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PURIFICATION AND CHARACTERIZATION OF LIPOXYGENASE EXTRACTS OF SELECTED MICROBIAL SOURCES

by

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Suggested Short Title

CHARACTERIZATION OF SELECTED MICROBIAL LIPOXYGENASES

This thesis is dedicated to

my parents

my husband George

my sister Anna

ABSTRACT

Barbara Bisakowski (Ph.D.)

Partially purified lipoxygenase (LOX) extracts were obtained from *Fusarium oxysporum*, *Fusarium proliferatum*, *Saccharomyces cerevisiae* and *Chlorella pyrenoidosa* by precipitation with ammonium sulfate at 20-80, 0-40, 20-80 and 40-80 % of saturation, respectively. The enzymatic extracts from *F. oxysporum* and *S. cerevisiae* both exhibited two optimal activities at pH 8.0 and 10.0 while those from *F. proliferatum* and *C. pyrenoidosa* showed one optimal pH at 6.0 and 4.5, respectively. The enzymatic extract from *F. proliferatum* showed the highest LOX activity while those from *F. oxysporum* and *S. cerevisiae* demonstrated only 27.8 and 16.5 % of the LOX activity at pH 8.0, and 61.2 and 9.7 % of the enzymatic activity at pH 10.0, respectively. The lowest LOX activity was exhibited in the *C. pyrenoidosa* extract. The results showed that, at pH 8.0, LOX activity from *F. oxysporum* and *S. cerevisiae* was inhibited by 31 and 49 %, respectively, at corresponding concentrations of 20 and 25 mM potassium cyanide (KCN), exhibiting a non-competitive inhibitory effect; however, at pH 10.0, KCN had relatively little effect on enzyme activity. In addition, KCN markedly inhibited LOX activity from *C. pyrenoidosa* by 58.2 % at 0.5 mM concentration and was an uncompetitive inhibitor. In contrast, the results showed that the enzymatic activity from *F. proliferatum* remained relatively stable at KCN concentrations as high as 60 mM. The addition of 5 mM sodium ethylenediaminetetraacetate was found to increase the enzymatic activity from *F. oxysporum* by 50.3 and 16.6 % at pH 8.0 and 10.0, respectively, from *F. proliferatum* by 50 %, and produced a noticeable eight-fold increase in the enzymatic activity from *C. pyrenoidosa*; however, LOX activity for the *S. cerevisiae* extract remained unaffected at both pH optimas. In addition, the use of 1.2 mM hydroquinone (HQ) resulted in a 2-fold increase in LOX activity from *F. proliferatum* whereas a competitive inhibitory effect on LOX activity from *S. cerevisiae* was observed at pH 8.0 at 0.5 mM HQ. The results also showed that the enzymes from the four microbial sources demonstrated an overall preference towards linoleic acid, followed by linolenic acid. In addition, the enzymatic extract from *F. proliferatum* showed a strong preference towards the glycerol fatty acid

esters. The microbial enzymatic preparations were assayed with linoleic acid, as substrate, which was bioconverted into 9- and 13-hydroperoxides (HPODEs) by all four extracts; in addition, the LOX activity in the *F. oxysporum* extract produced the 10- and 12-HPODEs from linoleic acid while that of the *C. pyrenoidosa* extract produced only the 10-HPODE. The partially purified extract from *F. proliferatum* was further purified by successive chromatography on size-exclusion and ion-exchange which resulted in fraction FIIIa and FIV, respectively. Fraction FIV showed the presence of one major protein band and two minor ones in both the native and sodium dodecyl sulfate polyacrylamide electropherograms. Optimal LOX activity was shown to be at pH 6.0 for fractions FIIIa and FIV; in addition, fractions FIIIa and FIV also exhibited maximal LOX activity at pH 10.0 and 10.5, respectively. The K_m values were 2.5×10^{-6} M (FIIIa, pH 6.0), 4.7×10^{-6} M (FIIIa, pH 10.0), 11.0×10^{-6} M (FIV, pH 6.0) and 3.9×10^{-6} M (FIV, pH 10.5). Fraction FIIIa demonstrated greater preference towards linoleic acid than linolenic and arachidonic acids at pH 6.0 and 10.0 and showed very little activity towards mono-, di- and trilinolein. Fraction FIV demonstrated approximately 2.5 times more activity towards mono- and dilinolein and 1.5 times more towards linolenic and arachidonic acids than that exhibited towards linoleic acid at pH 6.0; in contrast, at pH 10.5, the partially purified enzymatic fraction possessed an overall preference towards linoleic acid. The purified fractions FIIIa and FIV produced mainly the 13-HPODEs from linoleic acid at pH 6.0; however, the same fractions produced the 9- and 13-HPODEs at a ratio of approximately 1:1 at pH 10.0 and 10.5, respectively. In addition the presence of a LOX activity producing the 10- and 12-HPODEs was also suggested in fractions FIIIa (pH 6.0 and 10.0) and FIV (10.5); however, this activity was not detected in fraction FIV at pH 6.0.

RÉSUMÉ

Barbara Bisakowski (Ph.D.)

Des extraits partiellement purifiés en lipoxygénase (LOX) ont été obtenus de *Fusarium oxysporum*, *Fusarium proliferatum*, *Sacharomyces cerevisiae* et *Chlorella pyrenoidosa* par précipitation au ammonium sulfate à 20-80, 0-40, 20-80 and 40-80 % de saturation, respectivement. Les deux extraits enzymatiques de *F. oxysporum* et *S. cerevisiae* ont démontré deux activités optimales à pH 8,0 et 10,0 alors que ceux de *F. proliferatum* et *C. pyrenoidosa* n'avaient qu'un pH optimal à 6,0 et 4,5, respectivement. L'extrait enzymatique de *F. proliferatum* a montré l'activité LOX la plus élevée alors que ceux de *F. oxysporum* et *S. cerevisiae* n'ont démontré que 27,8 et 16,5 % de l'activité LOX à pH 8,0, et 61,2 et 9,7 % de l'activité enzymatique à pH 10,0, respectivement. L'activité LOX la plus faible a été exprimée par l'extrait de *C. pyrenoidosa*. Les résultats ont montré qu'à pH 8,0, l'activité LOX de *F. oxysporum* et *S. cerevisiae* a été inhibée de 31 et 49 % à des concentrations de cyanure de potassium (KCN) de 20 et 25 mM, respectivement, démontrant un effet inhibiteur de type non-compétitif; cependant, à pH 10,0, KCN avait un effet relativement faible sur l'activité enzymatique. De plus, KCN a fortement inhibé l'activité LOX de *C. pyrenoidosa* de 58,2 % à une concentration de 0,5 mM et était un compétiteur uncompétitif. En revanche, les résultats ont montré que l'activité enzymatique de *F. proliferatum* est restée relativement stable pour des concentrations de KCN aussi élevées que 60 mM. L'addition d'éthylènediaminetétraacétate de sodium à 5 mM a entraîné une diminution de l'activité enzymatique de *F. oxysporum* de 50,3 et 16,6 % à pH 8,0 et 10,0, respectivement, de *F. proliferatum* de 50 %, et a augmenté par huit fois l'activité enzymatique de *C. pyrenoidosa*; cependant, l'activité LOX de l'extrait de *S. cerevisiae* est restée inchangée aux deux pHs optima. De plus, l'utilisation d'hydroquinone (HQ) à 1,2 mM a provoqué une augmentation de deux fois de l'activité LOX de *F. proliferatum*, alors qu'un effet inhibiteur de type compétitif sur l'activité LOX de *S. cerevisiae* a été observé à pH 8,0 pour une concentration de HQ à 0,5 mM. Les résultats ont aussi montré que les enzymes des quatre sources microbiennes ont démontré une préférence pour l'acide linoléique, suivie par l'acide

linolénique. De plus, l'extrait enzymatique de *F. proliferatum* a montré une préférence marquée pour les esters d'acide gras glycéridiques. Les préparations enzymatiques microbiennes ont été testées avec l'acide linoléique, comme substrat, qui a été converti en 9- and 13-hydroperoxydes (HPODEs) par les quatre extraits; de plus, l'activité LOX de l'extrait de *F. oxysporum* a produit les 10- and 12-HPODEs de l'acide linoléique alors que celle de *C. pyrenoidosa* a seulement produit le 10-HPODE. L'extrait partiellement purifié de *F. proliferatum* a été davantage purifié par chromatographies successives de tamisage moléculaire et d'échangeurs d'ions pour donner les fractions FIIIa and FIV, respectivement. Les électrophorèses de polyacrylamide native et de dodécyl sulfate de sodium ont montré la présence d'une bande protéique majeure et de deux autres mineures pour la fraction FIV. L'activité LOX optimale a été mesurée à pH 6,0 pour les fractions FIIIa et FIV; de plus, les fractions FIIIa et FIV ont démontré une activité optimales à pH 10,0 et 10,5, respectivement. Les valeurs de K_m étaient de 2,5 x 10^{-6} M (FIIIa, pH 6,0), 4,7 x 10^{-6} M (FIIIa, pH 10,0), 11,0 x 10^{-6} M (FIV, pH 6,0) et 3,9 x 10^{-6} M (FIV, pH 10,5). La fraction FIIIa a démontré une plus grande préférence pour l'acide linoléique que pour les acides linolénique et arachidonique à pH 6,0 et 10,0 et une très faible activité pour les mono-, di- et trilinoléine. La fraction FIV a démontré approximativement 2 fois et demi plus d'activité pour les mono- and dilinoléines et 1 fois et demi plus pour les acides linolénique et arachidonique que celle exprimée pour l'acide linoléique à pH 6,0; en revanche, à pH 10,5, la fraction enzymatique partiellement purifiée avait une préférence pour l'acide linoléique. Les fractions fractions purifiées FIIIa et FIV ont principalement produit les 13-HPODEs de l'acide linoléique à pH 6.0; cependant, les mêmes fractions ont produit les 9- et 13-HPODEs à un ratio approximatif de 1:1 à pH 10,0 et 10,5, respectivement. De plus la présence d'une activité LOX produisant les 10- et 12-HPODEs a aussi été suggérée dans les fractions FIIIa (pH 6,0 et 10,0) et FIV (10,5); cependant, cette activité n'a pas été détectée dans la fraction FIV à pH 6,0.

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CLAIM OF ORIGINAL RESEARCH

1. This is the first study in which the partially purified lipoxygenase extracts from *Fusarium oxysporum*, *Fusarium proliferatum*, *Saccharomyces cerevisiae* and *Chlorella pyrenoidosa* were isolated and characterized with respect to pH, kinetic parameters, effect of inhibitors and activators, substrate specificity and electrophoretic profile.

2. This is the first study in which the partially purified lipoxygenase extracts from *Fusarium oxysporum*, *Fusarium proliferatum*, *Saccharomyces cerevisiae* and *Chlorella pyrenoidosa* were characterized with respect to end-product specificity, i.e. hydroperoxide isomers and carbonyl compounds, using linoleic acid as a model substrate.

3. This is the first time that lipoxygenase isozymes have been purified from *Fusarium proliferatum* and characterized with respect to pH, kinetic parameters, substrate and end-product specificity, and electrophoretic profile.

4. This is the first time that a method was developed for the separation of linoleic acid hydroperoxide isomers by capillary electrophoresis and compared with that used for high-performance liquid chromatography and gas-liquid chromatography/mass spectroscopy.

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CHAPTER I

INTRODUCTION

Lipoxygenase (linoleate: oxygen oxidoreductase; EC 1.13.1.13) is a dioxygenase which catalyzes as an initial reaction, the hydroperoxidation of linoleic acid and other polyunsaturated fatty acids (PUFA) and their esters containing a *cis*, *cis*-1,4-pentadiene moiety. Lipoxygenase is particularly relevant to the taste and flavor of food of plant origin. Lipoxygenase oxidizes the essential PUFA into stereo- and regio-specific hydroperoxides which can in turn be converted enzymatically by hydroperoxide dehydrase and hydroperoxide lyase to α- and γ-ketols as well as carbonyl compounds which are responsible either for the desirable, fresh-vegetable flavors associated with the normal metabolism of the growing plant or the undesirable off-flavors that occur due to degradation reactions as a result of instability after harvesting and during storage or processing. However, the mechanism of lipoxygenase for further bio-conversion of hydroperoxides of linoleic acid to ketols and carbonyl compounds does not appear to be consistent from one biological system to another. In addition, the literature reveals that two schools of thought still exist regarding the formation of ketols and the breakdown products from linoleic acid, one considering lipoxygenase as the sole cause and the other holding the combination of lipoxygenase and hydroperoxide dehydrase responsible.

Lipoxygenase can be found in plant, animal and microbial sources. Depending on the source, lipoxygenase may be found in the form of various isozymes which differ from each other with respect to pH optimum, substrate specificity and degree of inhibition by various agents. Each lipoxygenase isozyme has its unique optimal conditions for activity and by modifying the medium in which the isozyme resides, its activity is also altered. The type of substrate used in the reaction also alters the activity of the lipoxygenase isozymes due to preferences for different substrates. In addition, lipoxygenase isozymes differ from each other by producing from linoleic acid different ratios of optically active derivatives of 9- and 13-hydroperoxydienes which contribute to the formation of many aldehydes and alcohols that are responsible for desirable, fresh-vegetable flavors. Consequently, the production of a certain

flavor compound will depend on the initial conversion of the PUFAs such as linoleic acid by a lipoxygenase isozyme to the corresponding ratio of optically active derivatives of 9- and 13-hydroperoxydienes which will in turn be converted enzymatically to the corresponding flavor compound. The type of flavor compounds produced will depend on the lipoxygenase isozyme used, the substrate used and the optimal conditions provided for maximum enzyme activity. By altering the lipoxygenase isozyme, substrate or optimal conditions, the type of flavor compounds produced will also be altered. Flavor compounds ranging from six to nine carbons may be produced depending on the specificity of lipoxygenase isozymes. The elucidation of the role that lipoxygenase(s) may play in the bioformation of flavor precursors is therefore an important step in maximizing the production and recovery of selected flavors.

The use of natural flavors is becoming an important issue for the food industry in order to meet the consumer's demands for the use of "natural" additives in food products. The production of large quantities of flavoring materials or blends at relatively low cost by means of biotechnological processes is of major interest to the flavor industry. Inexpensive commercial edible oil such as Canola oil and unused fish oil may be employed as a source of fatty acids for the production of natural flavors thereby reflecting society's trend for natural flavors and preservatives. The biotechnological conversion of inexpensive raw materials or unused by-products into economically beneficial and commercially marketable products presents a great opportunity to the food industry and is also an environmental answer with respect to the usage of waste materials and a response to the consumer's concern of health and safety.

The bioconversion of raw materials into natural flavors may be performed by exo- and endo-enzymes. Enzymes play an important role in the production of flavor precursors. Fresh food aromas are produced by enzymes in living tissue and by degradative enzymes which begin acting when the tissue is cut or crushed. In addition, the flavors of cultured and fermented foods result from microbial enzyme activity. Industrial enzymes could be obtained from plant, animal and microbial sources. The source of an enzyme and its physical and chemical condition affects the efficiency of the extraction, purification process, stability and ultimately cost of the enzyme. Up to the 1970s, plant and animal material were considered to

be the best sources of enzymes since the bulk of enzyme production was aimed at the food processing industry and enzymes isolated from a plant or animal were assumed not to be tainted with problems of toxicity or contamination. Enzyme extraction from plant and animal tissue was also much simpler and less costly than that from micro-organisms due to the relative ease of disruption of plant and especially animal tissue. However, the demand for enzymes grew and animal sources of enzymes became limited and expensive, while plant sources were not a stable source since they were at the mercy of the weather and international politics. Consequently, microbial enzymes became more popular due to refinements occurring in fermentation technology and because microorganisms provide a stable, reliable source of enzymes.

Microbial enzymes may be produced in large quantities. The sheer quantity of product that can be produced in a short time in a small area vastly exceeds that of animal or plant enzymes. The second advantage is the ease of extraction. Since a large proportion of industrial enzymes from microbial sources are extracellularly excreted into the growth medium, there are no difficulties in extracting them. If the microbial enzyme is intracellular, the extraction procedure usually involves fewer steps than that for plant or animal enzymes. Since microorganisms are produced on the spot (with the exception of spent brewer's yeast), the cost of harvesting and transport involved in the production of plant enzymes is eliminated. The process of production and extraction of the microbial enzyme may also be integrated together. Equally important is the consideration that the plant or animal enzyme, unlike that of the microorganism, is usually specifically located in a particular tissue or organ and that this portion of the organism must first be separated from the rest and the remains disposed of. Microbial enzymes also provide a predictable enzyme yield and are not subject to seasonal variation while plant and animal enzyme sources are subject to wide variations of yield and may be available only at certain times of the year. Consequently, the latter problem requires either the facility for high volume storage of the plant or animal source or else leaving extraction equipment idle for long periods of time, both factors contributing significantly to increase in cost.

It is also important to note that animal and plant-derived enzymes have recently risen considerably in cost, while those from microbial sources have actually decreased in cost. Presently, plant enzymes are more prevalent and include the proteases papain, bromelain and ficin, cereal amylases, soybean lipoxygenase and some specialized enzymes from citrus fruits. These enzymes are mostly used in the food industries. The only major (in terms of volume of production) animal enzymes in use at present are trypsin, a variety of lipases and rennets. Microbial enzymes are by far the largest proportion by volume used in today's industries. However, despite the multitude of microorganisms available, few bacteria, yeasts and fungi, are actually used to produce virtually all of the microbial enzymes.

Microorganisms provide a natural and stable source of lipoxygenase isozymes. Each lipoxygenase isoenzyme provides unique end-products due to different substrate specificities. The production of end-products which produce good flavor could be an inexpensive source of flavor and could be termed "natural" thereby reflecting the changing trend of society towards healthier foods with non-artificial preservatives and flavors.

The present work is aimed at investigating the presence of lipoxygenase in selected microbial sources such as *Fusarium oxysporum* (Bisakowski *et al.*, 1995a), *Fusarium proliferatum* (Bisakowski *et al.*, 1995b), *Saccharomyces cerevisiae* (Bisakowski *et al.*, 1995c) and *Chlorella pyrenoidosa* (Bisakowski *et al.*, 1995d) and potential biotechnological applications for the bioconversion of lipid-rich by-products into highly desirable natural flavors. The objective of this work was to optimize a procedure for the extraction and partial purification of a lipoxygenase extract from the four microbial sources and to characterize the lipoxygenase extracts. The aim was also the development of a procedure for the recovery, purification and characterization of end-products, i.e. hydroperoxide isomers and carbonyl compounds, obtained from the enzymatic activity of partially purified extracts, using linoleic acid as well as the 9- and 13-hydroperoxides as model substrates. The ultimate objective was to further purify the selected partially purified lipoxygenase extracts, chosen on the basis of activity, specificity and nature of end-products, and to characterize the lipoxygenase fractions in terms of activity, specificity and nature of end-products, using linoleic acid as a model substrate. The purification and characterization of the selected microbial lipoxygenases will

establish the ground work of scientific knowledge necessary for the industrial application of selected microbial enzymes in the production of natural flavors.

The overall objectives of this research work were:

(i) Biomass production, partial purification and characterization of lipoxygenase extracts from selected microbial sources.

(ii) Characterization of end-products obtained from the partially purified microbial lipoxygenase extracts, using linoleic acid as a model substrate.

(iii) Further purification and characterization of the partially purified lipoxygenase extract, selected with respect to its activity and specificity.

(iv) Characterization of end-products obtained from the biocatalysis of the purified lipoxygenase isozymes, using linoleic acid as a substrate model.

This thesis consists of five chapters. Chapter one describes the general introduction. Chapter two covers the updated literature review, related topics and concepts used to undertake the research work. Chapter three describes the partial purification and characterization of the enzymatic extracts obtained from *F. oxysporum*, *F. proliferatum*, *S. cerevisiae* and *C. pyrenoidosa*. Chapter four deals with the purification and characterization of enzymatically-derived end-products, i.e. hydroperoxide isomers and carbonyl compounds, produced from linoleic acid by the partially purified extracts from *F. oxysporum*, *F. proliferatum*, *S. cerevisiae* and *C. pyrenoidosa*. Chapter five covers the further purification and characterization of the lipoxygenase isozymes from *F. proliferatum* as well as the analysis of hydroperoxide isomers from linoleic acid, using high-performance liquid chromatography, gas-liquid chromatography/mass spectroscopy and capillary electrophoresis chromatography.

CHAPTER II

LITERATURE REVIEW

2.1. Lipoxygenase

2.1.1. Definition of lipoxygenase

Lipoxygenase (linoleate: oxygen oxidoreductase; EC 1.13.1. 13) is considered to be a key enzyme in the oxidative degradation of lipids. It is a dioxygenase which catalyzes as an initial reaction, the hydroperoxidation of linoleic acid and other polyunsaturated fatty acids (PUFA) and their esters containing a *cis*, *cis*-1,4-pentadiene moiety (Axelrod *et al.*, 1981).

2.1.2. Role of lipoxygenase in the biogeneration of flavor

The production of many different volatile carbonyl compounds, important in the flavor of fruits and vegetables (Tressl and Drawert, 1973), is dependant on the initial conversion of PUFA such as linoleic acid by lipoxygenase (LOX) to stereo- and regio-specific hydroperoxides of linoleic acid (HPODEs). Figure 1 shows the lipoxygenase-catalyzed conversion of linoleic acid into the corresponding 8-, 9-, 10- and 13-HPODEs. These HPODEs can in turn be converted enzymatically by hydroperoxide isomerase (HPI), hydroperoxide dehydrase (HPD) and hydroperoxide lyase (HPL) to trihydroxy fatty acids, α- and γ-ketols as well as carbonyl compounds, respectively. Depending on the source, LOX may be found in the form of various isozymes which differ from each other with respect to pH optimum, substrate specificity and degree of inhibition by various agents. Each LOX isozyme has its unique optimal conditions for activity and by altering the medium in which the LOX isozyme resides, the activity of the isozyme is also altered. The type of substrate used in the reaction also alters the activity of the LOX isozymes since LOX isozymes have preferences for different substrates (Siedow, 1991). Hence, the production of a certain flavor compound will depend on the initial conversion of the PUFAs such as linoleic acid by a LOX isozyme to the corresponding regio- and stereo-specific HPODEs which will be converted enzymatically to the corresponding flavor compound.

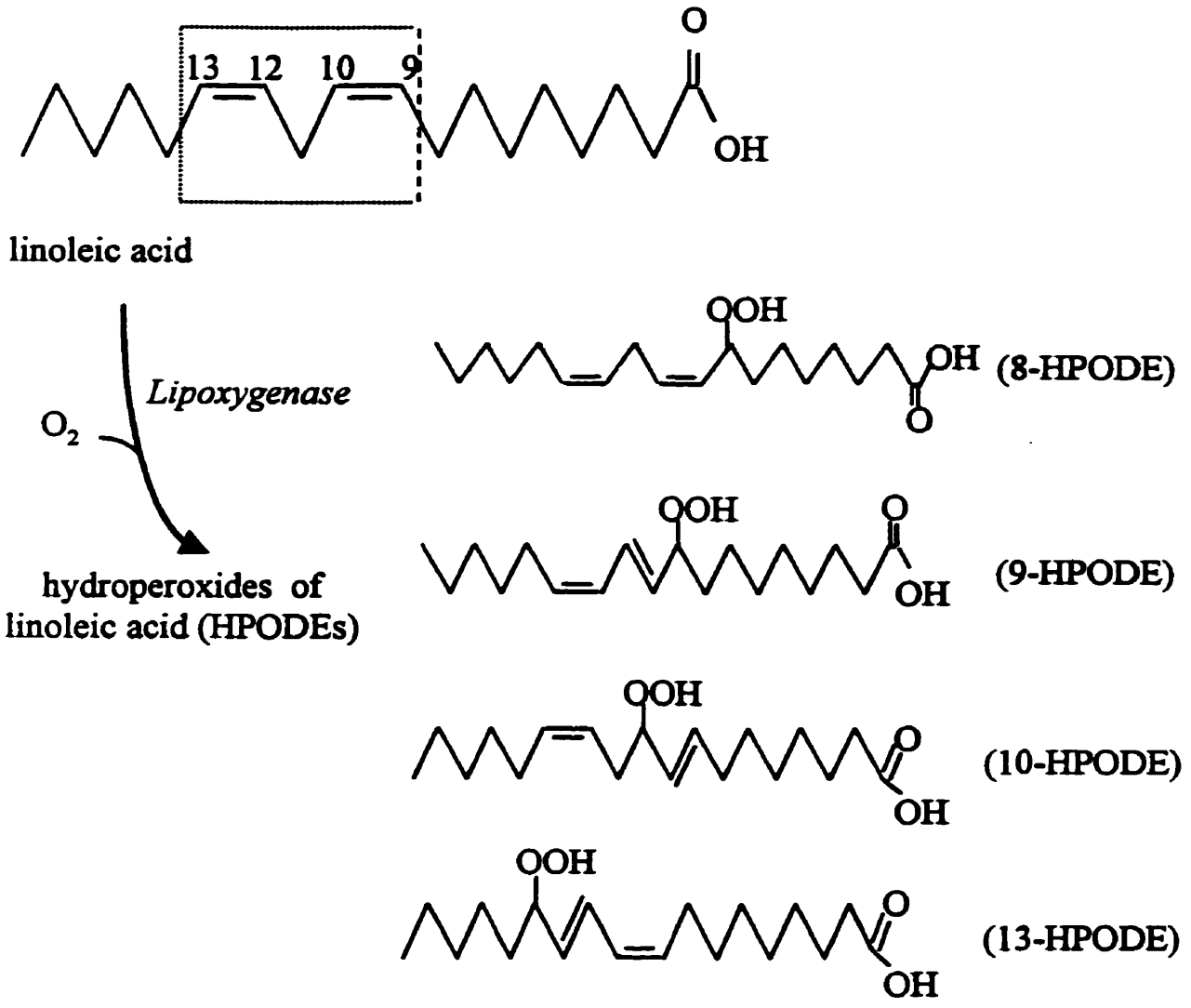

Figure 1. The bioconversion of linoleic acid into hydroperoxide isomers by lipoxygenase activity.

2.1.2.1. Hydroperoxide isomerase

The enzymatic conversion of the 13-HPODE into trihydroxy fatty acids by HPI activity is demonstrated in Figure 2. HPI catalyzes the transformation of the HPODE into an epoxyhydroxy fatty acid which in turn is followed by the subsequent hydrolysis of the epoxide resulting in the formation of trihydroxy fatty acids (Gardner, 1991).

The HPI activity from a *Saprolegnia parasitica* homogenate converted 15-hydroperoxyeicosatetraenoic acid (HPETE) into 11,12-epoxy-15-HETE and 13,14-epoxy-15-HETE which underwent subsequent hydrolysis of the epoxide group to produce four isomeric trihydroxyeicosatrienoic acids (Hamberg *et al.*, 1986a,b). In addition, the 13- and 9-HPODEs were also used as substrates by the HPI activity resulting in analogous products (Hamberg, 1989).

Brodowsky *et al.* (1992) reported the presence of a HPI activity from *Gaeumannomyces graminis* which catalyzed the conversion of 8-HPODE to 7S,8S-dihydroxylinoleic acid (HODE). A red marine alga *Gracilariopsis lemaneiformis* extract (Hamberg and Gerwick, 1993) containing both 12-LOX and HPI activity converted arachidonic acid into 12*S*-HPETE and 12*R*,13*S*-diHETE, a compound of principal biosynthetic interest because of its unique oxidation at C-13, an unusual position for oxidation in eicosanoids; in addition, incubations with linoleic acid-derived 13- and 9-HPODEs led to the formation of 13,14-diHODEs and 8,9-diHODEs.

2.1.2.2. Hydroperoxide dehydrase

HPD is a membrane-bound enzyme routinely located in the microsomal pellet. The presence of HPD has been reported in most cereal seeds or seedlings (Gardner, 1988), flaxseed, lettuce, sunflower, spinach, cotton seedlings (Vick and Zimmerman, 1987), *Vicia faba* (Vick and Zimmerman, 1983) and eggplant (Grossman *et al.*, 1983); however, with respect to microbial sources, HPD activity has only been found in *Chlorella pyrenoidosa* (Vick and Zimmerman, 1989).

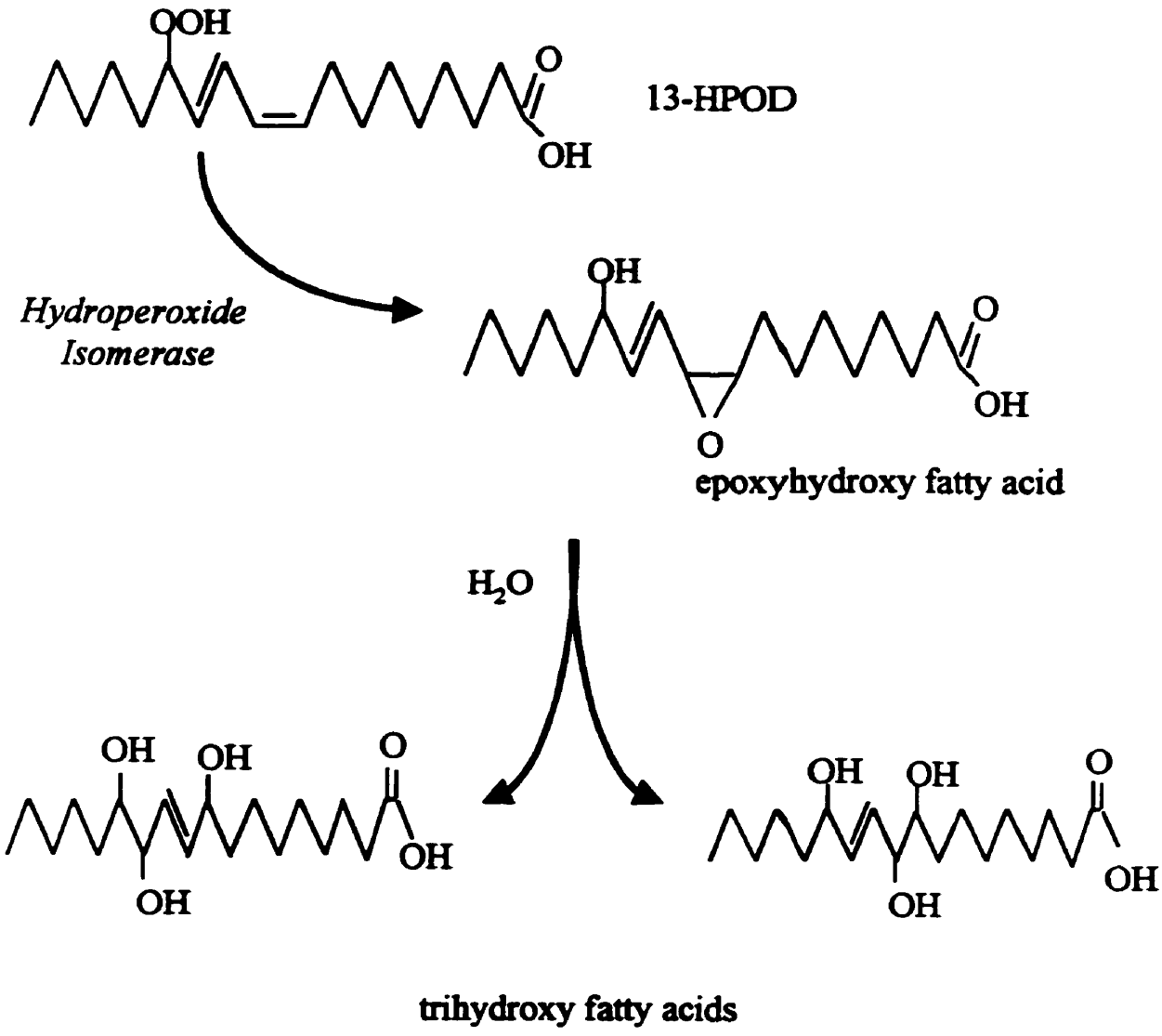

Figure 2. The enzymatic conversion of the 13-hydroperoxide isomer into trihydroxy fatty acids by hydroperoxide isomerase activity.

Figure 3 shows the conversion of the 13-HPODE into the respective allene oxide, 12,13-epoxy-octadecadienoic acid, by HPD activity which is followed by the spontaneous non-enzymatic hydrolysis to produce the corresponding α- and γ-ketols.

The French bean HPD (Kermasha *et al.*, 1986) converted the 13-HPODE to the respective ketols, 13-hydroxy-10-oxo-octadec-11-enoic acid and to a lesser amount , 13-hydroxy-12-oxo-octadec-9-enoic acid; this γ-ketol is similar to that observed in alfalfa (Esselman and Clagett, 1974), germinated corn seed (Gerristen *et al.*, 1976) and flaxseed (Veldink *et al.*, 1970). There was also some similarity in the findings of Galliard and Mercer (1975) and Stumpf and Conn (1980), who reported that the 13-HPODE was transformed mainly into 13-hydroxy-12-oxo-octadec-9-enoic acid (α-ketol) and 9-hydroxy-12-oxo-octadec-9-enoic acid (γ-ketol).

Yabuuchi and Amaha (1976) reported that a HPD from barley grains converted 9-HPODE to 9-hydroxy,10-oxo-12-octadecenoic acid and 13-hydroxy, 10-oxo-11-octadecenoic acid in the ratio of 2:1. Vick and Zimmerman (1981) reported the presence HPD in young cotton seedlings which preferred the 13-HPODE in preference to the 9-isomer to produce the . α-ketol (12-oxo-13-hydroxy-9,15-octadecadienoic acid) as a major product and γ-ketol (9-hydroxy-12-oxo-15,10-octadecadienoic acid) as a minor product.

Vick and Zimmerman (1989) reported a HPD activity from *C pyrenoidosa* which metabolized the 13-hydroperoxylinolenic acid into 13-hydroxy-9,11,15-octadecatrienoic acid and 12,13-epoxy-9-oxo-octadecadienoic acid.

2.1.2.3. Hydroperoxide lyase

The type of odor or flavor of a fruit or vegetable is largely determined by both the LOX oxidation specificity and the specificity of HPL for either the 13- or 9-HPODEs. Figure 4 indicates that HPLs from plant sources specific for the cleavage of 9-HPODEs furnish nine carbon aldehydes, such as nonenal, which tend to give cucumber, melon and violet odors

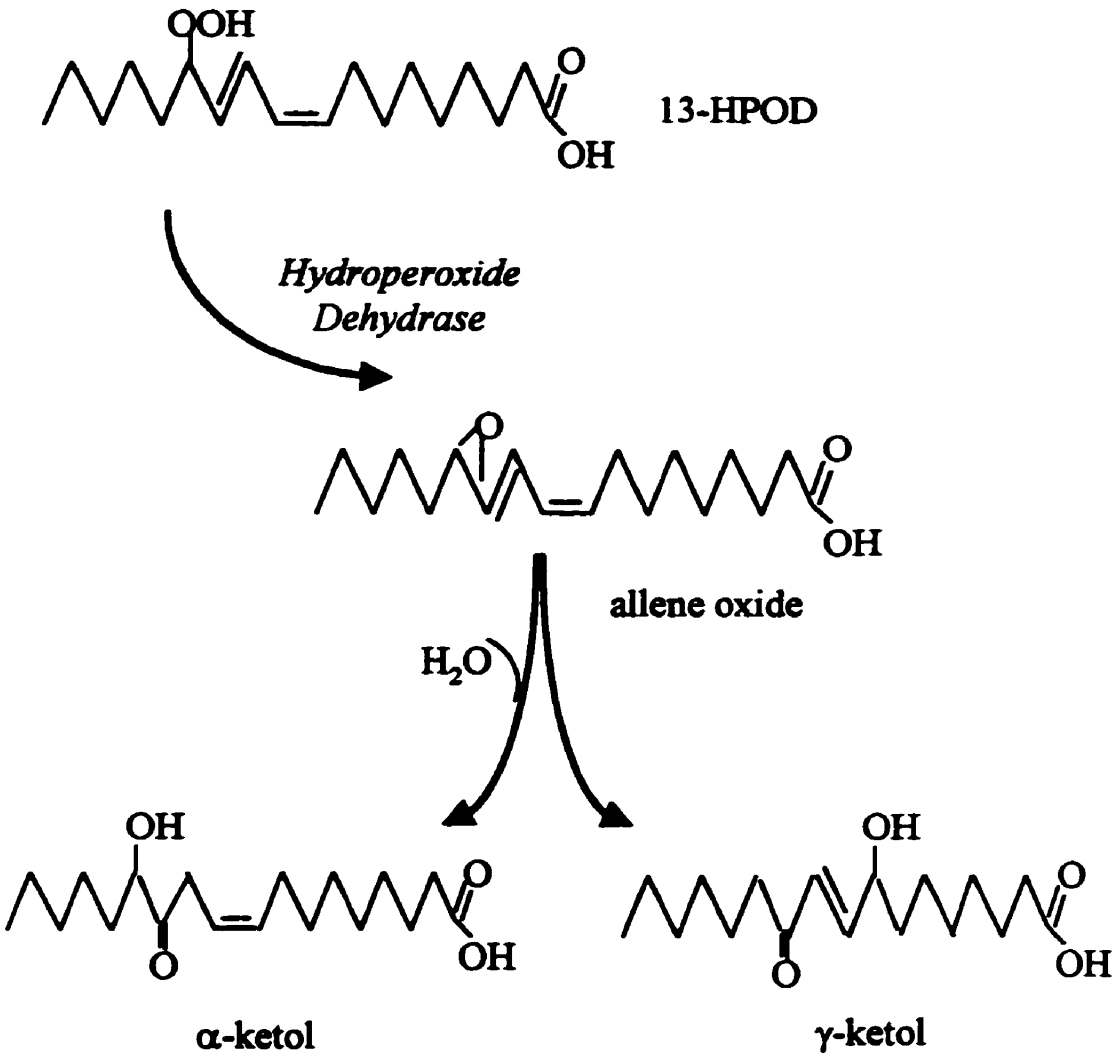

Figure 3. The bioconversion of the 13-hydroperoxide isomer into the allene oxide, 12,13-epoxyoctadecadienoic acid, by hydroperoxide dehydrase activity, followed by the spontaneous non-enzymatic hydrolysis to the corresponding alpha- and gamma-ketols.

whereas those specific for the 13-HPODEs produces six carbon aldehydes such as hexanal which have grassy/beany odors (Gardner, 1989).

A HPL isozyme specific for 13-HPODE was reported in tomato fruits (Kazeniac and Hall, 1970; Stone *et al.*, 1975; Galliard and Matthew, 1977). The presence of a HPL in germinating watermelon seedlings catalyzed the cleavage of 13-HPODE to 12-oxo-10-dodecenoic acid and hexanal (Vick and Zimmerman, 1976). The involvement of LOX and HPL from *Phaseolus vulgaris* in the conversion of 13-HPODE to the wound hormone traumatic acid (trans-2-dodecenedioic acid) (Zimmerman and Coudron, 1979) has been suggested (Vick and Zimmerman, 1981) along with the production of hexanal and 2-hexenal from 13-HPODE and 13-HPOTE, respectively (Vick and Zimmerman, 1987); these six-carbon volatile compounds characterize many freshly cut plant tissues (Tressl *et al.*, 1981; Grosch, 1982).

The HPL from pear fruit had a specificity for 9-HPODEs and 9-HPOTEs which resulted in the formation of nine carbon volatile aldehydes (Kim and Grosch, 1981). In cucumber fruits, HPL reacted with both 9-and 13-HPODEs (Galliard *et al.*, 1976); the enzymatic cleavage of 9-HPODE produced 9-oxononanoic acid and nonenal while that of 9-hydroperoxylinolenic acid (HPOTE) produced nonadienal. A HPL activity specific for 9- and 13-HPODEs was reported in cucumber fruits (Galliard *et al.*, 1976)

From microbial sources, different types of HPL specificities have been reported. Figure 4 shows a HPL activity in the protein fraction from *Psalliota bispora* (Wurzenburger and Grosch, 1986) which catalyzed the cleavage of the 10-HPODE but not the 13-, 12-, and 9-HPODE isomers into 1-octen-3-ol and 10-oxo-8-decenoic acid; in addition, the HPL acts with high regio- and stereo-selectivity by cleaving only the 10-*S*-HPODE and not the 10-*R*-HPODE (Wurzenburger and Grosch, 1984b). The production of two octadienols, i.e. 1,5-octadien-3-ol, 2,5-octadien-1-ol as well as 10-oxo-8-decenoic acid from linolenic acid or from 10-HPOTE suggests the presence of two HPL activities in the mushroom protein fraction.

Figure 4 also shows the cleavage of 13-HPODE by specific HPL activities from microbial sources which led to the production of different five-carbon compounds. A purified

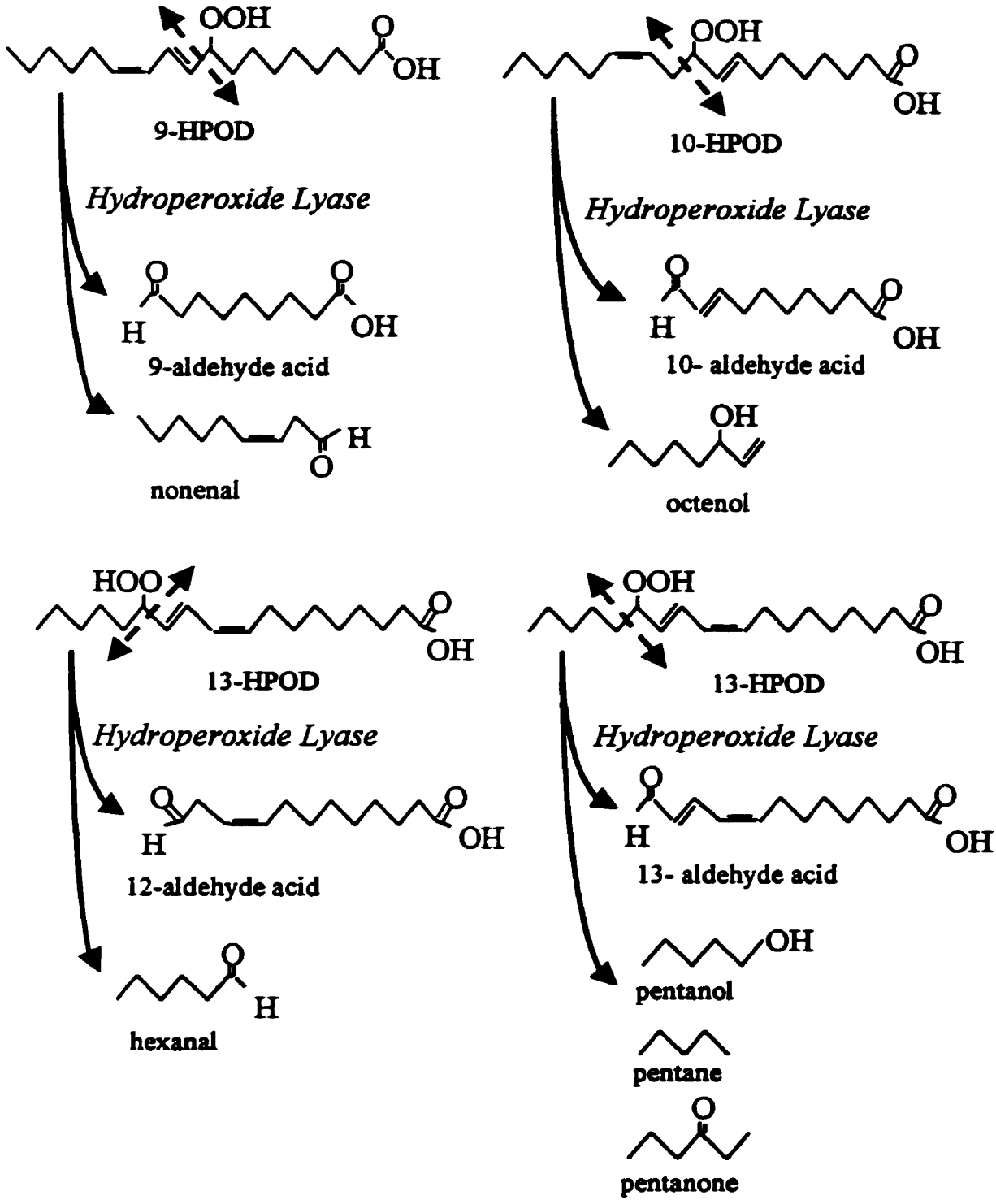

Figure 4. The bioconversion of the hydroperoxide isomers of linoleic acid into the corresponding compounds by hydroperoxide lyase activity.

HPL from the blue-green algae *Oscillatoria* sp. produced oxodienoic acid and pentanol from 13-HPODE (Andrianarison *et al.*, 1989). Vick and Zimmerman (1989) reported that HPL activity from *C. pyrenoidosa* cells cleaved the 13-HPODE and 13-HPOTE to form pentane or pentene, respectively, and 13-oxo-9,11-tridecadienoic acid; the products of the HPL activity from *Chlorella* differed from those reported for most lyase enzymes of higher plants (Vick and Zimmerman, 1987). Higher plant lyases cleave HPODE into 6- and 12-carbon fragments (hexanal and 12-oxo-dodecenoic acid) whereas the *Chlorella* enzyme produces 5- and 13-carbon fragments.

2.1.3. Mechanism of enzymatic action of lipoxygenase

The presence of one iron atom per molecule is believed to be a general property of LOXs and its removal results in the loss of enzymatic activity. Iron appears to be involved in the electron transfer during the incorporation of O_2 into unsaturated fatty acids containing *cis*, *cis*-1,4-pentadiene system. LOX must be in the oxidized (Fe^{3+}) form for the oxidation reactions to proceed (Hildebrand, 1989).

Figure 5 shows a proposed mechanism for LOX activity in plants (Galliard, 1991). The oxidized form of LOX catalyzes the stereospecific removal of hydrogen from the C-11 methylene group of linoleic acid which leads to the formation of a C-13 or C-9 radical and reduction of Fe^{3+} to the Fe^{2+} form. Under aerobic conditions, the LOX-fatty acid radical complex reacts with O_2, forming a lipid peroxy radical. This step is followed by the reduction of the fatty acid HPODE radical to a HPODE with the simultaneous re-oxidation of LOX to the Fe^{3+} state and the release of the fatty acid HPODE. The heterogeneity and the source of the enzyme are some of the factors which contribute to the variation in the proportion of 9- and 13-HPODEs (Kermasha and Metche, 1986).

The cytosolic 8*R*-dioxygenase of *G. graminis* (Brodowsky *et al.*, 1992) and *Laetisaria arvalis* (Brodowsky and Oliw, 1993) is a novel LOX catalyzing the conversion of linoleic acid to 8*R*-HPODE. The general LOX-catalyzed reaction (Fig. 5) involves hydrogen abstraction from the methylene carbon atom, followed by the formation of conjugated *Z,E*-double bonds,

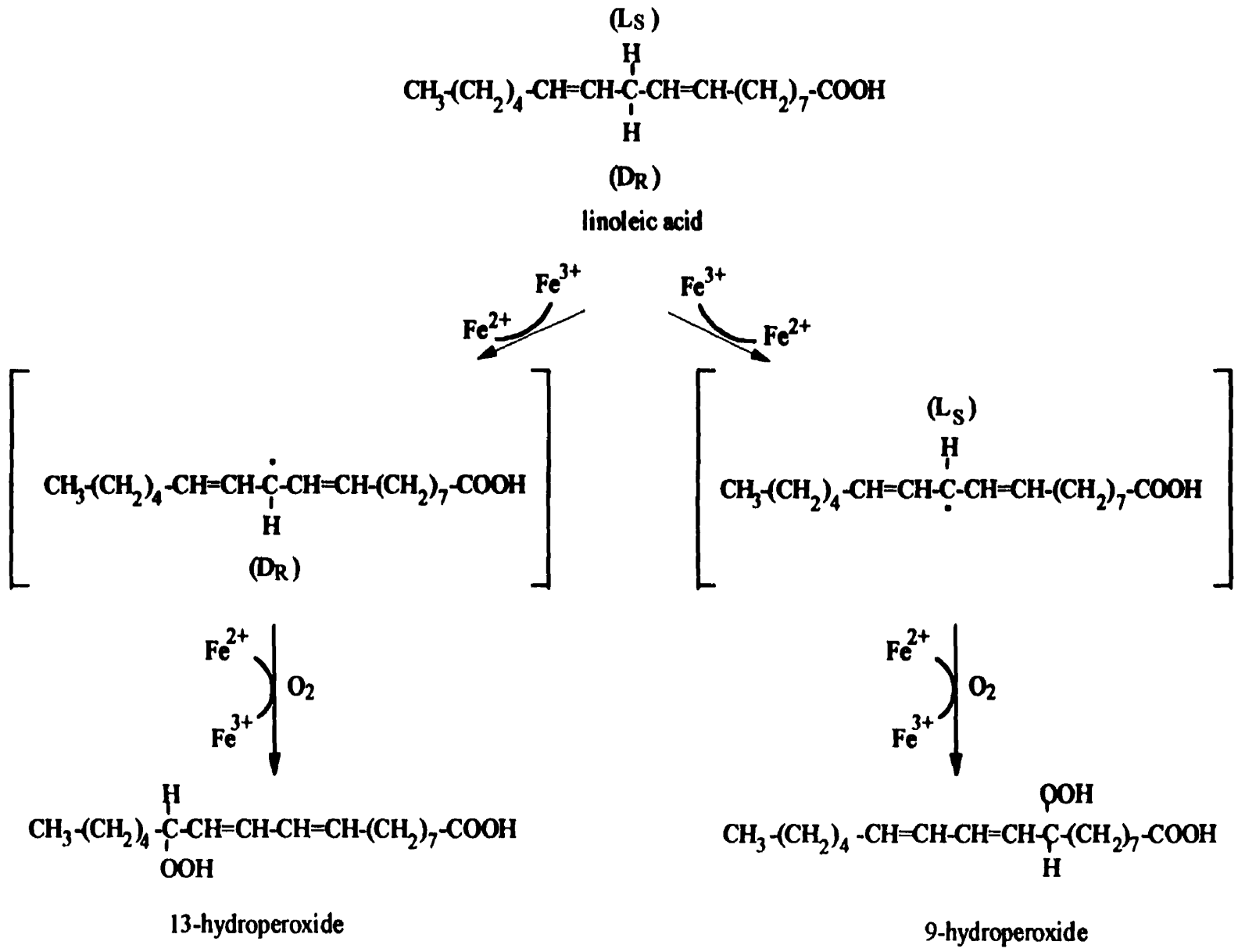

Figure 5. The proposed mechanism for lipoxygenase activity in plants (Gardner, 1991).

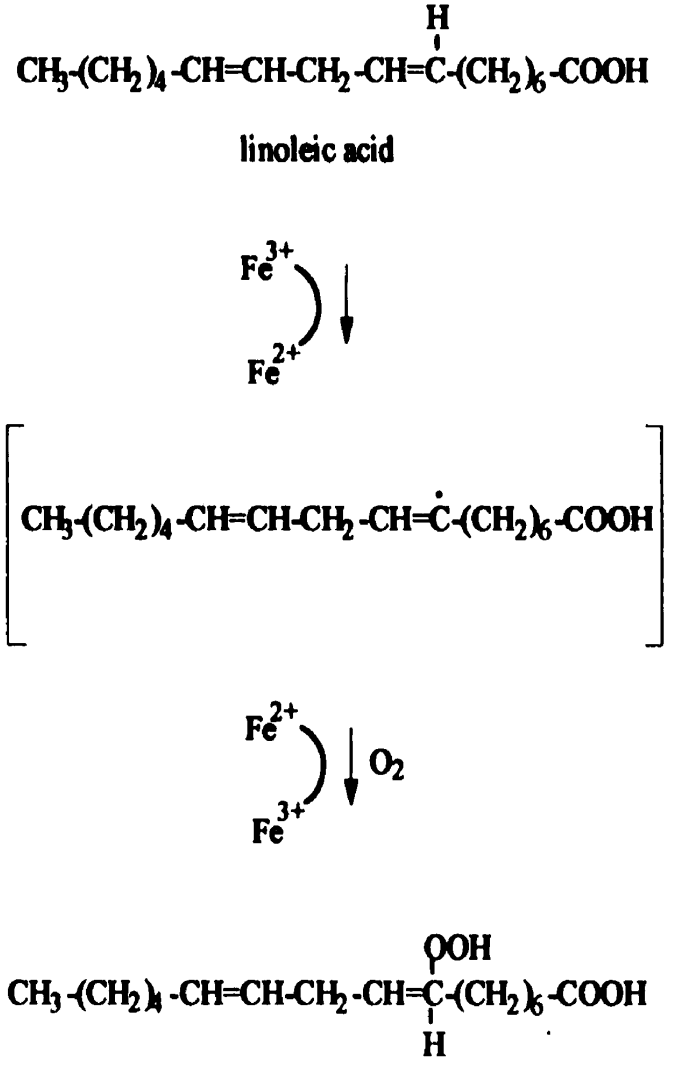

Figure 6. The proposed mechanism for lipoxygenase activity in *Gaeumannomyces graminis* and *Laetisaria arvalis* (Brodowsky *et al.*, 1992).

insertion of molecular oxygen, and the formation of a HPODE; however, the (8*R*)-dioxygenase reaction (Fig. 6) differs in that molecular oxygen is apparently inserted at carbon 8 after abstraction of a hydrogen, without any change in the position or configuration of the adjacent double bond (Brodowsky *et al.*, 1992). In general, LOXs require a fatty acid with one or more 1,4-*Z*,*Z*-pentadiene systems as a substrate; however, the (8*R*)-dioxygenase reacts with fatty acids possessing a 9*Z*-double bond and a saturated carboxyl side chain (Brodowsky *et al.*, 1992).

The production of 10-HPODEs from linoleic acid by the LOX activity in mushrooms was reported by Wurzenburger and Grosch (1984a,b,c,d); however, these authors did not suggest any mechanism for this bioconversion.

2.1.4. Sources of lipoxygenase

In 1928, lipoxygenase (LOX) was discovered when Bohn and Haas reported the presence of a carotene-destroying enzyme "carotene oxidase" in soybean. Andre and Hou (1932) also later reported that soybean contains an enzyme which oxidizes PUFA and subsequently named it "Lipoxidase". Summer and Summer (1940) had success in showing that partially purified LOX has a much lower activity in oxidizing carotene than the crude enzyme extract therefore raising the possibility that there is a second enzyme in addition to a LOX activity which oxidizes carotene (Kies *et al.*, 1969). The occurrence of LOX was originally thought to be confined to seeds of leguminous plants and some cereals (Tappel, 1963), but it is now known to be more widely distributed among higher plants (Pinsky *et al.*, 1971; Grosch, 1972) where they play a physiological role in such degradative processes as senescence, wounding, and infection (Hildebrand, 1989). Particularly rich sources of LOX are soybean (Wagenknecht and Lee, 1956; Axelrod *et al.*, 1981), potato tubers (Galliard and Phillips., 1971) and eggplant (Grossman *et al.*, 1972a). LOX also occurs in leaves (Grossman *et al.*, 1972b; Antis and Friend, 1972b) and avocado peels (Prusky and Kobiler, 1985).

The occurrence of LOX in animal tissues was first questionable, since lipid peroxidation in animal tissues was believed to be exclusively due to ubiquitous heme compounds (Boyd and Adams, 1955; Tappel, 1963). However, the unfolding prostaglandin

research in the mid 1970s led to the discovery of LOX activity in platelets (Veldink *et al.*, 1977) and leukocytes (Borgeat *et al.*, 1976). Recently, LOX has been found in chicken muscle (Grossman *et al.*, 1988), the oocytes of the frog *Xenopus laevis* (Hawkins and Brash, 1989) and fish tissues (Hsieh and Kinsella, 1989). In animals, it is well established that the products of the LOX reaction play a role in the pathway which leads to the formation of important regulatory molecules in inflammatory responses, leukotrienes, and lipoxins (Siedow, 1991).

The presence of LOX activity was first reported in microorganisms of *Aspergillus niger*, *Aspergillus flavoryzea*, *Aspergillus fumigatus* and *Penicillium glaucum* by Mukherjee (1951). A LOX-like enzyme was also reported in *Pseudomonas aeruginosa* by Shimahara and Hashizume (1973). Since then, LOX activity was exhibited in the fungus *Pleurotus pulmonarius* (Belinky *et al.*, 1994) and *Lagenidium giganteum* (Simmons *et al.*, 1987). Herman and Hamberg (1987) reported the presence of LOX activity in a partially purified preparation of *Saprolegnia parasitica*. Matsuda *et al.* (1976) reported the purification of a Co^{2+} requiring heme protein having LOX activity from *Fusarium oxysporum*. Moghaddam and Gerwick (1990) reported a highly active LOX-type activity in a crude fraction of *Gracilariopsis lemaneiformis*. A novel fatty acid dioxygenase was reported in the two fungi, *G. graminis* and *L. arvalis* (Brodowsky *et al.*, 1992; Brodowsky and Oliw, 1992, 1993; Su *et al.*, 1995).

In mushrooms such as *P. bispora*, a LOX activity was reported to be responsible for the conversion of linoleic acid into the 10-HPODE which was in turn cleaved by HPL to produce 1-octen-3-ol and 10-oxo-8-decenoic acid (Wurzenburger and Grosch, 1982, 1984a,b,c,d); linolenic acid was also converted into 1,5-octadien-3-ol, 2,5-octadien-1-ol and 10-oxo-8-decenoic acid (Wurzenburger and Grosch, 1986). The occurrence of LOX activity is suggested by the production of eight-carbon compounds in the fungi *Penicillium* and *Aspergillus* (Kaminski *et al.*, 1974) and edible mushrooms such as *Agaricus bisporus* and *Psalliota campestris* (Maga, 1981).

The presence of LOX activity was reported in the yeast *Saccharomyces vini* (Lyudnikova *et al.*, 1984) used in champagne production and in *Rhodotorula glutimus* (Collins

and Buick, 1989). Shechter and Grossman (1983) purified a LOX fraction from *Saccharomyces cerevisiae*.

Investigations of marine red algae have yielded a wide variety of oxylipins (Gerwick *et al.*, 1991), an encompassing term for oxygenated compounds which are formed from fatty acids by reactions involving at least one step of mono- or dioxygenase-catalyzed oxygenation (Gerwick *et al.*, 1991). The detection of such type of LOX-like activity was reported in the alga *Laminaria sinclairii*, *Laminaria saccharina* and *Laminaria setchellii* (Proteau and Gerwick, 1993), *Gracilariopsis lemaneiformis* (Jiang and Gerwick, 1991), *Lithothamnion corallioides* and *Lithothamnion calcareum* (Hamberg *et al.*, 1992), *Ptilota filicina* (Lopez and Gerwick, 1987), and *Farlowia mollis* (Solem *et al.*, 1989).

Beneytout *et al.* (1989) reported a purified LOX from the green algae, *Oscillatoria* sp. Zimmerman and Vick (1973) reported a LOX activity in a crude and partially purified extract from the green algae *C. pyrenoidosa*. Iny *et al.* (1993b) reported a purified LOX fraction from the thermophilic actinomycete, *Thermoactinomyces vulgaris*. Hamberg *et al.* (1992) reported the presence of a LOX activity in the red alga *Lithothamnion corallioides* extract. The presence of a 12-LOX-type activity reported in the acetone powder extract of the marine red alga *G. lemaneiformis* (Moghaddam and Gerwick, 1990).

2.2. Characterization of Lipoxygenase Isozymes

Characterization of the enzymes with respect to their properties shows that the LOXs comprise a large group of diverse enzymes (Axelrod, 1974).

2.2.1. Isozymes

Kies (1947), who questioned the identity of LOX and carotene oxidase, thought that the LOX activity was caused by more than one enzyme in soybean extracts, responsible for ß-carotene oxidation. Koch *et al.* (1958) subsequently reported that on the basis of measurement of peroxide-forming ability, soybeans contain two LOXs, one whose preference was for the triglyceride and the other for free acids. Christopher *et al.* (1970) purified to

homogeneity a second isozyme from soybean which differed from the first in pH optimum and ester-acid preference; hence,. the original enzyme (Theorell *et al.*, 1947) was designated as LOX-1 and the new isozyme as LOX-2. Yamamoto *et al.* (1970) also isolated an enzyme designated as "b" which may be identical to LOX-2. A third isozyme LOX-3 was isolated from soybean by Christopher *et al.* (1972); it was distinct from the other two isozymes in its elution profile from DEAE-Sephadex column, isoelectric point, pH-profile and effect of calcium ion on its' activity. The presence of four isozymes LOX-1, -2, -3 and -4 in soybean was subsequently reported (Hurt and Axelrod, 1977; Axelrod *et al.*, 1981); they differed from one another in reaction products, pH optima, substrate specificity and mobility in SDS gels (Axelrod *et al.*, 1981; Kitamura *et al.*, 1983; Matoba *et al.*, 1985).

The presence of LOX isozymes is not limited to soybean. Anstis and Friend (1974a) separated four isozymes from pea seed and five active fractions from pea seedlings. However, Arens *et al.* (1973) reported that only two to three isozymes were separated from fresh green beans, whereas Hale *et al.* (1969) showed the presence of one to two isozymes from green bean seeds. Two isozymes were isolated from faba beans (Beaux *et al.*, 1973; Eskin and Henderson, 1974), winged bean (Van Den *et al.* 1982) and wheat flour extract (Graveland, 1970; Wallace and Whealer, 1979).

Three isozymes LOX-1, -2 and -3 were reported in soybean (Engeseth *et al.*, 1987), the germ of bread wheat (Nicholas *et al.*, 1982) and immature English pea seeds (Chen and Whitaker, 1986). However, Ohta *et al.* (1986) reported the presence of only LOX-3 was reported in rice bran and its embryos. In contrast, Ida *et al.* (1983) showed the presence of LOX-1, LOX-2 and LOX-3 in rice bran; moreover, these authors demonstrated that rice LOX-1 and LOX-2 are similar to soybean LOX-2. A type-1 LOX was reported from pea seeds (Reynolds and Klein, 1982b).

2.2.2. Effect of pH on lipoxygenase isozyme activity

In general, enzymes are active only within a narrow pH range; the pH optimum is dependant on other factors such as time of reaction, temperature, type of substrate, substrate

concentration and the chemical properties of the medium in which the reaction is being carried out, such as ionic strength, type and source of the enzyme and its purity (Whitaker, 1972).

Almost all plant LOXs, except soybean LOX-1, have optimum activities at pH 6.5-7.0 (Siddiqi and Tapple, 1956; Eriksson and Svensson, 1970; Galliard and Phillips, 1971; Yoon and Klein, 1979; Galliard and Chan, 1980). Soybean LOX-1 and -2 (Christopher *et al.*, 1972) have optimum activity at pH 9.5 and 6.5, respectively, whereas LOX-3 ranged from pH 4.5-9.0. Axelrod *et al.* (1981) reported that soybean LOX-2 is a neutral plant enzyme because it has an optimum pH of 6.5-7.0. Davies *et al.* (1987) reported three isozymes in soybean seeds; LOX-1 has an optimum pH of 9.0-9.5 (Axelrod *et al.*, 1981) while LOX-2 and -3 have a pH optimum between 6.0 and 7.0. A partially purified Ca-stimulated LOX from soybean seeds (Dressen *et al.*, 1982) had optimum activity at 7.5 while the LOX-2 from soybean (Andrawis *et al.*, 1982) had a pH optimum of 6.5 and its range of activity was between pH 5.0-8.0.

The winged bean LOX (Mtebe and Gordon, 1987) was more active at pH 9 than at pH 7. The major isoenzyme from immature English pea seeds (Chen and Whitaker, 1986) and sweet corn germ (Theerakulkait and Barrett, 1995) exhibited maximal activity at pH 6.5-7.0. The LOX isozyme of mature maize germ (Gardner, 1988) which had a broad pH optimum between 6.0 and 7.2 as well as that from the embryos of inbred yellow dent corn (Belefant and Fong, 1991) exhibited optimal activity at pH 6.8-7.0. The partially purified enzyme extract from crude watermelon hypo-cotyl root exhibited two pH optima, at 4.4 and 5.5 (Vick and Zimmerman, 1976). The optimum pH for rice bran isozymes LOX-1, -2 and -3 was 4.5, 5.5 and 7.0, respectively (Ida *et al.*, 1983).

In contrast to plant LOXs, a much more broader range of optimum pH values was found for LOX activity from microbial sources. The isolated LOX of *P. aeruginosa* (Shimahara and Hashizume, 1973) showed optimal activity at a pH of 10.8-11.0 while that of *Bacillus* species (Shimahara, 1964) at the pH of 10.9. Three optima at pH 6.0, 10.0 and over 12.0 were reported for the LOX activity of *F. oxysporum* in its crude form (Satoh *et al.*, 1976) while the purified enzyme exhibited optimal activity at pH 12.0 with a shoulder peak at

pH 10 (Matsuda *et al.*, 1976). Beneytout *et al.* (1989) reported a purified LOX from the green algae, *Oscillatoria* sp. whose maximal activity was at pH 8.8.

The optimal dioxygenase activity of the fungus *G. graminis* (Brodowsky *et al.*, 1994) was between pH 7.2 and 7.6. Herman and Hamberg (1987) reported that the enzyme of the *S. parasitica* had a pH optimum at 7.5. Zimmerman and Vick (1973) reported a LOX activity in a crude and partially purified extract from the green algae *C. pyrenoidosa* demonstrated optimal activity at pH 7.4. A purified LOX from *S. cerevisiae* (Shechter and Grossman, 1983) showed maximal activity at 6.3 with very low activities with linoleate as substrate below pH 5.0 and above pH 7.0. Iny *et al.* (1993b) reported that the purified enzyme from *T. vulgaris* showed an optimum pH of 6.5 using arachidonic acid as substrate while with linoleic acid, a broad range of activity (pH 3.5 - 12.0) was observed with an optimum at pH 6.0 and a relatively lower one at pH 11.0; the activity found at pH 11.0 may be attributed to the presence of an isoenzyme in the purified preparation which is active at a basic pH.

2.2.3. Stabilizing factors on lipoxygenase isozyme activity

Certain compounds can enhance or promote the expression of enzyme activity and are called activators. Nearly 75% of all known enzymes require the presence of metal ions to express their full catalytic action. Metal ions can be either monovalent (Na^+, K^+) or divalent (Ca^{2+}, Mn^{2+}, Co^{2+}, Mg^{2+}, Zn^{2+}) (Whitaker, 1972).

There has been considerable work on the role of Ca^{2+} in stimulating LOX activity (Holman *et al.*, 1969; Christopher *et al.*, 1972; Verhue and Francke, 1972; Dreesen *et al.*, 1982). It is reported that Ca^{2+} may influence the physical state of the fatty acid substrate in solution, particularly at low pH (Zimmerman and Snyder, 1974), by eliminating the floating micelle fraction (Reynolds and Klein, 1982a). Hence, the role of calcium in activating soybean L-2 may be due to its interaction with the linoleate substrate, rather than a direct interaction with the enzyme, thereby making it more accessible to the enzyme (Zimmerman and Snyder, 1974). Andrawis *et al.* (1982) reported that the activity of LOX-2 from soybean increased

when calcium was added. Although, calcium stimulates the activity of LOX-2, it inhibits the activity of LOX-3 under similar conditions (Christopher *et al.*, 1972; Van Den *et al.*, 1982).

Hamberg and Gerwick (1993) reported that the purified LOX of *G. lemaneiformis* was activated more than 15-fold by the presence of 1 M NaCl; the stimulatory effect was almost identical using NaBr, whereas KCl lacked the stimulatory effect thereby concluding that the ion giving rise to the stimulatory effect was the sodium ion. Of the ions tested, Li^+ had about 50 % of the stimulatory effect at 1 M concentration compared to that observed with the sodium ion at the same concentration. In addition, Mg^+ appeared to have some stimulatory activity (ca. 10 % compared to that of Na^+). On the other hand, Ca^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+} and Co^{2+} did not give significant activation.

The *F. oxysporum* LOX requires Co^{2+} as an essential stabilizing factor for activity (Matsuda *et al.*, 1976) as its removal from the buffer caused a 30 % decrease in activity. In addition, when the enzyme was heated at 30°C for 15 min, activity was lost completely without the presence of Co^{2+} whereas 100 % activity was retained in the presence of Co^{2+} (Matsuda *et al.*, 1976).

2.2.4. Effect inhibitors on lipoxygenase isozyme activity

Enzyme inhibitors are important tools used to investigate the physiological functions of metabolic pathways and to characterize enzymes. Enzyme inhibitors may provide information on the degree of relationship between enzymes.

The iron atom of LOXs is the target of two major classes of LOX inhibitors, the redox and the iron ligand inhibitors (McMillan and Walker, 1992). Redox inhibitors reduce the iron from the catalytically active ferric form to the inactive ferrous form (Nelson *et al.*, 1991). Nordihydroguaiaretic acid (NDGA) has this mechanism of action (Nelson, 1988). Iron ligand inhibitors, e.g. o-phenanthroline and some hydroxamic acid derivatives, chelate ferrous and ferric iron, respectively (Pistorius and Axelrod, 1974; Corey *et al.*, 1984) which partially explains their inhibitory effect (Corey *et al.*, 1984; McMillan and Walker, 1992). Hydroxamic

acid derivatives may also reduce ferric iron to ferrous (Corey *et al.*, 1984; Falgueyret *et al.*, 1993) and may have other effects on the enzyme; for example, linoleate-hydroxamic acid is metabolized by soybean LOX and its metabolite causes suicide inhibition of the enzyme (Butovich *et al.*, 1990).

The partially purified LOX extract of *L. corallioides* (Hamberg *et al.*, 1992) was inhibited by 25 % using NDGA at 0.1 mM while 5,8,11,14-eicosatetraynoic acid (ETYA) was without effect at a concentration of 0.1 mM. Herman and Hamberg (1987) reported that the LOX activity in the crude preparation of *S. parasitica* was completely inhibited by 10^{-4} M of ETYA or NDGA. The purified LOX of *G. lemaneiformis* (Hamberg and Gerwick, 1993) was inhibited by 89 and 78 % in the presence of ETYA and NDGA, respectively while that from *Oscillatoria* sp. (Beneytout *et al.*, 1989) showed 50 % inhibition at 16.2 μM NDGA. The antioxidants NDGA, butylated hydroxytoluene (BHT), tertiary butylated hydroxytoluene (TBHT), and propyl gallate had IC_{50} values of 2.0, 0.8, 3.0 and 20.0 μM, respectively for the purified enzyme fraction from *T. vulgaris* (Iny *et al.*, 1993b).

o-Phenanthroline caused 50 to 100 % inhibition at 10^{-3} M for the LOX activity of *F. oxysporum* (Matsuda *et al.*, 1976). For the dioxygenase activity of *G. graminis* (Brodowsky *et al.*, 1994), the IC_{50} of BW A4C, a hydroxamic acid derivative, and the redox inhibitor, NDGA was 0.2 μM and 0.09 mM respectively. Linoleate-hydroxamic acid (30 μM) was found to inhibit the 8*R*-dioxygenase by over 80 % while ETYA and o-phenanthroline inhibited enzymatic activity by 50 % at 0.15 and 2.5 mM, respectively.

Cyanide inhibits LOXs by forming very stable complexes with the heavy metal located in the enzyme. Pistorius and Axelrod (1974) reported that all isozymes of soybean LOX contains one atom of iron per molecule; however, the nature of the binding of iron to the enzyme is not known (Eskin *et al.*, 1977). Cyanide has been used to distinguish between hemeprotein and LOX-catalyzed reactions (Ganthavorn and Powers, 1989).

For the LOX-like enzyme in *P. aeruginosa* (Shimahara and Hashizume, 1973), cyanide inhibited the enzyme reaction by 53 and 79 % at 0.4 and 4 mM, respectively. Zimmerman and

Vick (1973) reported that a LOX activity in the crude and partially purified extract from algae *C. pyrenoidosa* was not inhibited by 1.6 mM NaCN while 0.2 mM NDGA inhibited the activity by 100 %. A purified LOX from *T. vulgaris* (Iny *et al.*, 1993b) was not inhibited by 2.2 mM KCN whereas that from *S. cerevisiae* (Shechter and Grossman, 1983) was inhibited by 70 and 10 %, using propylgallate and KCN, respectively. Cyanide was a highly effective inhibitor (40 %) at 10^{-2} M for the LOX activity of *F. oxysporum* (Matsuda *et al.*, 1976).

Gibian and Galaway (1976) reported that the oxygenation of linoleate and arachidonate, catalyzed by soybean LOX, was subjected to competitive product inhibition which caused the reaction to cease before completion. This process was reversible upon addition of further substrate and was proposed to be a chemical (reversible) change of the enzyme. Iny *et al.* (1993b) reported that the purified enzyme activity from *T. vulgaris* was inhibited by the presence of 9- and 13-HPODEs at concentrations above 10 μM.

2.2.5. Molecular weight of lipoxygenase isozymes

The soybean LOX was first crystallized by Theorell *et al.* (1947) and had a molecular weight (MW) of 100 kDa. Vick and Zimmerman (1976) reported that LOX-1 and -2 from watermelon seedlings had a MW of 120 and 240 kDa, respectively while that from young cotton seedlings was 100 kDa (Vick and Zimmerman, 1981). Nicholas *et al.* (1982) identified three isozymes, LOX-1, -2, and -3 in the germ of bread wheat whose MW was estimated to be 90, 95 and 110 kDa, respectively. Reynolds and Klein (1982b) identified the presence of a type-1 LOX in pea seeds whose MW was 64 to 65 kDa. Chen and Whitaker (1986) reported that the major isoenzyme from immature English pea seeds, LOX-2 showed a MW of 100 kDa.

The MW of the partially purified LOX from lupin (Olías and Valle, 1988), avocado (Marcus *et al.*, 1988), potato tubers (Galliard and Phillips, 1971) and tomato fruit (Bonnet and Crouzet, 1977) was reported to be 92, 74, 100, and 87 kDa, respectively; in contrast, germinating sunflower seed LOX (Leoni *et al.*, 1985) had a MW of 250 kDa.

The MW of the LOX of *F. oxysporum* was estimated to be between 12 to 13.7 kDa (Matsuda *et al.*, 1976). A purified LOX from *Oscillatoria* sp. (Beneytout *et al.*, 1989) and *T. vulgaris* (Iny *et al.*, 1993b) possessed a MW of 124 and 160 kDa, respectively. The MW of the LOX from *S. parasitica* was found to be 145 to 150 kDa (Herman and Hamberg, 1987) while that from *G. lemaneiformis* (Hamberg and Gerwick, 1993) was 84-89 kDa.

2.2.6. Temperature stability of lipoxygenase isozymes

Temperature plays a very important role in all reactions; it affects the stability of the enzymes and their corresponding substrates, the availability of substrates and cofactors, the affinity of the enzyme towards the substrate, the activators and inhibitors as well as the formation of by-products (Whitaker, 1972).

The LOX of *F. oxysporum* was stable at 70°C and less for 15 min whereas activity was lost completely by heat treatment for 60 min at 100°C (Matsuda *et al.*, 1976). Beneytout *et al.* (1989) reported a purified LOX from the green algae, *Oscillatoria* sp. which maintained 50 % of its activity at 45°C for 5 min; however, activity was completely lost by a 5 min treatment at 70°C. The LOX activity of the crude and partially purified extracts of *C. pyrenoidosa* (Zimmerman and Vick, 1973) was destroyed by heating for 2 min at 100°C. The LOX activity in the partially purified preparation from *S. parasitica* (Herman and Hamberg, 1987) was stable at 0°C retaining 88 % of its activity after 6 hrs while at 37°C, all activity was rapidly lost.

The LOX from *F. oxysporum* (Matsuda *et al.*, 1976) showed optimal activity at 40°C while that from *P. aeruginosa* (Shimahara and Hashizume, 1973) was between 37°C and 40°C. The general temperatures found for maximal LOX activity of microbial sources are in the range of 35-40°C. However, Iny *et al.* (1993b) reported that a purified enzyme from *T. vulgaris* possessed an optimum temperature at 55°C and significant activity at 75°C. The purified thermophilic enzyme was also unstable at temperatures below zero, although at 4°C,

it kept its stability for a few days. These data show that the LOX from thermophilic bacteria has the properties of a classical thermophilic enzyme with a relatively stable protein conformation whereas LOXs from other microbial sources are less stable and at temperatures over 45°C.

2.2.7. Substrate specificity of lipoxygenase isozymes

Substrates for LOX may be straight chain fatty acids, esters, alcohols and hydroxymates (Haining, 1974) and halides (Blain and Shearer, 1965) which contain a *cis, cis*-1,4-pentadiene structure. The most common substrates used are essential fatty acids such as linoleic, linolenic and arachidonic acids (Hamberg and Samuelsson, 1965, 1967). Linoleic acid is probably the best substrate for LOX activity (Axelrod, 1974; Takagi *et al.*, 1987). However, the pH-dependent solubility of linoleic acid in the aqueous reaction medium causes many difficulties. The poor water solubility of linoleic acid can be improved by the addition of emulsifiers such as Tween-20 to the reaction mixture (Surrey 1964; Grossman *et al.*, 1969; Ben-Aziz *et al.*, 1970).

The three LOX isozymes in soybeans have slightly different substrate specificities; LOX-1 is most active on linoleic acid, while LOX-2 is more active on arachidonic acid than on linoleic acid and LOX-2 and -3 are somewhat more active on methyl linoleate than on linoleic acid (Bild *et al.*, 1977; Axelrod *et al.*, 1981). In addition, soybean LOX-1 shows a marked preference for charged fatty acids and therefore shows little reactivity with fatty acids that are esterified (i.e., as they would be found in a membrane). LOX-2 and -3 are more reactive toward neutral fatty acids but will react with free fatty acids, particularly at pHs below 7.0 (Siedow, 1991).

Arachidonic, linoleic and 5,8,11,14,17-eicosapentaenoic acids all served as good substrates for the LOX activity in the *S. parasitica* crude extract (Herman and Hamberg, 1987). The specific activity of the LOX of *F. oxysporum* was 19.4, 9.5, 1.46 and 0.95 units/mg for linoleic acid, methyl linoleate, linolenic acid and methyl linolenate, respectively (Matsuda *et al.*, 1976); the monoenoic oleic acid and the saturated stearic acid were inactive.

The LOX activity in the partially purified extract from *P. bispora* (Wurzenburger and Grosch, 1984c) oxidized α-linolenic acid at about one third of the rate as that found for linoleic acid while γ-linolenic, methyl linoleate and trilinolein were not accepted as substrates. In general, the free fatty acids-linoleic, linolenic and arachidonic serve as substrates for microbial LOXs; however, the LOX of the fungus *L. giganteum* is an exception since it oxidizes only eicosapentaenoic, arachidonic and docosahexaenoic acids in decreasing order (Simmons *et al.*, 1987).

The LOX activity in the crude and partially purified extract from *C. pyrenoidosa* (Zimmerman and Vick, 1973) was active with both linoleic and linolenic acids, the latter was approximately 30 % more active than the former. A LOX-like enzyme was reported in *P. aeruginosa* (Shimahara and Hashizume, 1973) which exhibited relative activities of 0.15 and 0.73 for methyl oleate and methyl linoleate, respectively, in comparison to linoleic acid.

The purified LOX from *S. cerevisiae* (Shechter and Grossman, 1983) demonstrated greater activity towards arachidonate and linoleate as substrates than linolenate. Iny *et al.* (1993b) reported that the purified enzyme from *T. vulgaris* showed substrate specificity towards linoleic acid (100 %), linolenic acid (33 %), arachidonic acid (22 %) 8,11,14 eicosatrienoic acid (43 %), 11,14,17 eicosatrienoic acid (38 %) but was not active when the substrate was linoleic acid ethyl ester (0 %).

2.2.8. End-product specificity of lipoxygenase isozymes

LOX isozymes are generally classified into three types according to the positional specificities of HPODEs produced when linoleic acid is used as substrate. This classification is based on soybean LOX isozymes. LOX-1 catalyses exclusively the production of 13-HPODEs (95%), LOX-2 catalyses the production of equal amounts of 9- and 13-HPODEs and LOX-3 produces more 9-HPODE (65%) (Axelrod *et al.*, 1981). Specificity for the site of O_2 insertion into the substrate during oxidation is affected by many factors including, source of LOX, type of isozymes, substrate and experimental conditions such as pH, temperature and O_2 tension (Eskin *et al.*, 1977).

The ratio of 13- to 9-HPODEs formed from linoleic acid was 95:5, 70:30, 40:10, 50:50 and 87.7:12.3 by LOX activity from potato (Galliard and Phillips, 1971), soybean (Hamberg and Samuelsson, 1965), flax seed (Zimmerman and Vick, 1970), dry immature English pea seed (Roza and Francke, 1973), and immature English pea (Chen and Whitaker, 1986), respectively.

The major isoenzyme from pea (Gardner, 1970) catalyzed the peroxidation of linoleic acid at the 13 and 9-positions with a ratio of 50:50. Grosch and Laskway (1975) stated that LOX-1 oxidizes linoleic acid only to the 13-HPODE whereas LOX-2 and -3 catalyze the formation of 9- and 13-HPODEs in an approximate ratio of 1:1.

A purified LOX from *F. oxysporum* converted linoleic acid into 9- and 13-HPODEs at a ratio of 70:30 at pH 9.0 and 56:44 at pH 12.0 (Matsuda *et al.*, 1978). An enzyme preparation from the red alga *L. corallioides* produced the 11-HODE as well as smaller amounts of 9- and 13-HODEs and 11-keto-9,12-octadecadienoic acid from linoleic acid (Hamberg *et al.*, 1992). Shechter and Grossman (1983) reported that the 9- and 13-HPODE isomers are produced by the purified LOX of *S.cerevisiae* with a quantitative ratio of 1:1.

2.3. Enzyme Assay

The primary products of the LOX enzymatic reaction are *cis*, *trans*-conjugated diene HPODEs. There are several methods that have been developed for determining the rate of the enzymatic reaction. The most commonly used methods are the polarographic method and the spectrophotometric technique.

The polarographic or oxygen electrode method measures oxygen uptake by the substrate in the presence of enzyme. It measures the reaction continually from the beginning and the whole reaction may be completed in a few minutes. This method is especially suitable for kinetic studies using the pure enzyme and the crude extract (Galliard, 1983) and does not require optically clear substrate solutions for measuring oxygen uptake. However, the oxygen uptake in the polarographic assay is not specific for LOX activity only (Nicholas and Drapron,

1981). Strict controls must be employed when the activity of crude extracts or particles is tested, since oxygen uptake can be caused by other systems in the respiratory chain. Hence, for the determination of the presence of a true LOX, the use of at least two criteria to assay the activity of extracts and fractions is recommended (Grossman and Zakut, 1979).

The polarographic method has been used to access the LOX activity in the crude *F. oxysporum* extract (Satoh *et al.*, 1976) and the disrupted *S. cerevisiae* yeast cells (Shechter and Grossman, 1983); the velocity was measured from the initial linear portion of the oxygen-consumption curve and the unit was defined as 1 μmole 0_2 consumed per minute at 25°C. In addition, the LOX assay of the purified cucumber cotyledon (Matsui and Kajiwara, 1995) and the bell pepper (Luning *et al.*, 1995) extracts was also performed using an oxygen electrode.

The direct spectrophotometric determination of enzyme activity is based on the absorbance at 234 nm formed when the *cis,cis*-1,4-pentadiene-containing substrate is oxidized (Surrey, 1964). The advantage of this method is that it is simple and rapid but it also requires optically clear solutions. When the method is operated at pH 9.0 or above, the unsaturated fatty acids are present in a soluble form as an ammonium or sodium salt; however, at lower pH values, the addition of the detergent , i.e. Tween-20 (polyoxyethylene sorbitan monolaurate) is necessary for the solubility of the PUFA (Grossman and Zakut, 1979). Tween-20 does not absorb light at any wavelength of the ultraviolet spectrum but has been reported to have competitive inhibitory effects when the amount of detergent exceeded that of linoleic acid; this effect is most striking at low substrate concentrations where the Tween-substrate ratio is the highest (Surrey, 1964; Grossman and Zakut, 1979). Another disadvantage is the difficulty in determining LOX activity of crude enzyme extracts due to the turbidity of the preparation (Eskin *et al.*, 1977); in addition, when preparations of low specific activity are used, interference from protein absorption can be a problem (Axelrod, 1974).

The spectrophotometric method was used to measure the conjugated diene absorption of the HPODEs at 234 nm produced by LOX activity from winged beans (Mtebe and Gordon, 1987), watermelon (Vick and Zimmerman, 1976), sweet corn germ (Theerakulkait and Barrett, 1995), the partially purified *C. pyrenoidosa* extract (Zimmerman and Vick, 1973), the

crude wine yeast *S. vini* extract (Lyudnikova *et al.*, 1984), the *S. cerevisiae* extract (Shechter and Grossman, 1983) and the *F. oxysporum* extract (Matsuda *et al.*, 1976).

Due to the different substrate specificity of LOX isozymes, LOX-1 activity from soybeans was determined by measuring conjugated diene absorption at 234 nm with linoleic acid as substrate at pH 9.0 whereas LOX-2 activity was determined at 238 nm with arachidonic acid at pH 6.1 while LOX-3 activity was determined at 234 nm and 280 nm (keto dienes) with linoleic acid at pH 6.5 (Frankel *et al.*, 1988). Axelrol *et al.* (1981) suggested that 280 nm was the preferable wavelength to measure ketodiene formation by LOX-3 activity as a secondary reaction product, as ketodiene formation is reasonably linear with time whereas the simultaneous HPODE production at 234 nm is not.

With respect to the polarographic procedure, the assay of LOX-1 at pH 9.0 with linoleic acid can be performed; however, the polarographic procedure is not well suited for measuring LOX-3 activity with linoleic acid as it fails to give good stoichiometry between enzyme concentration and specific activity (Christopher *et al.*, 1972). The LOX-2 activity with linoleic acid may be measured; however, when arachidonic acid is employed, the stoichiometric relationship between O_2 consumed and product(s) formed is more complicated (Axelrod *et al.*, 1981).

2.4. Cell Disruption

The frozen mycelia of *F. oxysporum* were homogenized with a Warring blender (Nihon Seiki Kogyo Co., Japan) for 1 min and the cells were disrupted by a two-cycle treatment with a Manton-Gaulin (Manton-Gaulin Manufacturing Co., USA) at 8000 lbs (Matsuda *et al.*, 1976). A crude LOX extract was prepared from *S. parasitica* (Herman and Hamberg, 1987), by homogenization in an ice-cold buffer with a Polytron. Moreover, the cells from *T. vulgaris*; were sonicated (three times for 1 min each) and also homogenized using a polytron homogenizer (Iny *et al.*, 1993b). The fungus *L. giganteum* (Simmons *et al.*, 1987) was frozen and homogenized for 2 min under CO_2 in phosphate buffer (0.2 M, pH 7.5) using a Braun homogenizer.

Pieces of the frozen algae *G. lemaneiformis* were homogenized using a Potter-Elvehjem type homogenizer (Hamberg and Gerwick, 1993). The fungus *G. graminis* (Brodowsky and Oliw, 1992; Brodowsky *et al.*, 1994) was disrupted by using a cell sonicator (Sonifier Cell disrupter, Branson) or by mincing the frozen mycelia in a mortar (at $^{+}4^{\circ}C$) with Al_2O_3 (Shoun *et al.*, 1985) and Tris-HCl buffer solution (0.05 M, pH 7.4 with 1 mM EDTA and 1 mM EGTA); the latter method was also used to obtain the enzymatic extract from the fungus *L. arvalis* (Brodowsky and Oliw, 1993). Su *et al.* (1995) homogenized the frozen mycelia of the fungus *G. graminis* with alumina type A-5 in potassium phosphate (pH 7.4).

The green algal cells of *C. pyrenoidosa* (strain 211-8B) were homogenized using glass beads (0.28 mm diameter) (Zimmerman and Vick, 1973) while those of *Oscillatoria* sp. (Beneytout *et al.*, 1989) were homogenized using an Ultra Turrax homogenizer (3000 rpm, 15 min). The red alga *L. corallioides* (Hamberg *et al.*, 1992) was crushed and ground into a powder using a mortar and liquid nitrogen; the powder was suspended in buffer and homogenized with an Ultra-Turrax (Janke and Kunkel, Staufen, Germany).

The yeast cells of *S. vini* (Lyudnikova *et al.*, 1984) were frozen and crushed in a press using the method of Lyubimov and L'vov while those of *S. cerevisiae* (Shechter and Grossman, 1983) were shaken thirty times for 30 sec using 0.2 mm Ballotini beads in 75-ml Pyrex bottles at $4^{\circ}C$ in a Braun shaker at 4,000 rpm.

2.5. Enzyme Extraction

Kermasha and Metche (1986) reported that LOX from *Phaseolus vulgaris* was defatted by successive washing with acetone and diethyl ether which resulted in a two-fold increase in enzyme activity when compared to that using hexane; this effect may be related to the polarity of the solvents, since both diethyl ether and acetone have a higher polarity index (2.8 and 5.1, respectively) than that of hexane (0.1). The effect of organic solvents on LOX activity was investigated by Allen (1968). Galpin and Allen (1977) have reported that the effect of *n*-alcohols and *n*-carboxylic acids on LOX activity was due to the physicochemical

interactions between these organic solutions and the substrate rather than their direct effects on the enzyme configuration.

Endogenous lipids were removed from peas by using acetone and diethyl ether (-30°C) and the crude enzyme was then extracted using Tris-HCl buffer solution (Eriksson and Svensson, 1970). Ganthavorn and Powers (1989) reported that lipids were extracted from asparagus using an acetone wash (-30°C) and the enzyme was then extracted from the defatted powder using potassium phosphate buffer at 4°C. The winged bean seeds (Mtebe and Gordon, 1987), soybean seeds (Axelrod *et al.*, 1981) and wheat germ (Bhirud and Sosulski, 1993) were ground into a fine powder and defatted with hexane. Singleton *et al.* (1976) prepared acetone powders of cured peanuts which were subsequently defatted using acetone and ether while Shiiba *et al.* (1991) defatted wheat germ using acetone.

2.6. Enzyme Purification

LOX was purified by conventional techniques of protein isolation, such as ammonium sulfate fractionations, ion-exchange chromatography, size-exclusion chromatography, isoelectric focusing, chromatofocusing, reverse-phase chromatography, hydrophobic chromatography, affinity chromatography and electrophoresis. These techniques are reliable for the isolation and separation of the enzyme from other proteins based on their respective physical and chemical properties (Eskin *et al.*, 1977).

2.6.1. Ammonium sulfate precipitation

Once the enzyme has been solubilized, an early purification step involves salt or alcohol mediated precipitation, followed by resolubilization and desalting. The objective of this procedure is two-fold; the first is to reduce the volume of the starting material to a level amenable to subsequent purification steps while the second is the removal of gross impurities such as carbohydrates, lipids and nucleic acids. Fractionation of protein mixtures by precipitation with organic solvents such as ethanol and acetone, ammonium sulfate and polyethylene glycol is used. The most frequently used salt is the ammonium sulfate and its

principal advantages are its high solubility (ca. 700 g/liter) which permits the precipitation of a wide scale of protein molecules, low cost and stabilizing effect on the activity of most enzymes (Lillehoj and Malik, 1989).

The first soybean LOX extract was partially purified by Theorell *et al.* (1947) using ammonium sulfate fractionation and the final product was crystallized. Since then, many partially purified preparations of LOX have been reported in the literature (Tappel, 1961; Yamamoto *et al.*, 1970; Verhue and Francke, 1972). LOX active proteins isolated from various sources were precipitated with ammonium sulfate using different levels of saturation. Olías and Valle (1988) reported that LOX was partially purified from lupin by ammonium sulfate at 25 % saturation. LOX from immature English pea (Chen and Whitaker, 1986) and pea (Eriksson and Svensson, 1970) was precipitated by ammonium sulfate at 25-50 % of saturation. Ammonium sulfate at 20 to 50 % of saturation was also used to precipitate the LOX of *P. vulgaris* (Kermasha and Metche, 1986) and pea seed (Haydar and Hadziyev, 1973). Klein (1976) indicated that LOX was isolated from dried split pea seeds, frozen raw peas and snap beans by ammonium sulfate fractionation at 30-50 % of saturation; this range was also used to isolate and purify LOX from dried winged beans seeds (Van Den *et al.*, 1982). LOX from peas (Arens *et al.*, 1973) and soybean seeds was isolated using 30-60 % saturation. Partially purified LOX from asparagus (Ganthavorn and Powers, 1989) and potato tubers (Galliard and Phillips, 1971) was obtained by precipitation with ammonium sulfate at 0-50 % saturation. Reynolds and Klein (1982b) indicated that LOX-1 was partially purified from pea seeds by 25-60 % whereas commercial soybean LOX (Stevens *et al.*, 1970) and green bean LOX (Adams and Ongely, 1989) were purified at 40-60 % of ammonium sulfate saturation. LOX was isolated from peanut seeds (Sanders *et al.*, 1975) and winged beans (Mtebe and Gordon, 1987) with ammonium sulfate at 40-65 % and 30-40 % of saturation, respectively. Ammonium sulfate at 60 and 40 % of saturation was used to precipitate LOX from a crude watermelon hypocotyl-root extract (Vick and Zimmerman, 1976) and the germ of bread wheat (Nicholas *et al.*, 1982).

Theerakulkait and Barrett (1995) used acetone to extract LOX activity from sweet corn germ, resulting in an acetone powder; ammonium sulfate precipitation at 40-60 % of

saturation was used to further partially purify the LOX extract. LOX from avocado (Marcus *et al.*, 1988) was partially purified by 66 % acetone precipitation from 20 % acetone supernatant.

The crude suspension of *F. oxysporum* (Matsuda *et al.*, 1976) and *S. parasitica* (Herman and Hamberg, 1987) was partially purified using ammonium sulfate at 10-100 and 25-55 % ammonium sulfate of saturation, respectively. The crude extract of the fungus *G. graminis* (Brodowsky *et al.*, 1994) was partially purified by acetone and ammonium sulfate precipitates (0-40 % of saturation); about 80 % of the total 8*R*-dioxygenase activity was lost during ammonium sulfate precipitation and acetone precipitation. A crude LOX extract from *G. lemaneiformis* (Hamberg and Gerwick, 1993) and *G. graminis* (Su *et al.*, 1995) was partially purified using ammonium sulfate precipitation at 30-55 % and 20-45 % saturation, respectively.

A partially purified LOX preparation from the red algae *L. corallioides* (Hamberg *et al.*, 1992) and the green algae *C.prenoidosa* (Zimmerman and Vick, 1973) was obtained by ammonium sulfate at 30-55 and 0-42 % of saturation, respectively. A LOX-like enzyme was partially purified from *P. aeruginosa* by (Shimahara and Hashizume, 1973) using ammonium sulfate at 35-65 % of saturation.

A LOX-type enzyme from an extract of mushrooms (Wurzenburger and Grosch, 1984a,b,c,d), was obtained by precipitation with polyethylene glycol (3-6 % saturation).

2.6.2. Liquid Chromatography

Protein separations are normally performed using liquid chromatography which employs a liquid mobile phase and a solid stationary phase packed into a tubular shaped column. The development of column packings with high performance, high resolution, speed, stability and rigidity has made it easier to isolate and purify proteins. Packings based on Sepharose resins provide high flow rates and good recovery of proteins while preserving biological activity, in comparison to conventional liquid chromatography techniques, thereby allowing separations to be performed on a time-scale of minutes (Lillehoj and Malik, 1989).

2.6.3. Ion-exchange chromatography

Ion-exchange chromatography is a technique which separates biomolecules according to their differences in charge; it involves the interaction of charged functional groups in the enzyme sample with the ionic functional groups of opposite charge on the absorbent surface. Anion-exchange chromatography is mediated by the interaction of negatively charged amino acid side chains (e.g. aspartic acid and glutamic acid) with positively charged supports; proteins with a net negative charge, i.e. in an aqueous solution with a pH above their isoelectric point will bind to these supports. Cation exchangers are prepared by polymerization of negatively charged groups to silica for interaction with positively charged residues (e.g. lysine and arginine); proteins in a solution with a pH below their isoelectric point will interact with these absorbents (Lillehoj and Malik, 1989). The binding energy between the sample and absorbent is dependent upon the solvent pH, ionic strength and selectivity of the counter-ion; the best native biological or enzymatic activity is maintained by constant conditions of pH and counter-ions with a gradient of increasing salt concentration.

There are several types of resins, the most commonly used being DEAE-cellulose, which were introduced by Peterson and Sober (1956). However, there are other ion-exchange resins that have also been used in the purification of LOX such as DEAE-Sephacel (Ida *et al.*, 1983) and DEAE-Sephadex (Sanders *et al.*, 1975; Sekiya *et al.*, 1977; Reynolds and Klein, 1982b; Van Den *et al.*, 1982). Siedow (1991) reported that anion-exchange chromatography (Christopher *et al.*, 1970; Axelrod *et al.*, 1981) was used to separate LOX-1 from LOX-2 and -3 due to the large charge difference between the isoelectric point of LOX-1 (5.68) and LOX-2 (6.25) and -3 (6.15) (Axelrod *et al.*, 1981).

Wallace and Whealer (1979) indicated that two isozymes of wheat germ LOX were separated and purified using DEAE-cellulose. Pea LOX has been purified using DEAE-cellulose (Eriksson and Svensson, 1970; Arens *et al.*, 1973; Haydar and Hadziyev, 1973; Chen and Whitaker, 1986). Commercial soybean LOX (Stevens *et al.*, 1970) was purified using DEAE-cellulose. The separation of a calcium-stimulated LOX from one active in the absence of calcium (Dressen *et al.*, 1982), was readily obtained using a DEAE-cellulose

column. Chen and Whitaker (1986) discovered three isoenzymes from immature English peas by DEAE-cellulose chromatography. LOX-1 from soybeans was purified using DEAE-Sephadex (Axelrod *et al.*, 1981). Sephadex DEAE-A50 column chromatography was used in the isolation of LOX-3 from soybean (Christopher *et al.*, 1972), of LOX-1, -2, and -3 from wheat germ (Nicolas *et al.*, 1982) and of LOX-2 and -3, from soybeans (Axelrod *et al.*, 1981).

A LOX-like enzyme preparation from *Pseudomonas aeruginosa* (Shimahara and Hashizume, 1973) was purified using DEAE-cellulose chromatography while that from *Oscillatoria* sp. (Beneytout *et al.*, 1989) was purified using a DEAE-Tris-acyl column (2.5 x 40 cm). The crude extract of *S. parasitica* (Herman and Hamberg, 1987) was purified using a DEAE Sephadex column (1.8 x 24.5 cm).

2.6.4. Size-exclusion chromatography

Size-exclusion chromatography is a simple and reliable technique used for the separation of biomolecules according to their size; macromolecules in the mobile phase differentially penetrate the pores of the stationary phase particles accordingly to their hydrodynamic volume such that the larger components are excluded from the interior of the particles whereas the smaller molecules are accessible to this volume. Size-exclusion is an excellent technique to employ early in a purification scheme, ideally as the first or second step, since it results in sample dilution; proteolysis due to chemical or enzymatic effects can therefore be minimized resulting in a higher yield in the final product. In addition, a wide variety of mobile phases can be used (Lillehoj and Malik, 1989).

Size-exclusion is suitable for the purification and size estimation of a wide range of biomolecules, with Sephadex being the most commonly used. LOX from pea, peanut, commercial soybean and winged beans LOX was purified by Sephadex G-150, (Eriksson and Svensson, 1970; Stevens *et al.*, 1970; Sanders *et al.*, 1975; Klein, 1976; Van Den *et al.*, 1982). LOX from pea seeds was purified by Sephadex G-200 (Yoon and Klein, 1979). LOX from lupin was purified by Phenyl Sepharose CL-4B (Olías and Valle, 1988) while that from apple (Kim and Grosch ,1979) was purified using Sepharose CL-2B.

A purified LOX was obtained from the concentrated enzyme suspension of *F. oxysporum* (Matsuda *et al.*, 1976) by using a SP-Sephadex C-25 column (5 x 25 cm), followed by a Sephadex G-100 column (1.5 x 75 cm) and then again the SP-Sephadex C-25 column. The partially purified extract of *S. parasitica* (Herman and Hamberg, 1987) was purified by a Sephacryl S-300 column (87.5 x 2.4 cm). The LOX extract of *Oscillatoria* sp. (Beneytout *et al.*, 1989) was further purified using a Sephadex G-150 column (4 x 46 cm) while that from *G. lemaneiformis* (Hamberg and Gerwick, 1993) was purified by using a Sephacryl S-300 column (85 x 2.6 cm) (Pharmacia LKB Biotechnology) or an Ultrogel AcA 44 (85 x 2.6 cm) column.

2.6.5. Isoelectric-focusing

The migration of proteins is determined by their isoelectric point (pI) and the development of a pH gradient which is formed by inclusion of highly mobile amphoteric components (ampholytes) in the gel matrix. Ampholytes consist of a collection of zwitterionic, small molecular weight polyaminopolycarboxylic acids with differing pIs. In the presence of an electric field, they arrange themselves in a manner that the most basic are located near the cathode and the acidic near the anode. A focusing effect is achieved when the proteins migrate to the position in the pH gradient where their net charge is zero. The resolving power of isoelectric focusing (IEF) is tremendous as proteins with a single charge difference can be separated on an analytical scale. However, a clear disadvantage relating to the use of IEF in a preparative mode is the tendency of proteins to precipitate out of solution at their pI where they are less soluble than in a charged state (Lillehoj and Malik, 1989). IEF techniques have been used successfully by Funk *et al.* (1985) to achieve a high degree of purification.

Reynolds and Klein (1982b) indicated that a type-1 LOX was purified from pea seeds using gel filtration, ion-exchange chromatography and preparative IEF in agranulated gel; however, extensive loss of enzyme activity occurred during preparative IEF. Dressen *et al.* (1982) partially purified a Ca-stimulated LOX from soybean seeds, having an pI of pH 5.9, by electrofocusing on flat beds of Sephadex G-75 with ampholytes of pH 3 to 11. Christopher *et*

al. (1972) purified three isoenzymes from soybean LOX with the use of an LKB column and LKB ampholytes of the pH range 3-10 at a final concentration of 1 %.

The isoelectric point of the LOX of *F. oxysporum* (Matsuda *et al.*, 1976) was estimated to be 9.46 by IEF electrophoresis. Su *et al.* (1995) reported that active fractions of 8*R*-dioxygenase from the fungus *G. graminis* were subjected to IEF with 1% ampholyte (3:10 or 5:8) and 20 % ethylene glycol, using a preparative IEF cell (Rotofor; Biorad). The apparent pI of 8*R*-dioxygenase was between 6.1 to 6.3, however, IEF led to a major loss of enzyme activity so that specific activity was not determined. For looking at unpurified mixtures containing more than one isozyme,

2.6.6. Chromatofocusing

Chromatofocusing is a relatively new technique that, like ion-exchange chromatography, separates macromolecules on the basis of their net ionic charge (Soderberg *et al.*, 1982). The sample is applied to the resin at high pH, followed by a buffer of lower pH containing various amphoteric components with a range of pKa values used to titrate both the absorbent and bound sample. The former results in a linear, descending pH gradient. As this pH gradient develops, the proteins at the rear of the sample zone are the first to be titrated to the same charge as the support and consequently desorbed from the support allowing them to migrate to the front of the sample zone where they are titrated back to the charge opposite that of the absorbent and reabsorb to it. Proteins comprising a crude mixture can be separated based upon their isoelectric point with those possessing isoelectric points near the initial column pH eluting first (Lillehoj and Malik, 1989).

Feiters *et al.* (1986) demonstrated the heterogeneity of soybean LOX-2 by chromatofocusing. Four major protein peaks with LOX activity at pH 6.6, were found when the pH range used was 5.0-7.0. The peak with the lowest pI was identical to the classical LOX-1. The other peaks belonged to type-2 LOX and were further characterized by isoelectric focusing.

Funk *et al.* (1985) reported that the separation of soybean LOX-2 and –3, whose isoelectric points are 6.25 and 6.15, respectively, was carried out by chromatofocusing using a column (10 x 500 mm) of PBE 94 (Pharmacia); the column was equilibrated with imidazole hydrochloride buffer (0.025 M, pH 7.4) and the proteins were eluted with Polybuffer 74 (Pharmacia (1:10 dilution,pH 5.0).

2.6.7. Reverse phase chromatography

Reverse phase chromatography employs a stationary silica phase bonded with nonpolar functional groups such as alkyl (butyl, C-4; octyl, C-8; octadecyl, C-18) or aromatic phenyl groups and a polar mobile phase such as acetonitrile, methanol, isopropanol or tetrahydrofuran. However, prediction of elution order is often limited by the secondary and tertiary structures that may prevent some hydrophobic amino acids from interacting with the support; although the native protein structure may be lost using standard organic solvents and acidic conditions of the mobile phase and hydrocarbon bonding in the stationary phase thus destroying biological activity, post-column conditions of lyophilization or dialysis, in many cases, allow some degree of renaturation to occur (Lillehoj and Malik, 1989).

Ramadoss and Axelrod (1982) reported that high performance chromatography was performed using a SynChropak AX-300 column (0.41 x 25 cm) (SynChrom) for the separation of soybean LOX-2 and -3 which could not otherwise be separated based on their charge as their pIs of 6.25 and 6.15 were so close.

2.6.8. Hydrophobic chromatography

Hydrophobic chromatography, like reverse phase chromatography, relies upon hydrophobic interactions between the protein sample and the absorbent. However, unlike reversed phase supports, the bonded phases contain lower ligand densities and shorter ligand lengths (e.g. phenyl and butyl groups). Consequently, surface hydrophobicity is reduced allowing bound proteins to be eluted under mild conditions, i.e. neutral pH and high ionic strength. Proteins initially bind to the support in the presence of high concentrations of salt such as 2 to 4 M ammonium sulfate. During a gradient of decreasing salt concentration, the

bound proteins will then be differentially desorbed from the absorbent accordingly to their increasing hydrophobicities. Due to the less disruptive elution conditions, hydrophobic chromatography is more effective in retaining enzymatic activity than reverse-phase chromatography (Lillehoj and Malik, 1989).

Olías and Valle (1988) purified LOX from lupin seed using a Phenyl Sepharose CL-4B column, resulting in a 24.7-fold increase in specific activity and a recovery of 73.6 % with respect to the crude extract.

2.6.9. Affinity chromatography

Affinity chromatography is founded on the principle of constituents being absorbed to and subsequently eluted from a solid support; the initial binding of the constituent to a conformationally complementary surface, i.e. an enzyme to its substrate, confers a level of specificity unparalleled by other methods so that the enzyme alone is eluted off the column (Lillehoj and Malik, 1989).

Soybean LOX-1 was separated from LOX-2 on a column containing polyclonal antibodies directed against LOX-1 covalently coupled to Sepharose 4B (Vernooy-Gerritsen *et al.*, 1982). Andrawis *et al.* (1982) isolated LOX-2 from soybean by affinity chromatography. LOX from avocado (Marcus *et al.*, 1988) was purified to near homogeneity by affinity chromatography.

Shechter and Grossman (1983) identified two LOXs located in the mitochondrial fraction of baker's yeast by affinity chromatography on linoleyl aminoethyl sepharose. A LOX extract from the thermophilic actinomycete, *T. vulgaris* (Iny *et al.*, 1993b) was also purified using a linoleyl aminoethyl sepharose column (2 x 10 cm); two fractions with similar recoveries were obtained but the purification factor was 3-fold higher in the fraction eluted with sodium borate as opposed to sodium acetate.

2.6.10. Electrophoresis

Electrophoresis is essentially based on the migration of molecules by their respective charge and the size through a support matrix such as paper, cellulose acetate, starch, agarose or polyacrylamide gel. The rate of migration of charged molecules in solution depends on the strength of the externally applied electric field, the net charge, size and shape of the molecules as well as the ionic strength, viscosity and temperature of the matrix. Polyacrylamide is formed by the polymerization of acrylamide monomers that are covalently crosslinked and has become a matrix of choice for resolving protein mixtures on a analytical scale; proteins can be visualized by staining with Coomassie blue which is sensitive to about 1 μg of protein or with silver or nickel (Giulian *et al.*, 1983; Yudelson, 1983) which provides sensitivity to approximately 10 ng of protein (Lillehoj and Malik, 1989).

Sodium sodecyl sulfate (SDS) is an anionic detergent that binds to proteins in a manner that overwhelms their individual charge and induces a conformational change to rod-like structures; all proteins therefore possess an equal charge density per unit length such that electrophoretic migration is determined by molecular weight. As an analytical tool, SDS-polyacrylamide gel electrophoresis (PAGE) offers a very high degree of resolution necessary to follow a purification scheme. Identification of the molecular weight of impurities that may contaminate the protein of interest can allow one to choose an appropriate chromatographic technique to achieve a greater degree of purity. However, it cannot be used as a preparative technique for the recovery of proteins due to the presence of SDS (Lillehoj and Malik, 1989).

The LOX extract from dried split pea seeds (Klein, 1976) was separated into one major and two trace bands using polyacrylamide gel electrophoresis while that from pea seed (Haydar and Hadziyev, 1973) was separated into three to four LOX bands. The gel patterns showed two to three bands in the pea seeds extract, four bands in the wheat extract (Hale *et al.*, 1969) and one protein band for soybean LOX-2 from soybean (Andrawis *et al.*, 1982). Germinating sunflower seed LOX (Leoni *et al.*, 1985) was separated on SDS-PAGE having a molecular weight of 250 kDa while the three LOX isozymes from wheat germ (Shiiba *et al.*, 1991) each exhibited one single band of molecular weight of 100 kDa.

Matsuda *et al.* (1976) reported that the purified *F. oxysporum* LOX formed only one band on PAGE at pH 4.3, regardless of the concentration of acrylamide, and in the presence of SDS. Two mitochondrial LOX fractions from *S. cerevisiae* (Shechter and Grossman, 1983) indicated the presence of three bands, a major one and two weak ones, on PAGE.

In addition to traditional non-specific staining techniques, LOX isozymes may be located in the gel using specific chromogenic staining. One method involves the separation of the LOX isozymes on a polyacrylamide gel which contains acidic potassium iodide and starch. The gel is then allowed to react with linoleic acid and the HPODEs produced oxidize the iodide to its elementary form. The oxidized iodide then reacts with starch at the active enzymatic fractions which results in a chromogenic reaction of blue and brown bands (Guss *et al.*, 1968a,b). The main disadvantages of this method are the several hours needed for the determination and the danger of oxidation of the iodine by oxygen in the air. Guss *et al.* (1967) reported, on the basis of a chromogenic disc electrophoresis reaction, that there are three to four LOXs existing in a soybean extract.

Another chromogenic reaction is based on the conversion of $Fe(CNS)_2$ to the colored $Fe(CNS)_3$ by HPODEs formed during the LOX reaction (Koch *et al.*, 1958). The cellulose acetate electropherogram containing the separated isozymes is incubated in a linoleic acid solution, then dipped in ferrous ammonium sulfate and ammonium thiocyanate; reddish brown bands of $Fe(CNS)_3$ indicate the location of the isozymes. This method can be performed in half an hour and there is no danger in the color development by oxygen in the air. However, the method offers less sensitive separation of proteins on cellulose acetate gel compared to polyacrylamide gel and the color is stable only for a few minutes. The presence of several isozymes in potato tubers (Pinsky *et al.*, 1973) was detected using this method. In addition, the HPODEs produced by the LOX activity in the *P. bispora* extract were also detected by this method (Wurzenburger and Grosch, 1984a).

The third method is based on the oxidation of the dye 3,3'-dimethoxybenzidine hydrochloride by the HPODEs of the LOX-catalyzed reaction. The electrophoretic gels,

containing the LOX isozymes, are reacted with linoleic acid (30 min) and then stained overnight with the dye. The gels have the advantage of a white background and can be stored for eighteen months without fading. DeLumen and Kazeniac (1976) reported the detection of the LOX isozymes of tomatoes and other fruits by this method.

2.7. Separation and Characterization of Products

The extraction of HPODEs, produced by the LOX-catalyzed reaction, from an aqueous environment by the use of organic solvents is an old but widely used procedure due to its simplicity and efficacy. The recovery rate of end-products is improved by acidification of the water phase; the extracts obtained by this manner are free from salts, proteins and carbohydrates but have to be purified from other lipids. The use of immiscible solvents such as ethyl ether or ethyl acetate have been used to selectively extract HPODEs (Bedetti and Cantafora, 1987).

2.7.1. Thin-layer chromatography

Thin-layer chromatography (TLC) is used for both analytical and preparative applications because of its simplicity and inexpensiveness. The commercial plates are coated on glass, aluminum foil or plastic; plates with a fluorescent indicator can be used to make UV absorbing compounds such as HPODEs visible as opposed to using destructive agents for detection. Large plates (20 x 20 cm) are developed in rectangular tanks containing 200 ml of solvent (Bedetti and Cantafora, 1987).

The HPODEs were separated using various organic solvents at different ratio of concentration; these include ethyl acetate/heptane (4:6), ethyl acetate/hexane (1:9 or 1:4 or 2:8, v/v), diethyl ether/heptane (1:1, v/v), toluene/dioxane/acetic acid/formic acid (82:14:1:1, v/v/v/v), isooctane/ether/acetic acid (50:50:1 or 60:40:0, v/v/v), petroleum ether/diethyl ether/acetic acid (60:40:1 or 50:100:1, v/v/v).

Precoated Kieselgel 60 F_{254} (0.25 mm) or silica gel 60A (0.25 mm) plates were used to separate the 8-HODE, 8-HPODE, 8,16-DiHODE, 8,17-DiHODE, and 9,10-Di-HOME

produced by the 8*R*-dioxygenase extract of *L. arvalis* (Brodowsky and Oliw, 1993); the latter was also used for the separation of 8-HPODE and 7,8-DiHODE, produced by the 8*R*-dioxygenase and HPI of *G. graminis* (Su *et al.*, 1995). Precoated Kieselgel 60 plates (0.25mm thick) were also used for the separation of the methyl 9-, 11-, and 13-HODEs from the methyl ester of 11-keto-9,12-octadecadienoic acid, produced by the incubation of linoleic acid with an enzyme preparation from *L. corallioides* (Hamberg *et al.*, 1992); however, the separation of 11-HODE from the 9- and 13-HOPEs was performed on Silica gel G/$AgNO_3$ (9:1, w/w) and that of 9-HOTE from 13-HOTE on plain silica gel.

The untreated or methylated 9- and 13-HPODEs, produced by the LOX activity in the *F. oxysporum* extract (Matsuda *et al.*, 1978) were separated using silica gel $60F_{254}$ plate; separation of the respective former 9- and 13-hydroxystearates was carried out by preparative TLC using Silica gel G plate. The 13- and 9-HPODEs, produced by the *C. pyrenoidosa* LOX extract (Zimmerman and Vick, 1973), were converted to the corresponding methyl hydroxystearates and analyzed on Adsorbosil-5-silica gel.

The 9- and 13-HPODEs produced by the LOX from *S. cerevisiae* (Shechter and Grossman, 1983) and *T. vulgaris* (Iny *et al.*,1993a) were analyzed using silica gel (0.2 mm thick). The 10-HPOD, produced by the LOX mushroom extract (Wurzenburger and Grosch, 1984a,b), was purified using silica gel (500 μm) containing 1 % oxalic acid.

The 10-HPODEs produced by the LOX activity from mushroom extract (Wurzenburger and Grosch, 1984a,b) and the 9- and 13-HPODEs produced by the LOX extract of *F. oxysporum* (Matsuda *et al.*, 1976) were located by spraying a small strip at each side of the plate with solutions of KI and starch (Satoh *et al.*, 1976)

The 8-HPODEs produced by the 8*R*-dioxygenase extract of *L. arvalis* (Brodowsky and Oliw, 1993) were also detected by spraying with 3.5 % phosphomolybdic acid (Merck) and charring while the 9-and 13-HPODEs produced by the *F. oxysporum* (Matsuda *et al.*, 1978) and the *C. pyrenoidosa* (Zimmerman and Vick, 1973) LOX extract were detected

under ultraviolet light or by spraying 0.1 % *N,N*-dimethyl-*p*-phenylenediamine in chloroform/acetic acid/water (5:5:1, v/v/v) (Vioque and Holman, 1962; Zimmerman, 1966),

The detection could also be performed by scanning the plates for radioactivity if the enzyme incubations were performed using radioactive substrates such as [^{14}C]linoleic acid; the HPODEs and diHODEs resulting from the LOX and HPI activity from *L. arvalis* (Brodowsky and Oliw, 1993) and *G. graminis* (Su *et al.*, 1995) were detected in this manner.

2.7.2. Gas-liquid chromatography

Gas-liquid chromatography (GLC) is used to separate mixtures of compounds which exhibit appreciable vapor pressures (volatilities). The GLC analysis of lipids involves the introduction of a small volume of a dilute solution (microgram to nanogram per microlitre, dissolved in a suitable organic solvent) into the column via a gas-tight injection port where the sample is volatilized into the inert carrier gas. Separation of the mixtures of volatile and semi-volatile lipids is performed according to their different vapor pressures, i.e. those compounds exhibiting the highest vapor pressures will spend a relatively longer time in the mobile phase and therefore pass through the column more rapidly whereas those compounds which possess a lower vapor pressure, due to their higher boiling points or interaction with the stationary phase, will progress through the column more slowly (Pomeranz and Meloan, 1994).

Wall-coated open tubular (WCOT) capillary columns are the most commonly used columns presently; flexible-fused silica capillary column coated externally with either polyimide or aluminum and internally with a polymeric stationary phase (0.1 μm to 10 μm film thickness, are employed. The advantages of the capillary column includes a high column efficiency, hence high resolving power, enhanced sensitivity due to improved signal/noise ratios as a result of narrow peak widths and compatibility with mass spectrophotometers due to the low flow rates used; disadvantages include long analysis times, low sample capacities, and being more expensive and fragile than packed columns (Pomeranz and Meloan, 1994).

Packed columns (typically 1 to 3 m x 4 mm i.d.) are usually comprised of fine particles of washed and deactivated earth (support), coated with polymeric stationary phase. These

columns are of robust nature, cheap and readily re-packed when chromatographic performance deteriorates, high sample capacity, analysis times less than 30 min; disadvantages include relatively low efficiencies and therefore limited separation power, and difficulty in interfacing with mass spectrometers (Pomeranz and Meloan, 1994).

The hydroperoxy and hydroxy derivatives of linoleic acid produced by LOX preparations from *F. oxysporum* (Matsuda *et al.*, 1978), *G. graminis* (Brodowsky and Oliw, 1992), *P. bispora* (Wurzenburger and Grosch, 1984a,b) and *Oscillatoria* sp (Beneytout *et al.*, 1989) were converted to the corresponding Me_3Si ether methyl ester derivatives before being analyzed by GLC. The columns used for separation included a stainless steel column (3 mm x 1m) packed with 3 % EGSS-X on Nbeopack 1A (Matsuda *et al.*, 1978), or a capillary GC column (30-m DB-5, J & WW Scientific, film thickness 0.25 μm, inner diameter 0.25-mm) (Brodowsky and Oliw, 1992, 1993).

The eight-carbon compounds, such as oct-1-en-3-one and 1-octen-3-ol, produced by the enzymatic extract of *A. bisporus* were analyzed using a Carbowax 20 M open-tubular stainless steel column (Cronin and Ward, 1971; Picardi and Issenberg, 1973; Kaminski *et al.*, 1974)

Alcohol and carbonyl compounds as well as the less volatile oxo and hydroxy acids were isolated from the *A. campestris* extract (Tressl *et al.*, 1982); the latter were subsequently methylated and the extracts were purified by preparative gas-liquid chromatography using a 3 m x 4 mm i.d. glass column packed with 60-mesh Chromosorb WAW coated with 15 % FFAP and identified on a 50 m glass capillary column (0.32 mm i.d.) coated with Carbowax 20M.

A Carbowax 20 M column (25 m x 0.25 mm i.d.) was also used to analyze pentanol and the methylated, reduced and TMS-derivative of 13-oxotrideca-9,11-dienoic acid produced from 13-HPODEs by the enzymatic extract from *Oscillatoria* sp (Andrianarison *et al.*, 1989).

1,5-Octadien-3-ol, 2,5-octadien-1-ol (Wurzenburger and Grosch, 1986) and 1-octen-3-ol and the methyl ester of 10-oxo-8-decenoic acid (Wurzenburger and Grosch, 1982, 1984a,d) were analyzed using the FFAP column (3 m x 2 mm stainless steel column packed

with 10 % FFAP on Chromosorb W, 80-100 mesh); 1,5-octadien-3-ol, 2,5-octadien-1-ol and 10-oxo-8-decenoic acid were identified using a 30 m x 0.3 mm glass capillary coated with SE 54 whereas 1-octen-3-ol was analyzed on a stainless steel column (3 m x 3.2 mm) of 15 % diethyleneglycol succinate on Chromosorb WAW (100-120 mesh). 1-octen-3-ol and methylated 10-oxo-8-decenoic acid (Wurzenburger and Grosch, 1984a) were also analyzed using a 25 m fused silica capillary column (CP Sil 5 B; Chrompak, Middelburg, The Netherlands).

2.7.3. Gas-liquid chromatography/mass spectroscopy

Gas-liquid chromatography is used to separate complex mixtures quantitatively into individual unidentified components while the mass spectrometer is excellent in the identification of these compounds. Mass spectroscopy produces ions by bombarding organic molecules with high energy electrons, then accelerating these ions in a definite direction so that they can be separated according to their mass/charge ratio. The separated ions are then detected and their relative intensity measured.

The mass spectrometer has three major components: an ion source, an analyzer, and a detector. The ion source emits high-energy electrons which strike sample molecules thereby producing positive ions (+), negative ions (-), electrons (e^-) and neutral molecules. The positive ions are separated from the mixture and subsequently separated from each other with respect to their different mass-to-charge (m/z) ratios by a magnetic field in the analyzer. The sample ions are detected by an electrode onto which they fall. Connected in series between this electrode and ground is a resistor. Electrons from ground rush to neutralize the positive charge on the collector. Current across the resistor causes a potential drop that is proportional to the current flow, so that by measuring the voltage the number of ions of each type can be determined (Pomeranz and Meloan, 1994).

Iny *et al.* (1983a) reported the presence of the 9- and 13- methylated, reduced, hydrogenated and silylated, i.e. methyl trimethylsiloxy (MTMS), derivatives of HPODEs by using a Finnigan 4021 mass spectrometer, by electron impact-mass spectral analyses. The

detection of 9- and 13-MTMS produced by a LOX preparation from *F. oxysporum* was also reported by using a Hitachi mass spectrometer model RMU-6L (Matsuda *et al.*, 1978).The mass spectra of the 9- and 13-MTMSs is indicated by the characteristic fragmentation patterns resulting from α-cleavage of both sides of the carbon atom to which the trimethylsiloxy group is attached; the results show the presence of the 9-MTMS as indicated by the m/e fragments at 230 and 260 and that of the 13-MTMS as exhibited by the strong signals at 174 and 316 (Beneytout *et al.*, 1989).

The 8-MTMS, produced by the LOX activity from *L. arvalis* (Brodowsky and Oliw, 1993) and *G. graminis* (Brodowsky *et al.*, 1992; Su *et al.*, 1995) was detected, using an ion trap mass spectrometer (ITS40, Finnigan MAT), by the strong signals of the respective 8-MTMS at m/e 243 and 245. Moreover, the presence of a 12-MTMS as shown by the m/e fragments of 188 and 302 (Wurzenburger and Grosch, 1984a) and that of a 10-MTMS as demonstrated by the strong m/e signals at 216 and 274 was detected using a mass spectrometer (MS 112, Fa. Varian).

2.7.4. High-performance liquid chromatography

High-performance liquid chromatography (HPLC) has been used extensively for the separation and purification of HPODEs. This method requires expensive equipment; however, it also allows the separation and the purification of closely related compounds with a degree of efficiency, selectivity and reproducibility not attainable by means of other techniques. Only two types of stationary phases are widely used; silica gel for the straight-phase (SP) HPLC stationary phase and the chemically bonded ODS or C18 stationary phase for reverse-phase (RP) HPLC. Both SP-HPLC and RP-HPLC may be used to achieve good purification of HPODEs which are molecules possessing both lipophilic chains and hydrophilic functional groups such as carboxyl, carbonyl and hydroxyl groups. Hence, the combination of SP and RP-HPLC in sequence provides an excellent method for achieving purification to the high degree required for mass spectrum analysis. The detection of HPODEs can be obtained by continuous monitoring of the UV spectrum. The structural identity of LOX products can often

be determined by their retention times as well as their complete UV spectrum profile in comparison with authentic standards. (Bedetti and Cantafora, 1987).

The 8*R*-HPODE and 7*S*,8*S*-DiHODE, produced by the 8*R*-dioxygenase and HPI of *G. graminis* (Su *et al.*, 1995), respectively, were analyzed using the 5-μm Nucleosil-C_{18}, (150 x 4.6 mm) RP-HPLC column with a solvent system of methanol/water/acetic acid (75:25:0.01, v/v/v) (Brodowsky and Oliw, 1992). A crude preparation of *G. graminis* (Brodowsky and Oliw, 1992) produced 8-HPODE, 8-HPOTE, and 18-hydroxyeicosatetraenoic acid (HETE), 19-HETE and traces of 17-HETE; these hydroxy metabolites were separated as methyl esters on SP-HPLC using 0.5 % 2-propanol in hexane (Oliw, 1989).

The 10-HPODE, produced by the mushroom LOX extract (Wurzenburger and Grosch, 1984a), was further purified using a stainless-steel column (0.9 x 25 cm) packed with LiChrosorb using a solvent mixture of hexane/2-propanol/acetic acid (990:10:1, v/v/v). The methyl ester of 10-oxo-8-decenoic acid (Wurzenburger and Grosch, 1982), produced from the cleavage of 10-HPODE by the HPL activity in the mushroom extract, was analyzed using a stainless steel column (25 cm x 4.6 mm) packed with LiChrosorb Si 60, 5μm (Merck) and a mobile phase of 0.35 % ethanol in hexane. The 13-oxotrideca-9,11-dienoic acid produced by the enzymatic cleavage of the 13-HPODE by the HPL activity from *Oscillatoria* sp (Andrianarison *et al.*, 1989) was separated using a μPorasil (10μm) Waters column (3.9 x 30 cm) and eluted with hexane/ethanol/acetic acid (98:1.9:0.1, v/v/v).

The methyl esters of the 9- and 13-HPODE, produced by the *F. oxysporum* LOX extract (Matsuda *et al.*, 1978), were separated using a stainless steel column (4.5 x 500 mm) packed with Partisil-10 with 0.5 % anhydrous ethanol in dried hexane (Chan and Prescott, 1975). The 13- and 9-HPODEs, obtained by the LOX preparation from *Oscillatoria* sp. (Beneytout *et al.*, 1989) were reduced and applied to SP-HPLC using a μPorasil (10 μm) Waters column (3.9 x 30cm) and eluted with hexane/ethanol/acetic acid (98:1.9:0.1, v/v).

A purified LOX preparation from *T. vulgaris* (Iny *et al.*, 1993a) oxidized linoleic acid into 13- and 9-HPODEs; the HPODEs were subsequently left untreated or reduced and

methylated, and analyzed by SP-HPLC; a Lichrosorb Si-60 column (Merck, 0.4 x 25 cm, 10μm) was used and a *n*-hexane:isopropanol:acetic acid solvent system for underived HPODEs (98.7:1.2:0.1, v/v/v) and methylated HPODEs (99.4:0.6:0.0, v/v/v).

2.7.5. Capillary electrophoresis

Capillary electrophoresis (CE) is typically performed using fused silica capillaries that are 30-100 cm long, 50 or 75 μm i.d., and 375 μm o.d. and with voltages of up to 30 kV. These capillaries provide very fast, high-efficiency separations and have an external coating of polyimide which makes them very strong. Electrophoresis is performed by filling the source vial, capillary, and destination vial with an electrolyte, which is usually an aqueous buffer solution. The capillary inlet is placed into a sample vial, the sample is introduced, then the capillary inlet is placed back into the source vial, and an electric field is applied between the source and destination vials (Baker, 1995).

In comparison to HPLC and GC, CE has very high efficiency; for typical capillary dimensions of 30-100 cm long and 50-100 μm i.d., hundreds of thousands of theoretical plates are common in CE compared to about 150,000 theoretical plates for a typical GC column, and at best, 20,000 theoretical plates for a typical HPLC column. In addition, advantages include short separation times due to the use of high voltages and short capillaries, small sample volumes and small quantities of reagents. Moreover, aqueous media is used so that aqueous samples may be directly injected and ambient temperature is employed thereby minimizing sample decomposition or denaturation (Baker, 1995). Volumes of the order of 2-10 nl can be injected into a capillary column with good reproducibility; thus precious material is not wasted. Although the mass limit can be very low because of the small volume, the concentration limit for UV detection is usually in the order of 10^{-6} M, which is several orders higher than the UV detection limits for HPLC. Laser-induced fluorescence also allows the detection of concentrations in the order of 10^{-9} M; however, most analytes do not fluoresce.

In chiral free solution capillary electrophoresis (FSCE), optically active reagents, such as cyclodextrins (CDs), proteins, carbohydrates, and crown ethers, are added to the

electrolyte in order to achieve enantioselective interactions with the solute molecules. The native CDs, α, β, γ, possess different numbers of glucose subunits, being six, seven, and eight, respectively. Enantioselective recognition is often explained by interaction between the CD and the guest enantiomers. By derivatization of the surface hydroxyl groups on the cyclodextrin, the solubility and enantioselective properties can be significantly altered. Hydroxypropyl- and dimethyl-cyclodextrins are two such derivatives, which when compared to the native forms, differ significantly (Rogan and Kevin, 1996a,b).

Schmitz and Gäb (1997) reported that capillary electrophoresis was used for the separation of several unsaturated fatty acids and the isomeric hydroperoxides derived from them. An untreated fused-silica capillary (L = 40/47 cm, i.d. 50 μm) purchased from Beckman was used for separations. Uncharged polyoxyethylene lauryl ether (Brij 35) was used as a surfactant and for the dynamic coating. A mixed micelle of Brij 35 and SDS made it possible to separate oleic, linoleic and linolenic acids and nearly all the isomeric HPODEs of these fatty acids in a single run of 15 minutes

CHAPTER III

PARTIAL PURIFICATION AND CHARACTERIZATION OF LIPOXYGENASE EXTRACTS FROM *FUSARIUM OXYSPORUM*, *FUSARIUM PROLIFERATUM*, *SACCHAROMYCES CEREVISIAE* AND *CHLORELLA PYRENOIDOSA*

3.1. Abstract

Crude lipoxygenases, obtained from the biomass cultures of *Fusarium oxysporum*, *Fusarium proliferatum*, *Saccharomyces cerevisiae* and *Chlorella pyrenoidosa* were partially purified by precipitation with ammonium sulfate at 20-80, 0-40, 20-80 and 40-80 % of saturation, respectively. The enzymatic extracts from *F. oxysporum* and *S. cerevisiae* both exhibited two optimal activities at pH 8.0 and 10.0 while those from *F. proliferatum* and *C. pyrenoidosa* showed one optimal pH at 6.0 and 4.5, respectively. The apparent K_m values, for the lipoxygenase (LOX) extract from *F. oxysporum*, at pH 8.0 and 10.0 were calculated to be 3.28×10^{-5} and 3.55×10^{-5} M, respectively, whereas those for the *S. cerevisiae* extract were 1.3×10^{-5} and 9.5×10^{-6} M, respectively. In addition, the LOX activity of *F. proliferatum* and *C. pyrenoidosa* exhibited K_m values of 5.15×10^{-5} and 9.12×10^{-5} M, respectively. The results showed that, at pH 8.0, LOX activity from *F. oxysporum* and *S. cerevisiae* was inhibited by 31 and 49 %, respectively, at corresponding concentrations of 20 and 25 mM potassium cyanide (KCN), exhibiting a non-competitive inhibitory effect; however, at pH 10.0, KCN had relatively little effect on enzyme activity. In addition, KCN markedly inhibited LOX activity from *C. pyrenoidosa* by 58.2 % at 0.5 mM concentration and was an uncompetitive inhibitor. In contrast, the results showed that enzyme activity from *F. proliferatum* remained relatively stable at KCN concentrations as high as 60 mM. The addition of 5 mM sodium ethylendiaminetetraacetate (EDTA) was found to increase the enzyme activity from *F. oxysporum* by 50.3 and 16.6 % at pH 8.0 and 10.0, respectively, from *F. proliferatum* by 50 %, and produced a noticeable eight-fold increase in the enzymatic activity from *C. pyrenoidosa*; however, LOX activity for the *S. cerevisiae* extract remained unaffected at both pH optimas. In addition, the use of 1.2 mM hydroquinone (HQ) resulted in a 2-fold increase in

LOX activity from *F. proliferatum* whereas a competitive inhibitory effect on LOX activity from *S. cerevisiae* was observed at pH 8.0 at 0.5 mM HQ. The results also showed that the enzymes from *F. oxysporum* and *S. cerevisiae* demonstrated an overall preference towards linoleic acid, followed by linolenic acid, at both pH 8.0 and 10.0; in addition, the enzymatic extracts showed higher activity towards the glycerol fatty acid esters at pH 10.0 compared to that at pH 8.0. The partially purified enzyme from *F. proliferatum* showed a three-fold substrate preference towards linoleic acid whereas that from *C. pyrenoidosa* demonstrated a preference for free linoleic acid and comparatively very little towards its fatty acid esters and free linolenic acid. Native polyacrylamide gel electropheorograms (PAGE) indicated the presence of one major and five minor bands of molecular weights of 67 to 140 kDa for the partially purified extract from *F. oxysporum* while that from *S.cerevisiae* showed two major bands. In addition, PAGE indicated the presence of one major band at 140 kDa and two minor bands at 67 and 232 kDa for the LOX extract from *F. proliferatum* whereas that from *C. pyrenoidosa* exhibited the presence of five bands varying between 67 and 140 kDa.

3.2. Introduction

Lipoxygenase (EC 1.13.11.12) is a dioxygenase which catalyzes, as an initial reaction, the regio- and stereo-specific addition of molecular oxygen to linoleic acid and other polyunsaturated fatty acids (PUFAs) and their esters containing a *cis*, *cis*-1,4-pentadiene moiety, resulting in the formation of hydroperoxides (Yamamoto, 1991). In cellular systems, the two most abundant unsaturated fatty acids are linoleic acid and arachidonic acid; linoleic acid can be hydroxylated in positions 9 or 13 resulting in corresponding 9- and 13-hydroxyoctadecadienoic acids, whereas arachidonic acid may be converted to six regio-isomeric products resulting in 5-, 8-, 9-, 11-, 12-, and 15-hydroxyeicosatetraenoic acids (Yamamoto, 1991).

The occurrence of lipoxygenase (LOX) was originally thought to be confined to the seeds of leguminous plants (Tappel, 1963) and among higher plants (Grosch, 1972; Hildebrand, 1989). The presence of the enzyme has also been reported in animals (Borgeat and Samuelsson, 1979; Hsieh and Kinsella, 1989; Yamamoto, 1989; Yamamoto and Ishimura,

1991). However, limited literature has been reported for the presence of LOX in microorganisms (Siedow, 1991).

LOX activity was reported in the fungus *Gaeumannomyces graminis* (Brodowsky *et al.*, 1992) and *Saprolegnia parasitica* (Herman and Hamberg, 1987). The fungus *Pityrosporum orbiculare* (Nazzaro-Porro *et al.*, 1986) was reported to possess a LOX activity capable of oxidizing linoleic acid and trilinolein. Simmons *et al.* (1987) reported that the fungus *Lagenidium giganteum* exhibited LOX activity selectively for arachidonic, eicosapentaenoic and docosahexaenoic acids. The fungus *Laetisaria arvalis* (Brodowsky *et al.*, 1994) metabolized linoleic acid to 8-hydroperoxylinoleic acid (HPODE) and 8-hydroxylinoleic acid (HODE) as major metabolites. The enzyme was also present in mushrooms including *Psalliota campestris* and *Psalliota bispora* (Grosch and Wurzenburger, 1984a,b,c,d), *Agaricus* bisporus (Mau *et al.*, 1992), and *Agaricus campestris* (Tressl *et al.*, 1982). In mushrooms such as *Pleurotus pulmonarius* (Belinky *et al.*, 1994), LOX activity was found to be involved in the formation of eight-carbon components from linoleic and/or linolenic acids.

Microbial lipoxygenases have been reported in the green algae *Oscillatoria sp.* (Beneytout *et al.*, 1989) and the thermophilic bacteria *Thermoactinomyces vulgaris* (Iny *et al.*, 1993a). Jiang and Gerwick (1991) reported the presence of a 12-LOX activity in a red algae *Gracilariopsis lemaneiformis* preparation responsible for the conversion of arachidonic acid to its corresponding 12-HPODE. A partially purified enzyme preparation from the red algae *Lithothamnion corallioides* was reported by Hamberg *et al.* (1992) which converted linoleic acid into 11-HODE as well as smaller amounts of 9-HODE, 13-HODE and 11-keto-ODE. LOX activity was reported in the pink yeast *Rhodotorula glutinis* (Collins and Buick, 1989), the wine yeast *Saccharomyces vini* (Lyudnikova *et al.*, 1984), and Baker's yeast otherwise known as *Saccharomyces cerevisiae* (Shechter and Grossman, 1983).

LOX is particularly relevant to the taste and flavor of food since the production of certain flavor precursors depends on the initial bioconversion of the PUFAs by LOX to corresponding optically active HPODEs which in turn are enzymatically converted by

subsequent enzymes, such as hydroperoxide isomerase and hydroperoxide lyase, into corresponding carbonyl compounds and alcohols (Gardner, 1989), responsible for the organoleptic quality of food (Sessa, 1979). In a single source, several LOX isoenzymes can exist and differ from each other by producing, from linoleic acid, different ratios of optically active derivatives of HPODEs (Gardner, 1991). The various ratios of HPODE derivatives contribute to the formation of different carbonyls and alcohol compounds responsible for desirable fresh-vegetable flavors. The enzymatic formation of HPODEs from PUFAs is a relatively well defined process; however, the bio-degradation of HPODEs to ketols and carbonyl compounds does not appear consistent from one biological system to another. Consequently, the production of certain flavor compounds depends on the initial conversion of PUFAs by LOX.

The specific objective of this work was to develop a procedure for the extraction and partial purification of LOX from *Fusarium oxysporum*, *Fusarium proliferatum*, *Saccharomyces cerevisiae* and *Chlorella pyrenoidosa*, and to characterize the enzyme extracts in terms of optimum pH, kinetic parameters, substrate specificity towards linoleic and linolenic acids as well as mono-, di- and trilinolein, enzyme inhibition and activation, and native electrophoretic profile.

3.3. Materials and Methods

3.3.1. Culture growth and harvesting conditions

The production of the biomass culture of *F. oxysporum* (MT-81-1) was performed in accordance to the procedure described by Satoh *et al.* (1976). The fungus was cultured under restricted aerobic conditions in a 5 liter conical flask charged with 3 liters of the basal medium (pH 8.0) containing 0.1 % $NaNO_3$, 0.1 % KH_2PO_4, 0.01 % $MgSO_4$ $7H_2O$, 0.1 % soybean flour and 90 ml soybean oil, with agitation of 150 rpm at 26.5°C for four days.

The biomass culture of *F. proliferatum* was grown for three days on 2 liters of modified Shoun medium, consisting of a mixture of 2 g $NaNO_3$, 0.2 g $MgSO_4$ $7H_2O$, 2 g soya

flour, 1 ml mineral salt solution, 0.5 g yeast extract, 1 g $(NH_4)_2HPO_4$, and 125 ml soya oil, at 27°C and 85 rpm (Shoun *et al.*, 1983).

Fleischmann Baker's yeast (*S. cerevisiae*) was obtained from the local supermarket and used throughout the study.

The biomass culture of *C. pyrenoidosa* (strain 211-8B) was grown and harvested according to the procedure described by Zimmerman and Vick (1973). The algal cells were grown in the light (10,000 lux) at 25°C in modified Arnon's medium (Budd *et al.*, 1969) which consisted of, per liter, 1 g KNO_3, 0.25 g $MgSO_4$ $7H_2O$, 0.03 g K_2HPO_4, 0.25 g KH_2PO_4 and micronutrients in the form of inner complex salts of ethylenediaminetetraacetate; the micronutrients were 5 μg/ml Fe and 1 μg/ml Co, Cu, Zn and Mn. The basal medium was also supplemented with 1 % glucose or galactose and the pH was adjusted to 5.85. The cells were harvested during the log phase of growth.

3.3.2. Preparation of crude extract

The microbial cultures of *F. oxysporum*, *F. proliferatum*, *S. cerevisiae* and *C. pyrenoidosa* were subsequently separated from the growth media and washed with deionized water and sodium phosphate buffer solution (0.01 M, pH 7.0). The mycelia of *F. oxysporum* were homogenized (1:4, w/v) in sodium phosphate buffer solution (0.01 M, pH 7.0) for a total of a 6 min period using a MSK cell homogenizer (Braun, Melsungen, Germany) whereas the algal cells of *C. pyrenoidosa* were homogenized (1:60, w/v) for 7 min and the *S. cerevisiae* yeast cells (1:4, w/v) for 3 min. The temperature of the homogenization medium was maintained at 4°C by a flow of stream liquid CO_2. The homogenized suspension was centrifuged (12,000 x*g*, 15 min) and the resulting supernatant was lyophilized whereas the pellet was discarded.

The fungal mycelia of *F. proliferatum* were suspended in sodium phosphate buffer solution (0.01 M, pH 7.0) to produce a 25 % suspension. For homogenization, a mixer mill

type MM2 (Retsch) was used according to the procedure described previously by Schütte and Kula (1988). Fifty-four ml of the cell suspension and 108 ml of glass beads were added to a 180 ml grinding vessel made of Nylatron GS Nylon (Morin *et al.*, 1993). Subsequently, two vessels were fixed to the mixer mill operated at 100 % of its oscillatory frequency for a period of 2 min. The homogenized suspension was then diluted with sodium phosphate buffer solution (0.001 M, pH 7.0) and centrifuged (12,000 x*g*, 15 min). The resultant supernatant was lyophilized; the pellet was discarded.

3.3.3. Defatting of crude extracts

The lyophilized enzyme extract was successively defatted with cold (-30°C) acetone and diethyl ether (Kermasha and Metche, 1986) in order to remove the lipids thereby eliminating their interference in the proceeding steps of purification. The defatted enzyme extract was then suspended (1:10, w/v) in sodium phosphate buffer solution (0.01 M, pH 7.0) and subjected to mechanical stirring for 16 h. All the purification steps were performed at a temperature of 4°C unless stated otherwise. The resulting suspension was centrifuged (12,000 x*g*, 15 min) and the pellet was discarded whereas the supernatant was subjected to DNA precipitation treatment, using protamine sulfate (Badaracco *et al.*, 1983). The subsequent suspension was centrifuged (40,000 x*g*, 10 min) and the supernatant, considered to be the crude enzymatic extract, was subjected to further purification.

3.3.4. Partial purification of lipoxygenase

The partial purification of LOX was first initiated by the addition of solid ammonium sulfate at 20 % of saturation. The suspension was allowed to stand for 30 min and then centrifuged (12,000 x*g*, 15 min) to obtain the precipitate (0-20 %). The resulting supernatant was saturated with 40 % solid ammonium sulfate and the precipitate (20–40 %) was obtained after centrifugation (12,000 x*g*, 15 min). The subsequent supernatant was further saturated with 60 % solid ammonium sulfate and the precipitate (40-60 %) was obtained by centrifugation (12,000 x*g*, 15 min). The same procedure was repeated twice to obtain the 60-80 and 80-100 % fractions by the addition of solid ammonium sulfate at 80 and 100 % of

saturation, respectively. The precipitated enzyme fractions were resuspended in a minimum amount of sodium phosphate buffer solution (0.01 M, pH 7.0) and dialyzed against sodium phosphate buffer solution (0.001 M, pH 7.0) for a period of 12 h. The desalted enzymatic fractions were then lyophilized and subjected to kinetic studies and electrophoretic analyses.

3.3.5. Protein determination

The protein concentration of the enzymatic fractions was determined according to a modification of the Lowry method (Hartree, 1972). Bovine serum albumin (Sigma Chemical Co., St-Louis, MO) was used as a standard for calibration.

3.3.6. Substrate preparation

Substrate standards used throughout this study, included linoleic acid (*cis*-9,*cis*-12-octadecadienoic acid), linolenic acid (*cis*-9,*cis*-12,*cis*-15-octadecatrienoic acid), monolinolein (1-mono[(*cis*,*cis*)-9,12-octadecadienoyl]-rac-glycerol), dilinolein (1,3-di[(*cis*,*cis*)-9,12-octadecenoyl]-rac-glycerol), and trilinolein (1,2,3-tri[(*cis*,*cis*,*cis*)-9,12,15-octadecadienoyl]-rac-glycerol), and were purchased from Sigma Chemical Co. The preparation of stock solutions of substrates (4×10^{-3} M) was performed according to the procedure described previously (Kermasha and Metche, 1986).

3.3.7. Enzyme assay

LOX activity was measured spectrophotometrically in accordance to the procedure outlined by Kermasha and Metche (1986). The reaction medium consisted of enzyme extract (12.5 μg protein, *F. oxysporum*; 20 μg protein, *F. proliferatum*; 25 μg protein, *S. cerevisiae*; 25 μg protein, *C. pyrenoidosa*;) and a sufficient amount of a selected buffer solution to adjust the final volume to 3 ml. For determination of enzyme activity in the crude extract and the partially purified fractions, the buffer used was sodium phosphate (0.1 M, pH 7.0). For optimum pH studies for the partially purified fraction, the enzyme assay was performed using the following buffers: sodium acetate (0.1 M, pH 5.0-5.5), sodium phosphate (0.1 M, pH 6.0-8.0), Tris-HCl (0.1 M, pH 8.5-9.0), sodium carbonate (0.1 M, pH 9.5-10.5), phosphate

hydroxide (0.1 M, pH 11.0-11.5) and hydroxide-chloride (0.1 M, pH 12.0). The enzyme reaction was initiated by the addition of linoleic acid (40 μM to 600 μM). A control solution, containing all the components minus the enzyme preparation, was run in tandem with these trials. The reaction time of all enzyme assays consisted of 3 min. The LOX activity was measured on a Beckman DU-650 spectrophotometer (Beckman Instruments, Inc., San Ramon, CA). The specific activity of LOX was expressed as the increase in A (mg of protein)$^{-1}$ min^{-1} (Surrey, 1964), where A is equal to 0.001 absorbance at 234 nm (Shastry and Rao, 1975; Klein, 1976; McCurdy *et al.*, 1983; Ali Asbi *et al.*, 1989; Ganthavorn and Powers, 1989).

3.3.8. Effect of inhibitors and activators on enzyme activity

The effect of potassium cyanide (KCN), sodium ethylenediaminetetraacetate (EDTA) and hydroquinone (HQ) on the LOX activity of the partially purified extract was determined using the enzyme assay procedure described above. The reaction medium consisted of enzyme extract, linoleic acid and buffer solution. KCN was added to the reaction medium up to a final concentration of 25 mM whereas EDTA and HQ were added up to final concentrations of 5 mM and 1 mM, respectively. The inhibitor was added to both the reaction medium and a control at the beginning of the reaction and the degree of inhibition was monitored for a period of 3 min at 234 nm on a Beckman DU-650 spectrophotometer (Beckman Instruments).

3.3.9. Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was performed using the PhastSystem Unit (Pharmacia LKB Biotechnology, Uppsala, Sweden). The electrophoresis run and the staining of the separated protein bands were performed in accordance to the procedure outlined in the manual (Pharmacia, 1992).

High molecular weight standards (Pharmacia) were run in tandem with the fraction samples and consisted of thyroglobulin (M_r 669 kDa), ferritin (M_r 440 kDa), catalase (M_r 232 kDa), lactate dehydrogenase (M_r 140 kDa), and albumin (M_r 67 kDa).

Native PAGE mini-gels (5 x 4 cm; 0.45 mm thickness) of 10 % polyacrylamide were precast in accordance to the procedure outlined in the manual (Pharmacia, 1992). The gels were run, on a PhastSystem for 30 min at a constant current of 10 mA, using PhastGel native buffer strips (Pharmacia); the buffer strips consisted of 0.88 M L-alanine, 0.25 M Tris, pH 8.8 in 2 % Agarose IEF. After the separation of the proteins, the gels were transferred to the development section of the PhastSystem Unit. Silver staining was performed on the gels as outlined in the manual (Pharmacia, 1992). The silver staining consisted of fixing and removing buffer ions, sensitizing the proteins in a glutardialdehyde solution, reacting the proteins with silver ions using a silver nitrate solution, developing in a basic formaldehyde solution and stopping the development with acetic acid. A final rinsing step in 10 % acetic acid/5 % glycerol was used to prevent gels from curling or cracking after drying.

3.4. Results and Discussion

3.4.1. Partial purification of lipoxygenase

Table 1 shows a scheme for the partial purification of the LOX extracts from *F. oxysporum*, *F. proliferatum*, *S. cerevisiae*, and *C. pyrenoidosa*. The partially purified extracts of *F. oxysporum*, *F. proliferatum*, *S. cerevisiae* and *C. pyrenoidosa* were obtained by precipitation with ammonium sulfate at different levels of saturation.

The results (Table 1) indicate that although the discarded fraction from *F. oxysporum*, precipitated by ammonium sulfate at 0-20 % of saturation, has a 2.6-fold purification compared to that of the crude extract, it contained only 11.8 % of recovery. The results also demonstrate that 70.4 % of the total activity and 69.9 % of the specific activity were found to be in the partially purified fraction precipitated by ammonium sulfate at 20 to 80 % saturation; this fraction also retained a recovery of 41.1 % and a 13.3-fold increase in purification with respect to the crude extract. The 20-80 % ammonium sulfate extract was therefore considered as the partially purified LOX extract of *F. oxysporum*.

The results (Table 1) also show that although the discarded fractions from *S. cerevisiae*, precipitated by ammonium sulfate at 0-20 and 80-100 % of saturation,

Table 1. Partial purification of lipoxygenase extracts from selected microbial sources.

Fraction	Total protein[a] (mg)	Specific activity[b]	Total activity[c]	Recovery (%)	Purification (fold)
F. oxysporum					
crude extract	248	467	115733	100.0	1.0
Ammonium sulfate precipitation at					
0-20%	11	1217	13645	11.8	2.6
20-80%	**22**	**6240**	**47742**	**41.1**	**13.3**
80-100%	4	1467	6476	5.6	3.1
F. proliferatum					
crude extract	184	1316	242144	100.0	1.0
Ammonium sulfate precipitation at					
0-40%	**1**	**3504**	**2277**	**1.0**	**2.7**
40-60%	12	26	303	0.1	0.0
60-80%	8	61	461	0.2	0.1
80-100%	4	182	740	0.3	0.1
S. cerevisiae					
crude extract	915	133	122117	100.0	1.0
Ammonium sulfate precipitation at					
0-20%	10	1190	11917	9.7	8.9
20-80%	**172**	**2343**	**110405**	**90.4**	**17.5**
80-100%	8	1493	12505	10.2	11.2
C. pyrenoidosa					
crude extract	687	32	21810	100.0	1.0
Ammonium sulfate precipitation at					
0-40%	240	8	2023	9.3	0.3
40-80%	**73**	**1645**	**119483**	**547.8**	**51.8**
80-100%	14	103	1482	6.8	3.2

[a]Protein was determined according to a modification of the Lowry method (Hartree, 1972), using bovine serum albumin as a standard.

[b]Specific activity is defined as A/mg protein/min, where A is equal to 0.001 absorbance at 234 nm.

[c]Unit of activity is defined as A/min, where A is equal to 0.001 absorbance at 234 nm.

respectively, have high specific activities, they did not exceed 20 % of the total LOX activity. In addition, the results indicate that 81.9 % of the total activity and 46.6 % of the specific activity were found to be in the partially purified extract, precipitated by ammonium sulfate at 20 to 80 % of saturation; this fraction also retained a recovery of 90.4 % and a 17.5-fold increase in purification with respect to the crude extract. Based on these findings, the LOX extract of *S. cerevisiae* was also purified at 20 to 80 % of ammonium sulfate saturation.

Rouet-Mayer *et al.* (1992) reported a 6-fold increase in the concentration of the soluble protein in the crude extract of carnation petals which was obtained by ammonium sulfate precipitation at 80 % of saturation. Zamora *et al.* (1987) reported that the partial purification of tomato LOX by ammonium sulfate precipitation at 25-70 % of saturation resulted in a 4-fold increase in purification and 83 % recovery. In addition, a LOX extract from tomato fruit was obtained by ammonium sulfate precipitation at 25-60 % of saturation, resulting in a 1.3-fold increase in purification (Bowser *et al.*, 1992). A partially purified LOX was obtained from barley (Van Aarlw *et al.*, 1992), snap bean and pea (Klein 1976) by ammonium sulfate precipitation at 30-60 % of saturation, resulting in a 4.4-, 9.1- and 2.7-fold increase in purification, respectively, with corresponding recoveries of 73, 400 and 47 %. LOX was partially purified by ammonium sulfate at 25-50 % of saturation from immature English peas (Chen and Whitaker, 1986) and peas (Eriksson and Svensson, 1970) resulting in a 3.24- and 4.2-fold increase in purification, respectively, and with corresponding recoveries of 85.5 and 93 %.

Table 1 shows that the LOX fraction from *F. proliferatum*, precipitated by ammonium sulfate at 0-40 % of saturation, had 92.9 % of the specific activity and 60.2 % of the total activity; this fraction also retained a higher recovery compared to those of the enzymatic fractions, precipitated by ammonium sulfate at 40-60, 60-80 and 80-100 % of saturation. In addition, the 0-40 % ammonium sulfate enzymatic extract showed a 2.7-fold increase in purification with respect to the crude extract and was therefore considered as the partially purified LOX of *F. proliferatum*.

The results (Table 1) indicate that although the fraction from *C. pyrenoidosa*, precipitated by ammonium sulfate at 0-40 % of saturation, had 73 % of the total protein, it possessed only 1.6 % of the total activity and was therefore discarded. In addition, the fraction, precipitated by ammonium sulfate at 80-100 % of saturation, was eliminated as it possessed only 4.2 and 1.2 % of the total protein and activity, respectively. The results also indicate that although the 40-80 % ammonium sulfate fraction had 22.3 % of the total protein, it possessed 97 % of the total activity with a recovery of 547.8 % and a 51.8-fold increase in purification as compared to the crude extract; the increase in enzyme activity could be due to the removal of inhibitors as a result of the partial purification process. On the basis of these findings, this fraction was selected as the partially purified LOX.

A partially purified enzyme was also obtained by ammonium sulfate precipitation at 40-80 % of saturation from maize (Poca *et al.*, 1990) with a recovery of 30.3 % and a 12.8-fold increase in purification. In addition, LOX was partially purified from peanut seeds (Sanders *et al.*, 1975) by ammonium sulfate at 40-65 % of saturation with a recovery of 135.9 % and an increase in purification of 6.1-fold. A partially purified enzyme was obtained from green beans (Adams and Ongely, 1989), chickpea (Sanz *et al.*, 1992) and cowpea (Van Den and Mendoza, 1982) by ammonium sulfate at 40-60 % of saturation. However, Zimmerman and Vick (1973) reported a partially purified LOX from *C. pyrenoidosa* obtained by precipitation with ammonium sulfate at 0-42 % saturation; in contrast, the partially purified fraction of *C. pyrenoidosa*, obtained by ammonium sulfate precipitation at 0-40 % of saturation (Table 1), contained only 1.6 % of the total enzyme activity.

3.4.2. Effect of pH on enzyme activity

The activity of LOX (Fig. 7) was determined over a wide pH range from 5.0 to 12.0. The results indicate that both in the acidic (pH 5.0-7.0) and alkaline range (pH 11.0-13.0), LOX activities from *F. oxysporum* and S. *cerevisiae* were relatively low and changed very little; however, Figure 7 demonstrates the presence of two points of maximum activity, respectively, at pH 8.0 and pH 10.0 which suggests the presence of two isozymes.

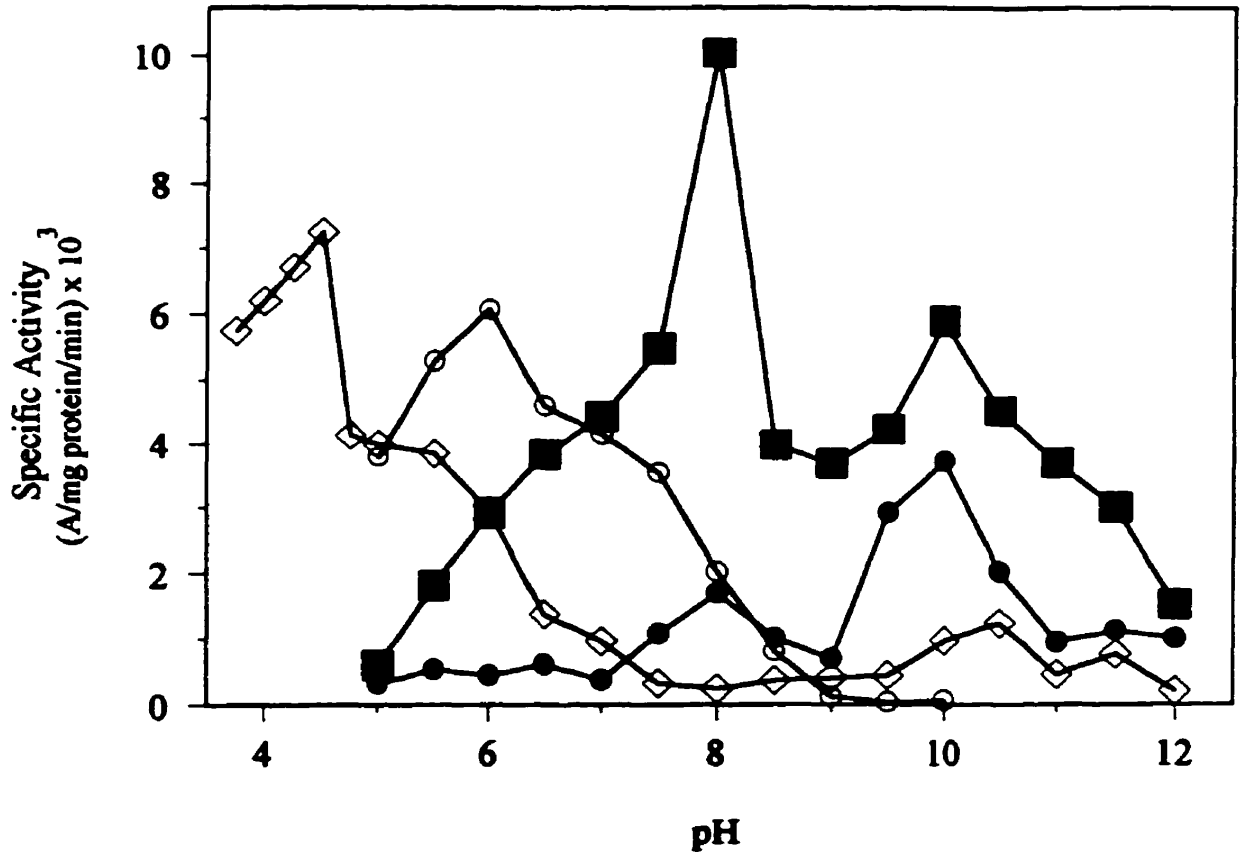

Figure 7. Effect of pH on the lipoxygenase activity of the partially purified enzymatic extracts obtained from *F. oxysporum* (—■—), *F. proliferatum* (—○—), *S. cerevisiae* (—●—) and *C. pyrenoidosa* (—◇—).

Satoh *et al.* (1976) reported two pH optimas, at pH 6.0 and 10.0, for the partially purified LOX of *F. oxysporum*. The same authors also indicated that autoxidation of linoleate proceeded at pH 12.0; the results reported in Figure 7 confirm the statement of Satoh *et al.* (1976) since the LOX activity of *F. oxysporum* decreased at pH 12.0 and above. In contrast, Shechter and Grossman (1983) reported a pH optimum of 6.3 for the purified LOX obtained from the mitochondrial fraction of *S. cerevisiae*. The difference in the observed pH optimas could be due to differences in the purification scheme used to obtain the partially purified enzyme.

With respect to other microbial sources of LOX, Beneytout *et al.* (1989) reported that a purified LOX preparation from the green algae *Oscillatoria* sp. had a pH optimum of 8.8. Partially purified LOX was reported to have two pH optima, 6.2 and 7.8-8.0 respectively, in kiwi fruit (Boyes *et al.*, 1992) and one pH optimum of 8.5 in rice bran (Shastry and Rao, 1975). Defatted crude LOX demonstrated optimum activity in small red bean, green pea and peanuts at pH 7.0, 7.5 and 8.1, respectively (Dillard *et al.*, 1960).

Figure 7 also indicates that the partially purified extract from *F. proliferatum* exhibited maximum LOX activity at pH 6.0. The results show that at pH 8.5, 13.2 % of the maximum enzymatic activity was observed while at pH 5.0, approximately 62.4 % was exhibited. These findings indicate that the enzyme appeared to be more active in an acidic reaction medium than an alkaline one. Brodowsky *et al.* (1994) recently reported that the LOX of the fungus *G. graminis* exhibited an optimum enzyme activity at pH 7.2. In addition, Iny *et al.* (1993b) reported that the LOX of *T. vulgaris* showed optimum LOX activity at pH 6.0. The pH optima of partially purified LOXs from tomato fruit (Bonnet and Crouzet, 1977), asparagus (Ganthavorn and Powers, 1989) and senescing carnation petals (Rouet-Mayer *et al.*, 1992) were 6.3, 5.5-6.0 and 6.1, respectively.

The results (Fig.7) also demonstrate that the enzyme activity from *C. pyrenoidosa* reached an optimum at pH 4.5; however, at pH values slightly higher than 4.5, activity decreased dramatically to a minimum at pH 7.0 and continued to remain comparably low throughout the alkaline pH region. Due to the limited solubility of free linoleic acid in the

acidic reaction medium of pH 4.5, enzyme assays for kinetic studies were performed at pH 7.0 thereby allowing better solubilization of the free linoleic acid (Rouet-Mayer *et al.*, 1992). However, Zimmerman and Vick (1973) reported a pH optimum of 7.4 for the partially purified LOX of *C. pyrenoidosa*. Wheat germ LOX-3 (Shiba *et al.*, 1991) and rice bran LOX-1 (Ida *et al.*, 1983b) had a pH optimum of 4.5-6.0 and 4.5, respectively, while a purified watermelon hypo-cotyl root LOX extract (Vick and Zimmerman, 1976) had two optima, at pH 4.4 and 5.5.

3.4.3. Kinetics of the partially purified lipoxygenase

Table 2 shows that the apparent K_m values at pH 8.0 and 10.0, for the *F. oxysporum* LOX extract, were calculated from the best straight line using Lineweaver-Burk plots (1934) to be 3.28×10^{-5} and 3.55×10^{-5} M, respectively. The results also indicate a slightly higher K_m of 5.15×10^{-5} M for the partially purified LOX extract from *F. proliferatum*. The LOX extract from *C. pyrenoidosa* exhibited the highest K_m value of 9.12×10^{-5} M while that from *S. cerevisiae* showed the lowest K_m values of 1.34×10^{-5} and 9.5×10^{-6} M, at pH 8.0 and 10.0, respectively.

Partially purified LOX was reported to have K_m values of 2.0×10^{-4}, 3.5×10^{-4} and 7.6×10^{-4} M from canola seed (Khalyfa *et al.*, 1990), rice bran (Shastry and Rao, 1975), and egg plant (Grossman *et al.*, 1972a), respectively. Shechter and Grossman (1983) reported a K_m value of 2.86×10^{-4} M for the purified LOX from the mitochondrial fraction of *S. cerevisiae*. A partially purified LOX from faba bean (Eskin and Henderson, 1974) and kiwi fruit (Boyes *et al.*, 1992) have reported K_m values of 5.7×10^{-4} and 2.4×10^{-4} M, respectively.

Slightly higher K_m values of 2.0×10^{-3}, 2.6×10^{-3} and 2.8×10^{-3} M were reported for pea seed LOX (Reynolds and Klein, 1982a; Chen and Whitaker, 1986) and partially purified

Table 2. Kinetic parameters of partially purified lipoxygenase extracts.

Microorganism	pH	K_m (10^{-5} M)	V_{max} [a]	K_i (10^{-5} M)	V_{maxapp} [a]	Type of inhibition
F. oxysporum	8.0	3.28	1.62	2.81	0.58	noncompetitive [b]
				-	-	no inhibition [c,d]
	10.0	3.55	1.36	-	-	no inhibition [b,d]
F. proliferantum	6.0	5.15	1.61	-	-	no inhibition [b,c,d]
S. cerevisiae	8.0	1.34	0.42	1.41	0.37	noncompetitive [b]
				2.10	0.45	competitive [c]
				-	-	no inhibition [d]
	10.0	0.95	0.61	-	-	no inhibition [b,d]
C. pyrenoidosa	7.0	9.12	0.40	4.75	0.25	uncompetitive [b]
				-	-	no inhibition [c,d]

[a] Values expressed in μmol hydroperoxide/mg protein/min.

[b] Potassium cyanide.

[c] Hydroquinone.

[d] Ethylenediaminetetraacetic acid.

broad bean LOX (Al-Obaidy and Siddiqi, 1981). The highest K_m of 1 mM was reported by Iny *et al.* (1993b) for the enzymatic extract from *T. vulgaris*.

Slightly lower K_m values were reported for partially purified tomato fruit LOX (0.015×10^{-6} M; Bonnet and Crouzet, 1977) and for the crude LOX from tomato fruit (2.53×10^{-5} M; Jadhav *et al.*, 1972). In addition, Bowsher *et al.* (1992) reported K_m values of 6.2×10^{-6} and 3.8×10^{-6} M for the membrane-associated LOX and the partially purified soluble LOX from tomato fruit, respectively. The discrepancy in the K_m values may be due to the differences in the sources used to obtain the enzyme, in the purification procedures and enzymatic assay methods (Klein, 1976).

3.4.4. Effect of KCN on enzyme activity

Figure 8 demonstrates the effect of KCN on LOX activity from the four microbial LOX extracts. The results show that the use of 20 mM of KCN decreased LOX activity from *F. oxysporum* at pH 8.0 and 10.0 by 30.8 and 8.4 %, respectively. However, the use of 10 mM KCN inhibited enzyme activity at pH 8.0 and 10.0 by 16.2 and 4.1 %, respectively. Matsuda *et al.* (1976) reported that, at 10 mM concentration of KCN and pH 7.3, 40 % of the LOX activity of *F. oxysporum* was inhibited. The results (Fig. 8) also show that at 25 and 5 mM KCN, the enzyme activity from *S. cerevisiae*, at pH 8.0, decreased by 51.4 and 16.2 %, respectively whereas it remained unaffected at pH 10.0. Hardy *et al.* (1991) reported that 10 and 20 mM NaCN inhibited the initial LOX activity in etiolated pea shoots by 0 and 15 %, respectively.

In addition, the results (Fig. 8) demonstrates that the relative activity of the enzyme from *C. pyrenoidosa* decreased by 58.2 and 67.1 % at 0.5 and 1 mM KCN concentration, respectively. However, Zimmerman and Vick (1973) reported that 1.6 mM sodium cyanide did not inhibit the partially purified LOX activity in the extract of *C. pyrenoidosa*. The activity of partially purified LOX from apple (Kim and Grosch, 1979) decreased by 42 % at 1 mM KCN whereas that from asparagus (Ganthavorn and Powers, 1989) decreased by

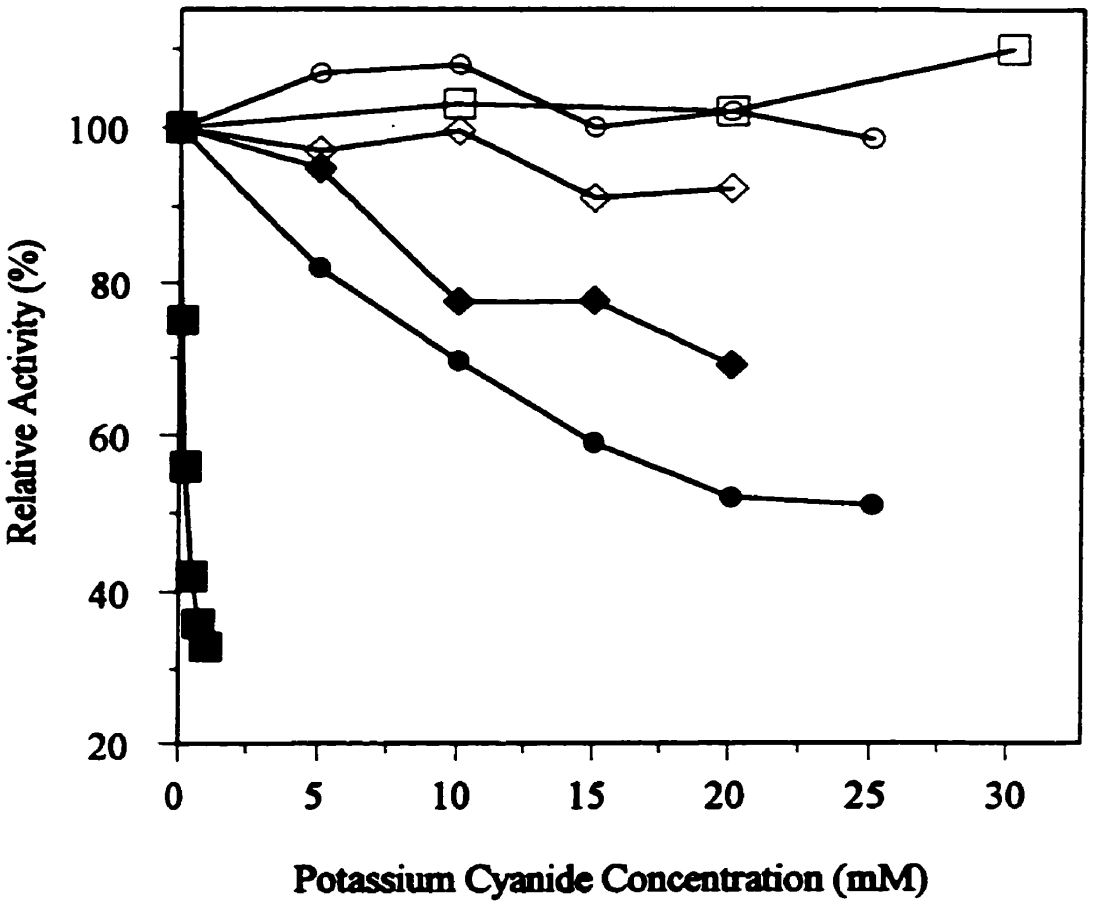

Figure 8. Effect of potassium cyanide on the lipoxygenase activity of the partially purified enzymatic extracts obtained from *F. oxysporum* at pH 8.0 (—◆—) and 10.0 (—◇—), *F. proliferatum* (—□—) at pH 6.0, *S. cerevisiae* at pH 8.0 (—●—) and 10.0 (—○—), and *C. pyrenoidosa* (—■—) at pH 7.0.

approximately 40 and 90 % at 1 and 5mM KCN, respectively. Flick *et al.* (1975) reported that 1 mM KCN completely inhibited the activity in the crude LOX extracts from three eggplant cultivars.

In contrast, Figure 8 also shows that the addition of 60 mM KCN had little effect on the LOX activity of the partially purified extract from *F. proliferatum*; however, at higher KCN concentrations (70-80 mM), the enzyme activity decreased dramatically. The experimental results (not shown) indicated that the decrease in LOX activity could be due to the increase in pH values from 6.0 to 9.0 upon the addition of 50 mM to 80 mM KCN, respectively.

St-Angelo and Kuck (1977) confirmed that the addition of KCN increases the pH of the reaction medium. In addition, Kermasha and Metche (1986) reported that the addition of 40 mM KCN resulted in a two-fold increase in the activity of partially purified LOX from *Phaseolus vulgaris*; however, these authors showed that higher concentrations of KCN caused a decrease in enzyme activity which may not have been due to the CN inhibitory effect but to the increase in pH values. The literature also showed that the use of 10 mM NaCN had no inhibitory effect on LOX activity of the partially purified extract from egg plant (Grossman *et al.*, 1972a) and tomato fruit (Bonnet and Crouzet, 1977). Iny *et al.* (1993b) reported that 2.2 mM KCN showed no significant inhibitory effect on the LOX activity from *T. vulgaris*. The use of 1 mM KCN had no inhibitory effect on LOX activity from cucumber (Wardale and Lambert, 1980), tomato (Jadhav *et al.*, 1972), and *Marchantia polymorpha* (Matsui *et al.*, 1990).

Table 2 shows that the K_m value, calculated from the best straight line using Lineweaver-Burk plots of 1/*v* versus 1/S (1934), demonstrates that, at pH 8.0, CN is a noncompetitive inhibitor for LOX activity from *F. oxysporum*. In addition, a plot (Fig. 9) of V_{max} versus the amount of total enzyme $[E]_{total}$ shows that the V_{max} of the enzyme-catalyzed reaction was lower in the presence of the inhibitor thereby indicating that KCN acts as a classical irreversible noncompetitive inhibitor by binding irreversibly to the active LOX residues and completely removing the enzyme from the system (Segal, 1976). The results

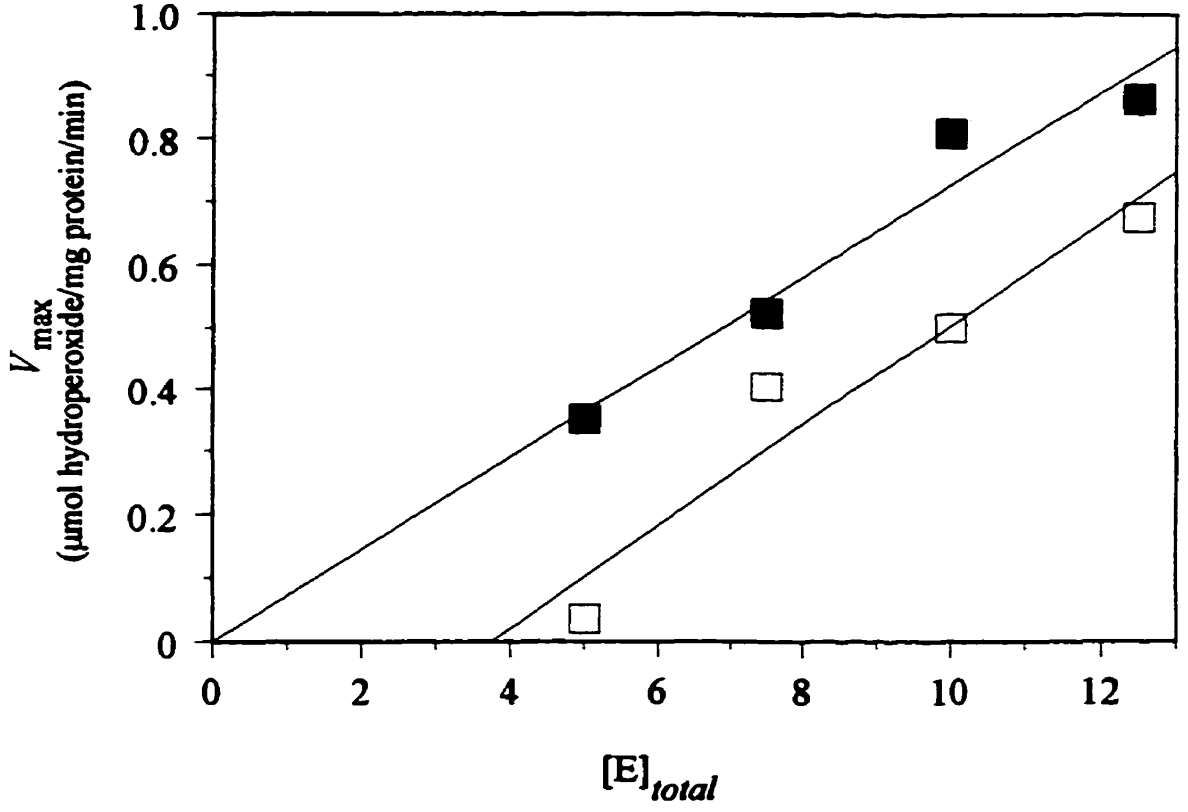

Figure 9. Plots (V_{max} versus $[E]_{total}$) of the inhibition of partally purified lipoxygenase from *F. oxysporum* by potassium cyanide, characterization without inhibition (—■—) and with inhibition (—□—), at pH 8.0.

(Table 2) also show that KCN was found to be a noncompetitive inhibitor for LOX activity from *S. cerevisiae* whereas it was an uncompetitive inhibitor for the *C. pyrenoidosa* enzymatic extract.

Earlier work on the inhibition of LOX by CN produced contradictory results; some researchers found little or no effect (Galliard and Phillips, 1971; Jadhav *et al.*, 1972; Eskin and Henderson, 1974; Bonnet and Crouzet, 1977) while others reported significant inhibition (De Lumen *et al.*, 1978; Van Den and Mendoza, 1982; Hidaka *et al.*, 1986; Kermasha and Metche, 1986; Khalyfa *et al.*, 1990). The difference in the degree of inhibition of LOX activity might be due to inaccessibility of the iron to KCN or the interaction of CN with other heme-proteins or CN inhibitors present in the less purified sample; indeed, CN is a well known inhibitor of other heme-proteins such as peroxidase and has been used to distinguish between heme-protein and LOX-catalyzed oxidation (Ganthavorn and Powers, 1989). These contradictory results may also be due to the fact that the degree of inhibition is influenced by several factors such as pH, temperature, nature and concentration of buffer, ionic strength, enzyme source, and enzyme, substrate and inhibitor concentration, reaction time (Whitaker, 1972) as well as the pre-incubation time of the enzyme with the inhibitor (Ganthavorn and Powers, 1989) and the effect of KCN on the pH of the reaction medium (St. Angelo and Kuch, 1977).

3.4.5. Effect of EDTA on enzyme activity

The results (Fig. 10) demonstrate that the addition of 5 mM EDTA increased the LOX activity from *F. oxysporum* by 16.6 and 50.3 % at pH 8.0 and 10.0, respectively. However, Matsuda *et al.* (1976) reported that the presence, at pH 7.3, of 1 mM EDTA did not cause appreciable inhibition of the activity of purified LOX of *F. oxysporum*. Figure 10 also shows that the addition of 1 mM EDTA had no effect on LOX activity from *F. proliferatum* and *S. cerevisiae* while 5 mM EDTA increased LOX activity of the former extract by 50 %. The results (Figure 10) also indicate that the addition of 1 and 5 mM EDTA noticeably increased the relative enzyme activity from *C. pyrenoidosa* by two- and eight-fold, respectively.

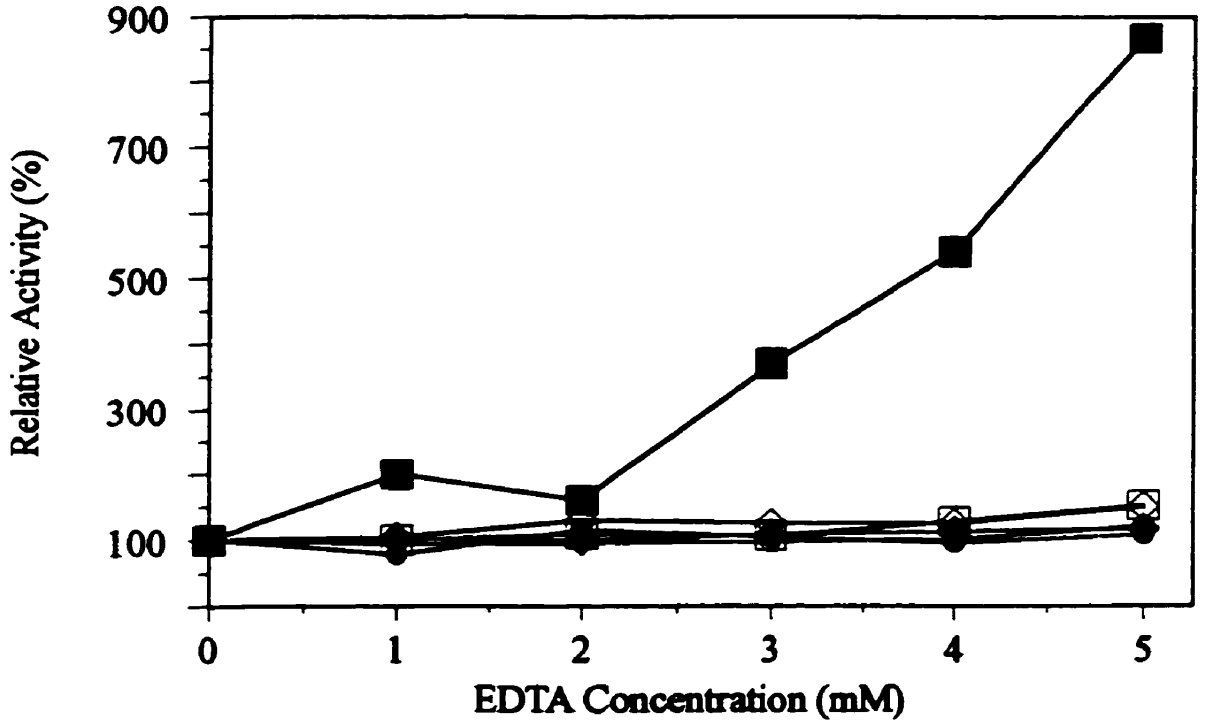

Figure 10. Effect of sodium ethylenediaminetetraacetate on the lipoxygenase activity of the partially purified enzymatic extracts obtained from *F. oxysporum* at pH 8.0 (—◆—) and 10.0 (—◇—), *F. proliferatum* (—□—) at pH 6.0, *S. cerevisiae* at pH 8.0 (—●—) and 10.0 (—○—), *and C. pyrenoidosa* (—■—) at pH 7.0.

In contrast, the LOX activity of *G. graminis* (Brodowsky *et al.*, 1994), *M. polymorpha* (Matsui *et al.*, 1990), sunflower (Leoni *et al.*, 1985) and cucumber (Wardale and Lambert, 1980) was not inhibited by the addition of 1 mM EDTA. Partially purified LOXs from egg plant (Grossman *et al.*, 1972a) and tomato fruit (Bonnet and Crouzet, 1977) were also not inhibited when EDTA was added to the reaction medium at a final concentration of 10 mM. In addition, EDTA was an ineffective inhibitor of crude LOX activity in raw peanut homogenates (Singleton *et al.*, 1976) and tomato fruit (Jadhav *et al.*, 1972). However, the ineffectiveness of EDTA as a LOX inhibitor could be explained by the fact that iron is strongly bound in native LOX-1, resisting removal by all high-affinity Fe(II) chelaters, such as EDTA, which do not contain divalent sulfur (Pistorius and Axelrod, 1974) due to the presence of two reported histidine residues which act as ligands to the iron in the LOX active site (Zhang *et al.*, 1992). Shastry and Rao (1975) stated that the activity of partially purified rice bran LOX was stimulated by 20 % after the addition of 1 mM EDTA; these authors postulated that this increase in activity may be due to the removal of the inhibitory divalent metal ions present in the preparation by the addition of EDTA.

The results (Fig. 10) also show that the residual activity of LOX from *F. oxysporum* was considerably higher at pH 10.0 where the chelating efficiency of EDTA is enchanced due to the dissociation of its carboxyl groups as a function of rising pH (Lindsay, 1985). The carboxylate ions of EDTA can then function as efficient donor groups thereby chelating to a greater degree with divalent metal ions in the reaction medium that might inhibit LOX activity. The ability of EDTA to act as a more efficient strong metal chelator in alkaline medium is seen by the greater enchancement of the relative activity of LOX at pH 10.0 in comparison to pH 8.0.

In contrast, the addition of 1 mM EDTA was reported to inhibit the activity of partially purified LOX from apples (Kim and Grosch, 1979) and etiolated pea shoots (Hardy *et al.*, 1991) by 67 and 6 %, respectively.

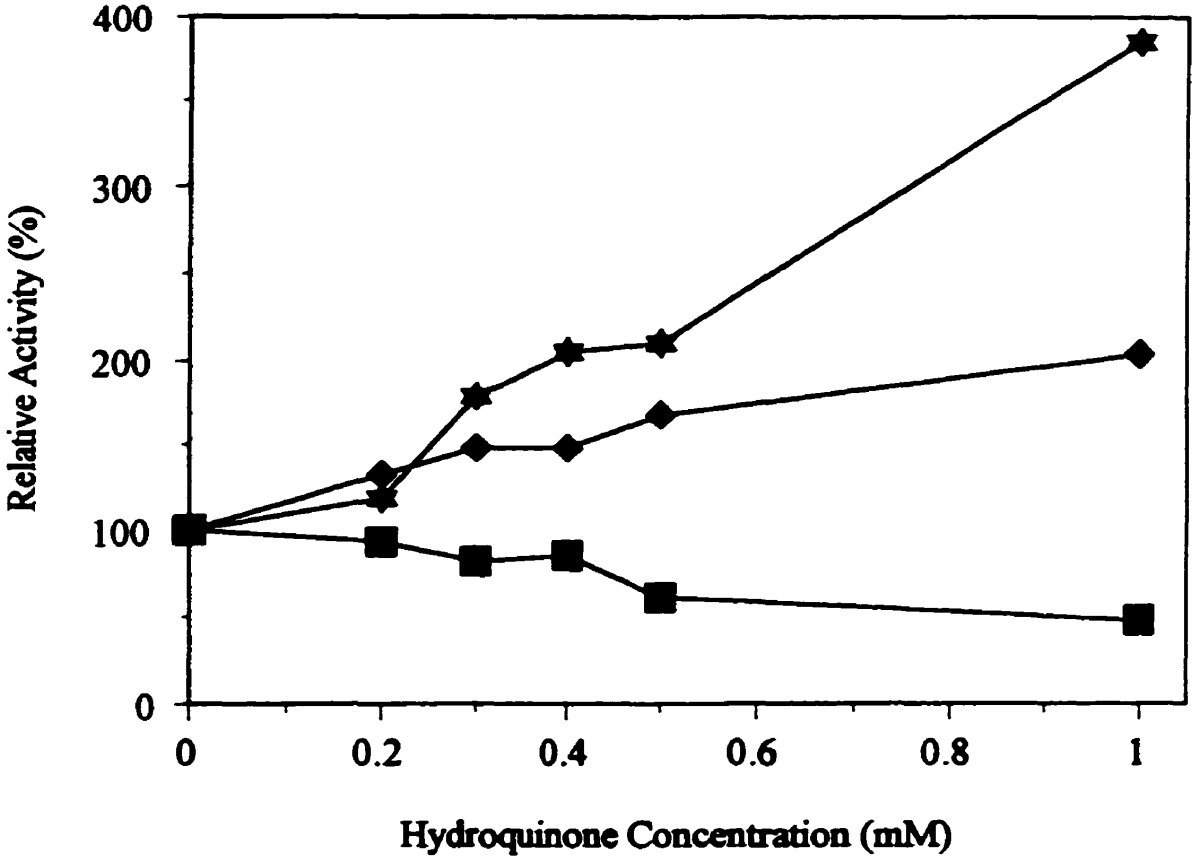

Figure 11. Effect of hydroquinone on the lipoxygenase activity of the partially purified enzymatic extracts obtained from *F. proliferatum* (◆), *S. cerevisiae* (■) and *C. pyrenoidosa* (★).

3.4.6. Effect of hydroquinone on enzyme activity

Figure 11 shows that the addition of 1 mM HQ resulted in a two- and four-fold increase in LOX activity from *F. proliferatum* and *C. pyrenoidosa*, respectively. In contrast, the addition of 1 mM HQ inhibited the LOX activity, at pH 8.0, from *S. cerevisiae* by 51.4 %. The effect of higher concentrations of HQ on LOX activity could not be determined due to the limits of absorbance measurements of the spectrophotometer; moreover, the oxidation of HQ was more pronounced at pH 10.0, thereby resulting in the formation of deep brownish-colored substances preventing absorbance measurements at 234 nm.

Yasumoto *et al.* (1970) showed, with the use of polarographic measurements, that 5.2 mM HQ had an inhibitory effect of 50 % on soybean LOX activity. In addition, Van Den and Mendoza (1982) reported that the use of 1 mM HQ inhibited the activity of cowpea LOX-1 and -2 by 100 and 82 %, respectively, whereas, Tappel *et al.* (1953) indicated that 2 mM HQ inhibited crude soybean LOX activity by 90 to 100 %.

In addition, the results (Table 2) indicate that the HQ has a competitive inhibitory effect on LOX activity of *S. cerevisiae*, since the V_{max} of the reaction, 0.44 µmol HPODE mg protein^{-1} min^{-1}, remained unchanged and the K_m values differed.

HQs are excellent hydrogen donors which can react with hydroperoxy radicals to form the corresponding hydroperoxides and the stable semiquinones resonance hybrids (Nawar, 1985). The LOX-catalyzed reaction could therefore be inhibited competitively by the accumulation of end-product, i.e. HPODEs. Moreover, the addition of a higher concentration of substrate, i.e. linoleic acid, to the reaction medium could decrease the competitive inhibitory effect of HQ.

3.4.7. Substrate specificity

The specificity of the partially purified LOX was assayed with a wide range of substrates, including free fatty acids and mono-, di- and trilinolein. The results (Table 3) show that the highest specificity of the partially purified LOX extracts from *F. oxysporum* and *S.*

Table 3. Substrate specificity of partially purified lipoxygenase extracts from selected microorganisms.

	Specific activity[a]					
	F. oxysporum		*F. proliferatum*	*S. cerevisiae*		*C. pyrenoidosa*
	pH		pH	pH		pH
Substrate	8.0	10.0	6.0	8.0	10.0	7.0
Linoleic acid	100.0	100.0	100.0	100.0	100.0	100.0
Linolenic acid	28.8	18.8	35.2	10.9	20.0	15.7
Monolinolein	0.3	8.9	40.7	2.3	10.5	10.6
Dilinolein	1.7	7.9	37.8	3.3	9.7	10.1
Trilinolein	0.6	7.5	36.2	2.7	8.7	9.7

[a] Specific activity is defined as A per mg protein per min, where A is equal to 0.001 absorbance at 234 nm.

cerevisiae, at both pH 8.0 and 10.0, was towards linoleic acid, followed by linolenic acid; the LOX extracts exhibited higher activity towards mono-, di- and trilinolein at pH 10.0 than that at pH 8.0. Table 3 also indicates that the enzymatic extract from *F. proliferatum* showed an overall preference for linoleic acid and exhibited approximately 37 % of its relative activity towards linolenic acid and mono-, di- and trilinolein. In addition, the results (Table 3) indicate that LOX from *C. pyrenoidosa* was more active towards free linoleic acid and comparatively less towards its fatty acid esters and free linolenic acid; the enzyme activity decreased in the order of linoleic acid > linolenic acid > mono- > di- > and trilinolein.

Iny *et al.* (1993b) reported that LOX from *T. vulgaris* demonstrated its maximum activity towards linoleic acid while exhibiting only a third of that with linolenic acid. Partially purified LOX from kiwi fruit (Boyes *et al.*, 1992) demonstrated preferential enzyme activity towards linoleic and linolenic acids but none towards trilinolein, whereas that from apple (Kim and Grosch, 1979) showed preferential specificity for linolenic acid in comparison to linoleic acid. Crude cucumber LOX (Wardale and Lambert, 1980) was most active towards linoleic acid followed by linolenic acid; however the enzyme was less than 80 % active towards monolinolein. Grossman *et al.* (1972a) indicated that the partially purified egg plant LOX exhibited less than 25 % activity towards di- and trilinolein than that observed with linoleic and linolenic acids. Partially purified LOX from *P. vulgaris* (Kermasha and Metche, 1986) and canola seed (Khalyfa *et al.*, 1990) had higher specificity towards linoleic acid followed by mono-, di-, and trilinolein while that from asparagus (Ganthavorn and Powers, 1989) exhibited similar specificities for linoleic acid and monolinolein but yielded 10 % or less activity towards di- and trilinolein. Eskin and Henderson (1974) reported that two partially purified lipoxygenases from faba bean showed preferential activity towards free linoleic acid but exhibited less than 50 % activity towards linolenic acid as well as less than 5 % towards mono-, di-, trilinolein.

3.4.8. Electrophoretic profile

The electrophoretic profile (Fig. 12A) of native PAGE of the enzyme fractions from *F. oxysporum* shows that the crude extract and partially purified fraction exhibit one major and

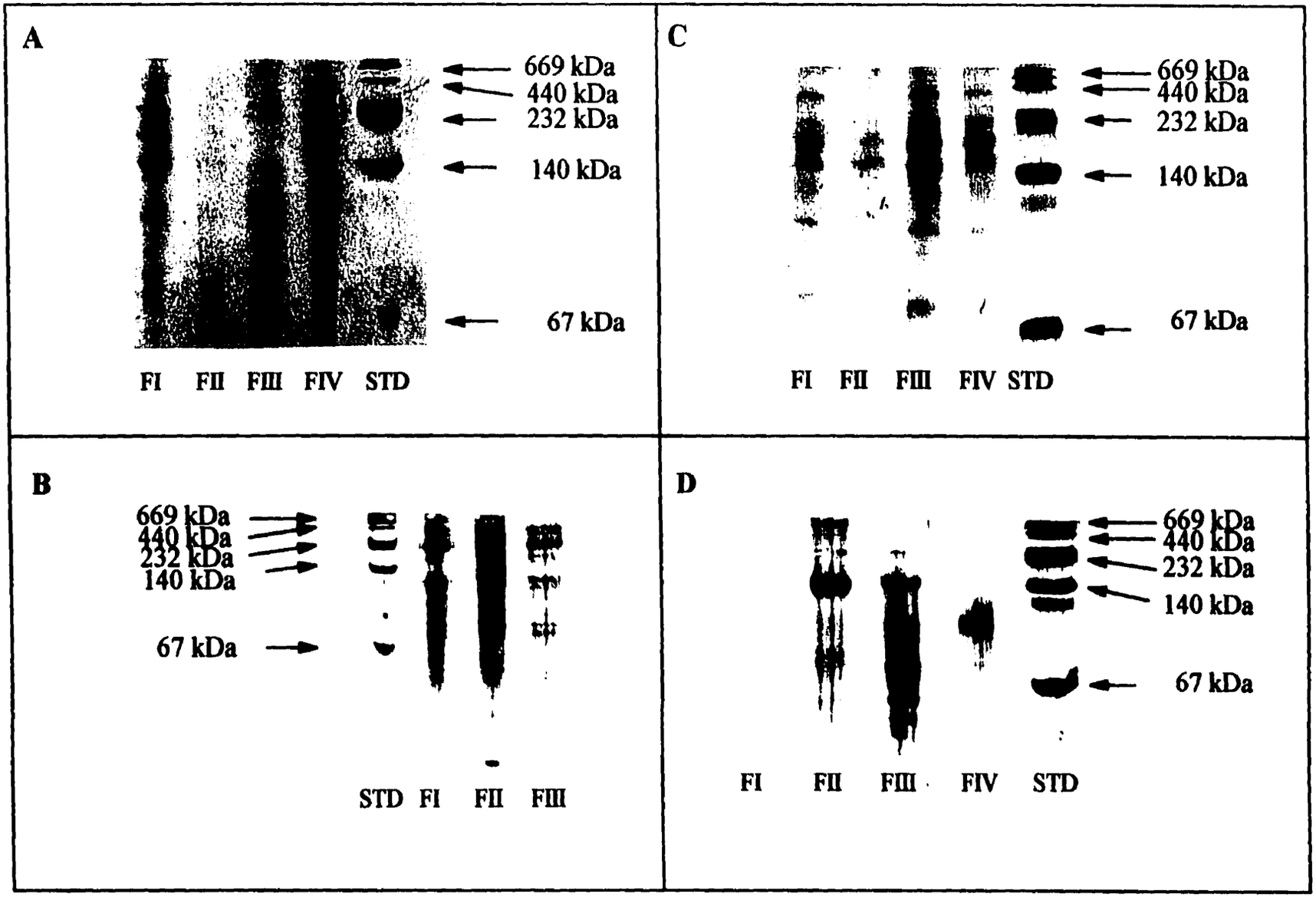

Figure 12. Electrophoreograms of the native polyacrylamide gel of the partially purified enzymatic extracts obtained from (A) *Fusarium oxysporum*, (B) *Fusarium proliferatum*, (C) *Saccharomyces cerevisiae*, and (D) *Chlorella pyrenoidosa*; FI is the crude extract; FII is the enzymatic fraction precipitated with ammonium sulfate (EFPAS) at 0-20% (A), 0-40% (B), 0-20% (C), and 0-40 % (D) of saturation; FIII is the EFPAS at 20-80% (A), 40-80% (B), 20-80% (C), and 40-80 % (D) of saturation; and FIV is the EFPAS at 80-100% (A,C,D) of saturation.

five minor bands with an approximate molecular weight (M_r) of 67 to 140 kDa while those from *F. proliferatum* (Fig. 12B) show the presence of one major band at 140 kDa and two minor bands at 67 kDa and 232 kDa. In addition, the results (Fig. 12C) demonstrate that the enzymatic extract from *S.cerevisiae* showed two major bands with an approximate molecular weight (M_r) of 67 to 140 kDa in the crude extract and partially purified fraction. The electrophoretic profile (Fig. 12D) of the *C. pyrenoidosa* enzymatic extract indicates that the crude fraction exhibits one band whereas the partially purified fraction exhibits five bands with an approximate molecular mass of 67 to 140 kDa. Although the exhibition of only one band on both native and sodium dodecyl sulfate PAGE is generally the result of extensive purification and indicative of a pure sample, the single band exhibited by the crude extract of *C. pyrenoidosa* does not represent a pure protein but the association of several proteins which are subsequently separated into several protein bands, by ammonium sulfate precipitation, based on their charge and molecular weight. The partially purified fractions derived from the crude extract are also representative of the increased solubility and protein purification of the sample as a result of the partial purification procedure that eliminated the interfering and insoluble material present in the crude extract.

The literature indicates that, depending on the source of extraction and the method of purification, the molecular mass of LOX isozymes varies greatly ranging from as low as 64 kDa for LOX-1 from pea seeds (Reynolds and Klein, 1982b) to as high as 250 kDa from germinating sunflower seeds (Lenoi *et al.*, 1985). Khalyfa *et al.* (1990) reported that partially purified LOX preparations from canola seed showed the presence of one major and four minor bands. Seven protein bands were exhibited by the partially purified LOX preparation of soybean meal (Yamamoto *et al.*, 1970) and alfalfa (Chang *et al.*, 1973). Shastry and Rao (1975) confirmed the presence of LOX as well as five proteins on PAGE. Klein (1976) reported the presence of one major and one to two trace protein bands in enzyme extracts from raw pea and split pea seeds, as well as one major and one trace protein band in that from snap bean preparations. Bonnet and Crouzet (1977) confirmed, by native electrophoresis, the presence of LOX and three protein stripes in the partially purified tomato extract.

3.5. Conclusion

The results gathered in this study indicated that the partial purification of the *F. oxysporum* and *S. cerevisiae* extracts resulted in two active LOX activities, at pH 8.0 and 10.0, respectively, whereas that from the fungus *F. proliferatum* and the algae *C. pyrenoidosa* demonstrated the presence of one optimal LOX activity at pH 6.0 and 4.5, respectively. Although the literature showed little information regarding the isolation and characterization of LOX from microorganisms, the present study demonstrated that LOX from *F. oxysporum*, *F. proliferatum*, *S. cerevisiae* and *C. pyrenoidosa* shared many of the same characteristics of those reported from other sources in terms of pH, kinetic parameters, substrate specificity, enzyme activation and inhibition studies, and electrophoretic profile.

CHAPTER IV

CHARACTERIZATION OF HYDROPEROXIDES AND CARBONYL COMPOUNDS OBTAINED BY LIPOXYGENASE EXTRACTS OF SELECTED MICROORGANISMS

4.1. Abstract

Partially purified lipoxygenase (LOX) extracts were obtained from *Fusarium proliferatum*, *Fusarium oxysporum*, *Saccharomyces cerevisiae* and *Chlorella pyrenoidosa*; the enzymatic extract for *F. proliferatum* showed the highest LOX activity while those of *F. oxysporum* and *S. cerevisiae* demonstrated only 27.8 and 16.5 % of the activity at pH 8.0, and 61.2 and 9.7 % of the enzyme activity at pH 10.0, respectively. The lowest LOX activity was exhibited in the *C. pyrenoidosa* extract. The microbial enzymatic preparations were assayed with linoleic acid, as substrate, which was bioconverted into 9- and 13-hydroperoxides (HPODEs) by all four extracts; in addition, the LOX activity in the *F. oxysporum* extract produced the 10- and 12-HPODEs from linoleic acid while that of the *C. pyrenoidosa* extract produced only the 10-HPODE. When assayed with the 9- and 13-HPODEs, as substrates, the selected microbial extracts exhibited secondary enzyme activities, one of which produced hexanal. The highest hexanal-producing activity was found to be 1.51 and 1.39 nmol hexanol/mg protein/min in the *F. proliferatum* and *C. pyrenoidosa* extracts, respectively, while those of *F. oxysporum* and *S. cerevisiae* exhibited approximately 15 % of the HPODE-cleaving enzyme activity. The *C. pyrenoidosa* extract showed the highest proportion of pentanone which was produced at only one-fourth the concentration by the HPODE-cleaving enzyme activity in the three other microbial enzymatic extracts.

4.2. Introduction

Lipoxygenase (EC 1.13.11.12) is an enzyme which metabolizes linoleic acid and other polyunsaturated fatty acids containing a *cis*, *cis*-1,4-pentadiene moiety to hydroperoxy fatty acids by hydrogen abstraction from the methylene carbon and antarafacial insertion of molecular oxygen (Yamamoto, 1992). Lipoxygenase (LOX) has been reported in plant (Mack *et al.*, 1987) and animal (Yamamoto, 1983) sources; however, limited literature has reported

its presence in micro-organisms. LOX-like activity was first described in several microorganisms, including, *Aspergillus* and *Penicillium* (Mukherjee, 1951), *Pseudomonas* and *Achromobacter* (Shimahara and Hashizume, 1973).

Matsuda *et al.* (1978) reported a purified *Fusarium oxysporum* LOX which converted linoleic acid into 9- and 13-hydroperoxyoctadecadienoic acids (HPODEs). Wurzenberger and Grosch (1984a) indicated that a *Psalliota bispora* LOX extract converted linoleic acid to 10-HPODE which was cleaved by a hydroperoxide lyase (HPL) thereby producing 1-octen-3-ol, responsible for the characteristic flavor of mushrooms (Cronin and Ward, 1971) and 10-oxo-8-decenoic acid. Su *et al.* (1995) reported that the purified 8*R*-dioxygenase and hydroperoxide isomerase (HPI) from *Gaeumannomyces graminis* converted linoleic acid into 8*R*-HPODE and 7,8-dihydroxyoctadecadienoic acid (DiHODE). Moreover, a *Laetisaria arvalis* extract converted linoleic acid into the 8-HPODE and the 8-HODE (Brodowsky and Oliw, 1993). Hamberg (1989) indicated that a HPI from *Saprolegnia parasitica* converted 13-HPODE into 11,12- and 9,10-epoxy-13-hydroxy-octadecenoic acid (HOME), respectively, and the 9-HPODE into 10,11- and 12,13-epoxy-9-HOME, respectively. A LOX activity which oxidized arachidonic, eicosapentaenoic and docosahexaenoic acids was reported in the fungus *Lagenidium giganteum* (Simmons *et al.*, 1987).

A LOX preparation from *Saccharomyces vini* (Lyudnikova *et al.*, 1984), *Saccharomyces cerevisiae* (Shechter and Grossman, 1983) and *Thermoactinomyces vulgaris* (Iny *et al.*, 1993a) was reported to catalyze the oxygenation of linoleic acid into the 9- and 13-HPODEs.

In algae, a LOX extract from *Oscillatoria* sp. produced the 9- and 13-HPODEs (Beneytout *et al.*, 1989); the 13-HPODE was converted by a HPL to 13-oxotridecadienoic acid and pentanol (Andrianarison *et al.*, 1989). The *Chlorella pyrenoidosa* LOX extract oxidized linoleic acid into the 13-HPODE and the minor product 9-HPODE (Zimmerman and Vick, 1973) while a HPL activity cleaved the 13-HPODE or 13-hydroperoxylinolenic acid (HPOTE) into 13-oxo-tridecadienoic acid and pentane or pentene, respectively; other metabolites of 13-HPOTE were 13-HOTE and 12,13-epoxy-9-HODE (Vick and Zimmerman,

1989). Hamberg *et al.* (1992) reported that an enzyme preparation of the red alga *Lithothamnion corallioides* produced 11-HODE as well as smaller amounts of 9-HODE, 13-HODE and 11-keto-octadecadienoic acid from linoleic acid. A HPI acetone powder extract of the red alga *Gracilariopsis lemaneiformis* converted 9- and 13-HPODE to the vicinal diol fatty acids 8,9- and 13,14-DiHODE, respectively (Gerwick *et al.*, 1991).

The specific objective of this work was the development of a procedure for the recovery, purification and characterization of end-products, HPODEs and carbonyl compounds, obtained from the enzymatic activity of partially purified extracts of *F. oxysporum* (Bisakowski *et al.*, 1995a), *Fusarium proliferatum*. (Bisakowski *et al.*, 1995b), *S. cerevisiae* (Bisakowski *et al.*, 1995c), *C. pyrenoidosa* (Bisakowski *et al.* 1995d) using linoleic acid as well as the 9- and 13-HPODEs as model substrates

4.3. Materials and Methods

4.3.1. Preparation of standards

The HPODE standards, the 9- and 13-HPODEs, were prepared according to the procedure described by Kermasha *et al.* (1986). The HPODEs were obtained by the incubation of commercial linoleic acid and soybean LOX (Sigma Chemical Co.). The reaction medium consisted of 22.4 mg linoleic acid in which the final concentration of the substrate in the reaction medium was equal to 2 mM, 0.6 % (v/v) polyoxyethylene sorbitan monolaurate (Tween-20) and sufficient Tris-HCl buffer solution (0.1 M, pH 7.3) to adjust the final volume of the mixture to 40 ml. The reaction medium was held in a temperature controlled water-bath at 25°C and shaked at 100 rpm. The reaction was initiated by adding preincubated soybean LOX suspension (3.3 mg protein) to the reaction medium. A control trial containing all the components except the enzyme preparation was run in tandem. The activity of the soybean LOX was 110,600 U/mg, with 1 U being equal to 0.001 absorbance units/min at pH 9.0 and 25°C. The formation of HPODEs was monitored by the increase in absorbance at 234 nm (Surrey, 1964), using a Beckman DU-650 spectrophotometer (Beckman Instruments). After

20 min, the enzymatic reaction was halted by the addition of 4 N HCl (pH 3.0) and immersed in an ice-bath (10 min).

The HPODEs were subsequently extracted with diethyl ether and all traces of protein and Tween-20 were eliminated by extraction with water. The diethyl ether was evaporated using a gentle stream of nitrogen, and the residual HPODEs were resolubilized in methanol. The presence of HPODEs was confirmed by thin-layer chromatography, using precoated silica gel RP-$18F_{254}S$ plates (5 x 20 cm, 0.25 mm thickness, Alltech Associates Inc., Deerfield, IL) with a solvent system of methanol/water (75:25, v/v). The HPODEs were revealed as brownish-red spots when sprayed with freshly prepared ferrocyanate reagent, a mixture of 10 ml aqueous solution of 4 % ferrous sulfate and 15 ml of 1.3 % ammonium thiocyanate in acetone (Kermasha *et al.*, 1986).

The aldehyde and ketone standards, ethanal, hexanal, 2-octenal, 2-nonenal, decanal, 2-propanone, 2-butanone, 2-hexanone, 4-hexen-3-one, 2-heptanone and 2-octanone, were purchased from Sigma Chemical Co. and converted to the corresponding 2,4-dinitrophenylhydrazones (DNPHs) according to the procedure described by Olías *et al.* (1990). The carbonyl compound solution (1 µl) was diluted in 20 ml sodium phosphate buffer solution (0.1 M, pH 7.0) and incubated at 25°C for 20 min. The mixture was subsequently acidified with 4 N HCl to pH 3.0 and an excess of a 0.4 % of 2,4-dinitrophenylhydrazine (Sigma Chemical Co.) solution (750 µl), prepared in 4 N HCl, was added. The mixture was shaken for 1 min and allowed to stand for 10 min at 25°C. The DNPH-derivatives of carbonyl compounds were then extracted three times with hexane (25 ml); the hexane was evaporated under a gentle stream of nitrogen and the residual extract was resolubilized in methanol.

4.3.2. Preparation of enzyme extracts

The partial purification of LOX extracts was carried according to the procedures described previously for *F. oxysporum* (Bisakowski *et al.*, 1995a), *F. proliferatum* (Bisakowski *et al.*, 1995b), *S. cerevisiae* (Bisakowski *et al.*, 1995c) and *C. pyrenoidosa* (Bisakowski *et al.* 1995d). All four crude microbial extracts were partially purified using

ammonium sulfate precipitation; the *F. oxysporum*, *F. proliferatum*, *S. cerevisiae*, and *C. pyrenoidosa* extracts were obtained at 20-80, 0-40, 20-80 and 40-80 % of saturation, respectively, which resulted in a corresponding 2.66-, 13.3-, 51.8- and 17.5-fold increase in purification. This procedure resulted in an overall higher LOX activity in all four enzymatic extracts; however, the presence of secondary enzymes such as HPL was also detected.

4.3.3. Protein determination

The protein concentration of the enzymatic fractions was determined according to a modification of the Lowry method (Hartree, 1972). Bovine serum albumin (Sigma Chemical Co.) was used as a standard for calibration.

4.3.4. Enzyme assay of lipoxygenase and hydroperoxide-cleaving activity

The LOX activity of the partially purified extracts of *F.oxysporum*, *F. proliferatum*, *S. cerevisiae* and *C. pyrenoidosa* was assayed spectrophotometrically (Beckman DU-650 spectrophotometer) according to the procedure described by Bisakowski *et al.* (1995a), using linoleic acid (33 to 267 μM) as substrate. A control solution, containing all the components minus the enzyme preparation, was run in tandem with these trials. The formation of 9- and 13-HPODEs by LOX activity was measured by an increase in absorbance at 234 nm (Surrey, 1964) due to the presence of a conjugated hydroperoxydiene moiety; however, the absence of this moiety in the 10- and 12-HPODEs allowed detection of these two products only as their respective methyl trimethylsilyloxystearate (MTMS) derivatives using the gas-liquid chromatography/mass spectroscopy (GLC/MS) analyses. The specific activity of LOX (U) was defined as the amount of enzyme giving rise to 0.001 absorbance per min at 234 nm (Ali Asbi *et al.*, 1989).

The partially purified extracts of *F. oxysporum*, *F. proliferatum*, *S. cerevisiae* and *C. pyrenoidosa* were assayed for the presence of secondary enzymes, such as HPI and/or HPL, in a similar way to that for LOX, with certain modifications; the substrate used was the 9- and 13-HPODE mixture (33 μM) and the activity of a cleaving-HPODE enzyme was monitored by the disappearance of HPODEs as indicated by a decrease in the absorbance at 234 nm

(Gardner *et al.*, 1991) as well as by the appearance of an oxodienoic acid as exhibited by an increase in the absorbance at 284 nm (Andrianarison *et al.*, 1989). A control solution, containing all the components minus the enzyme preparation, was also run in tandem with these trials.

4.3.5. Recovery of enzymatic end-products

In order to recover adequate quantities of HPODEs and carbonyl compounds by the activity of the partially purified enzymatic extracts, a scale-up of the enzymatic assay from 3 ml to 20 ml was performed. A control solution, containing all the components minus the enzyme preparation, was run in tandem with these trials.

4.3.6. High-performance liquid chromatography of end-products

The high-performance liquid chromatography (HPLC) system used for the analyses of HPODEs and DNPH-derivatives of carbonyl compounds was Beckman Gold (Beckman Instruments) with computerized integration and data handling (Beckman Model 126), equipped with a Beckman diode-array UV detector (Model 168). Injection was achieved through an automatic injector (Varian, Model 9095, Walnut Creek, CA) fitted with a 20 µl loop. The HPODEs were separated on a reverse-phase Econosil C_{18} column (250 mm x 4.6 mm I.D., Alltech Associates Inc.) and monitored by their specific absorption at 234 nm; the eluant system was a mixture of methanol/water/acetic acid (75:24.95:0.05, v/v/v) at a flow rate of 1 ml/min. The DNPH-derivatives of carbonyl compounds were also separated on the same column using an increasing gradient from 70 to 100 % methanol in water, at a flow rate of 1 ml/min and a specific absorption at 360 nm (Olías *et al.*, 1990).

4.3.7. Derivitization of linoleic acid hydroperoxides

The HPODEs were resolubilized in 100 µl methanol and reduced to HODEs by the addition of 2 ml of 5 % sodium borohydride ($NaBH_4$) solution, with continuous stirring, for 20 min at $0^{\circ}C$ followed by 40 min at room temperature. Deionized water (2 ml) was

subsequently added to the HODE solution and the excess $NaBH_4$ was eliminated by acidification to pH 3.0 with 4 N HCl; the HODEs were extracted with diethyl ether which was evaporated under a gentle flow of nitrogen (Hamberg and Samuelsson, 1967).

The carboxyl group of HODEs was methylated, using the Aldrich MNNG-Diazomethane Kit (Aldrich Chemical Co.). The residual HODEs were dissolved in diethyl ether (3 ml) and placed in the outside tube while 0.5 ml of water and one mmole of 1-methyl-3-nitro-1-nitrosoquanidine (MNNG) were placed in the inside tube. The tubes were clamped and immersed in ice water while 600 µl of a 5 N sodium hydroxide solution was injected via syringe through a septum. The addition of the alkali to the MNNG produced diazomethane gas in the inner tube which was allowed to pass into the outer tube and react for 45 min with the HODEs in diethyl ether. The diethyl ether was then evaporated under a gentle flow of nitrogen (Fales *et al.*, 1973).

The double bonds present in the methylated HODEs were subsequently hydrogenated using the method of Hamberg (1993); the residual products were dissolved in methanol (3 ml) and platinum dioxide (15 mg) was added as a catalyst for hydrogenation under a gentle flow of hydrogen gas and continuous stirring for 20 min at room temperature. The methanol was evaporated under a gentle flow of nitrogen.

Lastly, silylation of hydroxyl groups to trimethylsilanes was performed by resolubilizing the residual extract in pyridine (50 µl) and *N,N-bis* (trimethyl-silyl)-trifluoroacetamide (BSTFA) containing 1 % trimethyl chlorosilane (100 µl) and allowing the reaction to occur for 30 min at 40 ^{o}C (Kermasha *et al.*, 1986). The solution was evaporated under a gentle flow of nitrogen.

4.3.8. Gas-liquid chromatography/mass spectroscopy of end-products

The GLC/MS system used for the analyses of the treated HPODEs and DNPH-derivatives of carbonyl compounds was a HP 6890 Series GC System (Hewlett Packard) with computerized integration and data handling, equipped with a mass selective detector. Injection

was performed through an automatic liquid sampler; the volume analyzed was 1 µl by splitless mode injection set at a ratio of 1:100. Separation of the treated HPODEs and DNPHs was performed on a fused silica capillary SPB-1 column (30 m x 0.25 mm I.D., 0.25 µm film thickness, Supelco Inc., Bellefonte, PA); the initial column temperature was 150 °C and increased at a rate of 3 °C/min to a maximum of 290 °C where it was held for 5 min. Flow rates were set at 30 and 300 ml/min for the hydrogen and oxygen, respectively, while the carrier gas (He) flow rate was maintained at 1.5 ml/min. Injector and detector temperatures were at 290 °C.

4.4. Results and Discussion

4.4.1. Hydroperoxides formed by lipoxygenase activity

The results (Table 4) showed that among the four partially purified enzyme extracts, the specific activity of LOX was highest in the *F. proliferatum* extract. The results also indicate that the second-highest activity was exhibited by the *F. oxysporum* preparation, followed by that from *S. cerevisiae*. Moreover, the lowest enzymatic activity was found in the LOX preparation of *C. pyrenoidosa*; however, since the optimal pH of 4.5 for the *C. pyrenoidosa* extract limited the solubility of the substrate linoleic acid, the enzyme assays were run at the neutral pH of 7.0 where the specific activity was one-seventh of that obtained at the acidic pH.

Figure 13 presents the HPLC elution profile obtained for the HPODEs enzymatically produced by the partially purified extract of *F. oxysporum*; similar elution profiles were obtained for the 9- and 13-HPODE standards as well as the HPODEs produced by the partially purified extracts of *F. proliferatum*, *S. cerevisiae* and *C. pyrenoidosa*. The results show the presence of a major peak "a" at 24 min and two minor shoulder peaks "b" at approximately 25 to 29 min of elution time. Although the 9- and 13-HPODEs were detected by HPLC analysis, the 10- and 12-HPODEs were not as they showed no maximum

Table 4. Specific activity of partially purified lipoxygenase extracts from selected microorganisms.

Microorganism	pH[a]	Specific activity[b]
F. oxysporum	8.0	1691
	10.0	3715
F. proliferatum	6.0	6071
S. cerevisiae	8.0	1004
	10.0	589
C. pyrenoidosa	4.5	725
	7.0	96

[a]The optimal pH of the enzymatic reaction medium.

[b]Specific activity is defined as A/mg protein/min, where A is equal to 0.001 absorbance at 234 nm.

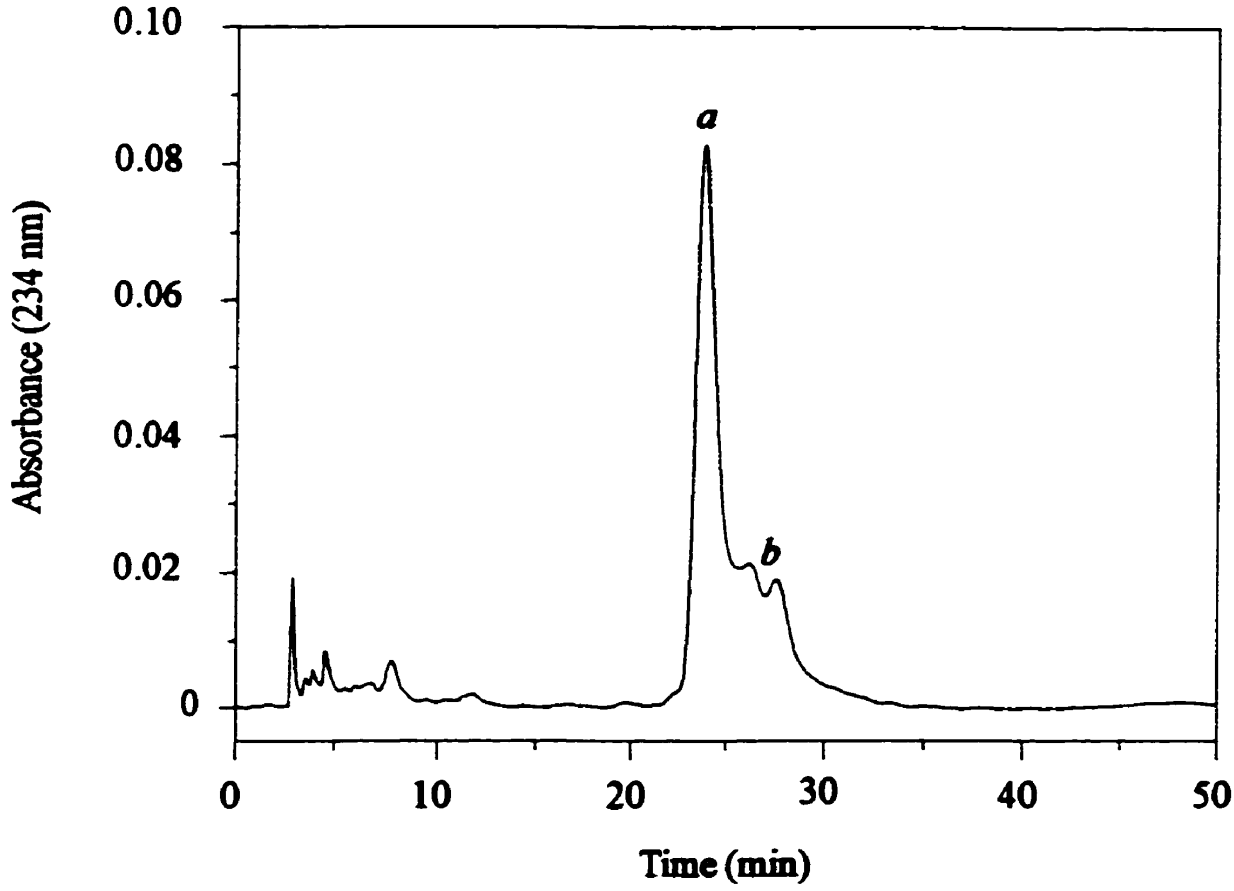

Figure 13. High-performance liquid chromatography elution profile of the hydroperoxides of linoleic acid, produced by the enzymatic extract of *F. oxysporum*.

absorbance in the ultraviolet region (205 to 300 nm) due to the absence of a conjugated diene system (Wurzenburger and Grosch, 1984a).

The GC elution profile of the MTMS derivatives of HPODEs obtained by the *F. oxysporum* LOX extract is shown in Figure 14; the 9- and 13-MTMS standards as well as those produced by the three microbial enzymatic extracts exhibited a similar elution profile. The results demonstrate that the MTMS isomers were eluted by numeric order starting with the 9-MTMS, closely followed by the 10-, 12-MTMS and lastly by the 13-MTMS at 21.30, 21.35, 21.54 and 21.70 min, respectively. The presence of the 10- and 12-HPODEs, as the respective 10- and 12-MTMSs, was first detected by the GC/MS analyses.

Figure 15 shows the mass spectra of the MTMS derivatives of HPODEs indicated in the GC elution profile (Fig. 14). Figures 15A and 15C demonstrate the mass spectra of the 9- and 13-MTMSs, obtained using the *F. oxysporum* extract, as indicated by the characteristic fragmentation patterns resulting from α-cleavage of both sides of the carbon atom to which the trimethylsiloxy group is attached; the results show the presence of the 9-MTMS as indicated by the m/z fragments at 230 and 260 and that of the 13-MTMS as exhibited by the strong signals at 174 and 316 (Beneytout *et al.*, 1989). The results (unshown) indicate that the 9- and 13-MTMSs produced from the 9- and 13-HPODE standards and by the LOX activity of the microbial extracts possessed the same characteristic mass spectra. The results also suggest the presence of a 12-MTMS (Fig. 15B) as shown by the m/e fragments of 188 and 302 (Wurzenburger and Grosch, 1984a) and that of a 10-MTMS (Fig. 15A) as demonstrated by the strong m/z signals at 216 and 274. A similar mass spectrum was also obtained for the 10-MTMS obtained using the *C. pyrenoidosa* extract.

Table 5 shows the relative qualitative production of HPODEs by the four microbial enzymatic extracts. The results indicate that the 9- and 13-HPODE isomers were produced by LOX activity in all four enzymatic extracts; the highest relative proportions were observed in extracts of *F. proliferatum* (45:55) and *S. cerevisiae* (47:53 at pH 8.0 and 45:55 at pH 10.0). The results also indicate that linoleic acid was converted into the 12-HPODE at a proportionate of 21 % by the enzymatic activity of *F. oxysporum* extract. Moreover, the

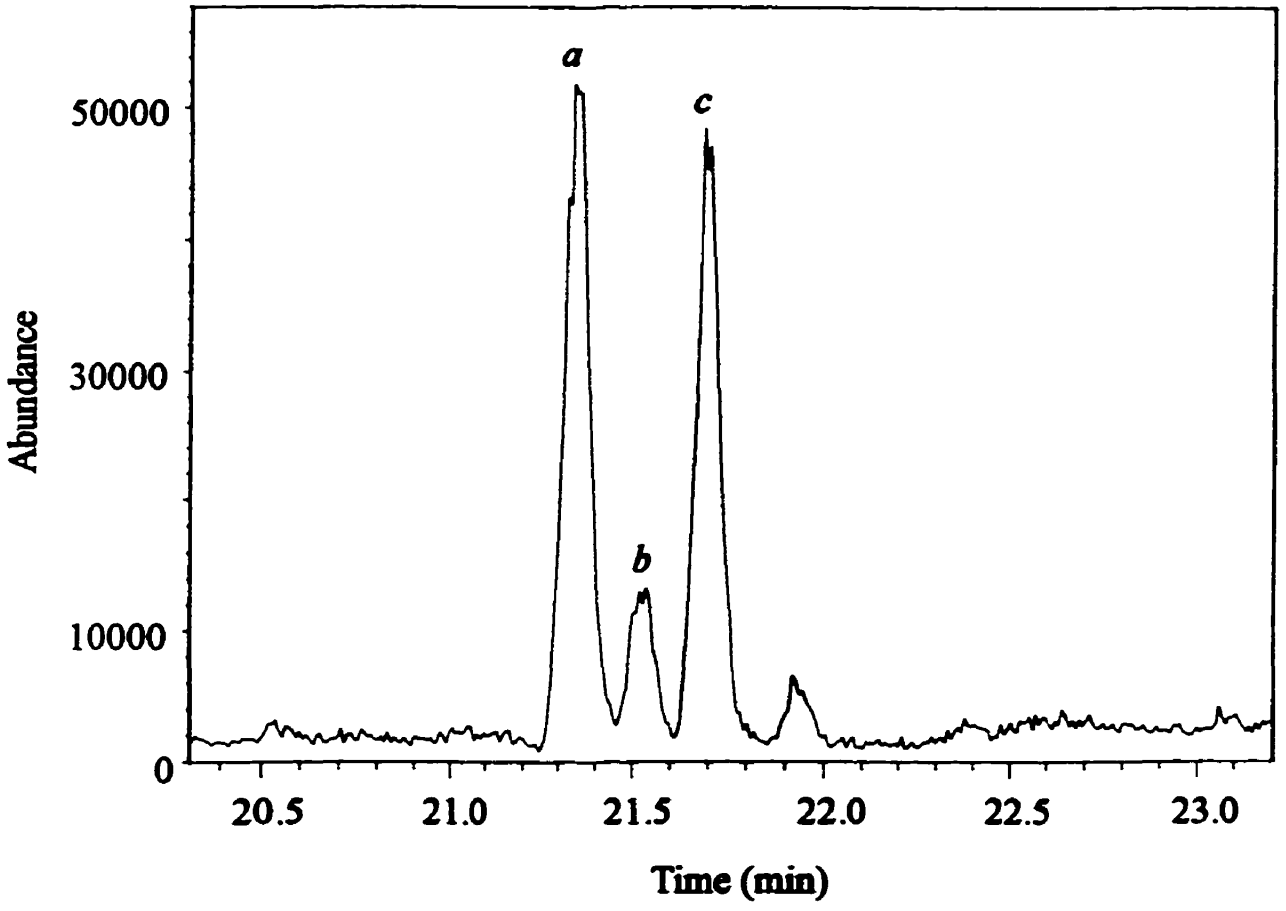

Figure 14. Gas-liquid chromatography elution profile of methyl trimethylsilyloxystearate (MTMS) derivatives of hydroperoxides of linoleic acid: (*a*) 9- and 10-MTMS, (*b*) 12-MTMS and (*c*) 13-MTMS, produced by the partially purified enzymatic extract of *F. oxysporum*.

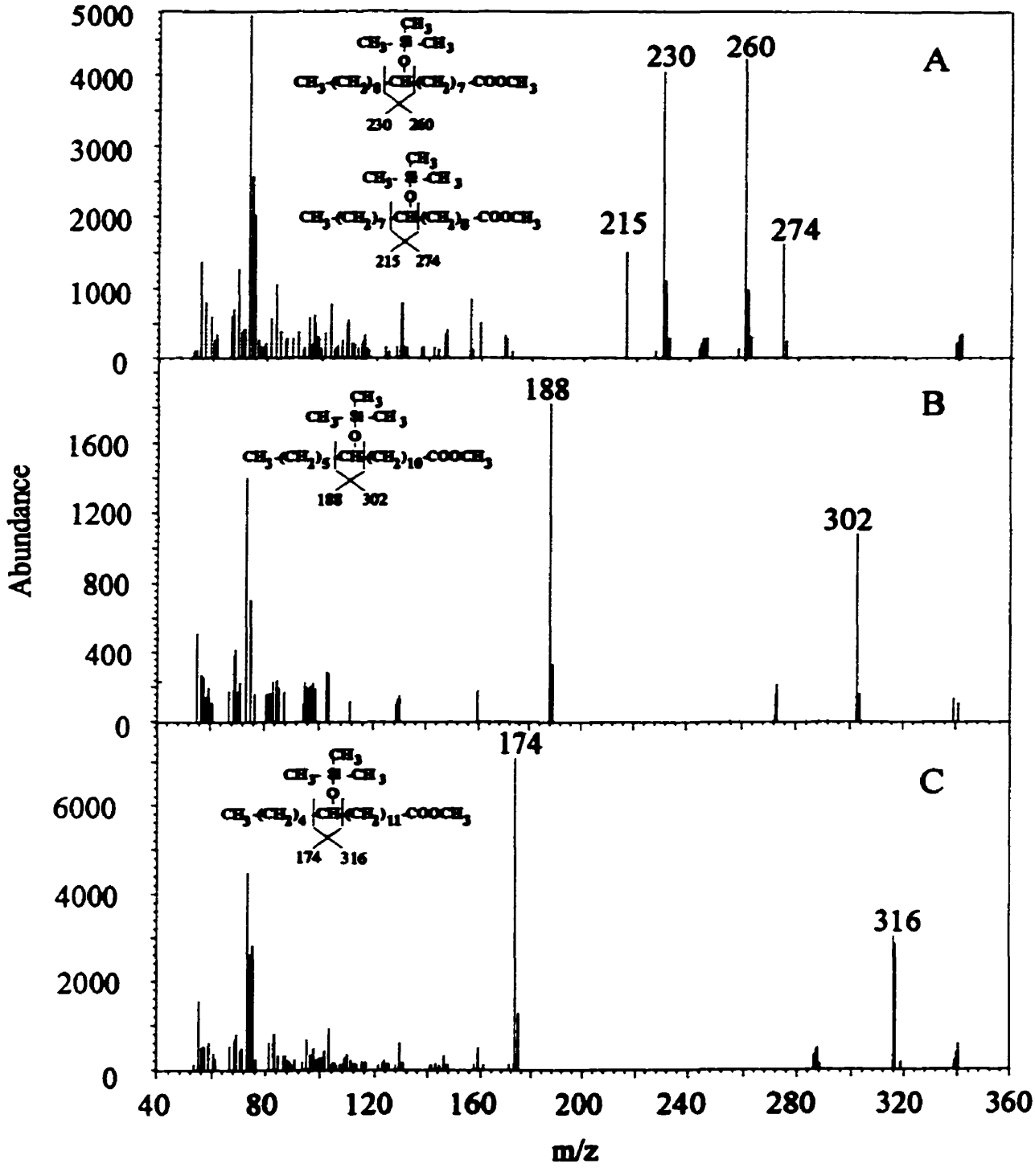

Figure 15. Mass spectrum of methyl trimethylsilyloxystearate (MTMS) derivatives of hydroperoxides of linoleic acid: (A) 9- and 10-MTMS, (B) 12-MTMS and (C) 13-MTMS, produced by the partially purified enzymatic extract of *F. oxysporum*.

Table 5. The gas-liquid chromatography analyses of hydroperoxide isomers produced by the partially purified lipoxygenase extracts from selected microorganisms.

			Relative peak area (%)[a]			
			Hydroperoxide isomers			
Microorganism	pH[b]	HPLC fraction[c]	9	9/10	12	13
F. oxysporum	8.0	a	-[d]	49.7	21.1	29.1
		b	-[d]	52.0	17.1	28.0
	10.0	a	-[d]	43.5	5.2	47.8
		b	31.2	-[d]	-[d]	68.8
F. proliferatum	6.0	a	45.3	-[d]	-[d]	54.7
		b	49.9	-[d]	-[d]	50.1
S. cerevisiae	8.0	a	47.1	-[d]	-[d]	52.9
		b	55.6	-[d]	-[d]	44.4
	10.0	a	45.3	-[d]	-[d]	54.7
		b	-[d]	-[d]	-[d]	-[d]
C. pyrenoidosa	7.0	a	-[d]	53.8	-[d]	46.2
		b	-[d]	-[d]	-[d]	-[d]

[a]The relative percentage peak area was defined as the peak area of the methyl trimethylsilyloxystearate isomer divided by the sum of the methyl trimethylsilyloxystearate isomers, multiplied by 100.

[b]The optimal pH of the enzymatic reaction medium.

[c]Hydroperoxide fractions collected by HPLC as indicated in Figure 13.

[d]No peak detected.

results suggest the presence of a 10-HPODE by LOX activity in the *C. pyrenoidosa* and *F. oxysporum* extracts; however, the 9- and 10-HPODEs were eluted closely together so that the latter could not be relatively compared by its peak area.

The conversion of linoleic acid into 9- and 13-HPODEs by a LOX preparation was reported for *F. oxysporum* (70:30 at pH 9.0 and 56:44 at pH 12.0) (Matsuda *et al.*, 1978) and the thermophilic actinomycete *T. vulgaris* (56:44) (Iny *et al.*, 1993a). However, a (8*R*)-dioxygenase from the fungus *G. graminis* (Brodowsky and Oliw, 1992) and *L. arvalis* (Brodowsky and Oliw, 1993) was found to catalyze the oxygenation of linoleic acid at the eighth carbon to produce a (8*R*)-HPODE, as indicated by the strong signals of the respective 8-MTMS at m/z 243 and 245. A LOX activity was also reported in several mushroom species, that specifically catalyzed the conversion of linoleic acid into a 10-HPODE (Wurzenburger and Grosch, 1984c).

A LOX yeast activity in *S. vini* (Lyudnikova *et al.*, 1984) and the mitochondrial fraction of *S. cerevisiae* (Shechter and Grossman, 1983) was reported to catalyze the bioconversion of linoleic acid into the 9- and 13-HPODEs.

In algal sources, a LOX extract converted linoleic acid into the 9- and 13-HPODEs (48:52) for *Oscillatoria* sp. (Beneytout *et al.*, 1989) and (20:80) for *C. pyrenoidosa* (Zimmerman and Vick, 1973). However, an enzyme preparation of the red *alga L. corallioides* produced the 11-HODE and smaller amounts of 9- and 13-HODE as well as the 11-ketodienone of linoleic acid (Hamberg *et al.*, 1992).

4.4.2. Absorbance spectrum of hydroperoxide conversion

The enzymatic conversion of the 9- and 13-HPODE standards (30:70) (Fig. 16), by the *F. proliferatum* extract, was monitored by a decrease in absorbance at 234 nm attributed to the decomposition of the conjugated hydroperoxydiene moiety and an increase in absorbance at 284 nm due to the formation of ketonic compounds by secondary enzyme activity (Andrianarison *et al.*, 1989); the extracts of *F. oxysporum*, *C. pyrenoidosa* and *S. cerevisiae* demonstrated similar absorbance profiles between 220 to 320 nm. The literature

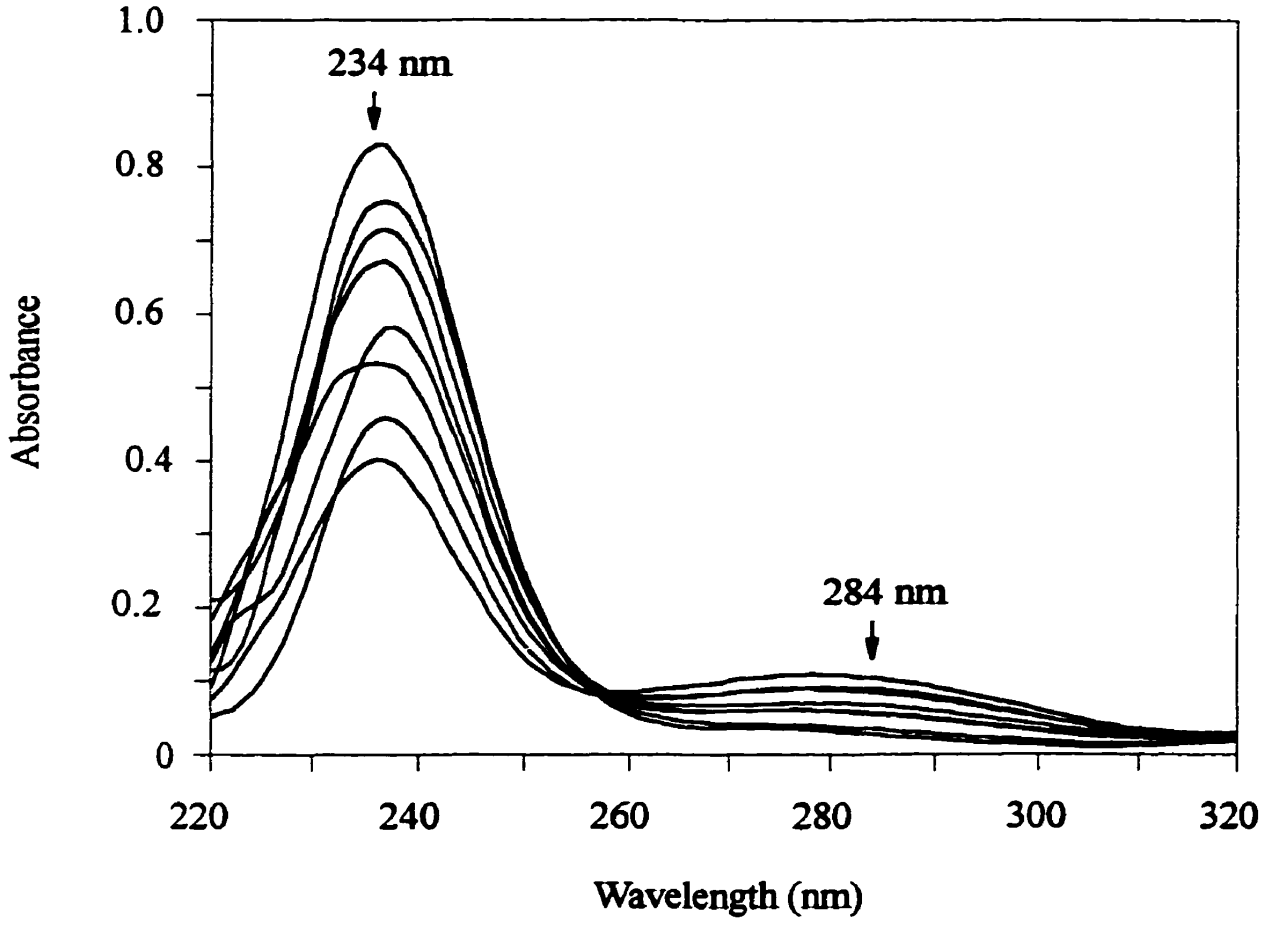

Figure 16. Spectroscopic scanning, at intervals of 0, 1, 5, 10, 15, 30, 45, and 60 min, of the enzymatic end-products obtained after incubation of the partially purified extract of *F. proliferatum* with a mixture (30:70) of 9- and 13-hydroperoxides.

indicated that absorption near 280 nm was characteristic of a conjugated dienone chromophore (Vioque and Holman, 1962) or an oxodienoic acid (Salch *et al.*, 1995).

The absorption profile (Fig. 16) was also reported by Andrianarison *et al.* (1989) for the algae *Oscillatoria* sp. extract, which metabolized linoleic acid into five-carbon compounds and 13-oxotrideca-9,11-dienoic acid; the C5,13-cleaving enzyme activity was attributed to the presence of a HPL in the *Oscillatoria* sp. and the *C. pyrenoidosa* extracts (Vick and Zimmerman, 1989).

The results indicate that the increase in absorbance at 284 nm corresponds to only 20 % of the decrease of that observed at 234 nm (Fig. 16). Consequently, the presence of secondary enzyme activities other than HPL (Gardner *et al.*, 1991) in the partially purified enzymatic extracts could also be responsible for the metabolization of the HPODEs. Kermasha *et al.* (1986) reported the presence of a HPI activity, in a *Phaseolus vulgaris* extract, which was measured by a decrease in absorbance at 234 nm.

4.4.3. Carbonyl compounds formed by hydroperoxide-cleaving activity

Figure 17 presents the HPLC chromatogram, obtained for the *F. oxysporum* enzymatic extract, which shows the elution profile of the DNPH-derivatives of the carbonyl compounds, ranging from four to nine carbons chain length; the DNPH-derivatized carbonyl standards and those produced by the microbial extracts exhibited a comparable elution profile. A similar HPLC separation of DNPH-derivatives of aldehydes was also obtained by Matoba *et al.* (1985), using a soybean enzymatic extract responsible for the synthesis of hexanal. The number of carbons in the DNPH-derivatives of the carbonyl compounds was determined with reference to the retention times of 14, 17, 20 and 26 min which correspond to the DNPH derivative standards of butanone, pentanal, hexanal and nonenal, respectively. The results (Table 6) show that the four enzymatic extracts produced hexanal when allowed to react with a mixture of 9- and 13-HPODEs (30:70) as substrates. The results also indicate that the highest proportion of hexanal was produced by *S. cerevisiae* extract, followed by those of *F. proliferatum* and *F. oxysporum*. Moreover, the results demonstrate that although the extract of *C. pyrenoidosa* indicated the lowest yield (39 %) of hexanal production, it displayed a

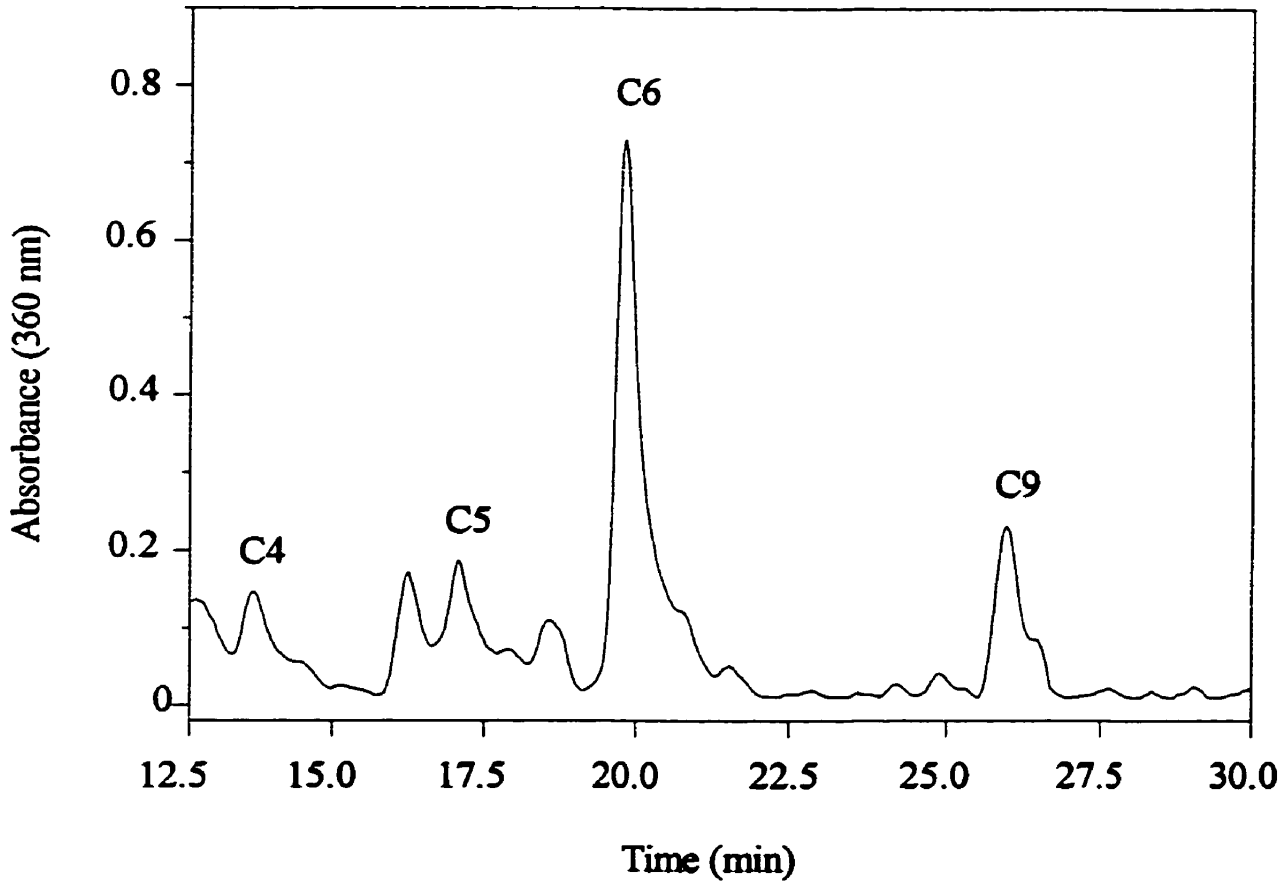

Figure 17. High-performance liquid chromatography elution profile of the dinitrophenylhydrazone-derivatives of carbonyl compounds produced by the partially purified enzymatic extract of *F. oxysporum*.

Table 6. The relative production of carbonyl compounds produced by partially purified lipoxygenase extracts from selected microorganisms.

Microorganism	pH[b]	Relative peak area (%)[a]			
		C_4	C_5	C_6	C_9
F. oxysporum	8.0	5.67	9.94	69.17	15.22
	10.0	5.85	18.61	71.43	4.11
F. proliferatum	6.0	6.75	17.16	76.09	-[c]
S. cerevisiae	8.0	6.87	-[c]	77.02	16.11
	10.0	-[c]	8.47	86.81	4.72
C. pyrenoidosa	7.0	2.67	58.60	38.83	-[c]

[a]The relative percentage peak area was defined as the peak area of the carbonyl compound divided by the sum of the carbonyl compounds, multiplied by 100.

[b]The optimal pH of the enzymatic reaction medium.

[c]No peak detected.

much higher proportion of five carbon-compounds in comparison to the other enzymatic extracts.

However, the results (Table 7) indicate that the highest HPODE-cleaving activity was found to be 1.51 and 1.39 nmol hexanol/mg protein/min in the *F. proliferatum* and *C. pyrenoidosa* extracts, respectively; approximately 15% of this activity was observed in the preparations of *F. oxysporum* and *S. cerevisiae*. The occurrence of nine-carbon carbonyl compounds (Table 6) was detected only in the enzymatic extracts of *F. oxysporum* and *S. cerevisiae* which produced four times the concentration at pH 8.0 than at pH 10.0; the presence of nine-carbon compounds suggests the occurrence of a HPODE-cleaving enzyme (Gardner, 1991) having a substrate specificity for the remaining 9-HPODEs (30 %).

Pradel and Adda (1980) reported that the generation of carbonyl compounds under acidic conditions may be due to the decomposition of HPODEs. Consequently, the quantity of carbonyl compounds, produced as a result of the acidic conditions (pH 3.0) used in our experiment, was taken into consideration by using a control trial containing all the components except the enzyme preparation. The results (unshown) indicated that the acid degradation of 13-HPODE did not produce appreciable yields of hexanal (Gardner *et al.*, 1984) in comparison to those obtained by enzymatic activity.

Figure 18 shows the GC elution profile of the DNPH-derivatized carbonyl compounds obtained using the *F. proliferatum* extract; the DNPH-derivatized carbonyl standards as well as those produced by the microbial extracts showed similar chromatograms. The results demonstrate that a five-carbon carbonyl compound and six-carbon isomeric carbonyl compounds were eluted at 20.23, 24.03 and 22.77 min, respectively.

Figure 19 shows the mass spectra of the DNPH-derivatized carbonyl compounds indicated in the GC elution profile (Fig. 18). The mass-spectrum of the DNPH-derivatized five-carbon carbonyl compound produced by the *F. proliferatum* extract is indicated in Figure 19A; the same mass spectrum was obtained for the DNPH-derivatized five-carbon carbonyl compound produced by the three other microbial extracts. The results suggest the presence of

Table 7. Production of hexanal by partially purified lipoxygenase extracts from selected microorganisms.

Microrganism	pH[a]	Production of hexanal[b]
F. oxysporum	8.0	0.31
	10.0	0.15
F. proliferatum	6.0	1.51
S. cerevisiae	8.0	0.20
	10.0	0.28
C. pyrenoidosa	7.0	1.39

[a]The optimal pH of the enzymatic reaction medium.

[b]Specific activity of a hydroperoxide-cleaving enzyme is defined as nmol hexanal/mg protein/min.

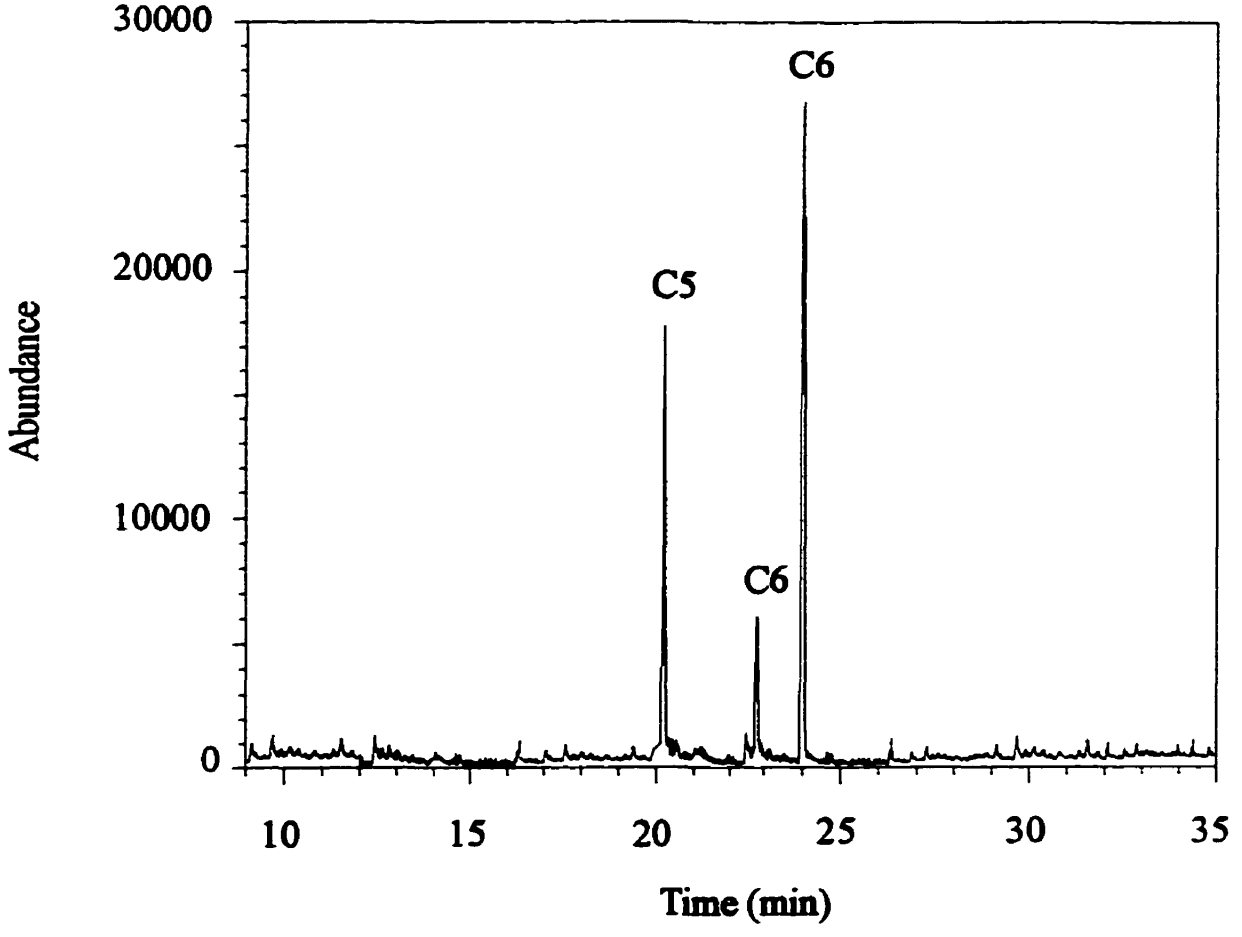

Figure 18. Gas-liquid chromatography elution profile of the dinitrophenylhydrazone-derivatives of carbonyl compounds, produced by the partially purified enzymatic extract of *F. proliferatum*.

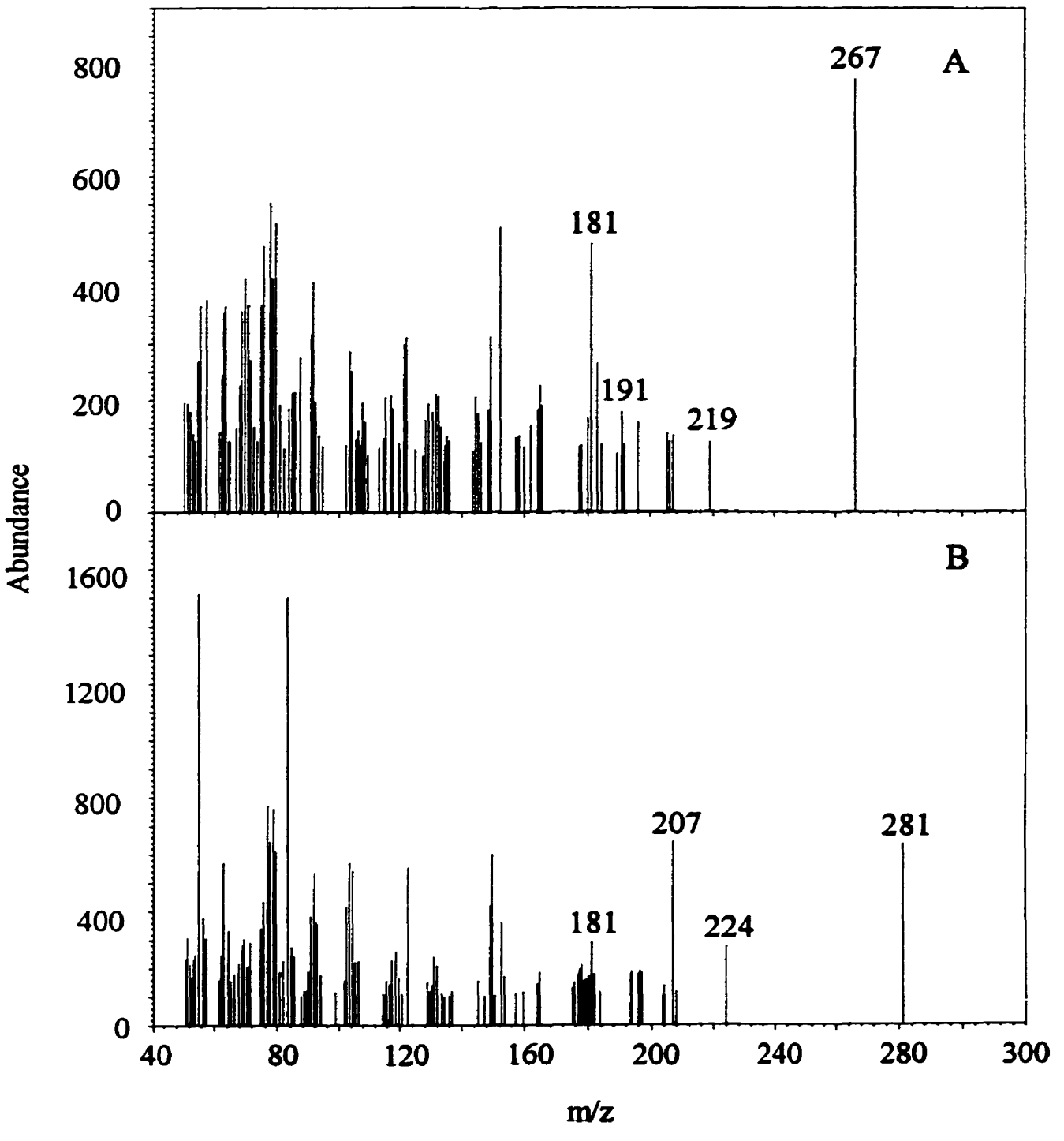

Figure 19. Mass spectrum of dinitrophenylhydrazone-derivatives: (A) a five carbon compound, and (B) a six carbon compound, produced by the partially purified enzymatic extact of *F. proliferatum*.

DNPH-pentanone as indicated by the strong m/z signal at 267 (M^+) and the fragmentation pattern which is identical to that of the DNPH-butanone standard, minus one CH_3-fragment. Further evidence of the presence of DNPH-pentanone is suggested by the GC elution profile of the standards; using the six-carbon carbonyl standards, DNPH-hexanal and DNPH-hexanone, it was observed that the aldehyde eluted one min after the ketone which was the elution pattern repeated for DNPH-pentanone and the DNPH-pentanal standard. The possibility of the five-carbon compound being 2-pentanal was eliminated as its mass spectra did not match that of the DNPH-derivatized pentanal standard. The results (Fig.19B) also show the mass spectra of the DNPH-derivatized six-carbon carbonyl compound produced by the *F. proliferatum* extract; comparable mass spectra were also obtained by the DNPH-derivatized hexanal standard and by those produced using the microbial enzymatic extracts. The results indicate the occurrence of DNPH-hexanal, as demonstrated by a major peak at m/z 281 (M^+). In addition, the results show that DNPH-hexanal had the same retention time and mass-spectra as its respective standard.

Our findings suggest that the HPODE-metabolizing activity in the four enzymatic extracts may be similar to a C6,C12-cleaving HPL activity found in higher plants; this HPL activity was reported to be specific for the 13-HPODE in tomato fruits (Galliard and Matthew, 1977), the 9-HPODE in pear fruit (Kim and Grosch, 1981) and the 9- and 13-HPODEs in cucumber fruit (Galliard *et al.*, 1976). Gardner (1991) reported that this type of HPL produced hexanal and a 12-oxo-9-dodecenoic acid from 13-HPODEs, and nonenal and a 9-oxo-nonanoic acid from 9-HPODEs.

To date, as the authors are aware, there is no report on this type of HPL-like activity in microorganisms. However, another type of HPL, namely a C5,C13-cleaving enzyme, has been reported in algae and two higher plants. In algae, the 13-HPODE was cleaved into pentanol or pentane, and a 13-oxodienoic acid by an enzyme preparation obtained from *Oscillatoria* sp. (Andrianarison *et al.*, 1989) and *C. pyrenoidosa* (Vick and Zimmerman, 1989), respectively. In grass *Agropyron repens* (Berger *et al.*, 1986) and soybean cotyledon (Kondo *et al.*, 1995) extracts, pentenol was identified as a product of a C5,13-cleaving HPL

activity. Another type of HPL, a C8,10-cleaving enzyme, was also reported in mushrooms, requiring a 10-HPODE as substrate. Wurzenberger and Grosch (1984a) reported a LOX extract from *Psalliota bispora* that converted linoleic acid to 10-HPODE which was then cleaved by a HPL thereby producing 1-octen-3-ol responsible for the characteristic flavor of mushrooms (Cronin and Ward, 1971) and 10-oxo-8-decenoic acid (Wurzenburger and Grosch, 1982). The presence of these two enzymes was also suggested by the formation of C8 volatile alcohols and ketones, such as 1-octen-3-ol, 3-octanol, 2-octen-1-ol and 1-octanol, and 3-octanone in extracts *Agaricus bisporus* (Mau *et al.*, 1992) and *Asperigillus flavus* (Kaminski *et al.*, 1972).

4.5. Conclusion

The results gathered in this study indicated that the partially purified extracts of *F. proliferatum*, *F. oxysporum*, *S. cerevisiae* and *C. pyrenoidosa* demonstrated the presence of a LOX activity which had certain similarities in terms of HPODE production to that reported from other microbial sources. However, the enzyme preparations also produced a more wider range of HPODE isomers. In addition, the occurrence of a HPODE-cleaving activity in the four microbial enzymatic extracts was also suggested by the production of hexanal.

CHAPTER V

CHARACTERIZATION OF PURIFIED LIPOXYGENASE EXTRACTS FROM *FUSARIUM PROLIFERATUM*

5.1. Abstract

A crude lipoxygenase (FI) extract from *Fusarium proliferatum* was partially purified by ammonium sulfate precipitation at 0-40 % of saturation (FII). The purification of fraction FII by size-exclusion chromatography resulted in three major peaks, FIIIa, FIIIb and FIIIc. Fraction FIIIa demonstrated the highest specific lipoxygenase activity as well as the highest recovery and was therefore further purified. The purification procedure resulted in one major fraction (FIV) by ion-exchange chromatography. Fraction FIV showed the presence of one major protein band and two minor ones in both the native and sodium dodecyl sulfate polyacrylamide electropheorograms. Optimal lipoxygenase activity was shown to be at pH 6.0 for fractions FIIIa, FIIIb, FIIIc and FIV; however, fractions FIIIa and FIIIc also exhibited an optimal pH at 10.0, while FIIIb and FIV at pH 7.5 and 10.5, respectively. The K_m values, obtained from Lineweaver-Burk plots, were 2.5×10^{-6} M (FIIIa, pH 6.0), 4.7×10^{-6} M (FIIIa, pH 10.0), 4.2×10^{-6} M (FIIIb, pH 6.0), 7.5×10^{-6} M (FIIIb, pH 7.5), 3.1×10^{-6} M (FIIIc, pH 6.0), 4.7×10^{-6} M (FIIIc, pH 10.0), 11.0×10^{-6} M (FIV, pH 6.0) and 3.9×10^{-6} M (FIV, pH 10.5). Fractions FIIIa and FIIIc demonstrated greater preference towards linoleic acid as substrate than linolenic and arachidonic acids at pH 6.0 and 10.0; in addition, these fractions showed very little activity towards mono-, di- and trilinolein. In contrast, fraction FIIIb possessed higher substrate specificity towards mono-, di- and trilinolein at pH 6.0 while at pH 7.5, it preferred trilinolein; with respect to the free fatty acids, fraction FIIIb preferred linoleic acid in comparison to linolenic and arachidonic acids at both pH optimas. Fraction FIV demonstrated approximately 2.5 times more activity towards mono- and dilinolein and 1.5 times more with linolenic and arachidonic acids than that exhibited towards linoleic acid at pH 6.0; however, at pH 10.5, this enzymatic fraction possessed an overall preference towards linoleic acid. The purified fractions FIIIa, FIIIb and FIV produced mainly the 13-HPODEs from linoleic acid at pH 6.0, 7.5 and 6.0 respectively; however, the same fractions produced

the 9- and 13-HPODEs at a ratio of approximately 1:1 at pH 10.0, 6.0 and 10.5, respectively. In addition the presence of a LOX activity producing the 10- and 12-HPODEs was also suggested in fractions FIIIa (pH 6.0 and 10.0), FIIIb (pH 6.0) and FIV (10.5); however, this activity was not detected in fractions FIIIb and FIV at pH 7.5 and 6.0, respectively.

5.2. Introduction

Lipoxygenase (EC 1.13.11.12) is an enzyme which metabolizes linoleic acid and other polyunsaturated fatty acids containing a *cis, cis*-1,4-pentadiene moiety to hydroperoxy fatty acids by hydrogen abstraction from the methylene carbon and antarafacial insertion of molecular oxygen (Yamamoto, 1992). Lipoxygenase (LOX) has been reported in plant (Mack *et al.*, 1987), animal (Yamamoto, 1983) and microbial (Mukherjee, 1951; Shimahara and Hashizume, 1973) sources. Theorell *et al.* (1947) were the first to isolate and purify the enzyme from soybeans; however, it was in the 1970s that Axelrod and coworkers first separated and purified individual LOX isozymes from soybean seeds (Axelrod, 1974). In most sources, LOX exists as several isozymes which can differ significantly in properties such as optimum pH, substrate specificity, and end-products (O'Connor and O'Brien, 1991).

LOX-like activity was first described in several microorganisms, including, *Aspergillus* and *Penicillium* (Mukherjee, 1951), *Pseudomonas* and *Achromobacter* (Shimahara and Hashizume, 1973); however, apart from studies performed on plant and animal sources, little is known about LOX isozymes in micro-organisms. Two LOX purified fractions were reported from *Saccharomyces cerevisiae* (Shechter and Grossman, 1983) and the thermophilic actinomycete, *Thermoactinomyces vulgaris* (Iny *et al.*, 1993a). A LOX isozyme was also purified from *Fusarium oxysporum* (Matsuda *et al.*, 1976). In addition, a purified LOX preparation was obtained from the green algae, *Oscillatoria* sp. (Beneytout *et al.*, 1989) and red algae *Gracilariopsis lemaneiformis* (Hamberg and Gerwick, 1993).

LOX plays an important role with respect to the taste and flavor of food since the resultant end-products of the LOX-catalyzed reaction, i.e. hydroperoxides (HPODEs), can be converted to volatile compounds such as alcohols, ketones and aldehydes that attribute to the organoleptic properties of food (Sessa, 1979).

The specific objective of this work was the development of a procedure for the purification and the characterization of LOX isozymes from *F. proliferatum* (Bisakowski *et al.*, 1995b) in terms of pH, kinetic parameters, substrate specificity towards linoleic, linolenic and arachidonic acids as well as mono-, di- and trilinolein, end-product specificity using linoleic acid as a model substrate, and the native and sodium dodecyl sulfate (SDS) electrophoretic profile.

5.3. Material and Methods

5.3.1. Culture growth and harvesting conditions

The biomass culture of *F. proliferatum* was grown for three days on 2 liters of modified Shoun medium, consisting of a mixture of 2 g $NaNO_3$, 0.2 g $MgSO_4$ $7H_2O$, 2 g soya flour, 1 ml mineral salt solution, 0.5 g yeast extract, 1 g $(NH_4)_2HPO_4$, and 125 ml soya oil, at 27°C and 85 rpm (Shoun *et al.*, 1983). The fungal mycelia were harvested and washed with deionized water followed by a sodium phosphate buffer solution (0.01 M, pH 7.0). The fungal mycelia were then suspended in sodium phosphate buffer solution (0.01 M, pH 7.0) and homogenized according to the procedure of Bisakowski *et al.* (1995b). The enzyme extract was successively defatted with cold (-20°C) acetone and diethyl ether (Kermasha and Metche, 1986) to remove the lipids thereby eliminating their interference in the proceeding steps of purification. The defatted enzyme extract was suspended (1:10, w/v) in sodium phosphate buffer solution (0.01 M, pH 7.0) and subjected to mechanical stirring for 16 h. All purification procedures were performed at 4°C unless otherwise indicated. The suspension was centrifuged (12,000 xg, 15 min) and the supernatant was subjected to DNA precipitation with the use of protamine sulfate (Badaracco *et al.*, 1983). The subsequent suspension was centrifuged (40,000 xg, 10 min) and the supernatant, considered to be the crude enzyme extract (FI), was subjected to partial purification by the addition of solid ammonium sulfate at 40 % of saturation (Bisakowski *et al.*, 1995b), thereby obtaining the partially purified LOX extract (FII).

5.3.2. Size-exclusion chromatography

The purification of the partially purified extract (FII) was carried out by size-exclusion chromatography, using a Superose-12 10/30 column (Pharmacia) and the Fast Protein Liquid Chromatography (FPLC, Pharmacia) system. The column was equilibrated with sodium phosphate buffer solution (0.01 M, pH 7.0) and the sample (50 mg protein/ml) was solubilized in 200 µl of the buffer solution. Elution was performed at a flow rate of 0.5 ml/min and 1 ml fractions were collected. The separated fractions were desalted by dialysis in sodium phosphate buffer solution (0.001 M, pH 7.0) and concentrated by lyophilization.

5.3.3. Ion-exchange chromatography

The main fraction obtained by size-exclusion chromatography was subjected to ion-exchange chromatography (IEC) on the Mono Q HR 5/5 ion-exchange column (Pharmacia) using the FPLC system. The column was pre-equilibrated with sodium phosphate buffer solution (0.005 M, pH 7.0; Buffer A), and the sample (125 mg protein/ml) was solubilized in 200 µl of the sodium phosphate buffer solution (0.005 M, pH 7.0). A linear gradient of buffer A and buffer B (Buffer A containing 1 M NaCl) was used for elution at a flow rate of 1 ml/min. The separated fractions of 1 ml were desalted by dialysis in sodium phosphate buffer solution (0.001 M, pH 7.0) and concentrated by lyophilization.

5.3.4. Protein determination

The protein concentration of the enzymatic fractions was determined according to a modification of the Lowry method (Hartree, 1972). Bovine serum albumin (Sigma Chemical Co.) was used as a standard for calibration.

5.3.5. Substrate preparation

The substrate selectivity of the LOX isozymes was determined using standards, purchased from Sigma Chemical Co. (St-Louis, MO), which included linoleic acid (*cis*-9,*cis*-12-octadecadienoic acid), linolenic acid (*cis*-9,*cis*-12,*cis*-15-octadecatrienoic acid), arachidonic acid (5,8,11,14-eicosatetraenoic acid), monolinolein (1-mono[(*cis*,*cis*)-9,12-

octadecadienoyl]-rac-glycerol), dilinolein (1,3-di[(*cis*,*cis*)-9,12-octadecenoyl]-rac-glycerol) and trilinolein (1,2,3-tri[(*cis*,*cis*,*cis*)-9,12,15-octadecadienoyl]-rac-glycerol). Stock solutions were prepared at a concentration of 4×10^{-3} M, as described previously (Kermasha and Metche, 1986).

5.3.6. Enzyme assay

The LOX activity of the purified extracts of *F. proliferatum* was assayed spectrophotometrically (Beckman DU-650 spectrophotometer) according to the procedure described by Bisakowski *et al.* (1995b) using linoleic acid (0.2×10^{-6} to 6×10^{-6} M) as substrate. A control solution, containing all the components minus the enzyme preparation, was run in tandem with these trials. The formation of 9- and 13-HPODEs by LOX activity was measured by an increase in absorbance at 234 nm (Surrey, 1964) due to the presence of a conjugated hydroperoxydiene moiety; however, the absence of this moiety in the 10- and 12-HPODEs allowed the detection of these two products only as their respective methyl trimethylsilyloxystearate (MTMS) derivatives using the gas-liquid chromatography/mass spectroscopy (GLC/MS) analyses. The specific activity of LOX was defined as the increase in U (mg of protein)$^{-1}$min^{-1}, where U is equal to 0.001 absorbance at 234 nm (Ali Asbi *et al.*, 1989).

5.3.7. Polyacrylamide gel electrophoresis

Native polyacrylamide gel electrophoresis (PAGE) was performed using the PhastSystem Unit (Pharmacia). The electrophoretic run and staining of the separated protein bands were performed in accordance to the procedure described previously by Bisakowski *et al.* (1995b). Sodium dodecyl sulfate (SDS) PAGE was performed in a similar manner as described above for the native PAGE; however, the standards and samples were treated with SDS (2.5 %) and mercaptoethanol (5 %) at 100°C (10 min) and PhastGel SDS buffer strips (Pharmacia), consisting of 0.20 M tricine, 0.20 M Tris (pH 8.1) and 0.55 % SDS in 3 % agarose isoelectric focusing buffer, were used.

5.3.8. Preparation of hydroperoxide standards

The HPODE standards, the 9- and 13-HPODEs, were prepared according to the procedure described by Kermasha *et al.* (1986). The HPODEs were obtained by the incubation of commercial linoleic acid and soybean LOX (Sigma Chemical Co.). The reaction medium consisted of 22.4 mg linoleic acid in which the final concentration of the substrate in the reaction medium was equal to 2 mM, 0.6 % (v/v) polyoxyethylene sorbitan monolaurate (Tween-20) and sufficient Tris-HCl buffer solution (pH 7.3, 0.1 M) to adjust the final volume of the mixture to 40 ml. The reaction medium was held in a temperature controlled water-bath at 25°C and shaked at 100 rpm and the HPODEs were subsequently extracted with diethyl ether.

5.3.9. Recovery of hydroperoxides

In order to recover adequate quantities of HPODEs by the activity of the purified enzymatic extracts, a scale-up of the enzymatic assay from 100 µl to 3 ml was performed. A control solution, containing all the components minus the enzyme preparation, was run in tandem with these trials.

5.3.10. High-performance liquid chromatography of end-products

The high-performance liquid chromatography (HPLC) system used for the analyses of HPODEs was Beckman Gold (Beckman Instruments) with computerized integration and data handling (Beckman Model 126), equipped with a Beckman diode-array UV detector (Model 168). Injection was achieved through an automatic injector (Varian, Model 9095, Walnut Creek, CA) fitted with a 20 µl loop. The HPODEs were separated on a reverse-phase Econosil C_{18} column (250 mm x 4.6 mm I.D., Alltech Associates Inc.) and monitored by their specific absorption at 234 nm; the eluant system was a mixture of methanol/water/acetic acid (75:24.95:0.05, v/v/v) at a flow rate of 1 ml/min.

5.3.11. Derivitization of linoleic acid hydroperoxides

The HPODEs were resolubilized in 100 µl methanol and reduced to HODEs by the

addition of sodium borohydride ($NaBH_4$) solution according to the procedure described by Bisakowski *et al.* (1997).

The carboxyl group of HODEs was methylated, using the Aldrich MNNG-Diazomethane Kit (Aldrich Chemical Co.) and the double bonds were subsequently hydrogenated using platinum dioxide (15 mg) and a gentle flow of hydrogen gas (Bisakowski *et al.*, 1997).

Lastly, silylation of hydroxyl groups to trimethylsilanes was performed by resolubilizing the residual extract in pyridine (50 µl) and *N,N-bis* (trimethyl-silyl)-trifluoroacetamide (BSTFA) containing 1 % trimethyl chlorosilane (100 µl) and allowing the reaction to occur for 30 min at 40°C (Kermasha *et al.*, 1986).

5.3.12. Gas-liquid chromatography/mass spectroscopy of end-products

The GLC/MS system used for the analyses of the treated HPODEs was a HP 6890 Series GC System (Hewlett Packard Co., Palo Alto, CA) with computerized integration and data handling, equipped with a mass selective detector. Separation of the treated HPODEs was performed on a fused silica capillary SPB-1 column (30 m x 0.25 mm I.D., 0.25 µm film thickness; Supelco Inc., Bellefonte, PA), using the conditions described by Bisakowski *et al.* (1997).

5.3.13. Capillary electrophoresis analyses

The capillary electrophoresis (CE) system used was the P/ACE System 5510 from Beckman Instruments Inc. (Beckman Instruments, Fullerton, CA). Electrophoresis separations were achieved using a capillary of 37 x 50 µm I.D., an applied voltage of 20.1 kV and UV detection at 230 nm, at a temperature setting of 15°C. The running buffer was a High pH buffer solution (pH 8.0) containing 15 mM β-cyclodextrin as outlined in the manual (Beckman, 1994). Samples were injected for 5 sec, using a pressure of 20 psi and each analysis lasted 30 min. Between each analysis, the capillary was washed with 0.1 M HCl for 0.5 min, followed by a 2 min wash with water and a 2 min rinse with buffer.

5.4. Results and Discussion

5.4.1. Enzyme purification

The results (Fig. 20) show that by using size-exclusion chromatography, the partially purified LOX extract was separated into nine fractions, FIIIa-FIIIi; however, the main LOX activity was exhibited in the first three enzymatic extracts, FIIIa, FIIIb and FIIIc possessing 88 % of the total activity (data unshown). Table 8 indicates that fraction FIIIa exhibited 80.1 % of the total activity present in fraction FIII and a 10-fold increase in purification with respect to the crude fraction FI. The results also show that FIIIa possessed 1.4 and 4 times higher specific activity than that for fractions FIIIb and FIIIc, respectively, as well as 4.9 and 23 times the respective recoveries. On the basis of these findings, fraction FIIIa was selected for further purification using IEC.

Figure 21 indicates that the IEC of fraction FIIIa resulted in one main fraction (FIV) which eluted at 11 min. In addition, fraction FIV had a specific activity of 6636 U/mg protein/min which was 13 times higher than that showed by the crude extract and an increase of 13-fold in purification.

Matsuda *et al.* (1976) reported a LOX isozyme from *F. oxysporum* which exhibited a 465-fold increase in purification and 21 % recovery. A LOX preparation from the green algae, *Oscillatoria* sp. (Beneytout *et al.*, 1989) was purified 81-fold and had a recovery of 0.089 %. Two active LOX fractions from *T. vulgaris* (Iny *et al.*, 1993b) were separated using acetate (pH 5.0) and borate (pH 9.0) buffers; the former had a recovery and purification factor of 10.1 % and 9.2-fold, respectively, while those of the latter were three times higher. In addition, the acetate fraction (pH 5.0) from *S. cerevisiae* (Shechter and Grossman, 1983) exhibited an overall purification of 6.5-fold and recovery of 26.9 % while the phosphate (pH 6.85) fraction was purified 9.7-fold and showed a recovery of 44.9 %. Shimahara and Hashizume (1973) reported a 25-fold purified LOX-like enzymatic fraction from *Pseudomonas aeruginosa*. The discrepancy regarding the various degrees of purification of LOX isozymes could be due to the different methods used for the extraction and purification

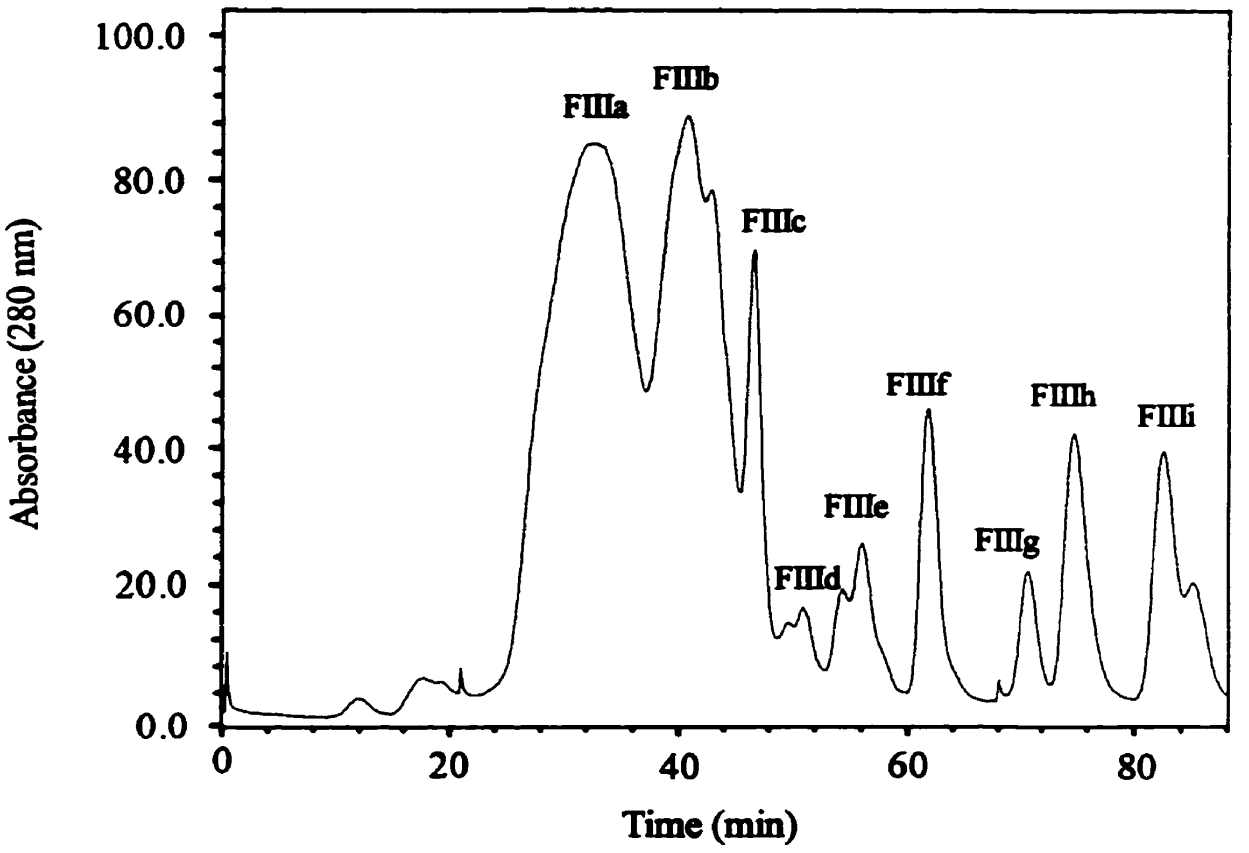

Figure 20. Profile of purification of the partally purified lipoxygenase FII from *F. proliferatum* on size-exclusion Superose-12 HR 10/30 column chromatography, using the FPLC system.

Table 8. Purification scheme of lipoxygenase from *Fusarium proliferatum*.

Fraction	Total protein (mg)[a]	Specific activity[b]	Total activity[c]	Recovery (%)	Purification (fold)
Crude (FI)	112706	506	57029236	100.00	1
Ammonium sulfate precipitation					
0-40 % (FII)	3026	3538	10705988	18.86	7
Size-exclusion chromatography					
FIIIa	77	5057	389389	0.69	10
FIIIb	23	3511	80753	0.14	7
FIIIc	13	1210	15730	0.03	2
Ion-exchange chromatography					
FIV	5	6636	33180	0.06	13

[a] Protein was determined according to a modification of the Lowry method (Hartree, 1972), using bovine serum albumin as a standard.

[b] Specific activity is defined as A/mg protein/min, where A is equal to 0.001 absorbance at 234 nm.

[c] Unit of activity is defined as A/min, where A is equal to 0.001 absorbance at 234 nm.

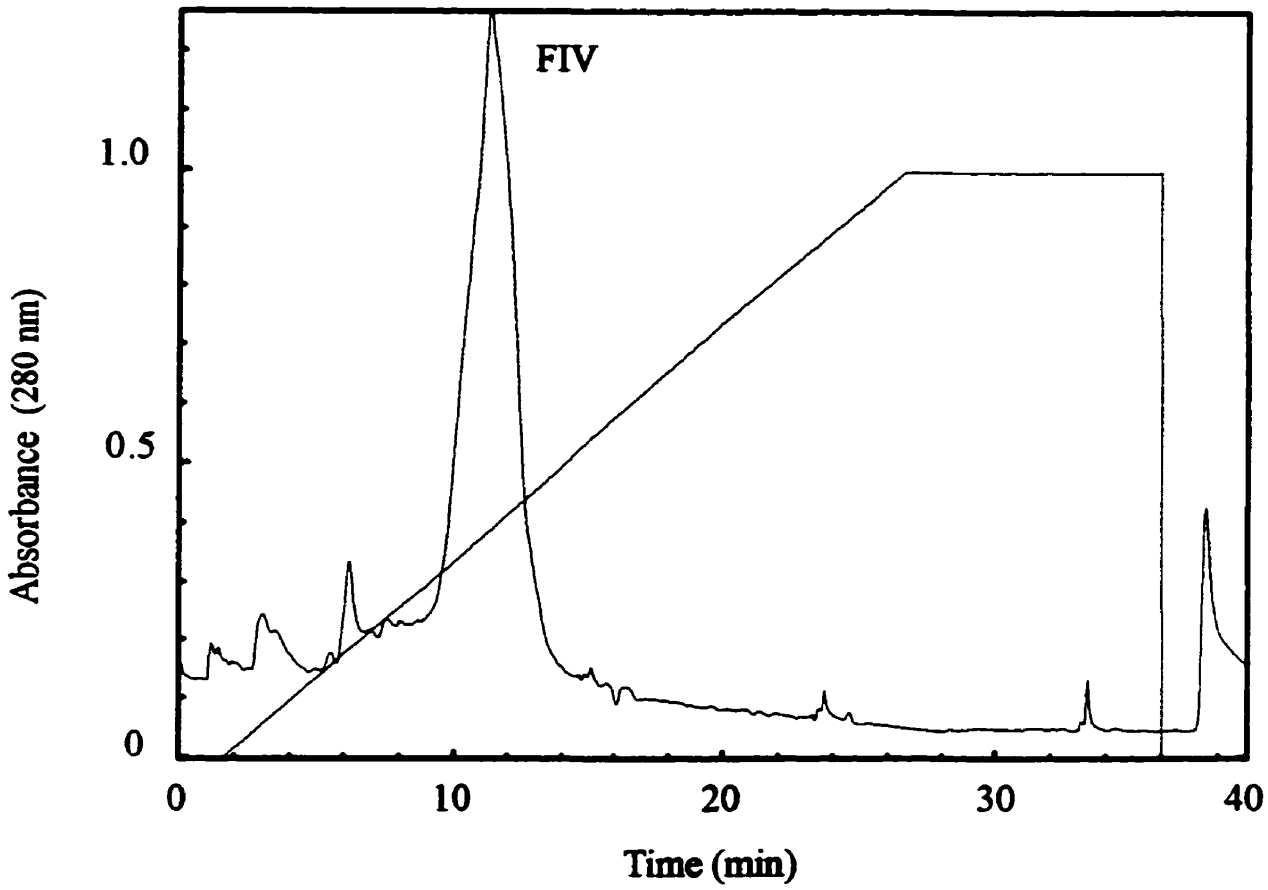

Figure 21. Profile of ion-exchange purification of fraction FIIIa, obtained from size-exclusion chromatography, on the Mono Q HR 5/5 column chromatography, using the FPLC system.

of these enzymes from various sources (Whitaker, 1972).

5.4.2. Optimum pH

Figure 22 demonstrates the influence of pH on the LOX activity of purified fractions. Fractions FIIIa, FIIIb, FIIIc and FIV demonstrated an optimal activity at the more neutral pH of 6.0; the partially purified fraction FII (Bisakowski *et al.*, 1995b) also exhibited maximal LOX activity at this pH. In addition, the presence of optimal LOX activity was exhibited at pH 10.0 for fractions FIIIa and FIIIc, 10.5 for fraction FIV, and pH 7.5 for fraction FIIIb. The enzymatic extract of fraction FIIIa possessed the highest specific activity at its two pH optimas, followed by that of the more purified fraction FIV; this relative loss of enzymatic activity during the course of purification is not straightforward due to the fragile nature of LOXs. The reason for enzyme activity loss is unknown, however, proteolysis and loss of catalytic iron after exposure to oxygen in the absence of reducing agents have been implicated (Percival, 1991). Some LOXs are therefore purified under anaerobic conditions (Carroll *et al.*, 1993).

A purified LOX extract from *T. vulgaris* (Iny *et al.*, 1993b) exhibited maximal activity at pH 6.0 and a relatively lower one at pH 11.0 which could be indicative of the presence of two isozymes. A purified LOX extract from *S. cerevisiae* (Shechter and Grossman, 1983) and *Oscillatoria* sp. (Beneytout *et al.*, 1989) exhibited maximal activity at pH 6.3 and 8.8, respectively. A pH of 10.8-11.0 was reported for the isolated LOX from *P. aeruginosa* (Shimahara and Hashizume, 1973) and the *Bacillus* species (Shimahara, 1964), while the purified isozyme from *F. oxysporum* exhibited an optimum of activity at pH 12.0, with a shoulder peak at pH 10.0 (Matsuda *et al.*, 1976).

5.4.3. Enzyme specificity

Table 9 shows that fractions FIIIa and FIIIc exhibited a strong substrate specificity towards linoleic acid at pH 6.0 and 10.0 in comparison with the other substrates. In contrast, fraction FIIIb showed an increased preference towards monolinolein (140 %), dilinolein (122 %) and trilinolein (115 %) at pH 6.0 and trilinolein (112 %) at pH 7.5 in comparison to

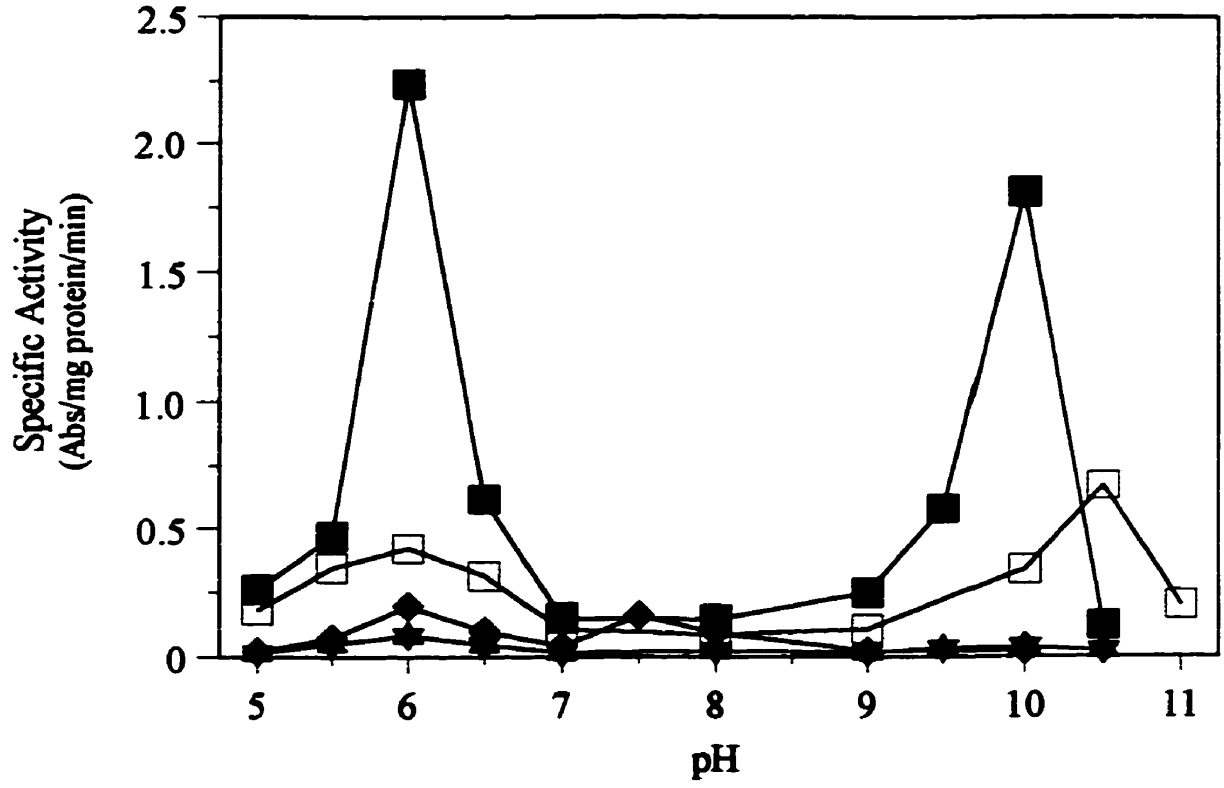

Figure 22. The effect of pH on the lipoxygenase activity of the purified isozymes from *F. proliferatum*: FIIIa (—■—), FIIIb (—◆—), FIIIc (—✱—) and FIV (—□—).

Table 9. Substrate specificity of the purified lipoxygenase fractions from *Fusarium proliferatum*.

		Relative lipoxygenase activity[a]					
		Substrate					
Fraction	pH	Linoleic acid	Linolenic acid	Arachidonic acid	Monolinolein	Dilinolein	Trilinolein
FIIIa	6.0	100	44	38	12	28	46
	10.0	100	52	11	3	9	1
FIIIb	6.0	100	65	92	140	122	115
	7.5	100	54	58	91	44	112
FIIIc	6.0	100	30	21	13	6	8
	10.0	100	74	10	21	6	19
FIV	6.0	100	167	154	250	222	67
	10.5	100	27	12	68	80	71

[a] Relative activity is defined as the percentage of specific activity compared to that obtained with linoleic acid, where specific activity is defined as A/mg protein/min, where A is equal to 0.001 absorbance at 234 nm.

linoleic acid (100 %); fraction FIIIb was also more active towards linoleic acid in comparison to linolenic and arachidonic acids, at both pH optimas. The more purified ion-exchange LOX fraction (FIV) demonstrated an overall increased activity, at pH 6.0, towards linolenic (167 %) and arachidonic (154 %) acids as well as monolinolein (250 %) and dilinolein (222 %), but was considerably less active towards trilinolein (71 %); however, at pH 10.5, fraction FIV showed a strong preference for linoleic acid (100 %) over linolenic (27 %) and arachidonic (12 %) acids but exhibited similar activity towards monolinolein (68 %), dilinolein (80 %) and trilinolein (71 %). The partially purified extract FII of *F. proliferatum* (Bisakowski *et al.*, 1995b) exhibited maximal activity towards linoleic acid and approximately 37 % of that activity towards mono-, di- and trilinolein.

The purified LOX extract from *S. cerevisiae* (Shechter and Grossman, 1983) demonstrated similar activity towards linoleic and arachidonic acids as substrates but only 40 % of that activity towards linolenic acid. The purified enzyme extract from *T. vulgaris* (Iny *et al.*, 1993b) showed substrate specificity towards linoleic acid (100 %) but little activity towards linolenic (33 %) and arachidonic (22 %) acids. The LOX isozyme from *F. oxysporum* possessed 13.3 times higher activity towards linoleic acid in comparison to linolenic acid (Matsuda *et al.*, 1976).

5.4.4. Kinetic studies

Table 10 shows the V_{max} and K_m values obtained for the purified LOX preparations using the best straight line determined by the Lineweaver-Burk plots (Lineweaver and Burk, 1934). Fraction FIIIa exhibited the highest V_{max} values of 13.9 and 20.6 μmol/mg protein/min at pH optimas of 6.0 and 10.0, respectively, followed by fraction FIV which showed V_{max} values of 8.4 and 6.6 μmol/mg protein/min at pH optimas of 6.0 and 10.0, respectively. The lowest values of 1.0 and 2.7 μmol/mg protein/min were displayed by the activity of fraction FIIIb, at pH 6.0 and 7.5, respectively. The purified factions FIIIa (pH 6.0 and 10.0), FIIIb (pH 7.5), FIIIc (pH 6.0 and 10.0) and FIV (6.0 and 10.5) exhibited higher V_{max} values in comparison to the V_{max} value of 1.6 μmol/mg protein/min of the partially purified extract. In

Table 10. Kinetic parameters, obtained by the Lineweaver-Burk plot, for the purified lipoxygenase fractions of *Fusarium proliferatum*.

Fraction	pH	K_m (10^{-6} M)	V_{max}[a]
Size-exclusion chromatography			
FIIIa	6.0	2.5	13.9
	10.0	4.7	20.6
FIIIb	6.0	4.2	1.0
	7.5	7.5	2.7
FIIIc	6.0	3.1	4.0
	10.0	4.7	3.4
Ion-exchange chromatography			
FIV	6.0	11.0	8.4
	10.5	3.9	6.6

[a]The V_{max} value is expressed as μmol hydroperoxide/mg protein/min.

addition, the purified fractions FIIIa, FIIIb, FIIIc and FIV all showed a considerably higher affinity towards linoleic acid as substrate as indicated by their lower K_m values in comparison to the partially purified extract whose K_m value was 5.15×10^{-5} M.

Higher K_m values of 1.69×10^{-3} and 1.0×10^{-3} M were obtained for the LOX isozyme from *F. oxysporum* (Matsuda *et al.*, 1976) and an enriched LOX preparation from *T. vulgaris* (Iny *et al.*, 1993b), respectively. Shechter and Grossman (1983) reported a K_m value of 2.86×10^{-4} M for the purified LOX extract from *S. cerevisiae*.

5.4.5. Electrophoresis

Figure 23A shows the native PAGE electrophoretic pattern of the enzymatic fractions. The purification of the *F. proliferatum* extract FII by size-exclusion chromatography resulted in the isolation of fraction FIIIa which is comprised of seven protein bands. In addition, IEC of fraction FIIIa resulted in the isolation of one major protein LOX fraction FIV; the efficiency of the purification of FIV is indicated by the presence of one major protein band and two minor faint ones in both native and SDS-PAGE (Fig. 23B).

The two purified LOX fractions from *S. cerevisiae* (Shechter and Grossman, 1983) showed three bands on polyacrylamide gel, a major one and another two weak bands. In addition, both purified LOX preparations from *T. vulgaris* (Iny *et al.*, 1993b) demonstrated the presence of one major protein and some traces of another protein on polyacrylamide gel electrophoresis. Matsuda *et al.* (1976) reported that the purified isozyme from *F. oxysporum* exhibited only one band on polyacrylamide gel in the presence and absence of SDS.

5.4.6. Enzymatic end-products

Figure 24 shows the typical HPLC elution profile of the HPODEs produced by the enzymatic fraction FIIIa; similar HPODE elution profiles were obtained for the purified fractions FIIIb and FIV as well as the partially purified extract (FII) of *F. proliferatum* (Bisakowski *et al.*, 1997). The HPODEs eluted as one major peak "*a*" and two minor peaks

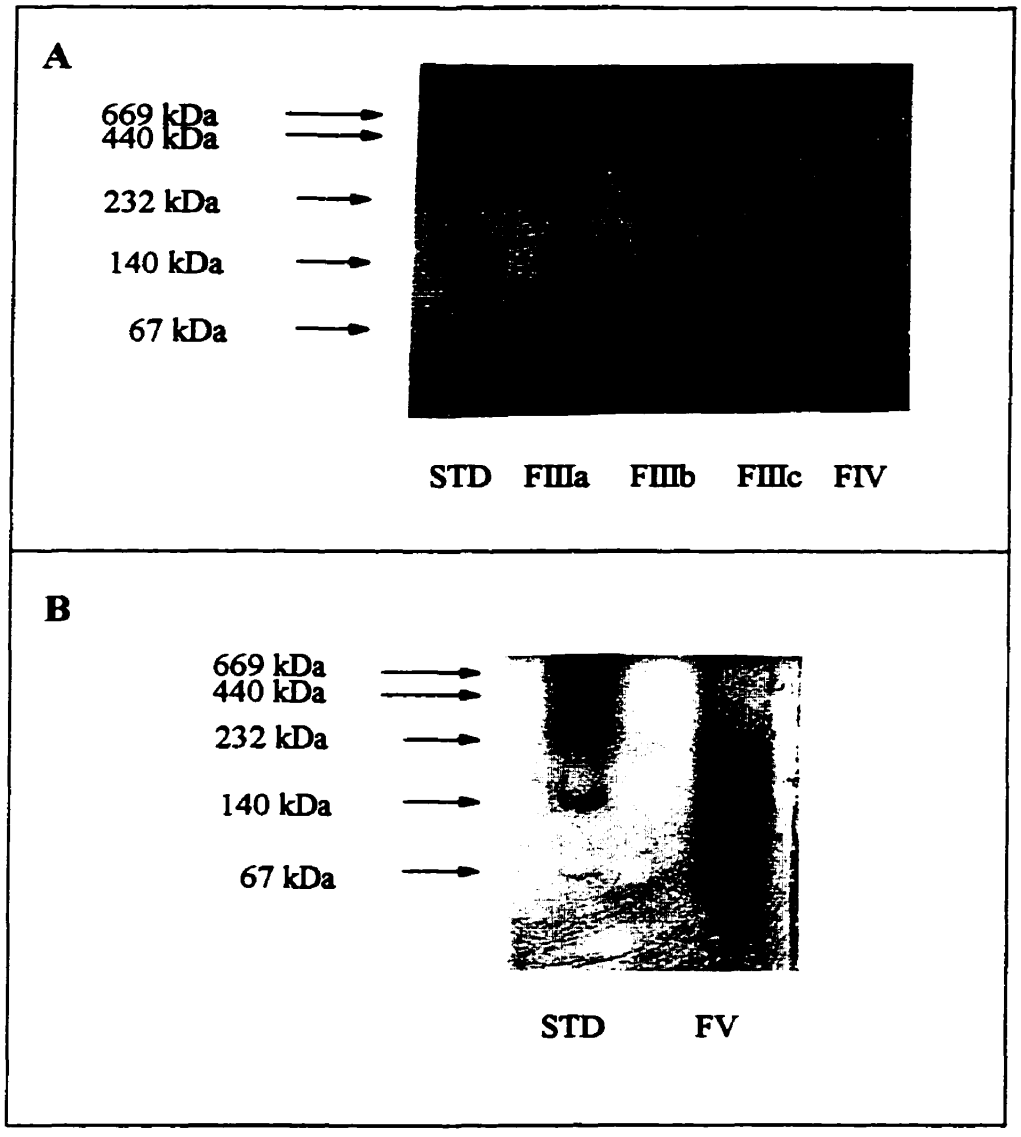

Figure 23. (A) Electrophoreogram of the native polyacrylamide gel and (B) electrophoreogram of the sodium dodecyl sulfate polyacrylamide gel of the purified lipoxygenase fractions obtained from *Fusarium proliferatum* by size-exclusion chromatography (FIIIa, FIIIb, FIIIc) and ion-exchange chromatography (FIV), using the FPLC system.

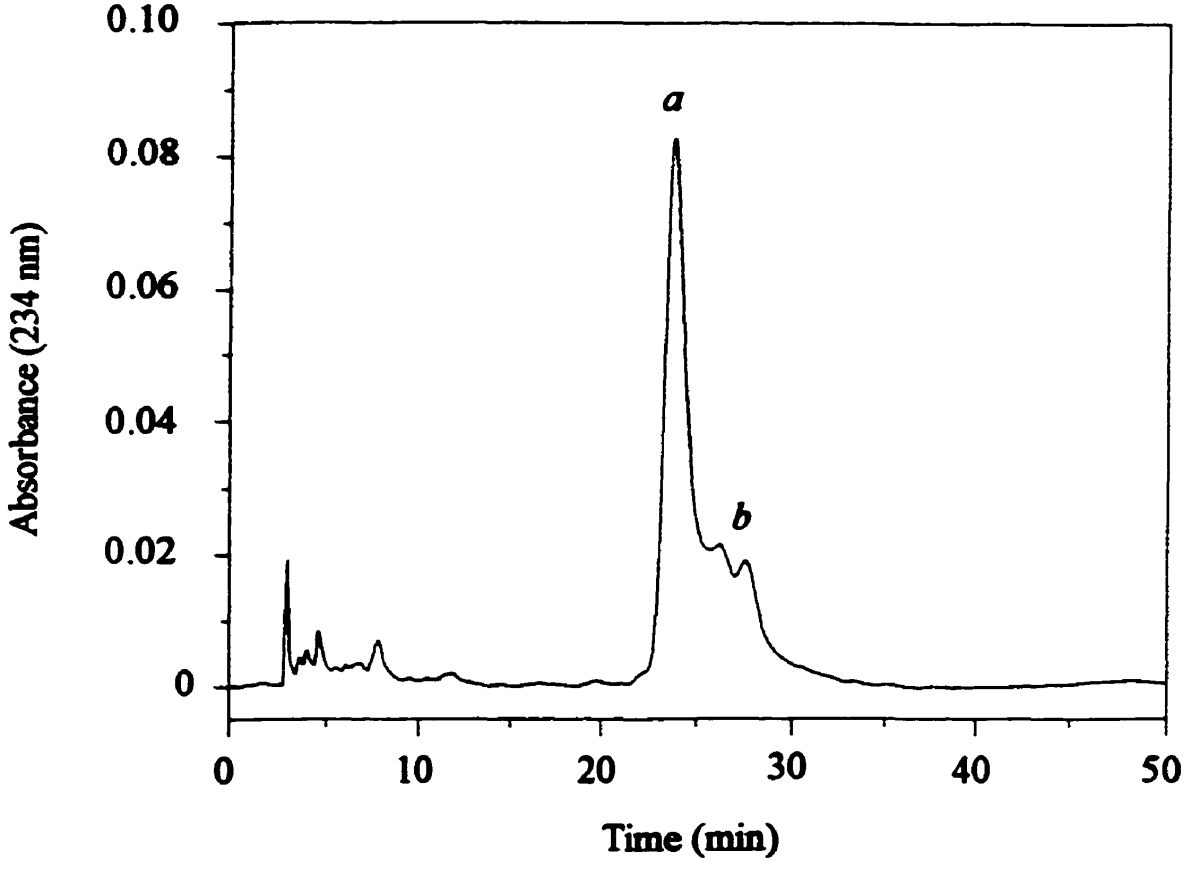

Figure 24. High-performance liquid chromatography elution profile of the hydroperoxides of linoleic acid, produced by the enzymatic extract FIIIa, obtained by size-exclusion chromatography, from *F. proliferatum*.

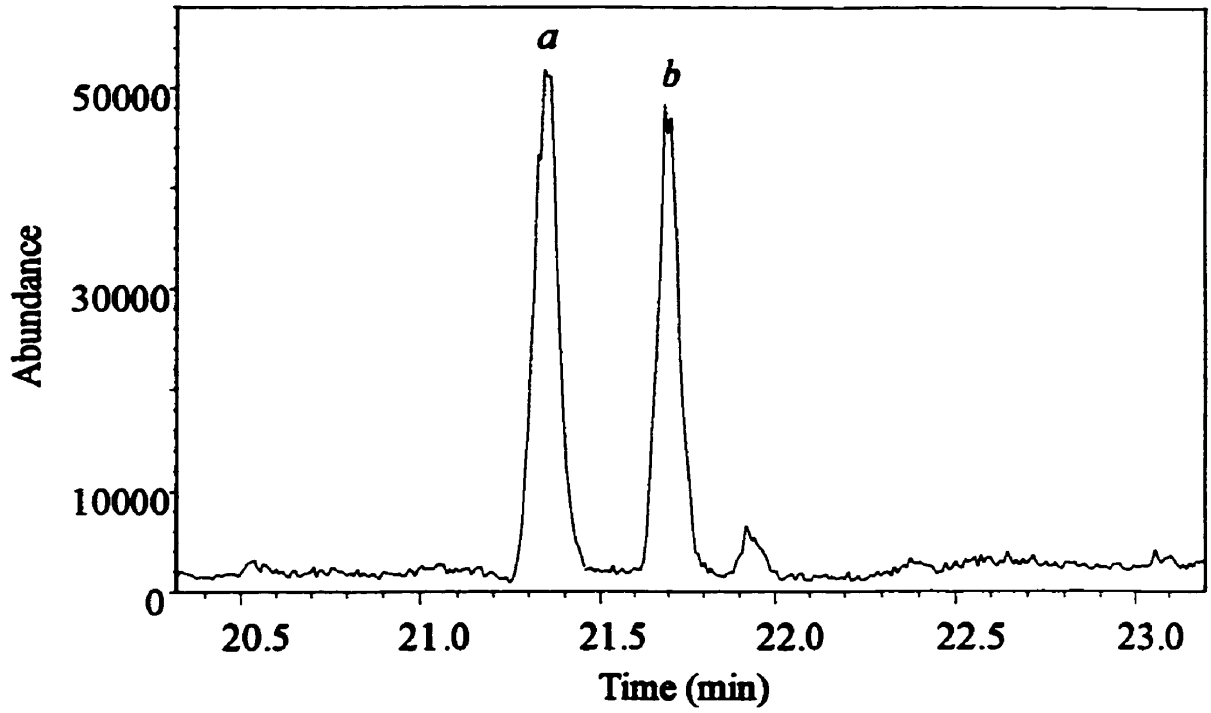

Figure 25. Gas-liquid chromatography elution profile of methyl trimethylsilyloxystearate (MTMS) derivatives of hydroperoxides of linoleic acid: (*a*) 9- and 10-MTMS and (*b*) 13-MTMS, produced by the enzymatic extract FIIIa, obtained by size-exclusion chromatography, from *F. proliferatum*.

"*b*" and "*c*"; the former was selected for further study as it was considered to be the major HPODE fraction.

The GLC elution profile of the MTMS derivatives of HPODEs obtained by the LOX activity of the purified fraction FIIIa is shown in Figure 25; the purified fractions FIIIb and FIV as well as the partially purified extract (FII) of *F. proliferatum* (Bisakowski *et al.*, 1997) exhibited a similar elution profile. The results demonstrate that the two MTMS derivatives of the HPODE isomers were eluted by numeric order starting with the 9-MTMS derivative of the HPODE and closely followed by the 13-MTMS of the HPODE at 21.30 and 21.70 min, respectively.

The mass spectra for the 9- and 13-MTMS derivatives of HPODEs, produced using the purified fractions FIIIa, FIIIb and FIV of *F. proliferatum*, was similar to those obtained by the partially purified extract (FII) (Bisakowski *et al.*, 1997). The presence of the 9- and 13-MTMS derivatives of the HPODEs was indicated by the characteristic fragmentation patterns resulting from α-cleavage of both sides of the carbon atom to which the trimethylsiloxy group is attached; the results show the presence of the 9-MTMS as indicated by the m/e fragments at 230 and 260 and that of the 13-MTMS as exhibited by the strong signals at 174 and 316 (Matsuda *et al.*, 1978; Beneytout *et al.*, 1989). The results (unshown) indicate that the 9- and 13-MTMSs produced from the 9- and 13-HPODE standards and by the LOX activity of the purified LOX fractions FIIIa, FIIIb and FIV possessed the same characteristic mass spectra.

Table 11 shows the relative qualitative production of HPODEs by the purified LOX fractions. The results show that fractions FIIIa, FIIIb and FIV produced the 9- and 13-HPODE isomers at a ratio of approximately 1:1 at pH 10.0, 6.0 and 10.5, respectively. The partially purified fraction FII (Bisakowski *et al.*, 1997) showed similar results to fractions FIIIa and FIV as it also produced the 9- and 13-HPODEs at a ratio of 45.3 to 54.7 %. However, the results also indicate a LOX activity in fractions FIIIa, FIIIb and FIV which produced the 13-HPODE as the predominant isomer at pH 6.0, 7.5 and 6.0, respectively. The production of the different ratios of HPODE isomers suggests the presence of at least two LOX activities in each purified fraction.

Table 11. The gas-liquid chromatography analyses of hydroperoxide isomers, produced by the purified extracts of *Fusarium proliferatum*.

Fraction	pH	Relative peak area (%)[a] Hydroperoxide isomers 9	13
Size-exclusion chromatography			
FIIIa	6.0	-[b]	100.0
FIIIa	10.0	46.2	53.8
FIIIb	6.0	39.1	60.9
FIIIb	7.5	-[b]	100.0
Ion exchange chromatography			
FIV	6.0	8.1	91.9
FIV	10.5	47.4	52.6

[a]The relative percentage peak area was defined as the peak area of the methyl trimethylsilyloxystearate isomer divided by the sum of the methyl trimethylsilyloxystearate isomers, multiplied by 100.

[b]No peak detected.

Matsuda *et al.* (1976) reported the conversion of linoleic acid into 9- and 13-HPODEs by a LOX preparation from *F. oxysporum* (70:30 at pH 9.0 and 56:44 at pH 12.0). Iny *et al.* (1993a) showed that the LOX extract from the thermophilic actinomycete *T. vulgaris* produced the 9- and 13-HPODEs at a ratio of 56:44. A LOX yeast activity in *Saccharomyces vini* (Lyudnikova *et al.*, 1984) and the mitochondrial fraction of *S. cerevisiae* (Shechter and Grossman, 1983) was reported to catalyze the bioconversion of linoleic acid into the 9- and 13-HPODEs. In addition, a LOX extract converted linoleic acid into the 9- and 13-HPODEs (48:52) for *Oscillatoria* sp. (Beneytout, 1989) and (20:80) for *Chlorella pyrenoidosa* (Zimmerman and Vick, 1973).

Figure 26 shows the CE elution profile of the HPODEs produced by the purified fractions FIIIa, FIIIb and FIV as well as the standards 9- and 13-HPODEs. The results show that the HPODEs were eluted in numeric order beginning with the 9-HPODE and ending with the 13-HPODE with respective retention times of 27.7 and 33 min. The results also indicate the presence of a 10- and 12-HPODE as suggested by the minor peak between the 9- and 13-HPODEs possessing a retention time of 31.5; the presence of this minor peak remained undetected by GC as its concentration was too low.

Table 12 shows the relative qualitative production of HPODEs by the purified LOX fractions as detected using CE. The results demonstrate that the relative proportions of HPODE isomers detected using CE is similar to that obtained using GC. However, due to the increased sensitivity of CE, fractions FIIIa (pH 6.0) and FIIIb (pH 7.5) also show the presence of the 9-HPODE isomer at a relative peak area of 34.9 and 27.9 %, respectively. In addition, purified fractions FIIIa (pH 6.0), FIIIa (pH 10.0), FIIIb (pH 6.0) and FIV (pH 10.5) also suggest the presence of a LOX activity which produced the 10- and 12-HPODE at a relative peak area of 12.8, 5.1, 16.3 and 10.2 %, respectively. However, fractions FIIIb (pH 7.5) and FIV (pH 6.0) did not exhibit the presence of such a LOX activity.

A LOX activity was also reported in several mushroom species, that specifically catalyzed the conversion of linoleic acid into a 10-HPODE (Wurzenburger and Grosch,

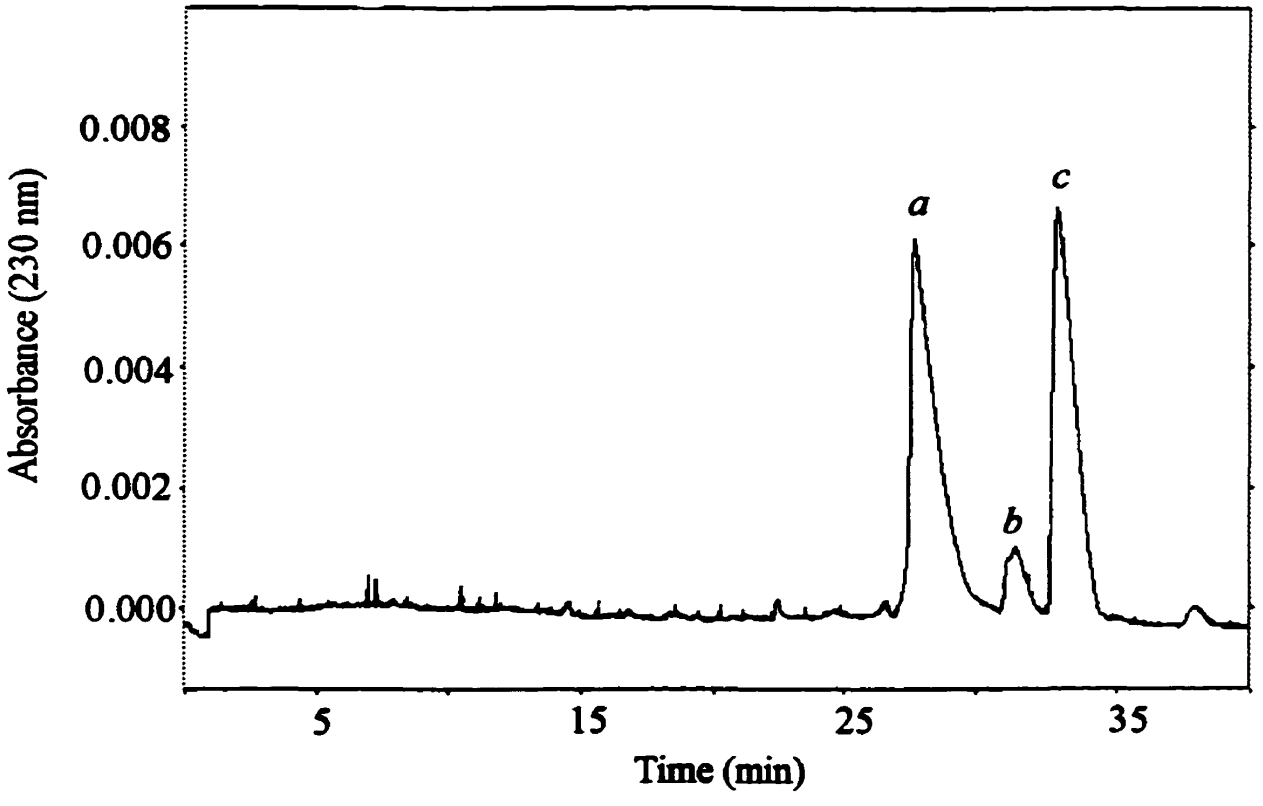

Figure 26: Capillary electrophoresis elution profile of hydroperoxides (HPODEs) of linoleic: (*a*) 9-HPODE, (*b*) 10- and 12-HPODE and (c) 13-HPODE, produced by th eenzymaticextract (FIIIa) from *F. proliferatum*

Table 12. The capillary electrophoresis chromatography analyses of hydroperoxide isomers, produced by the purified extracts of *Fusarium proliferatum*.

		Relative peak area (%)[a]		
		Hydroperoxide isomers		
Fraction	pH[b]	9	10/12	13
Size-exclusion chromatography				
FIIIa	6.0	34.9	12.8	52.3
FIIIa	10.0	45.4	5.1	49.4
FIIIb	6.0	35.0	16.3	48.7
FIIIb	7.5	27.9	-[b]	72.1
Ion exchange chromatography				
FIV	6.0	-[b]	-[b]	100.0
FIV	10.5	39.6	10.2	50.1

[a]The relative percentage peak area was defined as the peak area of the hydroperoxide divided by the sum of the hydroperoxide isomers, multiplied by 100.

[b]No peak detected.

1984c) while an enzyme preparation of the red alga *Lithothamnion corallioides* produced the 11-HODE and smaller amounts of 9- and 13-HODE as well as the 11-ketodienone of linoleic acid (Hamberg *et al.*,1992).

5.5. Conclusion

The results indicated that the purification of the partially purified extract of *F. proliferatum* resulted in the separation of several purified LOX activities which differed in terms of their pH optima, substrate specificity and end-product specificity. Several of these purified LOX fractions exhibited similar characteristics, in terms of HPODE production and a strong preference towards linoleic acid as substrate, to LOX extracts from other sources. However, the results also show that an unique LOX activity was isolated as suggested by the biocatalysis of various HPODE isomers and its strong substrate specificity towards mono-, di- and trilinolein.

General Conclusion

The results showed the presence of lipoxygenase (LOX) activity in the biomass cultures of *Fusarium oxysporum*, *Fusarium proliferatum*, *Saccharomyces cerevisiae*, and *Chlorella pyrenoidosa*. The partial purification of the crude enzymatic extracts resulted in an increase in LOX activity. The LOX activity in the partially purified extracts from *F. oxysporum* and *S. cerevisiae* exhibited two pH optimas while that from *F. proliferatum* and *C. pyrenoidosa* showed only one. The enzymatic extract for *F. proliferatum* showed the highest LOX activity followed by those of *F. oxysporum* and *S. cerevisiae* while the lowest LOX activity was exhibited in the *C. pyrenoidosa* extract.

The results also showed that the enzymatic activities from the four partially purified microbial extracts demonstrated an overall preference towards linoleic acid, followed by linolenic acid. In addition, the LOX activity from the *F. proliferatum* extract showed a strong preference towards the glycerol fatty acid esters.

The partially purified enzymatic preparations were assayed with linoleic acid, as substrate, which was bioconverted into 9- and 13-hydroperoxides (HPODEs) by the LOX activity in all four extracts; in addition, the LOX activity in the *F. oxysporum* extract produced the 10- and 12-HPODEs from linoleic acid while that of the *C. pyrenoidosa* extract produced only the 10-HPODE. When assayed with the 9- and 13-HPODEs, as substrates, the microbial extracts exhibited secondary enzyme activities, one of which produced hexanal. The highest hexanal-producing activity was found to be in the *F. proliferatum* and *C. pyrenoidosa* extracts, while those of *F. oxysporum* and *S. cerevisiae* exhibited the lowest activity. The *C. pyrenoidosa* extract also showed the highest production of pentanone.

The results showed that the LOX activity from *F. oxysporum* and *S. cerevisiae* was non-competitively inhibited by potassium cyanide (KCN). In addition, KCN was an uncompetitive inhibitor of the LOX activity from *C. pyrenoidosa*. In contrast, the results showed that the enzyme activity from *F. proliferatum* remained relatively stable at high KCN concentrations. The presence of sodium ethylenediaminetetraacetate was found to increase the LOX activity from *F. oxysporum*, *F. proliferatum* and *C. pyrenoidosa* extracts; however, the LOX activity from the *S. cerevisiae* extract remained unaffected. In addition, the use of

hydroquinone resulted in an increase in LOX activity from *F. proliferatum* whereas a competitive inhibitory effect was observed on the LOX activity from *S. cerevisiae* extract.

The characterization of the partially purified extracts, obtained from the four selected microbial sources, particularly in terms of LOX activity and substrate specificity showed that the enzymatic extract from *F. proliferatum* was the most appropriate extract to pursue further purification and characterization.

The successive purification of the enzymatic extract from *F. proliferatum* by size-exclusion and ion-exchange chromatographies resulted in one major fraction which demonstrated a LOX activity at two pH optima, 6.0 and 10.5. These findings suggest the occurrence of more than one isozyme of LOX. The electrophoretic analysis of the denatured purified enzymatic fraction showed the presence of one major protein band and two minor ones.

The purified LOX fraction demonstrated a strong substrate specificity towards mono- and dilinolein, as well as the linolenic and arachidonic acids in comparison to linoleic acid at pH 6.0. However, at pH 10.5, the purified fraction possessed an overall preference towards linoleic acid. These results could confirm the hypothesis of the presence of more that one LOX isozyme in the purified enzymatic fraction.

The purified LOX fraction produced, at neutral pH, mainly the 13-HPODE of linoleic acid while at the alkaline pH, the 9- and 13-HPODEs of linoleic acid were produced in equal proportions. In addition, the bioconversion of linoleic acid into the 10- and 12-HPODE isomers was also suggested by the LOX activity in the purified enzymatic fraction.

This work has provided a better understanding of the presence of purified LOX fractions obtained from four microbial sources, in particular *F. proliferatum*, as well as their involvement in the production of regio-specific HPODEs. The production of a wide range of end-products by the purified enzymatic extract may indicate the potentiality of using the LOX from *F. proliferatum* in the biogeneration of selected flavor precursors.

REFERENCES

Adams, J.B. and Ongely, M.H. 1989. The behavior of green bean lipoxygenase and isoelectric focusing. *Food Chem.* **31**, 57-71.

Al-Obaidy, H.M. and Siddiqi, A.M. 1981. Properties of broad bean lipoxygenase. *J. Food Sci.* **46**, 622-625, 629.

Ali Asbi, B., Wei, L.S. and Steinberg, M.P. 1989. Effect of pH on the kinetics of soybean lipoxygenase-1. *J. Food Sci.* **54**, 1594-1595, 1600.

Allen, J.C. 1968. Soybean lipoxygenase 1. Purification and the effect of organic solvents upon the kinetics of the reaction. *Eur. J. Biochem.* **4**, 201-208.

Andrawis, A., Pinsky, A. and Grossman, S. 1982. Isolation of soybean lipoxygenase-2 by affinity chromatography. *Phytochem.* **21**, 1523-1525.

Andre, E. and Hou, K.W. 1932. The presence of a lipoxidase in soybean seeds. *C.R. Acad. Sci.* [Paris] **194**, 6645, cited from *Chem. Abstr.* 1932, 3004.

Andrianarison, R.H., Beneytout, J.L. and Tixier, M. 1989. An enzymatic conversion of lipoxygenase products by a hydroperoxide lyase in blue-green algae (*Oscillatoria* sp.). *Plant Physiol.* **91**, 1280-1287.

Antis, P.J.P. and Friend, J. 1974a. Isozymes distribution of isolated pea seedlings lipoxygenase. *Planta* **115**, 329-331.

Antis, P.J.P. and Friend, J. 1974b. Lipoxygenase in dark-grown *Pisum staivum*. *Phytochem.* **13**, 567-573.

Arens, D., Seilmeier, W., Weber, F., Kloos, G. and Grosch, W. 1973. Purification and properties of a carotene co-oxidizing lipoxygenase from peas. *Biochim. Biophys. Acta* **327**, 295-305.

Axelrod, B. 1974. Lipoxygenase in food related enzymes. In *Advances in Chemistry Series*, Whitaker, J.R. (ed.), pp. 324-348.

Axelrod, B., Cheesbrough, T.M. and Laakso, S. 1981. Lipoxygenase from soybeans. *Methods Enzymol.* **71**, 441-451.

Badaracco, G., Plevani, P., Ruyechan, W.T. and Chang, L. 1983. Purification and characterization of yeast topoisomerase I. *J. Biol. Chem.* **258**, 2022-2026.

Baker, D.R. 1995. Introduction. In *Capillary Electrophoresis*, John Wiley & Sons, Inc., New York, N.Y., pp. 1-7.

Beaux, Y., Drapron, R. and Nicolas, J.M. 1973. Lipoxygenase from the horse bean (*Vicia faba*). 1. Isolation. *Biochimie* **55**, 253-255.

Beckman Instruments Inc. 1994. In *eCAP TM Chiral Methods Development Kit Guide*, Fullerton, CA, pp. 1-1 to 10-3.

Bedetti, C. and Cantafora, A. 1987. Extraction and purification of arachidonic acid metabolites from cell cultures. *Adv. Biochem. Eng.* **35**, 47-81.

Belefant, H. and Fong, F. 1991. Lipoxygenase in developing *Zea mays* kernels. *Plant Physiol. Biochem.* **29**, 99-104.

Belinky, P.A., Masaphy, S., Levanon, D., Hadar, Y. and Dosoretz, C.G. 1994. Effect of medium composition on 1-octen-3-ol formation in submerged cultures of *Pleurotus pulmonarius*. *Appl. Microbiol. Biotechnol.* **40**, 629-633.

Ben-Aziz, A., Grossman, S., Ascarelli, I. and Budowski, P. 1970. Linoleate oxidation induced by lipoxygenase and heme proteins: a direct spectrophotometric assay. *Anal. Biochem.* **34**, 88-100.

Beneytout, J.-L., Andrianarison, R.-H., Rakotoarisoa, Z. and Tixier, M. 1989. Properties of a lipoxygenase in green algae (*Oscillatoria* sp.). *Plant Physiol.* **91**, 367-372,

Berger, R.G., Kler, A. and Drawert, F. 1986. The C6-aldehyde-forming system in *Agropyron repens*. *Biochim. Biophys. Acta* **883**, 523-530.

Bhirud, P.R. and Sosulski, F.W. 1993. Thermal inactivation kinetics of wheat germ lipoxygenase. *J. Food Sci.* **58**, 1095-1098.

Bild, G.S., Ramadoss, C.S. and Axelrod, B. 1977. Effect of substrate polarity on the activity of soybean lipoxygenase. *Lipids* **12**, 732-735.

Bisakowski, B. Kermasha, S. and Klopfenstein, M.-L. 1995a. Partial purified lipoxygenase from *Fusarium oxysporum*: Characterization and kinetic studies. *Process Biochem.* **30**, 261-268.

Bisakowski, B. Kermasha, S., Lavorel, V. and Belin, J.-M. 1995b. Partial characterization of a lipoxygenase from *Fusarium proliferatum*. *Food Biotechnol.* **9**, 189-205.

Bisakowski, B., Kermasha, S. and Schuepp, C. 1995c. Partial purification and catalytic properties of lipoxygenase from *Saccharomyces cerevisiae*. *World J. Microb. Biotechnol.* **11**, 494-496.

Bisakowski, B. Kermasha, S. and Marsot, P. 1995d. Partial purification of lipoxygenase in *Chlorella pyrenoidosa*. *Biotechnol. Appl. Biochem.* **21**, 39-48.

Bisakowski, B., Perraud, X. and Kermasha, S. 1997. Characterization of hydroperoxides and carbonyl compounds obtained by lipoxygenase extracts of selected microorganisms. *Biosci. Biotech. Biochem. 61*, 1262-1269.

Blain, J.A. and Shearer, G. 1965. Inhibition of soya lipoxidase. *J. Sci. Food Agric.* **16**, 373-378.

Bohn, R.M. and Haas, L.W. 1953. In *Chemistry and Methods of Enzymes*, J.B. Summer and G.F. Summer (eds.), Academic Press, New York, N.Y.

Bonnet, J.L. andCrouzet, J. 1977. Lipoxygenase from tomato fruit: Partial purification and study of some properties. *J. Food Sci.* **42**, 625-628.

Borgeat, P. and Samuelsson, B. 1979. Arachidonic acid metabolism in polymorphonuclear leukocytes: Effects of ionophore A23187. *Proc. Natl. Acad. Sci. USA* **76**, 2148-2152.

Borgeat, P., Hamberg, M. and Samuelsson, B. 1976. Transformation of arachidonic acid and linolenic acid by rabbit polymorphonuclear leukocytes. *J. Biol. Chem.* **251**, 7816-7820.

Bowsher, C.G., Ferrie, B.J.M., Ghosh, S., Todd, J., Thompson, J.E. and Rothstein, S.J. 1992. Purification and partial characterization of a membrane-associated lipoxygenase in tomato fruit. *Plant Physiol.* **100**, 1802-1807.

Boyd, D.H.J. and Adams, G.A. 1955. An assay method for lipoxidase in animal tissues. *Can. J. Biochem. Physiol.* **23**, 191-198.

Boyes, S., Perera, C. and Young, H. 1992. Kiwi fruit lipoxygenase: Preparation and characteristics. *J. Food Sci.* **57**, 1390-1394.

Brodowsky, I.D. and Oliw, E.H. 1992. Metabolism of 18:2(*n*-6), 18:3(*n*-3), 20:4(*n*-6) and 20:5(*n*-3) by the fungus *Gaeumannomyces graminis*: Identification of metabolites formed by 8-hydroxylation and by w2 and w3 oxygenation. *Biochim. Biophys. Acta* **1124**, 59-65.

Brodowsky, I.D. and Oliw, E.H. 1993. Biosynthesis of 8*R*-hydroperoxylinoleic acid by the fungus *Laetisaria arvalis*. *Biochim. Biophys. Acta* **1168**, 68-72.

Brodowsky, I.D., Hamberg, M. and Oliw, E.H. 1992. A linoleic acid (8*R*)-dioxygenase and hydroperoxide isomerase of the fungus *Gaeumannomyces graminis*: Biosynthesis of (8*R*)-hydroxylinoleic acid and (7*S*,8*S*)-dihydroxylinoleic acid from (8*R*)-hydroperoxylinoleic acid. *J. Biol. Chem.* **267**, 14738-14745.

Brodowsky, I.D., Hamberg, M. and Oliw, E.H. 1994. BW A4C and other hydroxamic acids are potent inhibitors of linoleic acid 8*R*-dioxygenase of the fungus *Gaeumannomyces graminis*. *Eur. J. Pharmacol.* **254**, 43-47.

Budd, T.W., Tjostem, J.L. and Duysen, M.E. 1969. Ultrastructure of *Chlorella pyrenoidosa* as affected by environmental changes. *Am. J. Bot.* **56**, 540-545.

Butovich, I.A., Bridnya, V.P. and Kukhar, V.P. 1990. Linoleate-hydroxamic acid: a suicide inhibitor of lipoxygenase. *Biochemistry-Engl. Tr.* **55**, 908-915.

Carroll, R.T., Muller, J., Grimm, J., Dunham, W.R., Sands, R.H. and Funk, M.O. 1993. Rapid purification of rabbit reticulocyte lipoxygenase for electron paramagnetic spectroscopy of the non-heme iron. *Lipids* **28**, 241-244.

Chan, H.W.S. and Prescott, F.A.A. 1975. Separation of isomeric hydroperoxides by high performance liquid chromatography. *Biochim. Biophys. Acta* **380**, 141-144.

Chang, C.C., Esselman, W.J. and Clagett, C.O. 1973. The isolation and specificity of alfalfa lipoxygenase. *Lipids* **6**, 100-106.

Chen, A.O. and Whitaker, J.R. 1986. Purification and characterization of a lipoxygenase from immature English peas. *J. Agric. Food Chem.* **34**, 203-211.

Christopher, J., Pistorius, E. and Axelrod, B. 1970. Isolation of an isozyme of soybean lipoxygenase. *Biochim. Biophys. Acta* **198**, 12-19.

Christopher, J., Pistorius, E. and Axelrod, B. 1972. Isolation of a third isozyme of soybean lipoxygenase. *Biochim. Biophys. Acta* **284**, 54-62.

Collins, M.A. and Buick, R.K. 1989. Effect of temperature on the spoilage of stored peas by *Rhodotorula glutinis*. *Food Microbiol.* **6**, 135-141.

Corey, E.J., Cashman, J.R., Kantner, S.S. and Wright, S.W. 1984. Rationally designed, potent competitive inhibitors of leukotriene biosynthesis. *J. Am. Chem. Soc.* **106**, 1503-1510.

Cronin, D.A. and Ward, M.K. 1971. The characterization of some mushroom volatiles. *J. Sci. Food Agric.* **22**, 477-479.

Davies, C.S., Nielsen, S.S. and Nielsen, N.C. 1987. Flavor improvement of soybean preparations by genetic removal of lipoxygenase-2. *J. Am. Oil Chem. Soc.* **64**, 1428-1433.

De Lumen, B.O. and Kazeniac, S.J. 1976. Staining for lipoxygenase activity in electrophoretic gels. *Anal. Biochem.* **72**, 428-432.

De Lumen, B.O., Stone, E.J., Kazeniac, S.J. and Forsythe, R.H. 1978. Formation of volatile flavor compounds in green beans from linolenic acid. *J. Food Sci.* **43**, 698-702, 708.

Dillard, M.G., Henick, A.S. and Koch, R.B. 1960. Unsaturated triglyceride and fatty acid lipoxidase activities of navy beans, small red beans, peanuts, green peas and lima beans. *Food Res.* **25**, 544-553.

Dressen, T.D., Dickens, M. and Koch, R.B. 1982. Partial purification and characterization of a Ca^{2+}-stimulated lipoxygenase from soybean seeds. *Lipids* **17**, 964-969.

Engeseth, N.J., Klein, B.P. and Warner, K. 1987. Lipoxygenase isoenzyme in soybeans effects on crude oil quality. *J. Food Sci.* **52**, 1015-1029.

Eriksson, C.E. and Svensson, S.G. 1970. Lipoxygenase from peas, purification and properties of the enzyme. *Biochim. Biophys. Acta* **198**, 449-459.

Eskin, N.A.M. and Henderson, H.M. 1974. Lipoxygenase in *Vicia faba* minor. *Phytochem.* **13**, 2713-2716.

Eskin, N.A.M., Grossman, S. and Pinsky, A. 1977. Biochemistry of lipoxygenase in relation to food quality. *Critical Rev. Food Sci. Nutr.* **9**, 1-40.

Esselman, W.J. and Clagett, C.O. 1974. Products of a linoleic hydroperoxide-decomposition enzyme of alfalfa seed. *J. Lipid Res.* **15**, 173-178.

Fales, H.M., Jaouni, T.M. and Babashak, J.F. 1973. Simple device for preparing diazomethane without resorting to codistillation. *Anal. Chem.* **45**, 2302-2303.

Falgueyret, J.-P., Hutchinson, J.H. and Riendeau, D. 1993. Criteria for the identification of non-redox inhibitors of 5-lipoxygenase. *Biochem. Pharmacol.* **45**, 978-985.

Flick, J., St. Angelo, G. and Ory, R. 1975. KCN inhibition of lipoxygenase in purple, green, and white eggplants. *Lipids* **10**, 564-566.

Frankel, E.N., Warner, K. and Klein, B.P. 1988. Flavor and oxidative stability of oil processed from null lipoxygenase-1 soybeans. *J. Am. Oil Chem. Soc.* **65**, 147-150.

Funk, M.O., Whitney, M.A., Hausknecht, E.C. and O'Brien, E.M. 1985. Resolution of the isoenzymes of soybean lipoxygenase using isoelectric focusing and chromatofocusing. *Anal. Biochem.* **146**, 246-251.

Galliard, T. 1983. In *Lipids in Cereal Technology*, Barnes, P.J. (ed.), Academic Press, London, UK, pp. 403-407.

Galliard, T. and Chan, H.W-S. 1980. Lipoxygenase. In *The Biochemistry of Plants*, Stumpf, P.K. (ed.), Academic Press, New York, N.Y., pp. 131-161.

Galliard, T. and Matthew, J. 1977. Lipoxygenase-mediated cleavage of fatty acids to carbonyl fragments in tomato fruits. *Phytochem.* **16**, 339-343.

Galliard, T. and Mercer, E.I. 1975. Lipoxygenase catalyzed peroxidation of fatty acids in degradation of plant lipids by hydrolytic and oxidative enzymes. In *Recent Advances in the Chemistry and Biochemistry of Plant Lipids*, Galliard, T. and Mercer, E.I. (eds.), Academic Press, New York, N.Y., pp. 343-345.

Galliard, T. and Phillips, D.P. 1971. Lipoxygenase from potato tubers: Partial purification and properties of an enzyme that specifically oxygenates the 9-position of linoleic acid. *Biochem. J.* **124**, 431-438.

Galliard, T., Phillips, D.R. and Reynolds J. 1976. The formation of *cis*-3-nonenal, *trans*-2-nonenal and hexanal from linoleic acid hydroperoxide isomers by a hydroperoxide cleavage enzyme system in cucumber (*Cucumis sativus*) fruits. *Biochim. Biophys. Acta* **441**, 181-192.

Galpin, J.R. and Allen, J.C. 1977. The influence of micelle formation on lipoxygenase kinetics. *Biochim. Biophys. Acta* **488**, 392-401.

Ganthavorn, C. and Powers, J.R. 1989. Partial purification and characterization of asparagus lipoxygenase. *J. Food Sci.* **54**, 371-373.

Gardner, H.W. 1970. Sequential enzymes of linoleic acid oxidation in corn germ: lipoxygenase and linoleate hydroperoxide isomerase. *J. Lipid Res.* **11**, 311-321.

Gardner, H.W. 1988. Lipoxygenase pathway in cereals. In *Advances in Cereal Science and Technology*, Pomeranz, Y. (ed.), American Association of Cereal Chemists, MN, pp. 161-215.

Gardner, H.W. 1989. How the lipoxygenase pathway affects the organoleptic properties of fresh fruit and vegetables. In *Flavor Chemistry of Lipid Foods*, Min, D.B. and Smouse, T.H. (eds.), The American Oil Chemists' Society, Illinois, pp. 98-112

Gardner, H.W. 1991. Recent investigations into the lipoxygenase pathway of plants. *Biochim. Biophys. Acta* **1084**, 221-239.

Gardner, W.H., Weiskeder, D. and Plattner, R.D. 1991. Hydroperoxide lyase and other hydroperoxide-metabolizing activity in tissues of soybean, Glycine max. *Plant Physiol.* **97**, 1059-1072.

Gardner, W.H., Weisleder, D. and Nelson, E.C. 1984. Acid catalysis of a linoleic acid hydroperoxide: Formation of epoxides by an intramolecular cyclization of the hydroperoxide group. *J. Org. Chem.* **49**, 508-515.

Gerristen, M., Veldink, G.A., Vliegenthart, J.F.G. and Boldingh, J. 1976. Formation of α- and γ-ketols from ^{18}O-labelled linoleic acid hydroperoxides by corn hydroperoxide isomerase. *FEBS Letters* **76**, 149-152.

Gerwick, W.H., Moghaddam, M.F. and Hamberg, M. 1991. Oxylipin metabolism in the red alga *Gracilariopsis lemaneiformis*: Mechanism of formation of vicinal dihydroxy fatty acids. *Arch. Biochem. Biophys.* **290**, 436-444.

Gibian, M.J. and Galaway, R.A. 1976. Steady-state kinetics of lipoxygenase oxygenation of unsaturated fatty acids. *Biochemistry* **15**, 4209-4214.

Giulian, G.C., Moss, R.L. and Graeser, M. 1983. Improved methodology for analysis and quantitation of protein on one-dimensional silver stained slab gels. *Anal. Biochem.* **129**, 227.

Graveland, A. 1970. Enzymatic oxidation of linoleic acid and glycerol-monolinoleate in doughs and flour -wheat suspensions. *J. Am. Oil Chem. Soc.* **47**, 352-361.

Grosch, W. 1972. Enzyme lipoxygenase, its properties and action in foods. *Fette Seifen Anstrichm.* **74**, 375-380.

Grosch, W. 1982. Lipid degradation products and flavor. In *Food Flavors*, Morton, I.D. and Macleod, A.J. (eds.), Elsevier, London, pp. 254-265.

Grosch, W. and Laskawy, G. 1975. Differences in the amount and range of volatile carbonyl compounds formed by lipoxygenase isozymes from soybeans. *J. Agric. Food Chem.* **23**, 791-794.

Grosch, W. and Wurzenberger, M. 1984. Enzymatic formation of 1-octen-3-ol in mushrooms. In *Progress in Flavor Research*, Adda, S. (ed.), Elsevier Science Publishers B.V., Amsterdam, pp. 253-259.

Grossman, S. and Zakut, R. 1979. Determination of the activity of lipoxygenase (lipoxidase). In *Methods of Biochemical Analysis*, Glick, D. (ed.), Wiley, New York, N.Y., pp. 308-329.

Grossman, S., Ben Aziz, A., Budowski, P., Ascarelli, L., Gertler, A., Birk, Y. and Bondi, A. 1969. Enzymatic oxidation of carotene and linoleate by alfalfa: Extraction and separation of active fractions. *Phytochem.* **8**, 2287-2293.

Grossman, S., Bergam, M. and Skaln, D. 1988. Lipoxygenase in chicken muscle. *J. Agric. Food Chem.* **36**, 1268-1270.

Grossman, S., Bergman, M. and Sofer, Y. 1983. Purification and partial characterization of eggplant linoleate hydroperoxide isomerase. *Biochim. Biophys. Acta* **752**, 65-72.

Grossman, S., Trop, M., Avtation, R. and Pinsky, A. 1972a. Eggplant lipoxygenase: Isolation and partial purification. *Lipids* **7**, 467-473.

Grossman, S., Trop, M., Yaroni, S. and Wilchek, M. 1972b. Purification of soybean lipoxygenase by affinity chromatography. *Biochim. Biophys. Acta* **289**, 77-81.

Guss, P.L., Macko, V., Richardson, T. and Stahmann, M.A. 1968b. Lipoxygenase in early growth of wheat. *Plant Cell Physiol.* **9**, 115-122.

Guss, P.L., Richardson, T. and Stahmann, M.A. 1967. The oxidation-reduction enzymes of wheat. III. Isoenzymes of lipoxidase in wheat fractions and soybean. *Cereal Chem.* **44**, 607-610.

Guss, P.L., Richardson, T. and Stahmann, M.A. 1968a. Oxidation of various lipid substrates with unfractionated soybean and wheat lipoxidase. *J. Am. Oil Chem.* **45**, 272-276.

Haining, J.L. 1974. The mechanism of action of lipoxidase. *Adv. Chem. Ser.* **136**, 324-330.

Hale, S.A., Richardson, T., Von Elbe, J.H. and Hagedorn, H. 1969. Isozymes of lipoxidase. *Lipids* **4**, 210-215.

Hamberg, M. 1989. Fatty acid hydroperoxide isomerase in *Saprolegnia parasitica*: structural studies of epoxy alcohols formed from isomeric hydroperoxyoctadecadienoates. *Lipids* **24**, 249-255.

Hamberg, M. 1993. Oxidation of octadecatrienoic acids in the red alga *Liththamnion corallioides*: structural and stereochemical studies of conjugated tetraene fatty acids and *bis* allylic hydroxy acids. *J. Chem. Soc. Perkin Trans.* **1**, 3065-3072.

Hamberg, M. and Gerwick, W.H. 1993. Biosynthesis of vicinal dihydroxy fatty acids in the red alga *Gracilariopsis lemaneiformis*: Identification of a sodium-dependent 12-lipoxygenase and a hydroperoxide isomerase. *Arch. Biochim. Biophys.* **305**, 115-122.

Hamberg, M. and Samuelson, B. 1965. Specificity of the lipoxidase catalyzed oxygenation of unsaturated fatty acids. *Biochem. Biophys. Res. Commun.* **21**, 531-535.

Hamberg, M. and Samuelsson, B. 1967. On the specificity of the oxygenation of unsaturated fatty acids catalyzed by soybean lipoxidase. *J. Biol. Chem.* **242**, 5329-5335.

Hamberg, M., Gerwick, W.H. and Asen, P.A. 1992. Linoleic acid metabolism in the red alga *Lithothamnion corallioides*: Biosynthesis of 11(*R*)-hydroxy-9(*Z*),12(*Z*)-octadecadienoic acid. *Lipids* **27**, 487-493.

Hamberg, M., Herman, C.A. and Herman, R.P. 1986a. Novel biological transformations of 15-L_S-hydroperoxy-5,8,11,13-eicosatetraenoic acid. *Biochim. Biophys. Acta* **877**, 447-457.

Hamberg, M., Herman, R.P. and Jacobsson, U. 1986b. Stereochemistry of two epoxy alcohols from *Saprolegnia parasitica*. *Biochim. Biophys. Acta* **879**, 410-418.

Hardy, D.J., Violana Gallegos, M.A. and Gaunt, J.K. 1991. Extracts of Pisum sativum metabolize phospholipid via a lipoxygenase-like mechanism. *Phytochem.* **30**, 2889-2894.

Hartree, E.P. 1972. Determination of protein: a modification of the Lowry method that gives a linear photometric response. *Anal. Biochem.* **48**, 422-427.

Hawkins, D.J. and Brash, A.R. 1989. Lipoxygenase metabolism of polyunsaturated fatty acids in oocytes of the frog *Xenopus laevis*. *Arch. Biochem. Biophys.* **268**, 447-455.

Haydar, M. and Hadziyev, H. 1973. A study of lipoxygenase in pea seeds and seedlings. *J. Sci. Food Agric.* **24**, 1039-1053.

Herman, R.P. and Hamberg, M. 1987. Properties of the soluble arachidonic acid 15-lipoxygenase and 15-hydroperoxide isomerase from the oomycete *Saprolegnia parasitica*. *Prostaglandins* **34**, 129-139.

Hidaka, T., Katsuki, S., Nagata, Y. and Nakatsu, S. 1986. Partial purification and properties of pumpkin lipoxygenase with carotene-bleaching activity. *J. Food Biochem.* **101**, 55-73.

Hildebrand, D.F. 1989. Lipoxygenases. *Physiol. Planta* **76**, 249-253.

Holman, R.T., Egwim, P.O. and Christie, W.W. 1969. Substrate specificity of soybean lipoxidase. *J. Biol. Chem.* **244**, 1149-1511.

Hsieh, R.J. and Kinsella, J.E. 1989. Lipoxygenase generation of specific volatile flavor carbonyl compounds in fish tissues. *J. Agric. Food Chem.* **37**, 279-286.

Hurt, G.B. and Axelrod, B. 1977. Characterization of two isozymes of lipids from bush bean. *Plant Physiol.* **59**, 695-700.

Ida, S., Masaki, Y. and Morita, Y. 1983. The isolation of multiple forms and product specificity of rice lipoxygenase. *Agric. Biol. Chem.* **47**, 637-641.

Iny, D., Pinsky, A., Cojocoru, M. and Grossman, S. 1993a. Lipoxygenase of *Thermoactinomyces vulgaris*, purification and characterization of reaction products. *Int. J. Biochem.* **25**, 1313-1323.

Iny, D., Pinsky, A., Cojocoru, M. and Grossman, S. 1993b. Lipoxygenase of thermophilic bacteria *Thermoactinomyces vulgaris*-properties and study on the active site. *Int. J. Biochem.* **25**, 1325-1330.

Jadhav, S., Singh, B. and Salunkhe, D.K. 1972. Metabolism of unsaturated fatty acids in tomato fruit: linoleic and linolenic acid as precursors of hexanal. *Plant Cell Physiol.* **13**, 449-459.

Jiang,, Z. and Gerwick, W.H. 1991. An aldehyde-containing galactolipid from the red alga *Gracilariopsis lemaneiformis*. *Lipids* **26**, 960-963.

Kaminski, E., Libbey, L.M., Stawicki, S. and Wasowicz, E. 1972. Identification of the predominant volatile compounds produced by *Aspergillus flavus*. *Appl. Microbiol.* **24**, 721-726.

Kaminski, E., Stawicki, S. and Wasowicz, E. 1974. Volatile flavor compounds produced by molds of *Aspergillus, Penicillium, Fungi imperfecti*. *Appl. Microbiol.* **27**, 1001-1004.

Kazeniac, S.J. and Hall, R.M. 1970. Flavor chemistry of tomato volatiles. *J. Food Sci.* **35**, 519-530.

Kermasha, S, van de Voort, F.R. and Metche, M. 1986. Conversion of linoleic acid hydroperoxide by french bean hydroperoxide isomerase, *J. Food Biochem.* **10**, 285-303.

Kermasha, S. and Metche, M. 1986. Characterization of seed lipoxygenase from *Phaselous vulgaris* cv, haricot. *J. Food Sci.* **51**, 1224-1227.

Khalyfa, A., Kermasha, S. and Alli, I. 1990. Partial purification and characterization of lipoxygenase of canola seed (*Brassica napus* var. Westar). *J. Agric. Food Chem.* **38**, 2003-2008.

Kies, M.W. 1947. Complex nature of soybean lipoxidase. *Fed. Proc.* **6**, 267-267.

Kies, M.W., Haining, J.L., Pistorius, E., Schroeder, D.H. and Axelrod, B. 1969. On the question of the identity of soybean lipoxidase and carotene. *Biochim. Biophys. Res. Commun.* **36**, 312-315.

Kim, I. and Grosch, W. 1979. Partial purification of a lipoxygenase from apples. *J. Agric. Food Chem.* **27**, 243-246.

Kim, I.S. and Grosch, W. 1981. Partial purification and properties of a hydroperoxide lyase from fruits of pear. *J. Agric. Food Chem.* **29**, 1220-1225.

Kitamura, K., Davies, C.S., Kaizuma, N. and Nielsen, N.C. 1983. Genetic analysis of a null-allele for lipoxygenase-3 in soybean seeds. *Crop Sci.* **23**, 924-927.

Klein, R.P. 1976. Isolation of lipoxygenase from split peas seeds, snap beans, and peas. *J. Agric. Food Chem.* **24**, 938-942.

Koch, R.B., Stern, B. and Ferrari, C.G. 1958. Linoleic acid and trilinolein as substrates for soybean lipoxidase. *Arch. Biochem. Biophys.* **78**, 165-179.

Kondo, Y., Hashidoko, Y. and Mizutani, J. 1995. An enzymatic formation of 13-oxo-trideca-9,11-dienoic acid from 13-hydroperoxylinolenic acid by a homolytic hydroperoxide lyase in elicitor-treated soybean cotyledons. *Biochim. Biophys. Acta* **1255**, 9-15.

Lenoi, O., Iroi, R. and Palmieri, S. 1985. Purification and properties of lipoxygenase in germinating sunflower seeds. *J. Food Sci.* **50**, 88-92.

Lillehoj, E.P. and Malik, U.S. 1989. Protein purification. *Adv. Biochem. Eng.* **40**, 19-71.

Lindsay, R.C. 1985. Food additives. In *Food Chemistry*, Fennema, O.R. (ed.), Marcel Dekker, New York, N.Y., pp. 641-644.

Lineweaver, H. and Burk, D. 1934. The determination of enzyme dissociation constants. *J. Am. Chem. Soc.* **56**, 658-666.

Lopez, A. and Gerwick, W.H. 1987. Two new icosapentaenoic acids from the temperate red seaweed *Ptiota filicina*. *Lipids* **22**, 190-194.

Luning, P.A., Carey, A.T., Roozen, J.P. and Wichers, H.J. 1995. Characterization and occurrence of lipoxygenase in bell peppers at different ripening stages in relation to the formation of volatile flavor compounds. *J. Agric. Food Chem.* **43**, 1493-1500.

Lyudnikova, T.A., Rodopulo, A.K. and Egorov, I.A. 1984. Lipoxygenase activity of the yeast *Saccharomyces vini* Strain Msh-2. *Prikl. Biokhim. Mikrobiol.* **20**, 175-177.

Mack, A.J., Peterman, T.K. and Siedow, J.N. 1987. Lipoxygenase isozymes in higher plants: biochemical properties and physiological role. *Isozymes: Curr. Top. Biol. Med. Res.* **13**, 127-154.

Maga, J.A. 1981. Mushroom flavor. *J. Agric. Food Chem.* **29**, 1-4.

Marcus, L., Prusky, D. and Jacoby, B. 1988. Purification and characterization of avocado lipoxygenase. *Phytochem.* **27**, 323-327.

Matoba, T., Hidaka, H. and Kitamura, K. 1985. Contribution of hydroperoxide lyase activity to *n*-hexanal formation in soybean. *J. Agric. Food Chem.* **33**, 856-858.

Matoba, T., Hidaka, H., Narita, H., Kitamura, K., Kaizuma, N. and Kito, M. 1985b. Contribution of hydroperoxide lyase activity to *n*-hexanal formation in soybean. *J. Agric. Food Chem.* **33**, 856-858.

Matsuda, Y., Beppu, T. and Arima, K. 1978. Crystallization and positional specificity of hydroperoxidation of *Fusarium* lipoxygenase. *Biochim. Biophys. Acta* **530**, 439-450.

Matsuda, Y., Satoh, T. Beppu, T. and Arima, K. 1976. Purification and properties of Co^{2+} requiring heme protein having lipoxygenase activity from *Fusarium oxysporum*. *Agric. Biol. Chem.* **40**, 963-976.

Matsui, K. and Kajiwara, T. 1995. Cucumber cotyledon lipoxygenase oxygenizes trilinolein at the lipid/water interface. *Lipids* **30**, 733-738.

Matsui, K., Kajiwara, T. and Hatanaka, A. 1990. Physiological roles of plant lipoxygenase: lipoxygenase activity in *Marchantia polymorpha* cell. In *Plant Lipid Biochemistry, Structure and Utilization*. The Proceedings of the Ninth International Symposium on Plant Lipids, Quinn, P.J. and Harwood, J.L. (eds.), Portland Press Limited, London, pp. 292-294.

Mau, J.L., Beelman R.B. and Ziegler, G.R. 1992. 1-Octen-3-ol in the cultivated mushroom, *Agaricus bisporus*. *J. Food Sci.* **57**, 704-706.

McMillan, R.M. and Walker, E.H.R. 1992. Designing therapeutically effective 5-lipoxygenase inhibitors. *Trends Pharmacol. Sci.* **13**, 323-330.

McCurdy, A.R., Nagel, C.W. and Swanson, B.G. 1983. Isolation and characterization of lipoxygenase in pinto dry beans. *Can. Inst. Food Sci. Technol. J.* **16**, 179-184.

Moghaddam, M.F. and Gerwick, W.H. 1990. 12-Lipoxygenase activity in the red marine alga *Gracilariopsis lemaneiformis*. *Phytochem.* **29**, 2457-2459.

Morin, A., Géra, R. and Leblanc, D. 1993. Nylon-made 180 ml grinder vessel for laboratory-scale cell disruption. *J. Microbiol. Methods* **17**, 233-237.

Mtebe, K. and Gordon, M.H. 1987. Volatiles derived from lipoxygenase-catalyzed reactions in winged beans (*Psophocarpus tetragonolobus*). *Food Chem.* **23**, 175-182.

Mukherjee, S. 1951. Studies on degradation of fats by microorganisms. I. Preliminary investigations on enzyme systems involved in the spoilage of fats. *Arch. Biochem. Biophys.* **33**, 364-376.

Nawar, W.W. 1985. Lipids. In *Food Chemistry*, Fennema, O.R. (ed.), Marcel Dekker Inc., New York, N.Y., pp. 198-205.

Nazzaro-Porro, M., Passi, S., Picardo, M., Mercantini, R. and Breathnach, A. 1986. Lipoxygenase activity of *Pityrosporum in vitro* and *in vivo*. *J. Invest. Dermatol.* **87**, 108-112.

Nelson, M.J. 1988. Catecholate complexes of ferric soybean lipoxygenase 1. *Biochemistry* **27**, 4273-4278.

Nelson, M.J., Batt, D.G., Thompson, J.S. and Wright, S.W. 1991. Reduction of the active-site iron by potent inhibitors of lipoxygenases. *J. Biol. Chem.* **266**, 8225-8229.

Nicholas, J., Autron, M. and Drapron, R. 1982. Purification and some properties of wheat germ lipoxygenase. *J. Sci. Food Agric.* **33**, 365-372.

Nicholas, J. and Drapron, R. 1981. Lipoxygenase from plants. Current state of knowledge. 1. Biochemical aspects. *Sci. des Aliments* **1**, 91-168.

O'Connor, T.P. and O'Brien, N.M. 1991. Significance of Lipoxygenase in Fruits and Vegetables. In *Food Enzymology*, Fox, P.F. (ed.), Elsevier Applied Science, pp. 337-372.

Ohta, H., Ida, S., Mikami, B. and Morita, Y. 1986. Purification and characterization of rice lipoxygenase component 3 from embryos. *Agric. Biol. Chem.* **50**, 3165-3171.

Olías, J.M. and Valle, M. 1988. Lipoxygenase from lupin seed: Purification and characterization. *J. Sci. Food Agric.* **45**, 165-174.

Olías, J.M., Rios, J.J., Valle, M., Zamora, R., Sanz, L.C. and Axelrod, B. 1990. Fatty acid hydroperoxide lyase in germinating soybean seedlings. *J. Agric. Food Chem.* **38**, 624-630.

Oliw, E.H. 1989. Biosynthesis of 18(R_D)-hydroxyeicosatetraenoic acid from arachidonic acid by microsomes of monkey semical vesicles. *J. Biol. Chem.* **264**, 17845-17853.

Percival, M.D. 1991. Human 5-lipoxygenase contains an essential iron. *J. Biol. Chem.* **266**, 10058-10061.

Peterson, E.A. and Sober, H.A. 1956. Chromatography of proteins. 1. Cellulose ion-exchange adsorbents. *J. Am. Oil Chem. Soc.* **78**, 751-755.

Pharmacia LKB Biotechnology. 1992. In *Phastsystem Instruction Manual*, Pharmacia (ed.), Uppsala, Sweden, pp. 1-83.

Picardi, S.M. and Issenberg, P. 1973. Investigation of some volatile constituents of mushrooms (*Agaricus bisporus*): changes which occur during heating. *J. Agric. Food Chem.* **21**, 959-962.

Pinsky, A., Grossman, S. and Trop, M. 1971. Lipoxygenase content and antioxidant activity of some fruits and vegetables. *J. Food Sci.* **36**, 571-572.

Pinsky, A., Sporn, J., Grossman, S. and Rivlin, M. 1973. Lipoxygenase isoenzymes from *Solanum tuberosum*. *Phytochem.* **12**, 1051.

Pistorius, K.E. and Axelrod, B. 1974. Iron, an essential component of lipoxygenase. *J. Biol. Chem.* **249**, 3183-3186.

Poca, E., Rabinovitch-Chable, H., Cook-Moreau, J., Pages M. and Rigaud, M. 1990. Lipoxygenases from *Zea mays* L. Purification and physicochemical characteristics. *Biochim. Biophys. Acta* **1045**, 107-114.

Pomeranz, Y. and Meloan, C. 1994. Mass spectroscopy. In *Food Analysis: Theory and Practice*, Chapman & Hall, New York, N.Y., pp. 243-261.

Pradel, G. and Adda, J. 1980. Peroxides as sources of error in quantitative determination of monocarbonyls in cheese. *J. Food Sci.* **45**, 1058-1059.

Proteau, P.J. and Gerwick, W.H. 1993. Divinyl ethers and hydroxy fatty acids from the species of *Laminaria* (brown algae). *Lipids* **28**, 783-787.

Prusky, D. and Kobiler, I. 1985. Inhibitors of avocado lipoxygenase: their possible relationship with the latency of *Colletotrichum gloeosporioides*. *Physiol. Plant Pathol.* **27**, 269-279.

Ramadoss, C.S. and Axelrod, B. 1982. High-performance liquid chromatographic separation of lipoxygenase isozymes in crude soybean extracts. *Anal. Biochem.* **127**, 25-31.

Reynolds, P.A. and Klein, B.P. 1982a. Calcium interaction with soybean of type-2 pea lipoxygenase. *J. Food Sci.* **47**, 1999-2003.

Reynolds, P.A. and Klein, B.P. 1982b. Purification and characterization of a type-1 lipoxygenase from pea seeds. *J. Agric. Food Chem.* **30**, 1157-1163.

Rogan, M.M. and Kevin, D.A. 1996a. Application and limits of sample stacking in capillary electrophoresis. In *Capillary Electrophoresis Guidebook, Principles, Operation, and Applications*, Altria, K.D. (ed.), Humana Press, New Jersey, N.Y., pp. 211-226.

Rogan, M.M. and Kevin, D.A. 1996b. Chiral separations by capillary electrophoresis. In *Capillary Electrophoresis Guidebook, Principles, Operation, and Applications*, Altria, K.D. (ed.), Humana Press, New Jersey, N.Y., pp. 171-196.

Rouet-Mayer, M., Bureau, J. and Lauriere, C. 1992. Identification and characterization of lipoxygenase isoforms in senescing carnation petals. *Plant Physiol.* **98**, 971-978.

Roza, M. and Francke, A. 1973. Product specificity of soybean lipoxygenase. *Biochem. Biophys. Acta* **316**, 76-82.

Salch, Y.P, Grove, M.J., Takamura, H. and Gardner, H.W. 1995. Characterization of a C5,13-cleaving enzyme of 13(*S*)-hydroperoxide of linoleic acid by soybean seed. *Plant Physiol.* **108**, 1211-1218.

Sanders, T.H., Pattee, H.E. and Singleton, J.A. 1975. Lipoxygenase isozymes of peanut. *Lipids* **10**, 681-685.

Sanz, L.C., Perez, A.G. and Olias, J.M. 1992. Purification and catalytic properties of chickpea lipoxygenases. *Phytochem.* **31**, 2967-2972.

Satoh, T., Matsuda, Y., Takashio, M., Satoh, K., Beppu, T. and Arima, K. 1976. Isolation of lipoxygenase-like enzyme from *Fusarium oxysporum*. *Agric. Biol. Chem.* **40**, 953-961.

Schütte, H. and Kula, M.-R. 1988. Analytical disruption of microorganisms in a mixer mill. *Enzyme Microb. Technol.* **10**, 552-558.

Schmitz, O. and Gäb, S. 1997. Separation of unsaturated fatty acids and related isomeric hydroperoxides. *J. Chromatogr.* **767**, 249-253.

Segal, I.H. 1976. Enzymes. In *Biochemical calculations*, John Wiley and Sons, New York, N.Y., pp. 256-257.

Sekiya, J., Aoshima, H. and Kajiwara, T. 1977. Purification and some properties of potato tuber lipoxygenase and detection of linoleic acid radical in the enzyme reaction. *Agric. Biol. Chem.* **42**, 827-832.

Sessa, D.J. 1979. Biochemical aspects of lipid-derived flavors in legumes. *J. Agric. Food Chem.* **27**, 234-239.

Shastry, B.S. and Raghavendra Rao, M.R. 1975. Studies on lipoxygenase from rice bran. *Cereal Chem.* **52**, 597-603.

Shechter, G. and Grossman, S. 1983. Lipoxygenase from baker's yeast: Purification and properties. *Int. J. Biochem.* **15**, 1295-1304.

Shiiba, K., Negishi, Y., Okada, K. and Nagao, S. 1991. Purification and characterization of lipoxygenase isozymes from wheat germ. *Cereal Chem.* **68**, 115-122.

Shimahara, K. 1964. Peroxidation of soya oil with lipoxygenase-like bacterium (gram-negative *Bacillus*) separated from garbage. *Kogyo. Kajako. Zasshi.* **67**, 1164-1168.

Shimahara, K. and Hashizume, Y. 1973. Properties of a lipoxygenase-like enzyme produced by *Pseudomonas aeruginosa* A-4. *J. Ferment. Technol.* **51**, 183-189.

Shoun, H., Sudo, Y. and Beppu, T. 1983. Purification and properties of a cytochrome P-450 of a fungus, *Fusarium oxysporum*. *J. Biochem.* **94**, 1219-1229.

Siddiqi, A.M. and Tapple, A.L. 1956. Alfalfa lipoxidase. *Plant Physiol.* **31**, 320-321.

Siedow, J.N. 1991. Plant lipoxygenase: Structure and function. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **42**, 145-188.

Simmons, C.A., Kerwin, J.L. and Washino, R.K. 1987. Preliminary characterization of lipoxygenase from the entomopathogenic fungus *Lagenidium giganteum*. In *The Metabolism, Structure, and Function of Plant Lipids*, Stumpf, P.K., Mudd, J.B. and Nes, W.D. (eds.), Plenum Press, New York, N.Y., pp. 421-423.

Singleton, J.A., Pattee, H.E. and Sanders, T.H. 1976. Production of flavor volatiles in enzyme and substrate enriched peanut homogenates. *J. Food Sci.* **41**, 148-151.

Solem, M.L., Jiang, Z.D. and Gerwick, W.H. 1989. Three new and bioactive icosanoids from the temperate red marine alga *Forlowia mollis*. *Lipids* **24**, 256-260.

St-Angelo, A.J. and Kuck, J.C. 1977. Effects of cyanide on peanut lipoxygenase. *Lipids* **12**, 682-683.

Stevens, F.S., Brown, D.M. and Smith, A.L. 1970. Some properties of soybean lipoxygenase. *Arch. Biochem. Biophys.* **136**, 413-421.

Stone, E.J., Hall, R.M. and Kazeniac, S.J. 1975. Formation of aldehydes and alcohols in tomato fruit from U-^{14}C-labeled linolenic and linoleic acids. *J. Food Sci.* **40**, 1138-1141.

Stumpf, P.K. and Conn, E.E. 1980. In *Biochemistry of Plants, A Comprehensive Treatise*, Academic Press, New York, N.Y., pp. 571-572.

Su, C., Brodowsky, I.D. and Oliw, E.H. 1995. Studies on linoleic acid 8*R*-dioxygenase and hydroperoxide isomerase of the fungus *Gaeumannomyces graminis*. *Lipids* **30**, 43-48.

Summer, J.B. and Summer, R.J. 1940. The coupled oxidation of carotene and fat by carotene oxidase. *J. Biol. Chem.* **134**, 531-533.

Surrey, P.K. 1964. Spectrophotometric method for determination of lipoxidase activity. *Plant Physiol.* **39**, 65-70.

Takagi, T., Wakasa, N. and Miyashita, K. 1987. Formation of conjugated diene and triene products in lipoxygenase oxidation of C_{18}, C_{20}, C_{22} PUFAs. *J. Am. Oil Chem. Soc.* **64**, 1320-1323.

Tappel, A.L. 1963. Lipoxidase. In *The Enzymes*, Boyer, P.O., Lardy, H. and Myrback, K. (eds.), Academic Press, New York, N.Y., pp. 275-283.

Tappel, A.L., Lundberg, W.O. and Boyer, P.D. 1953. Effect of temperature and antioxidants upon the lipoxidase-catalyzed oxidation of sodium linoleate. *Arch. Biochem. Biophys.* **42**, 293-304.

Tapple, A.L. 1961. In *Autoxidation and Antioxidants*, Lundberg, W.O. (ed.), Interscience Publisher, New York, N.Y., pp. 325.

Theerakulkait C. and Barrett, D.M. 1995. Lipoxygenase in sweet corn germ: isolation and physicochemical properties. *J. Food Sci.* **60**, 1029-1040.

Theorell, H., Holman, R.T. and Akeson, A. 1947. Crystalline soybean lipoxidase system of alfalfa. *J. Biol. Chem.* **16**, 3-7.

Tressl, R. and Drawert, F.Z. 1973. Biogenesis of banana volatiles. *J. Agric. Food Chem.* **21**, 560-565.

Tressl, R., Bahri, D. and Engel, K. 1981. Lipid oxidation in fruits and vegetables. In *Quality of Selected Fruits and Vegetables of North America*, Teranishi, R. and Barrera-Benitez, H. (eds.), ACS Symposium Series, American Chemical Society, Washington, D.C., pp. 213-232.

Tressl, R., Bahri, D. and Engel, K. 1982. Formation of eight-carbon and ten-carbon components in mushrooms (*Agaricus campestris*). *J. Agric. Food Chem.* **30**, 89-93.

Van Aarlw, P.G.M., de Barse, M.M.J., Veldink, G.A. and Vliegenthart, J.F.G. 1992. A partially purified lipoxygenase from barley (*Hordeum distichum*) forms (*S*)-9-hydroperoxy-10-*trans*, 12-*cis*-octadienoic acid from linoleic acid. Catabolism of lipids. In *Plant Lipid Biochemistry, Structure and Utilization*, Quinn, P.J. and Harwood, J.L. (eds.), Portland Press Limited, London, pp. 289-291

Van Den, T. and Mendoza, E.M. 1982. Purification and characterization of two lipoxygenase isoenzymes from cowpea [*Vigna unguiculata* (L.) Walp.]. *J. Agric. Food Chem.* **30**, 54-60.

Van Den, T., Raymundo, L.C. and Mendoza, E.M. 1982. Winged bean lipoxygenase. Part 1: Isolation and purification. *Food Chem.* **8**, 277-288.

Veldink, G.A., Vliegenthart, J.F.G. and Boldingh, J. 1977. Plant lipoxygenases. *Prog. Chem. Fats Other Lipids* **15**, 131-166.

Veldink, G.A., Vliegenthart, J.F.G. and Boldinghg, J. 1970. The enzymatic conversion of linoleic acid hydroperoxide by flaxseed hydroperoxide isomerase. *Biochem. J.* **120**, 55-60.

Verhue, W.M. and Francke, A. 1972. The heterogeneity of soybean lipoxygenase. *Biochem. Biophys. Acta* **284**, 43-53.

Vernooy-Gerritsen, M., Bos, A.L.M., Veldink, G.A. and Vliegenthart, J.F.G. 1982. *Biochim. Biophys. Acta* **708**, 330-334.

Vick, B.A. and Zimmerman, D.C. 1976. Lipoxygenase and hydroperoxide lyase in germinating watermelon seedlings. *Plant Physiol.* **57**, 780-788.

Vick, B.A. and Zimmerman, D.C. 1981. Lipoxygenase, hydroperoxide isomerase and hydroperoxide cyclase in young cotton seedlings. *Plant Physiol.* **67**, 92-97.

Vick, B.A. and Zimmerman, D.C. 1983. The biosynthesis of jasmonic acid: A physiological role for plant lipoxygenase. *Biochem. Biophys. Res. Commun.* **111**, 470-477.

Vick, B.A. and Zimmerman, D.C. 1989. Metabolism of fatty acid hydroperoxides by *Chlorella pyrenoidosa*. *Plant Physiol.* **90**, 125-132.

Vick, B.A. and Zimmerman, D.C. 1987. In *The Biochemistry of Plants: A Comprehensive Treatise*, Stumpf, P.K. and Conn, E.E. (eds.), Academic Press, New York, N.Y., pp. 53-90.

Vick, B.A. and Zimmerman, D.C. 1987. Pathways of fatty acid hydroperoxide metabolism in spinach leaf chloroplasts. *Plant Physiol.* **85**, 1073-1078.

Vick, B.A. and Zimmerman, D.C. 1989. Metabolism of fatty acid hydroperoxides by *Chlorella pyrenoidosa*. *Plant Physiol.* **90**, 125-132.

Vioque, E. and Holman, R.T. 1962. Characterization of the ketodienes formed in the oxidation of linoleate by lipoxidase. *Arch. Biochim. Biophys.* **99**, 522-528.

Wagenknecht, A.C. and Lee, F.A. 1956. The action of lipoxygenase in frozen raw peas. *Food Res.* **21**, 605-610.

Wallace, J.M. and Whealer, E.L. 1979. Two lipoxygenase isoenzymes and an activator in wheat germ. *Phytochem.* **18**, 389-393.

Wardale, D.A. and Lambert, E.A. 1980. Lipoxygenase from cucumber fruit: localization and properties. *Phytochem.* **19**, 1013-1016.

Whitaker, J.R. 1972. In *Principles of Enzymology for the Food Sciences*, Fennema, O.R. (ed.), Marcel Dekker Inc., New York, N.Y., pp. 255-284.

Wurzenburger, M. and Grosch, W. 1982. The enzymatic oxidative breakdown of linoleic acid in mushrooms (*Psalliota bispora*), *Z. Lebensm. Unters. Forsch.* **175**, 186-190.

Wurzenburger, M. and Grosch, W. 1984a. The formation of 1-octen-3-ol from the 10-hydroperoxide isomer of linoleic acid by a hydroperoxide lyase in mushrooms (*Psalliota bispora*). *Biochim. Biophys. Acta* **794**, 25-30.

Wurzenburger, M. and Grosch, W. 1984b. Stereochemistry of the cleavage of the 10-hydroperoxide isomer of linoleic acid to 1-octen-3-ol by a hydroperoxide lyase from mushrooms (Psalliota bispora). *Biochim. Biophys. Acta* **795**, 163-165.

Wurzenburger, M. and Grosch, W. 1984c. Enzymic formation of 1-octen-3-ol in mushrooms. In *Progress in Flavor Research*, Adda, J. (ed.), Elsevier Science Publishers B.V., Amsterdam, pp. 253-259.

Wurzenburger, M. and Grosch, W. 1984d. Origin of the oxygen in the products of the enzymatic cleavage reaction of linoleic acid to 1-octen-3-ol and 1-oxo-trans-8-decenoic acid in mushrooms (*Psalliota bispora*). *Biochim. Biophys. Acta* **794**, 18-24.

Wurzenburger, M. and Grosch, W. 1986. Enzymatic oxidation of linolenic acid to 1,*Z*-5-octadien-3-ol, *Z*-2,*Z*-5-octadien-1-ol and 10-oxo-*E*-8-decenoic acid by a protein fraction from mushrooms (*Psalliota bispora*). *Lipids* **21**, 261-266.

Yabuuchi, S. and Amaha, M. 1976. Partial purification and characterization of the linoleate hydroperoxide isomerase from grains of *Hordeum distichum*. *Phytochem.* **15**, 387-390.

Yamamoto, A., Yasumoto, K. and Mitsuda, H. 1970. Isolation of lipoxygenase isozymes and comparison of their properties. *Agric. Biol. Chem.* **34**, 1169-1177.

Yamamoto, S. 1983. Enzymes in Arachidonic Acid Cascade. In *Prostaglandins and Related Substances*, Pace-Ascick, C. and Granstrom, E. (eds.), Elsevier, New York, N.Y., pp. 171-199.

Yamamoto, S. 1989. Mammalian lipoxygenases: molecular and catalytic properties. Prostaglandins leukotrienes and essential fatty acids. *Reviews* **35**, 219-229.

Yamamoto, S. 1991. Enzymatic lipid peroxidation: Reactions of mammalian lipoxygenases. *Free Radicals Biol. Med.* **10**, 149-159.

Yamamoto, S. 1992. Mammalian lipoxygenases: molecular structures and functions. *Biochim. Biophys. Acta* **1128**, 117-131.

Yamamoto, S. and Ishimura, Y. 1991. Lipoxygenases. In *Mechanism of Enzyme Action*, CRC Press, Boston, MA, pp. 322-327.

Yasumoto, A, Yamamoto, A. and Mitsuda, H. 1970. Effect of phenolic antioxidants on lipoxygenase reaction. *Agric. Biol. Chem.* **34**, 1162-1168.

Yoon, S. and Klein, B.P. 1979. Some properties of peas lipoxygenase isozymes. *J. Agric. Food Chem.* **27**, 955-962.

Yudelson, J. 1983. Visualization of protein electrophorgrams by physical development. Kodak Laboratories Chemicals Bulletin #74, Eastman Kodak Company, Rochester, New York, N.Y.

Zamora, R., Olias, J.M. and Mesias, J.L. 1987. Purification and characterization of tomato lipoxygenase. *Phytochem.* **26**, 345-347.

Zhang, Y.Y., Radmark, O. and Samuelsson, B. 1992. Mutagenesis of some conserved residues in human 5-lipoxygenase: Effects of enzyme activity. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 485-489.

Zimmerman, D.C. 1966. The new product of linoleic acid oxidation by a flaxseed enzyme. *Biochem. Biophys. Res. Commun.* **23**, 398-402.

Zimmerman, D.C. and Coudron, C.A. 1979. Identification of traumatin, a wound hormone, as 12-oxo-*trans*-10-dodecenoic acid. *Plant Physiol.* **63**, 536-541.

Zimmerman, D.C. and Snyder, H.E. 1974. A role of calcium in activating soybean lipoxygenase-2. *J. Agric. Food Chem.* **22**, 802-805.

Zimmerman, D.C. and Vick, B.A. 1970. Specificity of flaxseed lipoxidase. *Lipids* **5**, 392-397.

Zimmerman, D.C. and Vick, B.A. 1974. Lipoxygenase in *Chlorella pyrenoidosa*. *Lipids* **8**, 264-266.

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LIST OF PUBLICATIONS

REFEREED JOURNALS

Bisakowski, B. and Kermasha, S. 1997. Microbial lipoxygenases and their role in the formation of specific flavor precursors. (to be submitted)

Bisakowski, B., Kermasha, S. and Spinnler, H.-E. 1997. Characterization of purified lipoxygenase extracts from *Fusarium proliferatum*. (to be submitted)

Bisakowski, B., Perraud, X. and Kermasha, S. 1997. Characterization of hydroperoxides and carbonyl compounds obtained by lipoxygenase extracts of selected microorganisms. *Biosci. Biotech. Biochem.* **61**, 1262-1269.

Bisakowski, B., Kermasha, S. and Klopfenstein, M.L. 1995. Studies on partial purified lipoxygenase from *Fusarium oxysporum*. *Process Biochem.* **30**, 261-268.

Bisakowski, B., Kermasha, S. and Marsot, P. 1995. Partial purification of lipoxygenase in *Chlorella pyrenoidosa*. *Biotechnol. Appl. Biochem.* **21**, 39-48.

Bisakowski, B., Kermasha, S., Lavorel, V. and Belin, J.M. 1995. Partial characterization of a lipoxygenase from *Fusarium proliferatum*. *Food Biotechnol.* **9**, 189-205.

Bisakowski, B., Kermasha, S. and Schuepp, C. 1995. Partial purification and catalytic properties of lipoxygenase from *Saccharomyces cerevisiae*. *World J. Microb. Biotechnol.* **11**, 494-496.

Kermasha, S., Bisakowski, B., Ramaswamy, H. and van de Voort, F.R. 1993. Thermal and microwave inactivation kinetics of soybean lipoxygenase. *Lebensm.-Wiss. u.-Technol.* **26**, 215-219

Kermasha, S., Bisakowski, B., Ramaswamy, H. and van de Voort, F.R. 1993. Comparison of microwave, conventional and combination heat treatments on wheat germ lipase activity. *International J. Food Sci. Technol.* **28**, 617-623.

PRESENTATION AT SCIENTIFIC MEETINGS

Bisakowski, B. and Kermasha, S. (1997). Biocatalysis of selected partially purified microbial lipoxygenase using linoleic acid in a model system. 88th Annual Meeting of the American Oil Chemists' Society, Seattle, WA, USA, May 11-14.

Kermasha, S. and Bisakowski, B. 1997. The effect of immobilization on the hydrolytic/interesterification activity of lipase from *Rhizopus niveus*. 88th Annual Meeting of the American Oil Chemists' Society, Seattle, WA, USA, May 11-14.