOX/HPL enzyme preparation.

Immobilization of the extract from *P. camemberti* suggested that Eupergit®C and Eupergit®C250L-iminodiacetate (IDA) were the most appropriate supports for LOX and HPL activities, respectively. The results confirm that xylenol orange (XO) colorimetry is an effective method of determining LOX/HPL activities, however, care must be taken to ensure that (1) changes in color are not the result of support interferences and (2) the appropriate molar exctinction coefficient (MEC) is applied to the enzymatic assays. The effects of pH, reaction temperature and kinetic parameters suggested a similarity to other free and immobilized LOXs and HPLs characterized from other sources. Significant gains in thermostability were apparent for the immobilized trials versus their free counterparts. Further, the results suggested a good long-term stability for the immobilized enzyme exctracts, where 82.6 and 93.8% residual specific activities were determined after 4 and 8 weeks, for LOX and HPL, respectively.

It is hoped that the investigation into the microbial production of flavor producing enzymes and their stabilization will lead to a greater understanding of the biotechnological potential of such biocatalyses in the production of desirable natural food flavors.

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Colin Eric Hall

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