Partial characterization of lentil seed lipoxygenases and their impact in wheat (*Triticum aestivum L.*) bread making

by

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been subjected to any university for a degree.

_____   March 2017
_____

Stefan Hayward   Date

In loving memory of Jacobus Malan Hayward

"Learn from yesterday, live for today, hope for tomorrow. The important thing is not to stop questioning"

- Albert Einstein

SUMMARY

This study describes:

1. The evaluation of lentil seed flour as an alternative for soybean seed flour; as a source of lipoxygenase during the making of white bread.

2. The development of efficient, and reproducible methods for the purification of lentil seed lipoxygenase from flour.

3. An evaluation of the current methods for the quantification of products from lipoxygenase-catalyzed reactions, and the adaptation of these methods to enable high-throughput screening and kinetic analysis of lipoxygenase.

4. The kinetic and molecular characterization of lentil seed lipoxygenase with specific mention to the down-stream implications when used in the bread-making process.

5. The application of lentil seed extracts, and purified enzyme solutions in baking trials.

OPSOMMING

Hierdie studie beskryf:

1. Die evaluering van lensie saad meel as 'n alternatief vir sojaboon saad meel as bron van lipoksigenase tydens die bak van wit brood.

2. Die ontwikkeling van effektiewe, en herhaalbare metodes vir die suiwering van lensie saad lipoksigenase vanuit meel.

3. 'n Evaluering van die huidig-beskikbare metodes vir die kwantifisering van produkte afkomstig vanaf lipokigenase-gekataliseerde reaksies, en die aanpassing van hierdie metodes om hoe-deurset aktiwiteitsbepaling, en kinetiese karakterisering van lipoksigenase moontlik te maak.

4. Die molekulêre, en kinetiese karakterisering van lensie saad lipoksigenase met spesifieke melding van die implikasies van die gebruik van lipoksigenase tydens die bak-proses.

5. Die gebruik van lensie saad ekstrakte, en gesuiwerde ensiem oplossing in bak proewe.

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ABBREVIATIONS USED

Lipoxygenase	LOX
9S-hydroperoxy-octadecadienoic acid	9S-HPODE
13S-hydroperoxy-octadecadienoic acid	13S-HPODE
Ferrous oxidation-xylenol orange	FOX
nordihydroguaiaretic acid	NDGA
Methylene blue bleaching	MBB
Dichlorofluorescein	DCF
Dichlorofluorescein diacetate	DCF-DA
Excitation wavelength	E_x
Emission wavelength	E_m
Change in absorbance	$\Delta Absa$
Molar	M
Millimolar	mM
Well depth	h
Radius	R
Molar extinction coefficient	ε
Light path length	l
Milligram	mg
Microgram	µg
Unit	U
Minute	min
Nanomole	nMol
Bovine serum albumin	BSA
Volt	V
Dithiothreitol	DTT

Small unilammelar vesicles	SUVs
Isoelectric point	pI
Isoelectric focusing	IEF
SDS	Sodium dodecyl sulfate
PAGE	Polyacrylamide gel electrophoresis
Two dimensional	2-D
Center for proteomic and genomic research	CPGR
Liquid chromatography	LC
Mass spectrometry	MS
Liquid chromatography tandem mass spectrometry	LC-MS
Triscarboxyethyl phospine	TCEP
Trifluoracetic acid	TFA
Michealis constant	K_m
Maximum velocity	V_{max}
Sub-Saharan Africa	SSA
Alliance grain traders	AGT
Genetically modified	GM

Chapter 1

INTRODUCTION

There has never been such an abundance of readily available food as today. Yet, none has greater significance in human history than bread. Wheat, used to produce bread, was the first cultivated crop resulting in the establishment of agriculture some 10,000 years ago. The practice of agriculture in turn enabled humans to become farmers instead of nomadic hunters, leading to the formation of society. As such, apart from becoming one of the major global staple foods, bread has become an emotional symbol of prosperity.

Today, although the main ingredients of bread are still the same as in ancient times, there is an increasing consumer demand for consistency, quality and safety during bread manufacture. However, quite ironically, the most variable factor in the breadmaking process is also the main ingredient, namely wheat[1]. Wheat contains a wide range of endogenous enzymes, which influence the flour and resulting bread quality made from wheat flour. The levels and activities of these endogenous enzymes are influenced by the conditions during cultivation, harvesting, storage and milling. For example, during sprouting of wheat the activity of endogenous α-amylase is upregulated, rendering the flour unfit for breadmaking[1,2]. On the other hand, too low levels of α-amylase activity results in a reduction in product quality. A demand for greater consistency constituted the rationale for the current use of various chemical and enzyme additives. The use of additives allows for improved control of the bread-making process enhancing quality, stability and production efficiency.

The use of additives for wheat flour standardization, and improving the baking properties of wheat flour dough is well established. However, due to concerns about the effect of chemical additives on human health, enzymes have gradually replaced these compounds. The use of enzymes also has the advantage of full inactivation by heat-treatment eliminating their down-stream effects during storage. In the baking industry enzymes are routinely added to increase dough rheology, fermentation, stability, crumb color, etc. Lipoxygenases (LOX) in the form of enzyme active soybean flour is used during the production of white bread to give a whiter crumb, and increase dough rheology. However, soybean is a major food allergen which limits its use in various products[3–5]. Recombinant DNA technology could circumvent the limitations associated with the use of enzyme-active flour as source for LOX. However, due to an increasing concern

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among customers about the use of recombinant DNA technology for the production of enzymes, there is an increased demand for purified enzymes from natural sources.

Soybean seeds contain three different LOX isozymes namely LOX-1, LOX-2, and LOX-3 which differ in terms of optimal pH, substrate preference, product formation and stability[6–9]. Furthermore, results from baking trials with soybean null-mutants for LOX-1, LOX-2 and LOX-3, respectively, showed that all three isozymes are required for dough-strengthening and bleaching capacity[10,11]. As such, purification of each soybean seed LOX isozyme would not be financially viable for industrial application. A source expressing a single isozyme with comparable activity to soy is therefore required.

In a study by Chang and McCurdy[12], it was shown that among 14 legumes evaluated, lentil seeds contain a single LOX enzyme with similar activity to soy (Table 1.1). When assayed at a neutral pH, lentil seeds showed higher activity than soybean flour. Based on this data, lentils could potentially be an ideal source for the large-scale purification of LOX for application in the baking industry. However, lentil seed flour has not yet been evaluated as dough improver during the production of white bread.

Table 1.1: Protein content and LOX activity in fourteen legumes as presented by Chang and McCurdy[12]

Common name	Protein content (% dry basis)	Lipoxygenase activity		
		Flour (U/mg)	Crude extract (10^3 U/mL)	Extractable protein (10^6 U/mL)
Black bean	23.49	780	78	4.97
Great northern bean	27.92	1000	100	4.26
Navy bean	24.84	750	75	3.54
Pinto bean	24.93	970	97	4.78
Red kidney bean	27.00	1240	124	5.49
Adsuki bean	24.33	950	95	11.73
Chickpea (UC-5)	26.93	380	38	2.95
Chickpea (Mission)	18.75	430	43	3.61
Cowpea	26.70	2560	256	12.61
Faba bean (Diana)	30.90	700	70	6.87
Faba bean (Maris Bead)	32.58	740	74	4.24
Faba bean (Hertz Freya)	30.90	760	76	4.09
Field pea (Triumph)	27.75	840	84	5.76
Field pea (Century)	24.99	920	92	6.53
Field pea (Tara)	18.23	960	96	6.76
Lentil	24.95	3720	372	24.12
Baby lima bean	23.82	510	51	2.54
Large lima bean	23.35	350	35	1.94
Mung bean	26.69	280	28	1.68
Soybean (pH 6.9)	43.57	2370	237	12.28
Soybean (pH 9.0)	43.57	5570	557	28.86

Chang and McCurdy[12] showed that lentil seeds have comparable LOX activity to soybean seeds, and should therefore function as a valid alternative to soybean seeds, however, these authors did not specify which strain of lentil seed was used. Therefore, to establish whether lentil seed flour could function as an alternative source for LOX during the production of white bread, the effect of inclusion of various lentil seed flours on the baking characteristics of dough was evaluated in this study. The efficiency of each lentil flour as dough improver was gauged against soybean flour as control. Once the flour showing the best improvement was established, this flour was used for all subsequent studies.

Due to the benefits associated with the use of purified enzymes in the food industry, currently there is a greater demand for purified enzyme additives. Extraction of enzymes from natural sources could furthermore add value to the starting raw material by enabling the production of multiple value-added products from the same source. For example, soybean seed flour is extracted for oils, fats, protein and fiber which is used for the manufacturing of a wide range of different products[13–15]. The ability to produce multiple products from a single source, thereby increasing profitability of the raw material, resulted in the term value-added products. Although it has been suggested that lentil seeds contain at least two different LOX isoforms[16,17], this possibility has not yet been conclusively proven. Methods for the purification of lentil seed LOX from flour were therefore developed. The purified enzyme(s) were then characterized in terms of its kinetic- and molecular characteristics. In so doing proof for the presence of multiple isoforms was presented.

However, for purification and successful application of purified enzymes, efficient methods for the determination of enzyme activity are needed to ensure efficiency and reproducibility of the results obtained during baking. As will be discussed in more detail in Chapter 2, various methods for the determination of LOX activity have been described. However, most of these methods are difficult to use, and are not suited to accurate, high-throughput activity determination required during purification and commercialization[18–23]. The current methods for LOX activity determination were therefore evaluated for efficiency and adaptability to a high-throughput format. The most suitable assay was subsequently adapted to 96-well microtiter plate format and validated against the current standard method for LOX activity determination. This assay was used for all the studies conducted.

The effect of the purified lentil seed LOX enzymes on the baking characteristics of white bread dough was subsequently evaluated. In these trials, the results were compared to that obtained using soybean seed flours, and extracts. The effect of adding the substrate of LOX, namely linoleic acid, was also evaluated.

The studies conducted will be presented as follows:

Chapter 2 provides a detailed background to the LOX enzyme, and its application in the bread making industry. The history, purification, molecular characteristics, and reaction mechanism will be discussed in detail with focus on the current model for carotenoid bleaching and rheological improvement. Methods for LOX activity determination are also reviewed. This chapter concludes with a review of recombinant enzyme production and the possible use within bread manufacturing.

Chapter 3 evaluates the different methods used for activity determination of LOX, and their application in high-throughput activity assays. These assays were evaluated in 96-well microtiter plate format. The strengths and weaknesses of each method, as well as their potential use for peak identification during chromatography, are evaluated. The development and optimization of a lentil seed LOX purification strategy and the results obtained are discussed in detail.

In Chapter 4 the characterization of the lentil seed LOX enzyme is described in terms of temperature-, and pH-optima. Using the optimal conditions determined, the isolated enzyme(s) was kinetically characterized and discussed. Based on the results obtained using 2-dimensional polyacrylamide gel electrophoresis and tryptic digest mass spectrometric analysis, evidence for multiple lentil seed LOX isoforms are also presented.

In Chapter 5 the results of baking trials performed with lentil seed flour, extracts and purified enzymes are described and compared to results obtained using soybean seed flour as control. The difficulties associated with the use of extracts and purified enzymes are briefly outlined.

In conclusion, Chapter 6 presents a general overview and discussion of the results obtained in this study. The implication for the baking industry and the role of lentil seed LOX in bread whitening and rheological improvement is discussed. This chapter concludes with recommendations for future studies involving lentil seed LOX enzymes.

Chapter 2

LIPOXYGENASES: FROM ISOLATION TO APPLICATION

The content of this Chapter was published in "*Comprehensive reviews in food science and food safety* (doi: 10.1111/1541-4337.12239)". The paper is presented at the end of this thesis.

ABSTRACT

The positive effect of lipoxygenase, added as enzyme-active soy flour, during the production of white bread is well established. In addition to increasing the mixing tolerance and overall dough rheology, lipoxygenase is also an effective bleaching agent. It is known that these effects are mediated by enzyme-coupled co-oxidation of gluten proteins and carotenoids. However, the mechanism whereby these effects are achieved is not yet fully understood. In order to gain a better understanding into the reactions governing the beneficial effects of lipoxygenases in bread dough, an in-depth knowledge of the lipoxygenase catalytic mechanism is required. Until now no single review combining the molecular enzymology of lipoxygenase enzymes and their application in the baking industry has been presented. This review, therefore, focuses on the extraction and molecular characterization of lipoxygenases in addition to the work done on the application of lipoxygenases in the baking industry.

2.1 INTRODUCTION

The existence of an enzyme catalyzing the oxidative destruction of carotene was first reported by Bohn and Haas[24]. These authors discovered that the inclusion of small quantities of soybean flour in wheat dough resulted in the bleaching of wheat flour pigments. Since it was thought that color loss was solely due to oxidation of carotene, the enzyme was subsequently named carotene oxidase[24,25]. Around the same time period, Andre and Hou[26] showed that soybeans contained a "lipoxidase" enzyme which catalyzes the peroxidation of various unsaturated fatty acids[25,27]. Craig[28] subsequently showed that suspensions of *Lupinus albus* consumed large quantities of oxygen, compared to the amount of CO_2 produced, in the presence of lipids. This author designated the name unsaturated fat oxidase to the responsible enzyme. It was only 4 years later when Sumner[29] and Tauber[30], respectively, recognized that both reactions are catalyzed by the same class of enzyme. Following this, the names carotene oxidase,

unsaturated fat oxidase, and lipoxidase were consolidated and the responsible enzyme officially became known as lipoxygenase.

Lipoxygenase (linoleate: oxidoreductase, EC 1.13.11.12, LOX) is a group of non-heme metal-containing dioxygenases which catalyze the regio- and stereo-specific dioxygenation of polyunsaturated fatty acids to conjugated unsaturated fatty acid hydroperoxides[6,8,9,31–34]. Although these enzymes are widely distributed in the animal and plant kingdoms, they are particularly abundant in grain legume seeds and potato tubers[2,6,9,25,32,35]. Plants furthermore contain multiple isoforms of the LOX enzyme which differs in terms of its substrate preference, optimal pH, product formation, and stability[6–9,27,36]. Due to the high level of soybean LOX expression, most of the current knowledge on the enzymology and structural biology of lipoxygenase enzymes are derived from studies on soybean LOX isoforms[26,27,36–38].

The use of enzyme-active soy flour to fortify wheat flour, intended for the production of white bread, is well documented[6,7,33,39–41]. Apart from increases in protein content and nutritional value, enzyme-active soy flour enhances the baking properties and color of wheat flours fortified with it[33]. The latter of these effects are mediated by a complex interaction of LOX with native wheat flour lipids and carotenoids. In order to gain a better understanding of the mechanism whereby these effects are achieved, an in-depth knowledge on the reaction mechanism of LOX catalyzes is required. However, to the best of our knowledge, a review combining the molecular enzymology of LOX enzymes, and the application of LOX enzymes in the baking industry, has not yet been presented. This paper therefore aims to resolve this by reviewing the current knowledge on the molecular enzymology of the LOX family of enzymes. This will be followed by a discussion on the mechanism whereby exogenous LOX improves the baking properties of wheat flour dough. However, although LOX from many sources has been studied, throughout this work emphasis is on soybean LOX as this is the current commercial source for LOX in the baking industry.

2.2 MOLECULAR ENZYMOLOGY OF LOX

2.2.1 Classification and nomenclature

LOX isozymes were historically named based on their ease of purification, stability, and optimal pH of catalysis. However, owing to the diversity of the LOX enzymes currently known, this system is no longer practical. Higher plants produce multiple isoforms of the LOX enzyme. These isozymes differ in terms of optimal pH of catalysis, substrate preference, regiospecificity, and ability to bleach carotenoid pigments[8,9,35,42]. The natural substrate for plant LOX are the C18-polyunsaturated fatty acids linoleic and

α-linolenic acid[8,27,36]. The reaction of these substrates with LOX yields either 9S- or 13S-hydroperoxides depending on the LOX isozyme catalyzing the oxygenation[8,9,27,34,40,43]. The current nomenclature for LOX enzymes is therefore based on the specificity of the enzyme acting on its substrate[8]. Soybean seed LOX- 1 catalyzes the oxygenation of α-linoleic acid to, almost exclusively, 13S-hydroperoxy-octadecadienoic acid (HPODE)[8,27]. The LOX-1 enzyme is therefore designated 13-LOX. On the other hand, soybean seed LOX- 2, a 9/13-LOX, produces equal amounts of both 9S-HPODE and 13S– HPODE[36,38]. This isozyme is furthermore unique in its ability to utilize esterified unsaturated fatty acids in membranes, compared to LOX-1 which has an absolute requirement for free fatty acids[36,40]. LOX-3, on the other hand, has a moderate preference for the production of 9S-HPODE resulting in the classification as a 9-LOX. When comparing plant LOX with its animal counterparts, the chain lengths of the natural substrates (linoleic acid vs. arachidonate) result in the plant 13-LOX corresponding to a 15-LOX in animals[8]. However, both enzymes act on the ω-6 position of the fatty acid chain.

This system of nomenclature has, however, become somewhat confusing with growing family diversity. The major reason for this confusion is that the current nomenclature does not take evolutionary and functional relatedness into account[37,43]. This becomes especially apparent when comparing plant and animal LOX enzymes, since they do not use the same substrates. Further complications arise when multiple isoforms of, for example, mammalian 12-LOX, are present in the same organism[8,43]. Ivanov and others[43] therefore proposed a classification procedure which is based on phylogenetic relatedness. However, no unifying LOX nomenclature, which could overcome these difficulties, has been introduced. Currently, different LOX isozymes, catalyzing the same reaction within the same organism, are named after the prototypical tissue of their occurrence with reference to their regiospecificity[8,34]. For example, there are three isoforms of mammalian 12-LOX. These enzymes are therefore designated platelet, leukocyte or epidermal 12-LOX [8].

2.2.2 Extraction and purification

Soybean seeds are the richest known source of LOX contributing up to 2% of the total protein content[2,8,31,44]. As such, most of the current knowledge on LOX-catalyzed oxygenation of polyunsaturated fatty acids is based on studies using soybean LOX isoform 1. Since the time this enzyme was first described in the first half of the 20th century, the family diversity has expanded to include both the animal and plant kingdoms[6,25]. Interest was sparked in this enzyme for its biotechnological application in baking[24], and later

flavor production[45]. However, in order to study and optimize its application in industrialized settings, pure LOX isozymes are required.

Aqueous extraction of soybean flour yields a complex mixture of protein, peptides, carbohydrates, oligosaccharides, pigments, and other low-molecular-mass compounds[46]. Purification of LOX from this complex mixture is further complicated by the presence of multiple isoforms with similar molecular mass and pI values[6,25,27,41,47]. This close resemblance can lead to the misidentification of a specific LOX isoform. When the purified enzyme is intended for mechanistic studies, misidentification would cause errors during the interpretation of the results[6]. Two main types of LOX have been described. Type I LOX enzymes, such as LOX-1, have an optimal activity between pH 9 and 10, while type II LOX, which includes isoforms 2 and 3, are most active between pH 6.5 and 7[32]. Care should therefore be taken during identification of the purified enzyme.

Soybean LOX was first purified to considerable purity by Balls and others[21]. The enzyme was subsequently crystallized by the group of Theorell[48]. At that time, however, it was thought that a single LOX isozyme (LOX-1) was responsible for the carotene-bleaching ability of soybean flour. The second (LOX-2) and third isoforms (LOX-3), along with an isozyme designated "LOX-b", were subsequently purified by the groups of Christopher[49,50] and Yamamato[51]. In these studies it was shown that LOX enzymes can be purified by conventional techniques of protein isolation, including ammonium sulfate precipitation, ion-exchange, and size-exclusion chromatography[21,25,47]. Each LOX isoform can be extracted from ground soybean flour, with varying yields, using different buffers. In a study by Diel and others[47] the authors showed that LOX-1 is optimally extracted using 0.1 M sodium acetate, pH 4.5, while LOX-2 is most efficiently extracted using 0.05 M sodium phosphate, pH 6.0. However, others have reported using buffers in the pH range between 4.5 and 6.8 for extraction[51]. Active LOX enzymes can subsequently be precipitated using ammonium sulfate in the saturation range of 30 – 60%[47]. Following precipitation, LOX enzymes are recovered using successive chromatographic procedures. Each soybean seed isozyme has a distinct isoelectric point ranging between 5.7-6.4[47,49–51]. Based on these differences in pI, the enzymes can be purified from ammonium sulfate-concentrated flour extracts using successive anion and cation exchange chromatography steps[25,50]. Furthermore, soybean LOX isozymes are large monomeric proteins with a molecular mass ranging between 94-100 kDa[6,8,27,36,47]. Due to this relatively high molecular mass the enzyme can be resolved from smaller proteins and other low-molecular-mass compounds by gel permeation chromatography[41,52,53]. However, gel permeation chromatography has only a limited scope

separating large samples of similarly-sized proteins. This technique is therefore employed during the final clean-up of samples separated using ion exchange chromatography.

2.2.3 LOX reaction mechanism

Enzyme primary structure

LOX enzymes are non-heme iron-containing enzymes folded into a two-domain secondary structure[8,27,43]. The N-terminal region of 25- 30 kDa consists of a β-barrel domain which shares significant homology with the C-terminal domain of mammalian lipases[8,34,43,54]. Based on this homology, it is thought that this domain plays a role in membrane-binding and the acquisition of lipid substrates during catalysis[8,43]. The second larger α-helical catalytic domain contains a single non-heme iron molecule octahedrally coordinated by 3 histidine residues, the C- terminal isoleucine, an asparagine residue, and a hydroxide as shown in Figure 2.1[8,34,43,54,55]. This non-heme iron exists in one of two oxidation states, namely, Fe (II) or Fe (III)[56]. According to the current working mechanism, in the absence of substrate and autoxidized lipid species, the native enzyme remains inactive and the iron is in the low-spin Fe (II) state[56,57]. The enzyme is subsequently activated by oxidation of the active-site iron to the ferric form by hydroxylipids. Once activated, the enzyme cycles between the inactive (Fe (II)) and active (Fe (III)) states by product activation. In the active form, the enzyme catalyzes stereospecific hydrogen abstraction and oxygen insertion in polyunsaturated lipid species[43,56,57].
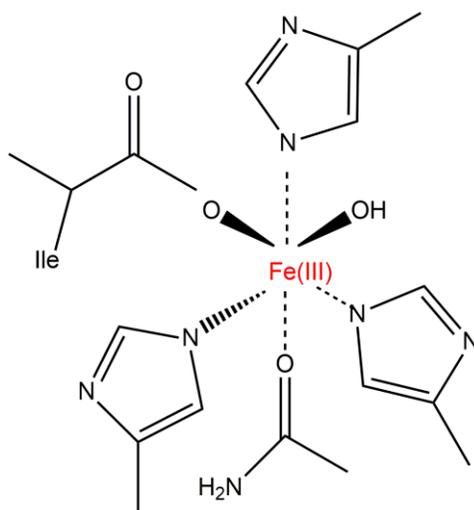
Figure 2.1 Octahedral coordination of the non-heme iron in the LOX active site. The figure was adapted from Brodhun and others[58].

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The active site of soybean LOX consists of an inter-domain-crevice containing 2 major cavities (cavities I and II) which intersect in close proximity of the non-heme iron[43,59]. Cavity II is furthermore subdivided into 2 sub-cavities, namely, cavity IIa and IIb by the side chains of Val354 and Arg707[43]. Cavity IIa is thought to function as the substrate-binding pocket since this cavity is intersected by a side channel, between Ile553 and Trp500, which is thought to direct oxygen to the active site[43]. Ile553 has been implicated in modulating the alignment of fatty acids entering the active site[43,60]. Once aligned inside the substrate binding site, a hydrogen is abstracted and oxygen is introduced at either the [+2] or [-2] position from the original site of abstraction[34,43]. Since different LOX isozymes produce either 9- or 13- HPODE, an orientation-related hypothesis for positional specificity, as depicted in figure 2.2, has been established. In this mechanism a fatty acid substrate penetrates the active site, methyl end first in the case of 13-LOX enzymes, whereas in the case of 9-LOX enzymes the substrate is forced into the binding pocket with its carboxyl group first[34,61]. In this way radical rearrangement may be facilitated by the same mechanism in both cases. This theory is supported by the observation that the position of oxygen insertion may be governed by pH[32,61]. LOX-1 exclusively produces 13-HPODE at a pH between 9 and 10. However, with a decrease in pH the formation of 9- HPODE is favored[62,63]. Formation of the product is therefore thought to be mediated by pH-dependent deprotonation of the carboxylic acid to form a more polar carboxylate which is not able to enter the hydrophobic active site[32,61]. The substrate subsequently enters the active site with the less polar methyl group first. This theory suggests that the regiospecificity of oxygen insertion is controlled by the carboxylate anion/carboxylic acid ratio of the substrate[32,63].
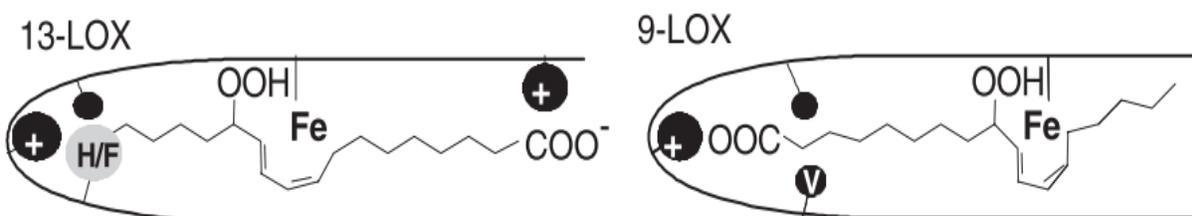
Figure 2.2 Depiction of the orientation-related hypothesis for the production of both 13- and 9-HPODE by a single LOX enzyme. In this figure the fatty acid substrate enters the active site methyl end first to produce 13-HPODE and carboxyl end first to produce 9-HOPTE. Based on observations from soy LOX-1 this mechanism is thought to be governed by a pH-dependent formation of a carboxylate which, based on its polarity, cannot enter the active site [34].

Reaction mechanism

Fatty acid oxygenation occurs in 4 consecutive steps: (i) hydrogen abstraction, (ii) radical rearrangement, (iii) oxygen insertion, and (iv) peroxyradical reduction as indicated in Figure 2.3[8,27,37,43,64,65]. Hydrogen

abstraction and oxygen insertion occurs in antarafacial sides in relation to the 1Z,4Z-pentadiene unit[27,36]. During catalysis hydrogen abstraction constitutes the rate-limiting step and corresponds to proton-coupled electron transfer[43,64,65]. In this reaction the electron is directly tunneled from the substrate to the ferric state iron[55,66,67]. In this way the enzyme is cycled between its active and inactive state by its substrate and products, as illustrated in Figure 2.3[8]. As such, the enzyme is product-activated and, in the absence of fatty acid hydroperoxides, the enzyme remains in an inactive state. It has furthermore been shown that the type of hydroperoxide, such as 9- or 13-HPODE, plays an important role during activation of different LOX isoforms[68]. These studies will be discussed in more detail below. Nevertheless, in kinetic studies, this "activation" step is observed as a lag phase that could be abolished by the addition of small quantities of fatty acid hydroperoxides[9,40,64]. In wheat flour dough, initial activation, by conversion of Fe (II) to Fe (III), is thought to occur via a small pool of autoxidized substrates leading to a chain reaction culminating in activation of the remaining inactive enzyme[9,64,69,70].
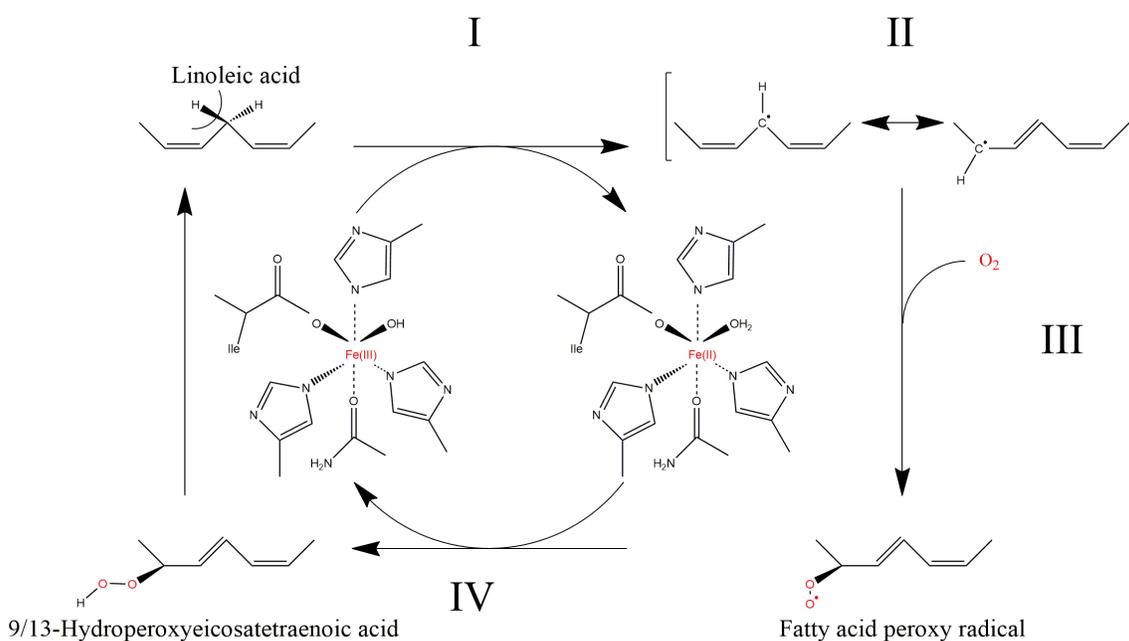
Figure 2.3 Mechanism of LOX catalysis. As depicted here, the LOX enzyme is cycled between the active Fe (III) and inactive Fe (II) states by the products and substrates of the reaction, respectively. Hydrogen abstraction results in the formation of a carbon radical, which is subsequently stabilized by electron rearrangement. This is followed by oxygen insertion and reduction to form a hydroperoxide product. Figure adapted from Brodhun and others[58].

Following hydrogen abstraction, a carbon radical is formed which is stabilized by a Z,E-double bond conjugation[27,37,43]. This is followed by the stereospecific insertion of molecular oxygen at the +2 or -2 position with relation to the original radical carbon to form a peroxy radical as well as a conjugated *trans*,

cis-diene chromophore[8,34,36,37,43]. This peroxyradical intermediate is subsequently reduced to form hydroperoxide in the fourth step of catalysis.

Under normal conditions this reaction may not be considered an effective source for free radicals since the intermediates remain enzyme-bound[43,56]. However, it has been shown that, under certain conditions, a considerable proportion of the reactive oxygen species may be prematurely released leaving the enzyme in an inactive Fe (II) state[35,56,57]. The group of Berry[57] showed that regiospecificity and radical release, as measured by oxidation of β-carotene, relies on the amount of dioxygen present. Based on these observations, Zoia and others[56] subsequently set up experiments using ^{31}P NMR spin trapping to obtain direct evidence for the radical-escaping mechanism in relation to dioxygen concentration. In this study the authors could define 3 distinct phases in terms of radical generation.

- An initial dioxygen consumption phase during which the dioxygen concentration is not limiting. In this phase the regiospecificity of the LOX reaction is maintained, and low levels of oxygen-centred radical species are released. The formation of oxygen-centered species relates to regiospecificity, as described by Berry and others[57].
- In the second phase, where the dioxygen concentration becomes limiting, an increased rate of carbon-centered radical release is observed. Carbon-centered radicals are correlated to a reduction in the regiospecificity of the LOX reaction. These results are in agreement with those in the earlier literature[57].
- In the third phase, where the dioxygen concentration is essentially zero, the enzyme generates radicals up to complete deactivation due to an inability to reactivate via hydroperoxides[9,71].

Once released from the enzyme active site the reactive oxygen species interact with, and oxidize, sensitive molecules, such as thiol-containing proteins, antioxidants, and pigments[35]. These interactions have important functions in dough rheology and bleaching.

Oxygen requirement of LOX catalysis

LOX also functions under anaerobic conditions, on condition that both polyunsaturated fatty acids and hydroperoxide products are present[72–74]. The anaerobic reaction, depicted in Figure 2.4, is initiated in a similar manner to the aerobic mechanism wherein a radical is formed due to hydrogen abstraction from the linoleic acid substrate[72]. However, since no oxygen is available for oxygenation, this reaction results in the formation of various carbonyl compounds. In order to maintain activity under oxygen-limiting

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conditions, the active-site iron is oxidized to Fe (III) by hydroperoxides instead of O_2[40,57,73]. In this reaction the hydroperoxide product is reductively cleaved into a hydroxide ion and an alkoxyl radical[40,72,74].
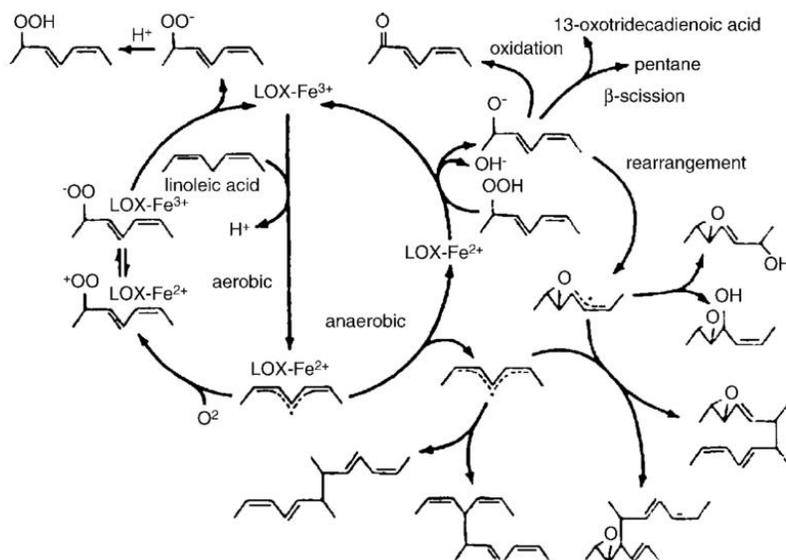
Figure 2.4 *LOX-catalyzed product formation during aerobic and anaerobic reactions with polyunsaturated lipid substrates as suggested by Gardner* [40].

The radicals produced have various downstream implications in the baking industry. Although thorough mixing is associated with aeration of the dough, oxygen concentrations may be reduced during fermentation by yeast metabolic processes. As such, radical formation may be augmented during the proving of the dough. It has been shown that these radicals have major implications in carotenoid bleaching and flavor development[68,75]. A reduction in oxygen concentrations have furthermore been associated with a decrease in the specificity of LOX-catalyzed oxygen insertion. In a study by Berry and others[57] it was shown that the products 13-HPODE and 9-HPODE are produced by LOX-1 in a 1:1 ratio under anaerobic conditions. This is in stark contrast to the high-level regiospecificity (13-HPODE: 9-HPODE 95:5) observed from LOX-1 catalysis under aerobic condition. As will become clear in the ensuing section, carotenoid bleaching, flavor development, and rheological improvement all rely on combinations of the activity of the LOX isozymes. However, the activities of these enzymes are modulated by their reaction products. These effects will be discussed in more detail in the following sections.

Chiral-specificity of oxygenation

In most cases, especially in plants, the reaction of LOX with polyunsaturated fatty acids produces hydroperoxy products which are in the S configuration, irrespective of the carbon which is oxygenated[27,32].

However, LOX enzymes catalyzing the formation of HPODE products in the R configuration have been found among invertebrates, plants, and mammals[8]. The reason for this chiral specificity is still unknown. It has, however, been reported that the antarafacial relationship between the abstracted hydrogen and the inserted oxygen determines the chirality of the resulting product[32]. This relation is thought to be due to the orientation of the oxygen connected to the iron atom in the active site.

2.2.4 Lox activity determination

For the successful industrial application of enzymes, details of their activity and reaction mechanism are required. For this purpose, a wide array of activity assays has been developed. In the ensuing section, the major assays, and some of their drawbacks, will briefly be discussed.

Spectrophotometric assays

As discussed previously, the presence of LOX in the seeds of legumes was based on the ability to react with pigments, mainly xanthophylls, in the presence of unsaturated fatty acids[24,25]. As such, one of the first methods for the detection of LOX activity was based on the co-oxidation of carotene in the presence of polyunsaturated fatty acids[21]. In this method the rate of carotenoid bleaching is determined in timed intervals. However, determination of bleaching efficiency is not an accurate activity measure, since this assay has a limited range over which activity is proportional to enzyme concentration. The substrate mixture is furthermore inherently unstable, and large variations between carotene oxidase of different LOX enzymes, compared to their peroxidizing activities, are common[18,25].

In 2 separate studies the groups of Holman[76] and Theorell[22], observed an increase in the absorbance at 234 nm of LOX-oxidized fats. These authors independently showed that the increase in absorbance was due to the formation of a conjugated diene in linoleic acid. As such this method has become known as the diene conjugation method[22,25]. The diene conjugation method is more sensitive than the carotene co-oxidation assay, since it measures product formation directly. This assay could furthermore be applied for kinetic studies when a recording spectrophotometer is used[23,25]. However, although this method is superior in sensitivity, it has the disadvantage of a tendency towards turbidity due to a limited solubility of linoleic acid in aqueous suspensions[18]. Surrey[23] subsequently optimized this method with the introduction of "Surrey's" substrate mixture wherein linoleic acid is dissolved and clarified using 0.25% Tween® 20 and 1 M NaOH, respectively. To date, adaptations of this substrate mixture are still the most commonly used substrate. The conjugate diene method, performed with Surrey's substrate mixture, is

the current standard method for LOX activity determination based on sensitivity and reproducibility. However, due to the low molar absorbance of the reaction products, this assay remains susceptible to interference by protein absorption when preparations of low specific activity are assayed[25].

Based on the above-mentioned drawbacks, it was subsequently shown that interference could be overcome by determination of downstream products of the formed hydroperoxides. One of the oldest of these methods relies on the interaction of hydroperoxide products with ferrous thiocyanate[77]. During this reaction the formed hydroperoxides oxidize Fe (II) to Fe (III), which subsequently reacts with thiocyanate to form a colored product which can be determined spectrophotometrically[77,78]. This colored product is, however, unstable which limits its use. Waslidge and Hayes[79] subsequently substituted thiocyanate with xylenol orange, a quantitative cation indicator[80]. The use of xylenol orange, in the ferrous oxidation-xylenol orange (FOX) method, yields a stable colored product which has a high molar absorption, eliminating the need for specialized equipment[78–80]. This assay is furthermore highly reproducible, rapid, and not sensitive to oxygen[80]. However, due to the requirement of an acidic environment for color development, this assay can only be performed in end-point styled assays. As such, it is not suited for kinetic studies and can only account for the presence of LOX, and is not an accurate representation for the relative amount of LOX unless compared to a known standard. False positives are also possible when the sample can chelate iron[81].

Spectroscopic assays

The hydroperoxides formed during LOX catalysis are also able to oxidize a variety of electron-donating probes in reactions catalyzed by heme-containing compounds such as hematin, hemoglobin, and cytochrome C[78]. Such coupled assays provide colorimetric[82], fluorometric[83], and chemiluminescent[18] methods for the detection of hydroperoxides formed by LOX[78]. For this purpose, various probes and dyes have successfully been applied. The group of Okawa[84] has shown that thiobarbituric acid reacts with linoleic acid hydroperoxides to form malondialdehyde, a red pigment which can be determined spectrophotometrically. However, this assay has the drawback that color development is optimal at pH 4.0, well below the optimum pH for LOX, and the assay is sensitive to variations in pH, thus limiting its use for kinetic characterization of the enzyme. The use of fluorescent probes, which can be oxidized by fatty acid hydroperoxides, can overcome the difficulties associated with background interferences in spectrophotometric assays. Whent and others[81] showed that addition of fluorescein to solutions containing linoleic acid and LOX results in high fluorescence. Fluorescein has previously been used as a

probe in free radical-scavenging assays, as this probe is degraded by peroxy radicals to yield a fluorescent product[81,85]. However, this assay is susceptible to interferences by Tween® 20 and secondary lipid oxidation products which can contribute to fluorescence. Therefore, although this assay is highly sensitive, care must be taken when the assay is performed, since higher LOX concentrations may influence the total assay time. The data from large amounts of samples, with varying concentrations of LOX, are therefore not readily comparable. This method could, however, be developed into a high-throughput assay[81]. Chemiluminescent assays have also been developed. Lilius and Laakso[18] showed that free radical processes during lipid peroxidation result in the emission of low-level chemiluminescent light. These authors showed that this low-level chemiluminescent light could be amplified by the addition of luminol. It was furthermore shown that, when performed under properly selected conditions, this method is comparable in sensitivity to the spectrophotometric assay. Luminescence could furthermore be enhanced by the addition of cytochrome C[86,87].

Oxygen consumption

Based on the requirement for oxygen during the LOX-catalyzed oxygenation of polyunsaturated fatty acids, activity can be determined by monitoring O_2 usage in an attempt to overcome the difficulties associated with spectrophotometric assays. This method is as sensitive as the conjugated diene method, however, the results from these assays do not always agree. Holman[88] noted that when secondary reactions occur, as is the case with crude soybean extracts, spectrophotometric assays fall short on theoretical yield based on oxygen uptake. This observation is due to reactions which consume the hydroxylipids during catalysis, and cannot be accounted for when using the spectrophotometric assays. Activity assays which are based on O_2 consumption are, therefore, superior in accuracy. However, based on equipment requirements, this method has limited value as a high-throughput activity assay for large numbers of samples.

2.3 LOX IN THE BAKING INDUSTRY

Various chemicals are frequently added to flour in order to improve its bread-making performance. The benefits for the use of chemical additives, such as potassium bromate and azodicarbonamide, on dough rheology are well established[89,90]. However, due to doubts about the effect on health such additives have been gradually replaced with additives generally regarded as safe[89]. As such, the use of enzymes as replacements for chemical improvers have recently received substantial attention due to restrictions on the use of chemical additives[2,91,92]. Enzyme catalysis is furthermore highly specific, and limits the

production of nonspecific by-products. Efficient application could therefore enable the production of products with predetermined qualities. Various enzymes are currently used in order to improve the quality, and shelf-life, of the final product. For a review of these enzymes, and their purposes, please see references shown by Martínez-Anaya[93], Miguel and others[2], and Popper and others[892,89,93]. As discussed earlier, enzyme-active soy flour has been used in the baking industry for almost 100 years[24]. The ensuing section will discuss the role of LOX during dough rheological improvement and bleaching in more detail.

2.3.1 Gluten

The production of bread requires dough to be elastic, so as to enable inflation during fermentation. The ability to retain CO_2, resulting from fermentation, relies on the viscoelastic properties of dough, a property which is reliant on the protein content of the wheat flour. The mixing characteristics of wheat flour and the rheological properties of the resulting doughs are largely determined by the properties of the major storage proteins of wheat, the prolamins[94]. When mixed with water, these proteins interact to form a cohesive protein network known as gluten[95]. The term gluten therefore does not describe a single protein, but the proteinaceous network formed upon interaction of the wheat storage proteins described below.

The proteins comprising the prolamins can be broadly classified based on their extractability in aqueous alcohols into either the extractable gliadins or unextractable glutenins[96–98]. The extractability of these proteins relies largely on their ability to form inter-chain disulfide bonds, with glutenins consisting of high-molecular-weight polymers entirely stabilized by disulfide bonds[96,98]. Reduction of the glutenin polymer results in the liberation of, predominantly, low-molecular-weight glutenin subunits[99]. These low-molecular-weight glutenins contain 8 cysteines, 6 of which partake in intrachain disulfide bonds [100,101]. Due to steric hinderance, the remaining 2 cysteine residues are not able to form intrachain disulfide bonds and, consequently, only form interchain disulfide bonds with other glutenin proteins[99]. This intra- and interchain disulfide bonding results in the formation of the high-molecular-weight glutenin polymers [101]. Based on the formation of such disulfide bond stabilized polymers, glutenins contribute to the elasticity of dough. In contrast to glutenins, gliadins are monomeric proteins which contain either no cysteine (ω5- and ω1,2-gliadins) or only intramolecular disulfide bonds (α- and γ-gliadins), and they contribute towards viscosity[101]. Due to these properties the prolamins confer the property of viscoelasticity, an important factor in determining the suitability of a flour for its end use[91,97,102]. For instance, bread-making requires highly elastic "stronger" doughs containing more glutenins, while doughs for cakes and biscuits should be more extensible, consisting of more gliadins[96,98,102].

The proteins comprising gluten can be separated by SDS-PAGE analysis into high- and low-molecular-weight fractions which differ in terms of gene composition [95,103]. The high-molecular-weight proteins are encoded by genes located at the Glu-A1, Glu-B1 and Glu-D1 loci on the long arms of chromosomes A1, B1, and D1, respectively [103,104]. The short arms of the same chromosomes contain the loci Gli-A1, Gli-B1 and Gli-D1 which, respectively, encode for ω-gliadins, γ-gliadins and low-molecular-weight glutenins[104]. The remaining low-molecular-weight gliadins are encoded by genes located at the Glu-A3, Glu-B3 and Glu-D3 loci[103]. Since both glutenins and gliadins contain low-molecular-weight proteins, which differ in terms of gene composition, confusion can arise when ascribing low-molecular-weight proteins to either the glutenins or gliadins. Recent research therefore caused a gradual move from the terminology glutenin and gliadin to high- and low-molecular weight proteins, though the terms glutenin and gliadin remains valid. Although both the high- and low-molecular-weight fractions are important in determining the functional properties of wheat dough, the high-molecular-weight fraction of glutenins are the key determinants of wheat dough quality[103,105,106]. Furthermore, the composition and size distribution of this group of proteins play a major role in the functional properties of the resulting doughs[94,96,97,102]. Early experiments have shown that the addition of reducing agents such as mercaptoethanol or dithiothreitol results in a weaker dough with a decreased tolerance to overmixing[94,96]. Conversely, addition of oxidizing agents is associated with an increase in the average molecular weight of dough proteins and an increased tolerance to overmixing[31,94,107]. As such, addition of compounds broadly known as improving agents to flour is common practice during the production of breads and rolls of all types[6]. These additives not only play an important role in flour maturation, but also accelerate dough development, improve dough strength and workability, and increase reproducibility of the final product[96,107]. These effects are mainly facilitated by the oxidation of cysteine residues and the concurring formation of intramolecular disulfide bonds between glutenin proteins during gluten formation[31,89]. Disulfide bond formation is regarded as the major force binding wheat storage proteins incorporated into the integrated gluten network[31].

LOX is considered an oxidative improving agent, since the products formed during the oxidation of flour fatty acids have a cross-linking effect on the flour proteins by oxidation of the glutenin thiol groups[6,31,33]. As such, the use of LOX, in the form of enzyme-active soybean flour, as dough improver is commonplace during the production of white bread[6,7]. In addition to improving mixing tolerance and dough rheology, inclusion of enzyme-active soybean flour also bleaches the wheat flour carotenoid pigment to yield a whiter crumb[2,6,10,31,41]. However, although it is now generally accepted that rheological improvement and dough bleaching is mediated by processes of co-oxidation, the mechanism of co-oxidation is complex and remains obscure[6,39]. The major reason for this is that lipid oxidation and carotenoid bleaching and/or

sulfhydryl oxidation, although linked, are not the same activities[6,32]. Various groups have shown, using enzyme extracts, that pigment destruction could be inhibited by the addition of antioxidants, such as ascorbic acid, without a reduction in diene formation[108,109]. Likewise, Frazier and others[39] showed that inhibiting the appearance of lipid peroxidation products, by addition of nordihydroguaiaretic acid (NDGA), also did not have an effect on dough rheology. In these studies, ascorbic acid and NDGA, respectively, removed the intermediate and the product of the LOX reaction with lipids. These results suggested that it is a reactive intermediate in the LOX reaction that is involved in co-oxidation, and not the final hydroperoxide product. This theory is strengthened by the observation that addition of exogenous hydroperoxides have no effect on dough rheology or pigment destruction[6,39].

2.3.2 Rheological improvement

Historically wheat flour was matured by exposure to oxygen in order to improve its baking properties. Dahle and Sullivan[110] suggested that the efficiency of natural aging was, at least in part, due to the destruction of antioxidants. Among these, the -SH groups of glutenins may also be considered as an antioxidant, although it is not as efficient as tocopherols and pigments which are also present. The improving effect of natural aging has also been associated with an increase in the free lipid content and a concurrent decrease in the -SH content[110,111]. Tsen and others[112] argued that the -SH groups are oxidized by fatty acid peroxides, which are formed during LOX-catalyzed peroxidation of free lipids[110]. However, Frazier[39] showed that replacing the flour-free lipids with peroxidized lipids had no effect on the rheological properties of the dough when baked. Based on these results, it was suggested that the radical intermediates, formed during LOX-catalyzed lipid oxygenation, were responsible for oxidation of -SH groups in dough during mixing. According to Graveland and others[113], normal wheat contains between 5 and 7 µmol thiol per gram flour, of which 0.5 – 1.5 µmol is oxidized during mixing[40]. Bloksma[114] consequently concluded that a conversion of a relatively few sulfhydryls to disulfide bonds could significantly increase a dough's resistance to deformation and overmixing.

In addition to the formation of disulfide bonds, the oxidized thiyl moieties can also cross-link with water soluble pentosans, via ferulic acid esterified to arabinoxylans, to form viscous gels in a process known as oxidative gelation[115–117]. During oxidative gelation the double bond of ferulic acid is activated by free radicals, enabling oxidative phenolic coupling of arabinoxylan molecules with adjacent arabinoxylans, protein tyrosine residues, and thiyl residues[115–118]. This cross-linking results in the formation of a high-molecular weight entity with multiple cross-linkages, which, in aqueous suspensions, translates to

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increases in viscosity[118]. Cross-linking only occurs in the presence of oxidizing agents capable of producing free radicals, and not in the presence of the common oxidants potassium iodate, potassium bromate, or ascorbic acid[115,116,118]. Oxidative gelation is therefore an alternative mechanism whereby LOX improves dough rheology based on the formation of reactive intermediates during the oxygenation of linoleic acid[117,119].

2.3.3 Pigment destruction

As discussed earlier, the LOX family of enzymes was first discovered based on the catalysis of wheat flour pigment destruction[24,25,49]. Carotenoids are among the most common naturally occurring pigments, with over 600 molecular species being fully characterized[27,120]. In photosynthetic organisms these compounds play an important role in the protection against photo-oxidative stresses and are essential structural components of the photosynthetic antenna[121]. Here these compounds are efficient antioxidants which are able to scavenge singlet molecular oxygen and peroxy radicals[120]. Protection against molecular oxygen depends largely on physical quenching and involves energy transfer between both molecules resulting in a ground-state oxygen and a triplet excited carotene[120,122]. The carotenoid subsequently dissipates the energy by an interaction with the surrounding solvent. However, this reaction is of minor importance in terms of pigment destruction since the carotenoid remains intact following return to ground-state energy.

Of the known carotenoids, β-carotene is the most prominent[123]. It has been shown that β-carotene is capable of inhibiting various free-radical reactions[124,125]. As such, it has been hypothesized that β-carotene, as well as other carotenoids, contribute toward protection of membranes from lipid peroxidation products[120,123]. In wheat flour dough, fortified with LOX, this antioxidant activity relates to pigment destruction. The mechanism of peroxyl radical inactivation is based on the formation of resonance-stabilized carbon-centered radical adducts[120,123,125]. In this reaction, peroxy radical attack on carotenoids usually occurs at the terminal double bond, resulting in epoxide formation at the site of addition[125]. This is followed by homolytic cleavage of the peroxide bond resulting in the formation of an epoxide and release of an alkoxyl radical[125]. This reaction may also occur via abstraction of an electron directly from the polyene by a peroxy radical resulting in the formation of an intermediate ion pair consisting of a peroxide ion and a radical cation of β-carotene[125]. Irrespective of the route of formation, these products subsequently combine to form the epoxide radical addition product. As such, interaction of β-carotene with peroxy radicals results in the formation of a β-carotene radical which subsequently reacts with oxygen to form carotenoid peroxyl radicals[32,126,127]. Following their formation, these radicals

initiate attack on other β-carotenoids, resulting in a chain reaction culminating in pigment destruction and the formation of 5,6 epoxides, β-apo-13-carotenone, and conjugated polyene ketones[126–128]. A simplified schematic for the formation of volatile flour compounds, as presented by Martínez-Anaya[93], is presented in Figure 2.5.
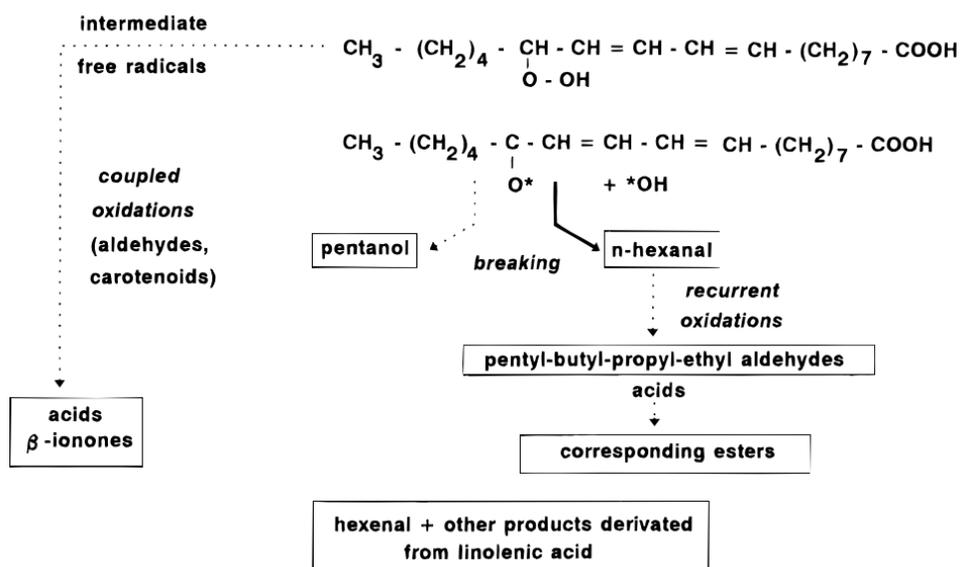
Figure 2.5 LOX catalyzed volatile formation originating from coupled oxidation reactions. Linoleic acid-derived products, such as hexanal, have various implications in the food industry[93].

2.3.4 LOX and flavor

Since flavor is one of the most appreciated sensory attributes of food products, the formation of volatile flavor compounds is of major importance to food scientists. However, although most volatile flavor compounds are perceived as having a positive contribution to the sensory experience, some compounds may have a deleterious effect, even at low levels. For example, short-chain C_6- or C_9-aldehydes and alcohols, formed by hydroperoxide lyases, isomerases, and dehydrogenases, are responsible for the natural aromas of fruits and vegetables[32,35]. However, in some cases, these products may be perceived as serious off-flavors. The use of soy is a good example of such a case. Although the use of soy in various food products is well established, if used in excess, the addition of soybean may result in the formation of volatile off-flavors. For use in bread, soy flour is therefore only used up to 0.5% to keep the adverse effect of volatile flavor down[10,33]. Fujimaki and co-workers[129] showed that hexanal, derived from LOX-catalyzed hydroperoxidation of linoleic acid, is primarily responsible for flavors perceived as grassy or beany[45,130,131]. The flavor produced by hexanal furthermore has an extremely low taste threshold. The use of soybean in other food products has also been associated with the formation of off-flavors during storage[7]. On the

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other hand, in some applications these compounds, known as green leaf volatiles, are perceived as fresh natural flavors[45]. Depending on the application, care should therefore be taken when using soybean flour, for its LOX function, in order to limit off-flavor production.

2.3.5 Co-oxidation potential of LOX isoforms

Studies on the co-oxidation of β-carotene have shown that the efficiency of co-oxidation is dependent on the LOX isoform catalyzing the reaction. During early experiments Kies[132] showed that heat treatment of partially purified preparations of soybean LOX resulted in a loss of the ability to co-oxidize β-carotene without having an effect on the peroxidation of linoleic acid. However, at that time it was not yet known that soybean has multiple LOX isoforms. This author subsequently concluded that catalysis of the coupled oxidation reaction relies on a heat-sensitive entity which is distinct from the one purified by Theorell[48]. Although various LOX isoforms were subsequently discovered, these results indicated that each of the different isoforms plays a significant role during catalysis of β-carotene co-oxidation. Moreover, these results indicate that the enzymes display synergism with relation to co-oxidation of β- carotene.

Evidence for a synergistic mechanism of the LOX isozymes was presented by the group of Ramadoss[68] when they showed, using purified LOX isozymes, that none of the LOX isozymes are individually efficient catalysts for co-oxidation of β-carotene. Bleaching capacity was only regained using combinations of LOX-1 or LOX-2 and LOX-3, respectively. However, bleaching capability was only observed for isoforms 2 and 3. LOX isoform 1 is not an efficient catalyst for enzyme-coupled oxidation of carotenoids. These results suggested that LOX-1 and 2, respectively, produce a product which is able to facilitate carotene-bleaching by LOX-3. In an analysis of this process, it was discovered that addition of 13-HPODE, produced by LOX-1 and to some extent LOX-2, to inactive LOX-3, recovered bleaching ability[68,127]. Conversely, addition of 9-HPODE was much less efficient in promoting carotenoid-bleaching[32,133]. As discussed earlier, depending on the incubation conditions, LOX-2 produces mixtures containing different ratios of 13- and 9-HPODE. Based on these results it can be assumed that activation of LOX isozymes relies strongly on activation by 13-HPODE. Similar work by Weber and others[134] showed that crocin was only bleached in the presence of either LOX-2 or 3, but not LOX-1, suggesting the formation of especially active radicals in the presence of LOX-2 and 3.

2.3.6 Studies evaluating the effect of LOX isozymes on baking

Multiple attempts have been made to isolate the various soy LOX isozymes and to investigate the rheological and baking properties of doughs fortified with them[10,31,33]. Van Ruth and others[11] studied the effect of stored soybean preparations on the baking properties of wheat flour dough. During these experiments it was shown that LOX isoforms 2 and/or 3 were primarily responsible for bleaching and the formation of volatile flavor compounds. Studies by Addo and others[33] subsequently showed that soybean LOX-2 is also the major isoform responsible for the production of the undesirable n-hexanal aroma compound[32]. However, these studies did not elaborate on the effect of individual LOX isozymes on other baking properties of wheat flour dough. Cumbee and others[10] subsequently used soy mutant isolines and purified LOX enzyme preparations to investigate the effect on wheat flour dough rheological and bread-baking properties. In this study addition of a null mutant containing only isoforms 1 and 2 resulted in an increase in dough tenacity (resistance to extension), consistent with the addition of control soy flour containing all three isoforms. Furthermore, dough extensibility decreased when null mutants for isoforms 1 and 3 were added. Contrary to the increase in dough strength observed when soy flour containing all three isoforms were added, addition of the isoform 3 null mutant did not have any effect, while a decrease was observed upon addition of null mutants for isoforms 1 and 2. As a result, loaf volume of bread loaves baked using mutants containing only isoforms 2 and 3 and the mutant containing only isoforms 1 and 2 were increased over the control. Among these, the isoform 1 null mutant had the greatest effect on loaf volume. In contrast to the results obtained using null mutants, addition of purified LOX isozymes resulted in an increase in dough extensibility and strength. These results were in agreement with that obtained by Shiiba and others[41] using wheat flour LOX suggesting that LOX-1 plays an accessory role rather than an active role in dough improvement.

Taken together, these results indicate that, in terms of dough rheology, isoforms 1 and 2 are required for dough strengthening. However, of these isoform 2 is the most important since omission of this isoform results in a reduction in tenacity, extensibility, and dough strength. In terms of carotenoid bleaching ability, Isoforms 2 and 3 are most essential, since isoform 1 does not display significant bleaching capacity. On the other hand, LOX isoform 2 is also responsible for the formation of undesirable volatile flavor compounds, and isoform 3 relies on the products of isoform 1 for bleaching capacity. Based on the data presented it is safe to assume that, when using soybean LOX, the ratio of each isoform present plays a significant role in product quality. However, apart from catalyzing the oxygenation of polyunsaturated fatty acids, LOX-2 is also capable of using ester lipids bound to membranes and lipoproteins[37]. It is

therefore possible that the overall positive effects of LOX-2 are due to an ability to use substrates which are inaccessible to LOX-1 and 3.

2.3.7 Recombinant LOX as improver

In most cases it is possible to use enzyme-active soy flour as an improver. However, soybean seeds contain multiple enzymes which reduce polyunsaturated substrate availability, or metabolize the hydroperoxide products. The benefit of added LOX can therefore be modulated by the presence of these enzymes and/or the products of their catalysis[35]. In addition to volatile off-flavor production, and possible reaction modulation by secondary reactions, soybeans are considered a major food allergen warranting labeling of its use[3–5]. These limitations could be circumvented with the use of recombinant enzyme technology. The use of recombinant enzymes could overcome the allergenicity of soy, by production of LOX in the absence of soybean flour. Among others, heterologous expression of soybean LOX-1[135], soybean LOX-3[136], rice LOX-2[137], *Arabidopsis thaliana* LOX-1[138], pea seed LOX-2 and 3[139], barley seed LOX-2[140], and lentil LOX-1[53], along with stress-induced LOX from potato[141] and rice[142] have been successfully achieved[139]. However, many of these proteins have been expressed as fusion or truncated proteins for use in mechanistic studies. Furthermore, application of recombinant soy LOX proteins to baking may be difficult since synergism exists between these LOX isozymes. The positive effects conveyed by enzyme-active soybean flour are therefore reliant on the presence of all the LOX isozymes. LOX isozymes, which are capable of carotenoid bleaching and dough strengthening, from other sources should therefore be evaluated. The group of Zhang[143] succeeded in expression and purification of LOX from *Anabaena* sp. PCC 7120 in *Bacillus subtilis*. Following on these results, the authors showed that a single recombinant enzyme could be used for the improvement in both whiteness and loaf volume, as compared to benzoyl peroxide and potassium bromate. This study illustrated the potential of recombinant enzyme technology. However, this study did not compare their results against enzyme-active soy flour as an improver but against benzoyl peroxide. No conclusions for its effectiveness when compared against the current standard, could therefore be reached. LOX isozymes from other sources may therefore be better suited to the baking industry.

2.4 CONCLUSION

The LOX enzyme family is diverse and isozymes often differ in terms of activity, substrate specificity, and product formation, even in the same organism. Although enzyme-active soybean flour has been used for its improving effect on dough rheology and crumb color for about a 100 years, its use remains limited to 0.5% due to off-flavor production and allergenicity. Recombinant enzyme technology offers a solution to

these problems. However, due to synergy of soybean LOX isozymes a single recombinant soybean LOX may not be feasible as an improving agent. A single recombinant LOX enzyme capable of bleaching and rheological improvement is therefore required. Various authors have succeeded in the isolation and purification of both native and recombinant LOX enzymes. However, of these only a few have been evaluated as active ingredients in baking, and very few studies resulted in significant improvement. Therefore, although recombinant enzyme technology is more economically viable than purification of enzymes from its native source, the current information regarding the LOX reaction mechanism, and the effect of individual enzyme on dough quality is not yet sufficient to enable the use of recombinant enzyme technology. Due to the difficulties, alternative natural sources for LOX isolation and purification were investigated.

It has been shown that lentil seeds have LOX activity comparable to that of soybean seed flour[12]. Although it has been suggested that lentil seeds may contain multiple LOX isoforms, to date it has not yet been conclusively proven. It therefore remains possible that lentil seeds may contain only a single LOX isoform. In such an instance, the availability of a single natural LOX isozyme capable of improving dough quality would permit better insights into the mechanistic details of reactions catalyzed by LOX in bread dough. Lentil seed flour was therefore evaluated as alternative to soybean flour for use in bread manufacture. The enzyme(s) was purified and characterized in terms of pH-, and temperature optima and the kinetic parameters K_m, and V_{max} were determined. The number of lentil seed LOX isoforms was determined by tryptic digest mass spectrometric analyses after two-dimensional polyacrylamide gel electrophoreses. Finally, the effect of purified enzyme(s) on dough rheology was evaluated in baking application trials using standardized commercial recipes.

Chapter 3

ISOLATION AND PURIFICATION OF LENTIL LIPOXYGENASES

3.1 INTRODUCTION

Since ancient times, enzymes have played an indispensable role in food production. There are mainly two scenarios for the use of enzymes in food production[2]. Enzymes are either applied to produce the final product directly from the raw material, or they are added to improve the functional characteristics of the final product. The production of cheese by addition of rennet to milk, and the use of lipoxygenases (LOX) for the rheological improvement of bread dough are examples of the former- and latter scenarios, respectively. Although various enzymes are added as purified preparations, many are added as a constituent of a more complex or even crude enzyme mixtures. LOX is added to white bread flour in the form of full-fat enzyme-active soybean flour. When enzymes are added as part of a more complex mixture it is often difficult to establish the role of individual constituents on the final quality of the product. The problem becomes more complex when synergism exist between the different enzymes and/or constituents are present. As discussed in Chapter 2, synergism often exist between different isoforms of the same enzyme, as is the case with soybean LOX.

In this study, an alternative source for LOX was investigated. Lentil seed flour was selected based on the relative amount, and total amount of LOX activity when compared to soybean seeds. Although different authors have described the presence of multiple isoforms of LOX in lentil seeds, consensus on the amount and molecular properties of each isozyme has not yet been reached[16,17]. As such, it remains a possibility that lentil seeds may contain only a single LOX isoform, reducing the difficulties associated with synergism. The purification of lentil seed LOX will therefore be discussed in this chapter.

For the successful application of enzymes as processing aids in any industry, methods for quality assurance are essential. As discussed in Chapter 2, various methods for LOX activity determination have been described. However, no accurate high-throughput method exists whereby activity could be determined in multiple samples simultaneously. Besides the use in quality control of the final purified product, such assays are also required for the identification of LOX activity within different fractions collected during chromatography.

This chapter describes the evaluation, and development, of LOX activity assays which can be used for high-throughput activity determination of chromatography, and purified samples. These methods were also used for activity determination prior to the trials conducted in Chapter 5. The purification of lentil seed LOX via a multistep fractionation method is described and the purified protein obtained in this study was applied in the baking trials presented in Chapter 5.

3.2 MATERIALS AND METHODS

Unless stated otherwise, all reagents, chemicals, resins, and solvents used in this study were of analytical grade and were obtained from reputable suppliers. Whatman DE52 and DE32 anion exchange cellulose, and GE Healthcare Q Sepharose fast flow chromatography resins were obtained from Sigma-Aldrich (St. Louis MO, USA). Fractogel® TSK CM-650(S) cation exchange resin was purchased from Merck (Darmstadt, Germany). Protein concentrations were determined using the Pierce BCA protein quantification kit (Pierce, Rockford III, USA), unless stated otherwise. Linoleic acid was obtained from Sigma-Aldrich (St. Louis MO, USA). Type I-B Lipoxygenase from *Glycine max*, used as LOX standard, was obtained from Sigma-Aldrich (St. Louis MO, USA). Greiner UV-Star® 96 well plates were purchased from Sigma-Aldrich (St. Louis MO, USA). AGT PulsePlus™ Lentil Flour V-6000™ was obtained from DuPont® SSA Innovation Center Cape Town, South Africa.

3.2.1 Flour extraction

Throughout the course of extraction, 1 mL aliquots were collected, and stored at -20°C, at each successive step during extraction and purification. The protein concentration and LOX activity of each fraction was determined by the methods described below. The values determined were used to calculate the purification factor and yield.

Crude extraction

Lentil V-6000 and Soybean flour were extracted by suspension in 10 volumes of 10 mM phosphate buffer pH 7.0. This suspension was stirred for 1 hour at 4°C. The crude protein extract was recovered by centrifugation at 15000 x g at 4°C for 30 min. The supernatant, containing active LOX enzyme(s), were collected and the pellet discarded. For chromatography 20g of flour was extracted with 200 mL phosphate buffer.

Determination of total extractable material

To determine the total amount of extractable material in the lentil flour, 25 g lentil flour was suspended in 250 mL distilled water (0.1 mg/mL) and stirred for 1 h at 4°C. This suspension was subsequently centrifuged at 15000 x g for 30 min at 4°C and the supernatant collected. The supernatant fraction was then filtered through a Millipore 0.45 μM pore size type HVHP filter. Prior to weighing, round bottom flasks were dried at 100°C in a vacuum oven. Once dry, the flasks were cooled in a desiccator filled with silica beads. The weight of each flask was then accurately determined to the nearest milligram. Of the filtered extract, exactly 100g was then transferred to the pre-weighed round bottom flask and freeze-dried. Once completely dry, the flasks, containing the total extracted protein, were weighed and the value noted. The % extractable material was calculated by dividing the mass obtained by 10g, the total amount of dry flour per 100 mL distilled water, multiplied by 100. Extractions and freeze-drying was performed in duplicate and the values obtained were averaged.

For comparison 25g lentil flour was extracted in the same way as described above using 10 mM sodium phosphate buffer, pH 7.0, as the extraction solvent. Following extraction, the sample was recovered, filtered and freeze dried as described above. The extractions were compared based on the amount of dry powder and activity recovered following freeze-drying. In order to account for the contribution of buffering salts to the total % extractable material, 0.140 g was subtracted from the mass obtained from each extraction using phosphate buffer.

Precipitation

Following extraction, the sample was concentrated by addition of solid ammonium sulfate. In order to determine the optimal % ammonium sulfate saturation, solid ammonium sulfate, corresponding to the degrees of saturation listed in Table 3.1, was added to 20 mL crude extract. Each sample was mechanically shaken for 10 min at 1000 RPM on an IKA Vibrax VXR shaker. Following agitation, the samples were kept at 4°C for 1 hour. Each sample was subsequently centrifuged at 20000 x g, 4°C for 30 min. Following centrifugation, the supernatant of each sample was decanted into clearly marked vials. The pellet was patted dry using a paper towel and resuspended in 20 mL 10 mM sodium phosphate buffer pH 7.0. LOX activity of the precipitate and supernatant fractions was subsequently determined using the microtiter plate conjugated diene method described later in the text. The optimal amount of ammonium sulfate thus determined was used for the remainder of the study.

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Table 3.1: *Degrees of saturation used for the optimization of ammonium sulfate saturation. The g/L column refers to the gram of solid ammonium sulfate required to obtain the % saturation listed*

% Saturation	g/L	g/20mL
10	53	1.06
20	110	2.20
30	170	3.40
40	233	4.66
50	301	6.02
60	373	7.46
70	450	9.00

Following precipitation, the pellet, containing the active LOX enzyme(s), was collected by centrifugation at 10000 xg, 4°C for 30 min. The pellet was subsequently dissolved in 10 mM sodium phosphate buffer pH 7.0 in 25% of the extraction volume. Therefore, for chromatography, samples were dissolved in 50 mL buffer. Once dissolved the sample was dialyzed overnight against 10 volumes 10 mM sodium phosphate buffer, pH 7.0 at 4°C. The resulting dialysate was subjected to subsequent chromatography procedures.

3.2.2 Activity assays

As discussed in Chapter 2, several assays for LOX activity have been described. However, most of these assays are not practical for use during high-throughput screening of multiple samples. During column chromatography, each of the collected fractions must be assayed in order to determine where LOX elutes.

In the following section, various assays were evaluated for ease of use, accuracy and usefulness during peak identification following chromatography. The assays were also evaluated for their suitability for the kinetic characterization of LOX enzymes.

Preparation of the substrate

The substrate solution used throughout this study was prepared using an adapted method originally described by Surrey[23]. The substrate solution was prepared in 50 mL batches in order to limit freeze-thaw cycles of the pure linoleic acid stock. Following use, the stock solution was purged of oxygen and stored under nitrogen at -20°C.

For the preparation of 10 mM linoleic acid substrate solution, 99% pure linoleic acid was dispersed in 5 mL deionized water containing 125 μL Tween® 20. The content was thoroughly mixed to form a fine emulsion. This suspension was subsequently clarified by the addition of 500 μL 1M NaOH. This solution was brought to a final volume of 50 mL with deionized water. The resulting substrate solution contained 10 mM linoleic acid and 0.25% ($^v/_v$) Tween® 20 and was stored under nitrogen at -20°C in 1mL aliquots.

FOX-LOX assay

In order to increase the throughput of the FOX assay, the method originally described by Waslidge and Hayes[79] was adapted to a 96 well microtiter plate format. The FOX reagent used consisted of 25 mM sulphuric acid, 100 µM xylenol orange, 100 µM iron(II) sulphate and methanol prepared in a 50 Mm Tris-HCl, pH 7.4 buffer in a 9:1 methanol to Tris-HCl ratio. The iron(II) sulphate and sulphuric acid was prepared together in order to prevent oxidation of the iron(II). The FOX reagent was prepared fresh prior to each assay.

To determine LOX activity, the reaction was initiated by adding 50 µL of the substrate solution, described above, to 50 µL of the test sample in the wells of a 96 well microtiter plate. In order to account for possible substrate oxidation by exposure to oxygen and light, 10 mM Tris-HCl, pH 7.0, was included as control. The plate was subsequently incubated for 15 min at 37°C. Following incubation 100 µL FOX reagent was added to each well. The absorbance of each sample/control was determined at 620nm in 1 min intervals for a total of 30 minutes on a BioTek Powerwave 340 microplate reader.

Methylene blue bleaching assay

The methylene blue bleaching (MBB) assay, as described by Romero et al[144], was adapted to 96 microtiter plate format by reducing the total assay volume to 200 µL. Initially, the assay was evaluated using dilutions of a 1 mg/mL solution of soybean LOX 1-B. The substrate mixture used for this assay consisted of 80 mM sodium phosphate buffer, pH 7.0, 10 µM methylene blue, 20 mM 1,4-dithiothreitol, 1.5 mM linoleic acid and 10 % $^v/_v$ acetone. The optimal wavelength of absorbance for methylene blue was determined by performing a spectral scan between 800 and 340 nm. Once the optimal absorbance wavelength was established, all subsequent assays were performed using this wavelength.

For the assay, 170 µL of the substrate solution was added to 30 µL sample/control using a multichannel pipette. Immediately after addition of the substrate solution to the sample, the absorbance of each well was determined at 660 nm in 10-second intervals for a total of 10 minute using a BioTek Powerwave microtiter plate reader. Phosphate buffer was used as control.

The adapted MBB assay was also evaluated for use in peak identification during chromatography. An acitve sample of lentil LOX was injected onto a 10 x 300 mM column packed with Toyopearl HW 55 gel permeation chromatography resin. The column was eluted with 2 column volumes of 20 mM Tris-HCl, pH 7.0, at 1 mL/min. Fractions of 1 mL each were collected througout the entire run. The activity of each

fraction was subsequently determined using the MBB assay. This data was compared with that obtained using the conjugated diene method.

Dichlorofluorescein-linked fluorescent assay

As discussed in Chapter 2, the reaction products of LOX can be detected with the use of fluorescent probes. The reaction between LOX and linoleic acid results in the formation of reactive oxygen species which can oxidize the reactive oxygen probe, dichlorofluorescein (DCF). Oxidation of DCF results in a fluorescent molecule with an excitation wavelength at 485 and an emission wavelength at 518nm. DCF was subsequently used in the development of a high-throughput assay for LOX activity.

End-point DCF-DA assay

In the assay, 150 µL substrate solution (containing 0.5 µM linoleic acid in 200 mM sodium phosphate buffer, pH 7.0, containing 0.05% Tween® 20 and 20 µM DCF-DA) was added to 50 µL sample/ control in the wells of a Corning 96-well black polystyrene microtiter plate. Following addition of the substrate solution, the plate was incubated at 25°C for 30 min. After incubation, the fluorescence of each well was determined at E_x = 485 nm and E_m = 518 nm using a Tecan Spark 10M microtiter plate reader. For activity determination of chromatography fractions, the fluorescence values determined, in relative fluorescence units, were plotted against fraction volume. This yielded a peak activity profile for the chromatographic run.

Continuous DCF-DA assay

This assay was also performed in kinetic mode. For kinetic analysis of LOX activity, the assay was performed in a similar manner described above. However, immediately after addition of the substrate solution, the fluorescence of each well was determined in 30 second intervals for a total of 30 minutes. The fluorescence value for each time-interval was subsequently plotted against time in order to obtain a progress curve.

An attempt was made to validate this newly developed fluorescent assay against the microtiter plate conjugated diene method described later in the text. Validation experiments were performed using a Greiner UV-Star® 96 well plate. In these assays, the absorbance (λ = 234nm) and fluorescence (E_x = 485 nm and E_m = 518 nm) values of each well was determined in 10 second intervals for a total of 30 min. The progression curves obtained were overlaid for comparison.

Conjugated diene method

As discussed in Chapter 2, the conjugated diene method is the current standard for LOX activity determination and this assay was used to gauge the efficiency of all the assays evaluated. The standard assay used throughout this study is based on the one described by Guido *et al.,*[145].This assay was subsequently adapted to 96 well microtiter plate format in order increase the throughput. The microtiter plate assay was validated against the one originally published by Guido *et al.,*[145] using soybean LOX-1B (Sigma-Aldrich, St. Louis MO, USA). During the initial evaluation phase, it was found that when the 10 mM linoleic acid stock solution is diluted in assay buffer, the substrate solution becomes turbid resulting in a decrease in assay sensitivity and 0.05 % ($^v/_v$) Tween ® 20 was subsequently added to all assay buffers used.

Full scale conjugated diene method

For determination of LOX activity in test samples, 160 µL of each diluted sample was added to 840 µL of assay buffer. Dilutions were determined based on the change in absorbance (ΔAbs) obtained when LOX was assayed with 0.775 mM linoleic acid at pH 7.0 and 25 °C. at 234nm while the assay buffer relies on the pH at which the specific LOX isozyme is optimally active. The spectrophotometer was blanked using this solution, and the reaction was subsequently initiated by addition of 16.8 µL of a 5 mM linoleic acid solution, yielding a final assay concentration of 0.775 mM linoleic acid. Following the addition of linoleic acid, the ΔAbs was measured for a total of three minutes. According to the definition provided in the Sigma Aldrich catalogue, one LOX unit will cause an increase of 0.001 in absorbance when assayed at 234 nm. As such, enzyme activity was calculated by dividing the slope of the linear section of the progress curve by 0.001 .

Microtiter plate conjugated diene method

For high-throughput screening of multiple LOX samples, the full-scale conjugated diene method was scaled to a final volume of 200µL. In this assay 150 µL substrate solution (1mM linoleic acid in 200 mM Tris-HCl, pH 8.0, containing 0.25% Tween® 20) was added to 50 µL sample in the wells of a Greiner UV-Star® 96 well plate. Immediately after addition of the substrate solution, the absorbance of each well was determined at 234 nm in 10 second intervals at 25°C for a total of 30 min using a Tecan Spark 10M microtiter plate reader. The final concentrations of linoleic acid and Tween® 20 in this assay was 0.750 mM and 0.19 % ($^v/_v$) respectively.

In order to convert the absorbance to concentration, using the Beer-Lambert equation, the path length for the microtiter plates was required. The path length was subsequently calculated to be 0.5672 cm when the final volume was 200 μL as described in Equation 3.1 below. The concentration of the formed hydroperoxides was subsequently calculated using a molar extinction coefficient of 25000 $M^{-1}.cm^{-1}$.

$$h = \frac{V}{\pi r^2}$$

Equation 3.1 Equation used for the calculation of the path length (h = 0.5672 cm) of a microtiter plate well containing 200 μL final. In this equation V = 0.2 cm^2, and r = 0.335 cm. The values for r and h were obtained by measuring the dimensions of 5 separate wells of 5 different plates. The values were then averaged.

Using the data obtained from activity assays and that presented in Equation 3.1, activity in units/mg protein was calculated using Equation 3.2.

$$\frac{U}{mg} = \frac{\left(\left(\frac{Abs_{234}}{\varepsilon \times l} \right) \times assay\ volume\ (\mu L) \times unit\ (\frac{nmol}{min}) \right)}{mg\ protein}$$

Equation 3.2 Equation for the calculation of enzyme activity in units/mg protein. In this equation Abs_{234} is the value obtained from the activity assay, ε = molar extinction coefficient (25000 $M^{-1}.cm^{-1}$), l = the path length determined using Equation 3.1 (l = 0.5672), the assay volume was 200 μL, units is equal to the oxidation of 0.12 nmole linoleic acid per minute, and mg protein refers to the amount of protein used in the assay.

Equation 3.2 was subsequently simplified to:

$$\frac{U}{mg} = \frac{\left(\left(\frac{Abs_{234}}{14180} \right) \times 1.7 \times 10^6 \right)}{mg\ protein}$$

Equation 3.3 Simplified equation for calculation of LOX activity.

3.2.3 Purification of Lentil LOX

All buffer solutions used during the purification of the LOX enzyme(s) were of reagent grade and were prepared using deionized water with a resistivity of 18.2 $m\Omega.cm^{-1}$. Buffer solutions were filtered prior to use through Millipore 0.45 μM pore size type HVHP filter, and degassed under vacuum for 20 min at room temperature. All chromatographic separations were performed using a Bio-Rad NGC Quest 10 under the control of Bio-Rad Chromlab version 4.0.0.25 software, unless stated otherwise.

Optimization of chromatography conditions

For small-scale evaluation of chromatography resins, buffers, flow rates, elution gradients and loading conditions, a 0.7 x 2.5 cm column (1 mL final volume) was packed with the applicable resin. Unless stated otherwise, the column was run at 37.30 cm.h^{-1} (0.3 mL/min) and 500 µL fractions were collected and assayed as described. In order to enable direct comparison of the results obtained during optimization of the elution conditions, the same elution profile, and linear flow rate was used for each parameter evaluated. Chromatography conditions were optimized based on peak shape, peak resolution and width of the active peak.

Resin selection

For this study two chromatographic resins were evaluated based on total dynamic binding capacity using a 100 mg/mL solution of bovine serum albumin (BSA) prepared in equilibration buffer as protein standard. This was achieved by increasing the injection volume in 100 µL increments from an initial injection volume of 500 µL (50mg) to a final injection volume of 2 mL. Following loading, the column was washed with two-column volumes equilibration buffer. The bound BSA was subsequently eluted with 0.5 M NaCl. Maximal loading was established once BSA eluted during the wash step. Resins for the remaining chromatography steps were selected based on peak shape and resolution following injection of a known standard.

Buffer optimization

Various authors have described the purification of LOX, mostly from soybeans, using anion exchange chromatography as initial capture step[21,49–51,53]. During these purifications 10 mM sodium phosphate, with a pH between 6.5 and 7.2, was the most commonly used chromatography buffer. However, phosphates are anionic species capable of binding cationic resins. As a result, the total binding capacity of the column could be significantly reduced. For this reason, Tris-HCl was also evaluated as primary buffer salt.

Following binding to the stationary phase, elution of the bound proteins was optimized by evaluating different elution salts. These salts are categorized, based on their elution strength, in the Hofmeister series. The elution strengths of the anions evaluated are as follows: $SO_4^{2-} > HPO_4^{2-} > CH_3COO^- > Cl^- > I^- > SCN^-$. All elution buffers were prepared in 20 mM Tris-HCl, pH 7.5, containing 1 mM EDTA to a final concentration of 500 mM. Prior to injection the column was washed with 20 column volumes of the elution buffer followed by re-equilibration with 20 mM Tris-HCl, pH 7.5, containing 1 mM EDTA. Once the most efficient buffer was established, the elution gradient, and final concentration of elution buffer,

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was adjusted based on retention of the active fraction and peak shape. Each elution buffer was also evaluated in terms of compatibility with the subsequent activity assay.

It has been shown that individual soybean LOX isoforms can be resolved, subsequent to initial capture and cleanup, by cation exchange chromatography. In this study cation exchange was also used as a final polishing step during purification. For this reason, different buffering systems were also evaluated. However, there were no marked differences between different buffering systems. All subsequent cation exchange separations were therefore performed using 50 mM sodium acetate, pH 5.0, since this is a widely used, and economical, buffering salt.

Purification of lentil LOX

Once the chromatographic conditions were optimized, lentil LOX was purified to relative homogeneity using a three-column purification procedure. The protein capture and secondary purification was performed using anion exchange chromatography, while polishing was done using cation exchange chromatography. Aliquots were collected prior to- and after all chromatographic runs. Prior to equilibration of all columns, each column was first washed with 5 column volumes 500 mM NaCl, in the appropriate equilibration buffer.

Protein capture

For the initial capture a 32 x 190 mm column was packed with Whatman DE32 weak anion exchange resin. Prior to injection, the column was equilibrated with 10 column volumes equilibration buffer (20 mM Tris-HCl, pH 7.5, containing 1 mM EDTA) at 4°C. A constant flow rate of 37.30 cm.h^{-1} was maintained throughout the run. Of the precipitated lentil extract described above, 50 mL was loaded onto the column. Unbound proteins were then eluted from the column by washing the column with 800 mL equilibration buffer. LOX, and other contaminating proteins, was subsequently eluted over 6 column volumes with a linear gradient from 0 to 500 mM NaCl in equilibration buffer. Strongly bound proteins were eluted from the column by passing 2-column volumes 500 mM NaCl, in equilibration buffer, through the column. The column was subsequently re-equilibrated with 2-column volumes equilibration buffer. Starting immediately after column loading, fractions of 15 mL each were collected for the duration of the run.

After completion of the first anion chromatography step, LOX activity of all fractions were assayed using the microtiter plate conjugated diene assay. All active fractions were pooled, and concentrated by ammonium sulphate precipitation (60% saturation) for 1 hour at 4°C. The active fraction was collected by

centrifugation at 15000 xg for 30 min at 4°C. The resulting pellet was dissolved in 30 mL 20 mM Tris-HCl, pH 7.5, buffer. Once dissolved, the solution was dialyzed overnight against 100 volumes 20 mM Tris-HCl, pH 7.5, containing 1 mM EDTA. Following dialysis, the total volume of the solution was brought to 50 mL with distilled water. This solution was subsequently filtered through a Millipore 0.45 µM pore size type HVHP filter under vacuum. Prior to all chromatographic procedures, 0.5 % Triton X100 was added to disrupt protein-lipid interactions.

Intermediate purification

The second column used was an 18 x 200 mm column packed with Q Sepharose fast flow strong anion exchange resin. Prior to injection, the column was equilibrated with 5 column volumes equilibration buffer consisting of 20 mM Tris-HCl, pH 7.5, containing 1 mM EDTA at a flow rate of 36.67 cm.h^{-1}. This flow rate was used throughout the chromatographic run.

Following equilibration, 50 mL of the dialysate from the first chromatographic run was injected onto the Q Sepharose column. During injection, the fraction collector was set to collect 6 mL fractions of peaks with an absorbance larger than 20 mAu. Following injection, 6 mL fractions were collected throughout the entire chromatography run. Loosely bound proteins were subsequently washed from the column with 2 column volumes 25 mM NaCl in equilibration buffer. Two separate active peaks were eluted over 6 column volumes with a linear gradient of 25 – 250 mM NaCl. Strongly bound proteins were then eluted by stepping the concentration of NaCl to 500 mM. This concentration of NaCl was maintained for 2 column volumes, followed by re-equilibration of the column with 3-column volumes equilibration buffer. All collected fractions were assayed for LOX activity as before. The active peaks were pooled separately and dialyzed against 50 mM sodium acetate buffer, pH 5.0. These samples were injected separately onto the next column.

Cation exchange chromatography

Prior to injection onto an 18 x 200 column packed with Fractogel® TSK CM-650(S) cation exchange resin, the samples were concentrated to a final volume of 30 mL using Amicon Ultra-15 centrifugal filter units equipped with a 50 kDa MWCO membrane. For comparison, aliquots were collected prior to- and following concentration. The total activity of each aliquot was compared in order to determine protein recovery following ultrafiltration. A total of 30 mL was subsequently injected onto the cation exchange column.

Following injection, unbound proteins were eluted from the column with 3 column volumes of a 20 mM sodium acetate buffer, pH 5.0. The active protein was subsequently eluted over 5 column volumes with a linear gradient of 0 -250 mM NaCl in equilibration buffer. The concentration of NaCl was then stepped to 500 mM for a total of 2 column volumes. The column was subsequently re-equilibrated with 3-column volumes equilibration buffer. Fractions of 5mL were collected throughout the run. Following completion of the chromatography run, each fraction was assayed for LOX activity.

Each active fraction was then dialyzed against distilled water overnight at 4°C and the protein content subsequently determined. Aliquots of each fraction were then freeze-dried. The resulting powder was dissolved in a sufficient volume of 10 mM Tris-HCl buffer, pH 7.0, to yield a final protein concentration of 100 µg/mL. These fractions were used in subsequent characterization experiments.

3.3 RESULTS AND DISCUSSION

Unless stated otherwise, all experiments were performed at least in triplicate and the data is presented as the mathematical mean. Data presentation, analysis and statistics were performed using GraphPad Prism® 5 software.

3.3.1 Flour extraction

In order to purify the LOX from lentil flour, the soluble proteins were extracted by suspension of full-fat lentil flour in a buffered solution followed by centrifugation. However, after centrifugation the sample remained cloudy, even after prolonged centrifugation. Upon further investigation, it was found that the suspension could be clarified by heat or the addition of detergents. Figure 3.1 illustrates the effect of temperature on the clarity of the lentil extract. The sample on the left was stored a 4°C while the sample on the right was stored at room temperature. These results suggest that an emulsion, which cannot be removed by centrifugation, is formed by the native flour lipids during extraction at 4°C. The presence of insoluble particles resulted in a reduction in filtering efficiency prior to chromatography and, thus, reduced protein recovery.

Figure 3.1 (A) Lentil extract at 4°C, and (B) an extract at room temperature. In this figure, it can be seen that at reduced temperatures, lentil extracts become cloudy.

Non-ionic detergents, such as Tween® 20 and Triton X-100, are frequently used for sample clarification prior to chromatography, however, these detergents often absorb at 280 nm thereby reducing detector sensitivity during chromatography. Furthermore, the detergents most often used during chromatography cannot be removed by dialysis due to a low critical micelle concentration. Moreover, the presence of detergents can result in errors during down-stream sample analyses such as protein determinations and activity assays. As such, alternative methods for sample clarification were investigated.

Although the formation of lipid emulsions has not been described in literature, soybean flour is often defatted using organic solvents prior to extraction. However, it has been reported that defatting of soybean flour resulted in significant losses in LOX 2 activity[33]. As illustrated in Chapter 2, each LOX isoform plays a different, but integral, role in dough rheology. It has been suggested, but not yet conclusively proven, that lentil seeds may have multiple LOX isoforms[16,17]. Since this study is centered around finding a possible suitable replacement for soy-derived LOX, maximal amounts of the full complement of all possible lentil LOX isozymes were required. Any losses during the extraction and purification of the lentil LOX enzyme(s) should therefore be limited. Due to the shortcomings associated with the use of detergents and defatting of the flour, alternative clarification methods were investigated.

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Upon investigation, it was found that the formation of emulsions could be prevented by addition of 1% glycerol to the extraction buffer. However, this resulted in an increase in sample viscosity and a decrease in filtering efficiency. During chromatography, the added glycerol also resulted in an increase in column backpressure limiting the flow rate, thereby increasing run times. When dialyzing against a buffer containing less than 1% glycerol, the sample once again became cloudy. Therefore, although glycerol was effective in suppressing the formation of lipid emulsions, it reduced filtering efficiency and increased the total run time during chromatography.

In order to establish the role of extraction time on emulsion formation, a sample was extracted by suspension in buffer with stirring for 1h. Following stirring the sample was incubated for an additional 16h at 4°C prior to centrifugation. This resulted in a clearer solution when compared to a sample extracted for 1h. However, as can be seen in Table 3.2, when comparing the total activity and purification factor, prolonged extraction resulted in losses in active protein. Furthermore, samples extracted for 1h yielded a higher degree of purity following precipitation than those extracted for 16h.

Table 3.2 Comparison of the extraction efficiencies of prolonged extraction and step-wise precipitation protocols. The data presented in this table show that both prolonged extraction, and step-wise precipitation protocols result in protein loss

Sample	Step	Activity/mL	Total activity	Specific activity (U/mg)	%Recovery	Purification factor
	Crude extraction	29855	6866742	5862	100.00%	1.00
1 Hour extraction	Before dialysis	104800	3982418	7163	58.00%	1.22
	After dialysis	76404	3820216	7286	55.63%	1.24
	Crude extraction	26379	6067223	5936	100.00%	1.00
16 Hour extraction	Before dialysis	76833	3457477	5275	56.99%	0.90
	After dialysis	67941	3397055	5653	55.99%	0.96
	Crude extraction	27337	6287495	5845	100.00%	1.00
Stepped precipitation	Before dialysis	78221	3363493	4830	53.49%	0.82
	After dialysis	67038	3351900	5871	53.31%	1.00

Based on these results, samples were extracted for a maximum of 2 h followed by clarification using a detergent. Triton X-100 was chosen since it is often used for membrane solubilization at low concentrations (< 1 %) and it can be removed by stirring with Amberlite XAD-2 beads[17]. Furthermore, although Triton X-100 is known to interfere with the BCA protein determination method, it does not affect the Bradford protein determination.

Determination of total extractable material

Following extraction, the % extractable material was determined by lyophilization of an aliquot of the extract. For comparison, flour samples were extracted in water and phosphate buffer. Interestingly, extractions performed using distilled water resulted in the extraction of 18.78 $\pm$ 0.002% $^m/_v$ while extractions with phosphate buffer yielded 20.745 $\pm$ 0.37% $^m/_v$ extractable material. However, a comparison based on extractable material alone is not sufficient since this study relates to LOX. As such specific activity for LOX was also compared following extraction. These results also showed that more of the active protein is extracted with phosphate buffer than with distilled water.

The extracted proteins were subsequently concentrated by precipitation with ammonium sulfate. In literature a stepwise precipitation is often employed during the purification of soybean LOX[47]. In this procedure contaminating proteins are first removed with ammonium sulfate in the saturation range of 0-30% followed by centrifugation. The ammonium sulfate concentration of the supernatant is then increased to a final saturation range of 30 - 60% in order to precipitate the active LOX enzyme(s). Active soybean LOX enzymes are then recovered by centrifugation. However, using this procedure for the concentration of lentil LOX resulted in an average loss of 3.25 % in recovery. Performing a stepped precipitation protocol furthermore did not increase the purification factor in the same way as for the single step precipitated samples. Since multistep precipitation protocols have only been described for the extraction of soybean proteins, it was necessary to evaluate the efficiency of the ammonium sulfate precipitation steps.

The data presented in Figure 3.2 indicate that the reduction in protein recovery observed during stepped precipitation protocols is due to a loss of LOX at ammonium sulfate saturation ranges 0-50%. As can be seen from Table 3.2, although ammonium sulfate saturation below 30% resulted in removal of contaminating proteins, as indicated by an increase in specific activity, active lentil LOX is lost with the precipitate when % recovery is considered. Full recovery of active lentil LOX was only achieved at ammonium sulfate saturation above 50%. Therefore, in order to ensure full recovery, extracted lentil LOX was henceforth concentrated with ammonium sulfate in the saturation range of 0-60% for all subsequent studies.
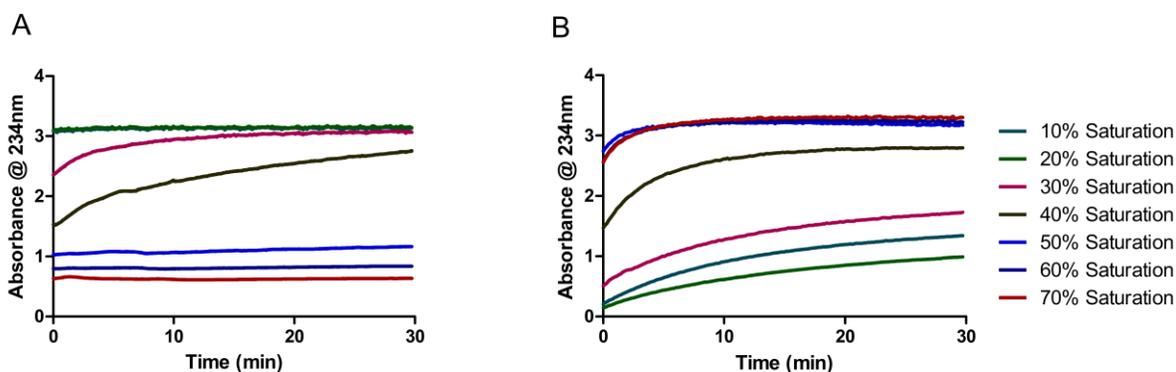
Figure 3.2 Comparison of LOX activity in (A) the supernatant of samples precipitated with the saturations depicted, and (B) the activity remaining in the supernatant of the extracted samples.

3.3.2 Activity assays

For the successful purification of proteins from crude extracts, and to minimize protein loss, all fractions collected during chromatography should be assayed for activity. This is especially important when it is not known if the protein of interest is present in more than one isoform. For this purpose, high-throughput activity assays are required. As discussed in Chapter 2, various assays for LOX activity have been developed. However, although many of these assays have been shown to be accurate, not all are readily scalable to enable high-throughput screening of multiple samples. Table 3.3 lists the assays most often cited in literature, and compares these assays based on scalability, usefulness during kinetic studies, accuracy, and ease of use. This comparison is based on an intensive literature search (see Chapter 2), and the author's own experience.

Table 3.3 *LOX activity assays most often cited in literature. Except for assays that rely on the measurement of O_2 production, and those using carotene as substrate, each of the assays listed was evaluated and compared in this study*

Assays based on	High throughput	Kinetics	Accuracy	Ease of execution
Oxygen consumption		√	√	
β-carotene oxidation	~			
Iron oxidation state – FOX-LOX	√		~	
Dye degradation – Methylene blue	√	~		
Intermediate/Product probes – DCF Fluorescence	√	~	√	~
Conjugated diene formation	√	√	√	√

Of the assays listed in Table 3.3, assays based on oxygen consumption have been shown to be most accurate since it is not affected by secondary reactions. However, due to the need for specialized equipment this assay is neither easy to perform, nor can it be adapted for high-throughput screening. The

β-carotene assay is also not suited to high-throughput kinetic screening due to the instability of β-carotene in aqueous suspensions. Based on these shortcomings, only the remaining assays were evaluated in this study.

FOX-LOX assay

The ferrous oxidation-xylenol orange (FOX) assay relies on the ability of lipid hydroperoxides to oxidize Fe(II) to Fe(III). Under acidic conditions the Fe(III) subsequently oxidizes xylenol orange to a product which absorb strongly at 590 nm (Figure 3.3). In this study the FOX assay, described by Waslidge and Hayes[79], was evaluated for use during high-throughput screening of fractions collected during chromatography.
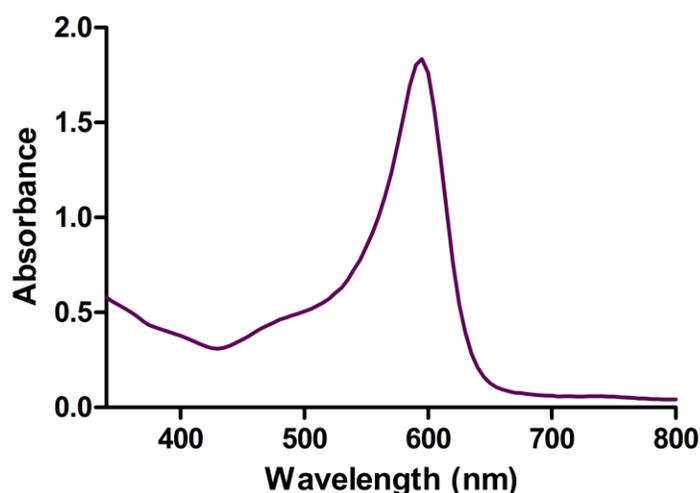
Figure 3.3 Absorption spectra of oxidized xylenol orange when scanned from 800 to 340nm.

The FOX-LOX assay was performed in two steps. Each test sample was first incubated with linoleic acid at 37°C for 30 min prior to addition of the FOX reagent. Following incubation, FOX reagent was added and color development was monitored at 590 nm in 10-second intervals for a total of 10 min. In this format this assay is not capable of kinetic evaluation of the LOX reaction. However, it has been shown that although the assay itself is not a kinetic assay, color development is dependent on the amount of LOX present[146]. Based on this evidence, Nieuwoudt[146] subsequently showed that this assay could be adapted, by inclusion of LOX activity standards, for the quantitative determination of LOX. This method could, however, not compensate for the presence of compounds, other than LOX, capable of reducing iron.

In the current study the FOX-LOX assay was shown to be useful during screening of large amounts of samples resulting from chromatography. However, during the initial chromatography step EDTA, a metal chelator, is added to inhibit metalloproteases present in plant tissue. The EDTA present in the buffer of

chromatography samples resulted in a complete elimination of LOX detectability by the FOX-LOX assay. Although the EDTA could easily be removed prior to the assay by dialysis or desalting, performing these techniques on large scale is not practical.

Furthermore, the FOX reagent is prepared in a Tris-buffered methanol solution (9:1 methanol:50 Mm Tris-HCl, pH 7.4). Addition of the FOX reagent to concentrated protein solutions resulted in protein precipitation. This caused high background absorbance levels and a concurrent reduction in detectability. As such, all samples first had to be diluted prior to the activity assay. Based on these shortcomings, the FOX-LOX assay was of limited application in this study.

Methylene blue bleaching assay

Methylene blue is a well-known redox indicator, used to indicate the presence of oxidizing agents. Figure 3.4 shows that methylene blue, when oxidized, absorbs strongly at 660 nm. Upon reduction, aqueous solutions of methylene blue turn colorless. As discussed in Chapter 2, the reaction of LOX with linoleic acid results in the formation of reactive oxygen species capable of oxidizing a variety of substrates, such as β-carotene, via a coupled-oxidation reaction. As such, it seems intuitive that the reaction between lipid hydroperoxides and methylene blue would result in the formation of the blue chromophore. However, addition of methylene blue to a solution containing LOX and linoleic acid results in bleaching of methylene blue, albeit after an initial delay[147]. It has been shown that the Toyosaki[148] delay length is inversely related to LOX activity. Based on this, the methylene bleaching (MBB) assay could be used to determine the relative levels of LOX present in a sample.
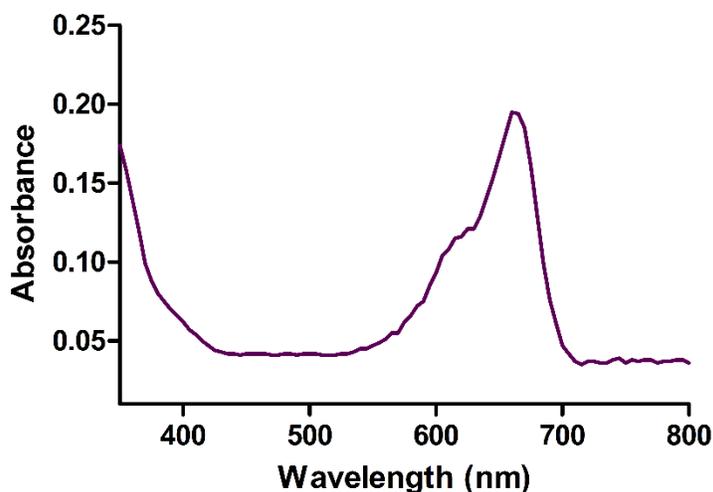
Figure 3.4 Absorption spectra of oxidized methylene blue. The methylene blue spectra were determined using blank sample during the methylene blue bleaching assay.

In this study the MBB assay described by Romero[144] and co-workers was adapted to 96-well plate format to enable high-throughput screening of multiple samples. During evaluation, using 0.5 mg/mL soybean LOX 1-B, a delay prior to initiation of bleaching was also observed. However, as can be seen in Figure 3.5.A, in contrast to that described by Toyokasi[148], this delay was marked by an increase in absorbance at 660 nm. Once maximal absorbance had been reached, bleaching was initiated leading to a rapid decline in absorbance. Moreover, dilution of the enzyme stock resulted in an elimination of bleaching as shown in Figure 3.5.B. When the assay time was extended, bleaching did occur but only at protein concentrations above 0.1 mg/mL. Based on these results, the use of the MBB method in kinetic studies was evaluated. All kinetic studies were performed with a substrate concentration ranging between 0 and 5 mM linoleic acid. A total of 3 µg protein was used in each of these assays.
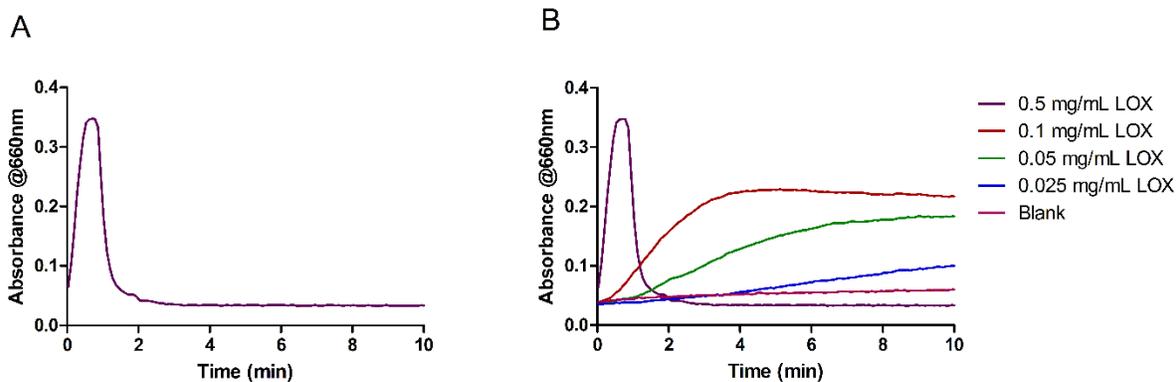
Figure 3.5 Results of the methylene blue bleaching assay when performed with (A) relatively concentrated protein solutions, and (B) assays performed using diluted protein solutions. From this figure, it can be seen that methylene blue oxidation occurred in a dose-dependent manner.

The data obtained from kinetic studies performed using the MBB assay is presented in Figure 3.6. By using this method a Michealis-Menten type kinetic profile for the LOX enzyme was obtained. However, difficulties arise when attempting to compare the kinetic parameters obtained with this assay to that presented in literature. The MBB assay, applied in this manner, has not yet been validated, and no definition for enzyme activity has been established. The data presented does, however, indicate that the adapted MBB assay could possibly be used for LOX kinetics. Although various assays have been developed whereby kinetic analysis could be performed, the MBB assay has the advantage of ease, reproducibility and no absolute requirement for specialized equipment.
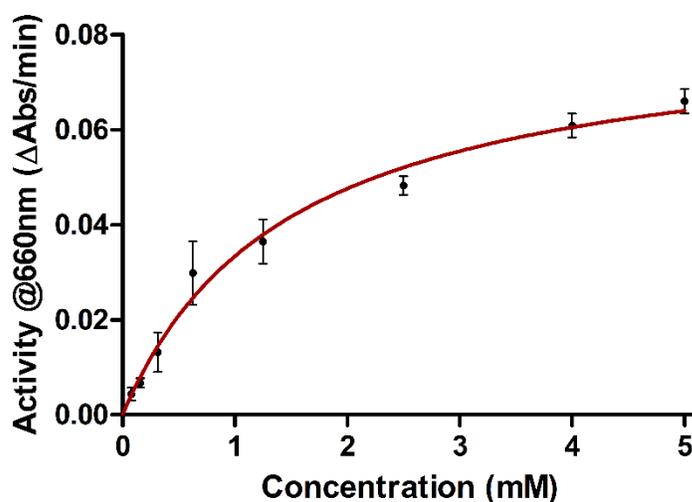
Figure 3.6 Michealis-Menten type kinetics obtained using the methylene blue bleaching method with a LOX concentration of 50 µg/mL.

The MBB assay was also evaluated for use of peak identification following chromatography. The data obtained was compared to that obtained with the use of the standard conjugated diene method. From Figure 3.7 it can be seen that the MBB assay was not sufficiently sensitive to detect LOX activity in all the chromatography fractions. Activity was only detected in the fraction with the highest activity when determined using the conjugated diene method. Using the conjugated diene method, activity was detected in two additional fractions.
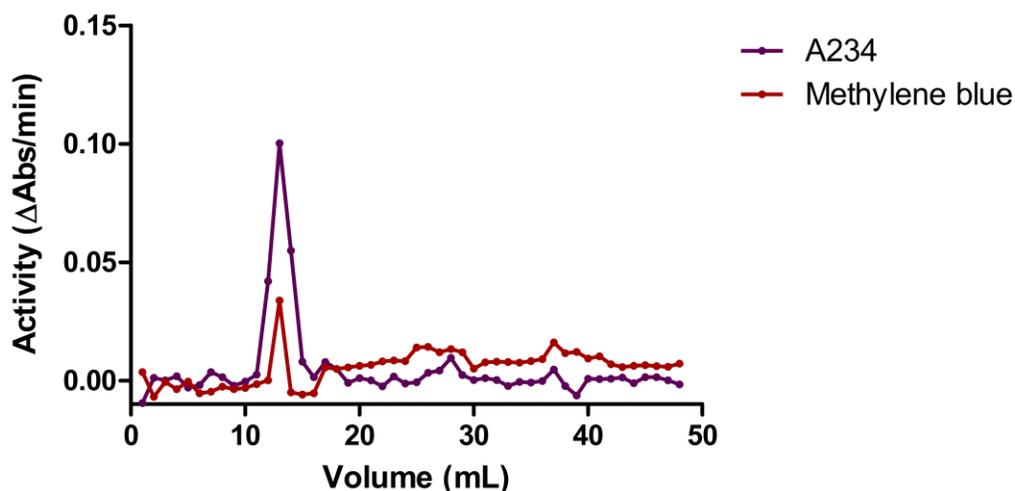
Figure 3.7 Activity of chromatography fractions when determined using the conjugated diene and methylene blue bleaching assays.

Based on these results the MBB assay found limited use during peak identification of chromatography fractions. However, although it was beyond the scope of this study, the results presented indicate that this assay could potentially be applied during kinetic characterization of LOX enzymes. The assay must, however, first be validated, and a unit of enzyme activity defined. Based on the work by Suda *et al.,*[147] it appears possible to optimize this assay in order to increase the sensitivity for low-level detection of LOX. However, for the purpose of this study the MBB assay was not sufficiently sensitive. As such, alternative methods were investigated.

Dichlorofluorescein-linked fluorescent assay

As discussed earlier, colorimetric assays for LOX activity often suffer from interference from native plant lipids and pigments. In 2010 Whent *et al.,*[81] showed that the fluorescent probe fluorescein, can be used to overcome the difficulties associated with colorimetric assays. These authors showed that when incubated in the presence of LOX and linoleic acid, fluorescein fluorescence is quenched in a dose-

dependent manner by the reactive intermediates formed during the LOX reaction. However, this method has the disadvantage that fluorescein fluorescence is not stable at pH values below 7. This probe could therefore not be used to assay the activity of LOX isoforms (Type II) with an optimal pH below pH 7.0.

Although the LOX isozymes of various plants have been fully characterized, little information is available on the lentil seed LOX enzyme(s). An ideal assay should therefore be effective, and accurate, over a wide pH and temperature range. Due to the limited use of fluorescein in the determination of type II LOX activity, the use of dichloroflourescein diacetate (DCF-DA) as fluorescent probe was evaluated. DCF-DA is one of the most widely used probes for the detection of intracellular H_2O_2 and reactive oxygen species[149]. Fluorescence is furthermore stable at pH values below 7[150]. Upon oxidation by the hydroxyl radicals produced during LOX-catalyzed hydroxylation of linoleic acid, DCF-DA is converted to the fluorescent product, DCF.

Figure 3.8 shows that, although addition of DCF-DA resulted an increase in fluorescence, the development of fluorescence was not linear. Moreover, maximum fluorescence could not be reached, even after prolonged incubation. This data would suggest that the concentration of DCF-DA was not limiting. This figure also shows that an increase in DCF-DA concentration did not have any marked effect on maximal fluorescence, except for increasing the initial fluorescence value. As such, the DCF-DA concentration was maintained at 20 µM in all subsequent studies to prevent saturation of the fluorescent detector used.
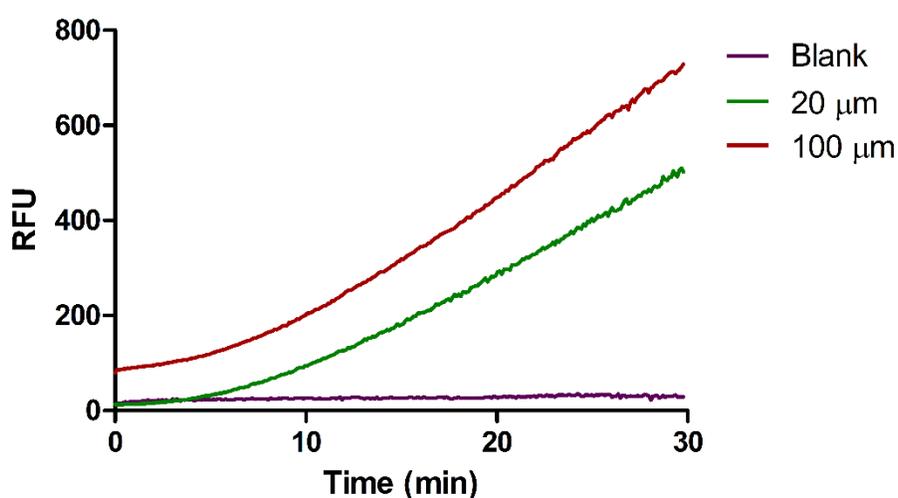
Figure 3.8 Activity assays performed using DCF-DA as a fluorescent probe.

During development of the assay it was observed that, although fluorescence development was not linear, there was a dose-dependent increase in the slope of the curve for the first 4 min of the reaction. In order to assess the use of DCF-DA during kinetic evaluation of LOX enzymes, kinetic analysis was performed with linoleic acid concentrations ranging between 0.078 and 5 mM. Figure 3.9.A shows that the development of fluorescence was dose-dependent. However, the maximal rate of fluorescence development was decreased at substrate concentrations in excess of 2.5 mM. When comparing these results with that obtained by monitoring the formation of the conjugated diene presented in Figure 3.9.B, it is evident that the decrease in fluorescence development is not due to a reduction in product formation.

The pKa values for linoleic acid and DCF-DA are 4.77 and 4.8, respectively. Both species are therefore similarly charged under assay conditions. It is therefore possible that these compounds repel each other at high concentrations, resulting in a reduced rate of fluorescence development. On the other hand, as discussed in Chapter 2, the LOX reaction under normal assay conditions is a poor source for reactive oxygen species. It therefore remains a possibility that when the reaction occurs at maximum velocity, the relative amount of reactive oxygen species formed is comparatively less than the conjugated diene formed. Gauging activity based on the formation of reactive oxygen species would therefore result in an underestimation of enzyme activity.
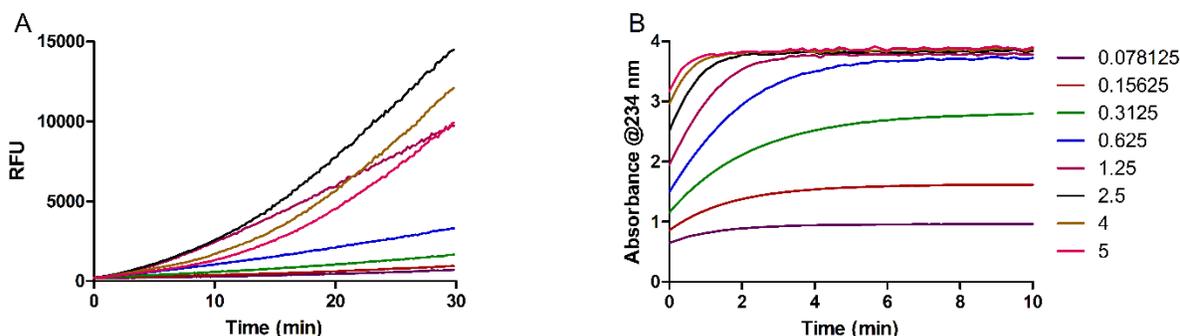
Figure 3.9 Reaction curves produced when measuring (A) fluorescence development due to oxidation of DCF-DA and (B) the formation of a conjugated diene in the primary structure of linoleic acid. These curves were produced by assaying the oxidation of linoleic acid using 2.5 µg soybean LOX I-B.

The use of fluorescent probes, as opposed to direct monitoring of product formation, often has the advantage of a reduced susceptibility to interference. As such, the DCF-DA microplate assay was evaluated for use during peak identification after chromatography. In this assay chromatography fractions were

evaluated using the DCF-DA assay. For this purpose, the assay was performed in end-point style. As can be seen in Figure 3.10, activity was only detected in the active fractions collected from chromatography runs.
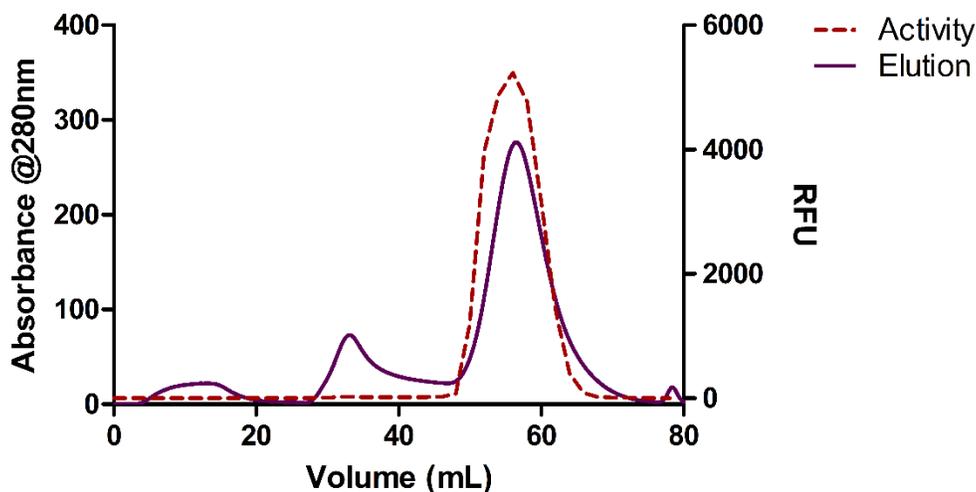
Figure 3.10 Chromatogram obtained by injection of 1 mL lentil extract onto a CM-sephadex fast flow column. In this run 1 mL fractions were collected. The activity of each fraction was subsequently determined and the activity data was overlaid with the elution profile.

With the use of DCF-DA as fluorescent probe, the difficulties associated with fluorescein could be overcome to produce an assay with stable fluorescence development at a pH below 7.0. The use of DCF-DA furthermore allows for peak identification following chromatography. However, the result presented here show that kinetic characterization could not be performed using DCF-DA as probe since fluorescence development is reduced when approaching maximal velocity. As such, this method was only used for peak identification prior to development of the more accurate microtiter plate conjugated diene method which will be described in the next section.

Conjugated diene method

As discussed in Chapter 2, the conjugated diene method is the current standard whereby LOX activity is determined. Besides assays based on oxygen consumption, the conjugated diene method is the most accurate assay currently available since it measures lipid oxygenation directly. As the name suggests, this assay relies on the determination of the conjugated diene produced by the reaction of LOX with linoleic acid. Formation of a conjugated diene yields a chromophore which can be monitored spectrophotometrically at 234 nm using a recording spectrophotometer. It has previously been

established that an increase of 0.001 absorbance units per min at 234nm is equal to one LOX activity unit[17,145,151,152]. When activity is determined in a 1 cm path length, an increase of one absorbance unit at 234 nm is equal to the oxidation of 0.12 μmole of linoleic acid. One unit of LOX activity (0.001 absorbance units per min) therefore results in the oxidation of 0.12 nmole linoleic acid per minute.

During initial evaluation, the full-scale assay was sufficiently sensitive to detect activity in chromatography fractions and control samples. However, since only a single assay could be performed at a time, this assay was not suitable for use during high-throughput activity screening. The full-scale assay was also labor-intensive, and had an absolute requirement for the analyst's presence during the assay. Moreover, due to the sensitivity of the assay, each fraction had to be sufficiently diluted prior to activity determination, further increasing the assay time. Based on these drawbacks, it was concluded that the full scale conjugated diene method, although sensitive, is not suitable for the high-throughput screening required in this study.

The full-scale assay was subsequently scaled to a final assay volume of 200 μL for use in microtiter plate assays. However, the major drawback of the conjugated diene method is the low molar absorbance, $\lambda_{max} = 234$ nm, of the reaction products formed. As can be seen in Figure 3.11, normal polystyrene microtiter plates absorb in the UV range below 300 nm and non-absorbing UV-star microtiter plates had to be used. Based on these requirements, previous authors suggested that downscaling the conjugated diene assay had limited potential, especially in an industrial settings[146]. Nevertheless, as will become clear, in this study the microplate conjugated diene method proved extremely valuable.
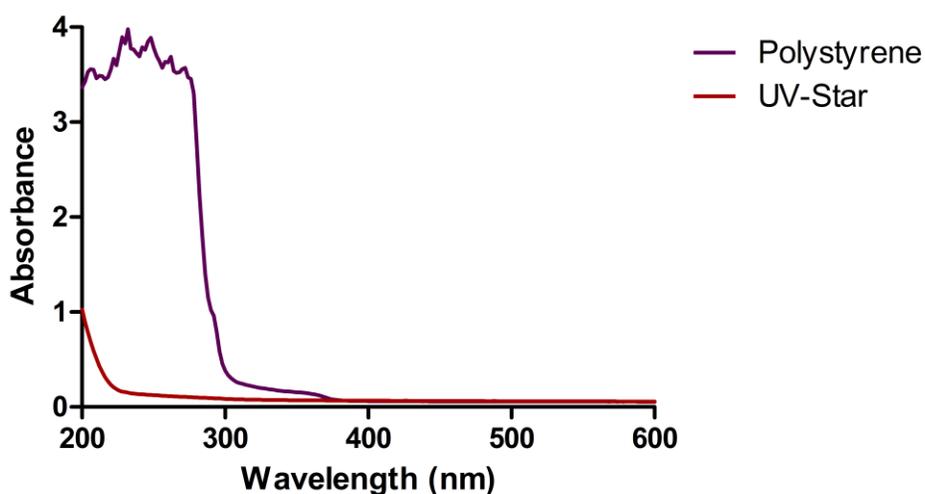
Figure 3.11 Absorption spectra of polystyrene microtiter plates compared to the spectra for UV-Star low-absorbing microtiter plates when scanned from a wavelength of 600 nm to 200 nm.

To determine the optimal substrate concentration required to ensure substrate saturation, a substrate concentration range between 0.078 and 5 mM linoleic acid was evaluated. As can be seen in Figure 3.12, saturation was reached at substrate concentrations in excess of 1 mM linoleic acid. This substrate concentration was therefore used for all subsequent activity assays. Figure 3.12 furthermore illustrates the use of the newly developed microtiter plate method during kinetic studies. The results presented were obtained in a single run, in triplicate. With the use of this method, up to 96 samples could be screened simultaneously. The level of simplicity and reproducibility achieved with the newly developed assay also allowed for automation using a Tecan Spark 10M equipped with automatic injectors. In this assay, samples were first added to each well, and the substrate solution was subsequently added to the wells by the instrument injectors. Due to this versatility, this assay could have major implications in industrial settings, and breeding programs.
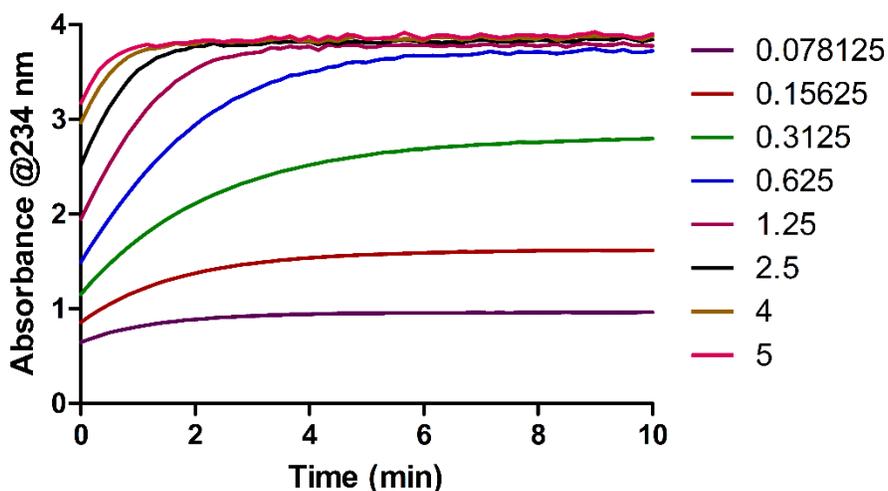
Figure 3.12 Reaction curves for soybean LOX I-B when assayed using the newly developed microtiter plate conjugated diene method.

In order to enable full kinetic characterization of LOX enzymes using the newly developed assay, a unit of enzyme activity first had to be defined for the microtiter plate assay. This was achieved by comparison to the full-scale assay. When comparing the change in absorbance with relation to protein concentration, Figure 3.13.A, the assays are not directly comparable. However, as shown in Figure 3.13.B, once the absorbance values were converted to µmol product produced per min, using Equation 3.3 in the materials and methods section, the calculated activities of the two methods were in good agreement. Therefore, the data presented here shows that, by comparison to the full-scale assay, the microplate assay could be

used to accurately determine LOX activity. The results presented here furthermore indicate that the newly developed microplate assay could also be used for kinetic analysis of LOX isozymes.
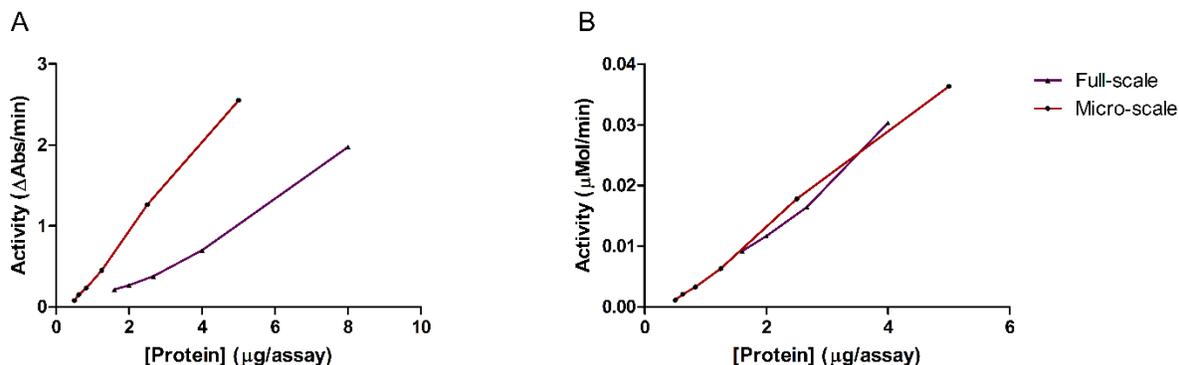
Figure 3.13 Comparison of the full- and microplate scale conjugated diene activity assays. (A) LOX absorbance change in relation to µg protein in the assay (B) activity in µMol product produced per minute per µg protein used.

By converting the data presented in Figure 3.12 to concentration product produced per min, the kinetic parameters K_m and V_{max}, for soybean LOX 1-B, was also determined. The initial rates of the reactions presented in Figure 3.12 were used to construct the kinetic curve presented in Figure 3.14. As can be seen, soybean LOX 1-B follows Michealis-Menten type kinetics and the kinetic parameters, K_m and V_{max}, were determined to be 0.1956 mM and 83745 U/min/mg protein, respectively.
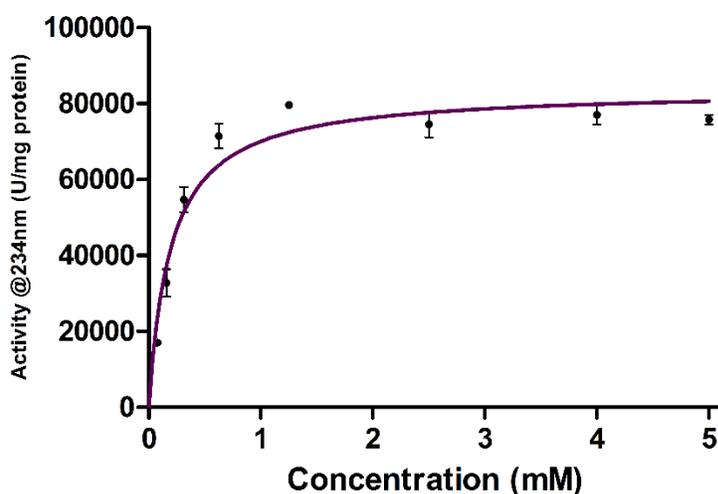
Figure 3.14 Kinetic curve of soybean LOX I-B showing a Michealis-Menten kinetic profile. The data for this curve was produces using the microtiter plate conjugated diene methods. The ΔAbs/min was converted to U/mg protein using Equation 3.3.

Finally, the microtiter plate assay was also evaluated for use in peak identification following chromatography by assaying all the fractions collected during chromatography for LOX activity. As can be seen in Figure 3.15, this assay could also be used in end-point style for rapid identification of LOX in chromatography fractions. Although the values presented here are not quantitative, LOX activity could be determined by inclusion of protein concentration data for each of the samples. The ability to rapidly identify peaks as LOX containing fractions following chromatography, had major implications for purification efficiency and throughput. Furthermore, with the use of this assay, fraction volumes could be decreased limiting cross-contamination from closely eluting peaks.
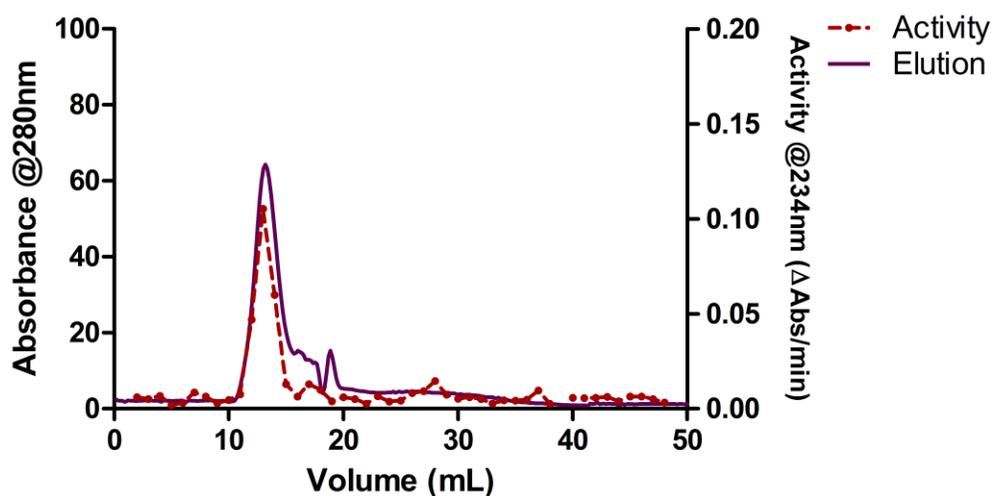
Figure 3.15 Activity profile for a 1 mL sample of lentil LOX following chromatography. In this figure the activity profile is overlaid with the chromatogram.

The results presented here show that the adapted microplate conjugated diene method could be used for accurate activity determination, kinetic characterization and peak identification following chromatography. During validation of the assay it was determined that this newly developed assay could detect activity in samples with a protein concentration of 0.1 µg/mL. Based on the accuracy, low detection limit, reproducibility and efficiency, this assay was used for all subsequent activity determinations.

3.3.3 Purification of Lentil LOX

It has been reported previously that lentil seeds may contain multiple isoforms of LOX[16,17]. However, in not one of these studies were the "newly discovered" isozymes fully characterized. Furthermore, very little kinetic information is available for lentil LOX isozymes. The goal of this study was to evaluate the potential use of lentil LOX in the baking industry. However, in order to optimize the positive effect of LOX

in the baking industry the enzyme needed to be characterized. For characterization, pure protein is required. This section describes the purification of lentil LOX from commercial lentil flour currently used in the baking industry.

Optimization of chromatography conditions

For application on an industrial scale, the procedures used for purification of the LOX enzyme should be efficient, reproducible and cost-effective. The methods used in this study were therefore optimized to limit the chromatographical procedures and reagents required to obtain sufficiently homogeneous preparations of lentil LOX. However, compared to the wealth of information regarding the purification of soybean LOX isozymes, little information is available on the purification of lentil LOX enzymes. It has been reported that lentil LOX has a pKa of 6.3[17]. Since the lentil flour is extracted at neutral pH, anion exchange chromatography was chosen as the technique for initial capture. Fractions were collected throughout all chromatographical procedures, and the activity of each fraction was determined using the newly developed microtiter plate conjugated diene method described earlier in the text.

Resin selection

For efficient capture of the protein of interest, the chromatography resin used must have a high binding capacity. The total amount of protein, which could bind to the resin under operating conditions, is referred to as the dynamic binding capacity of the resin. During initial chromatographic procedures, a high dynamic binding capacity is required. The resin to be used for the initial chromatography step in this study, was evaluated based on the dynamic binding capacity of the resin using BSA as protein standard. Two chromatography resins, Whatman® DE32 and DE52, were evaluated, based on their availability and theoretical binding capacity as supplied by the manufacturer. Overloading, due to limited binding capacity, was observed by an appearance of a peak in the flow-through (peak 1) of the chromatograms presented in Figure 3.16. As can be seen in Figure 3.16.A, Whatman® DE32 had a higher dynamic binding capacity than the DE52 resin (Figure 3.16.B). Whatman® DE52 resin only retained a maximum of 50 mg protein per mL resin while the DE32 resin retained up to 80 mg per mL resin. The first column used had a bed volume of 150 mL and, therefore, a total dynamic binding capacity of 12000 mg protein when packed with Whatman® DE32 resin. When packed with the DE52 resin, the column would have the capacity to bind 7500 mg protein. The total binding capacity for these two resins therefore differ by 37.5%. Based on these results, Whatman® DE32 resin was used for the initial purification step in all subsequent studies.
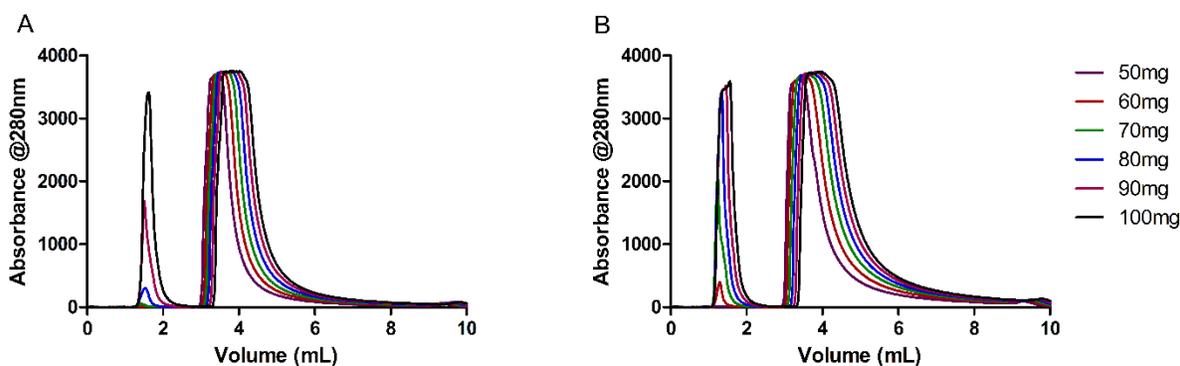
Figure 3.16 Dynamic loading capapcity of (A) Whatman DE32 and (B) Whatman DE 52 resins when increasing concentration of BSA was injected. The BSA was eluted with a 20 mM Tris-HCl buffer (pH 7.5), using a stepped increase in the concentration of NaCl from 0 to 500 mM. Once maximal loading capacity was reached, unbound BSA eluted in the first peak.

Buffer optimization

The composition of the buffers used is one of the most important considerations during ion exchange chromatography. For purification of soybean LOX isozymes, phosphate buffers are often used during anion exchange chromatography. However, once dissociated, phosphates are anionic species which have a high efficiency at displacing other anionic species from the cationic resin. In order to maintain maximal binding capacity, Tris-HCl (pKa = 8.07) was chosen as the buffer for all subsequent purifications. allowing for efficient buffering capacity at a working pH of 7.5.

Following binding, the bound proteins can be eluted with the use of various salts. These salts have different eluting strengths and, based on this property, can be ranked in a series correlating to the Hoffmeister series. In this series, as presented below, anionic species are ranked from strongest to weakest eluent. It is important to note that the strongest eluting salt is not always the best option for use during elution. For this reason, salts containing the anions highlighted in the series below were evaluated for elution efficiency of the protein of interest. These anions were selected based on compatibility with the subsequent activity assays and reactivity. Salts containing iodides and bromides have strong absorbance in the UV range while salts containing nitrates, chlorates, or perchlorates are highly reactive and salts containing these anions were therefore not evaluated.

$$F^- \sim SO_4^{2-} > HPO_4^{2-} > CH_3COO^- > Cl^- > NO_3^- > Br^- > ClO_3^- > I^- > ClO_4^- > SCN^-$$
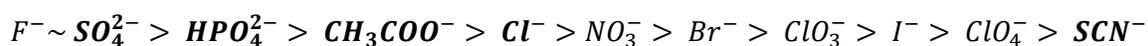
Figure 3.17.A shows the elution profiles of lentil extract when eluted with salts containing the anions listed. As can be seen, there was no marked increase in retention times. However, peak resolution and

total number of peaks observed differed considerably when using different eluents. With sulfate as the counter ion, a reduction in column binding capacity, as indicated by activity in the flow through (Figure 3.17.B), was observed. None of the other elution salts tested caused a reduction in binding capacity while the peak shape was unaffected. In contrast thiocyanate and acetate resulted in a reduction in peak resolution. The data presented in Figure 3.17.B suggest that eluting with thiocyanate results in a decrease in the total elution volume of the active protein. However, thiocyanate resulted in high background absorbance in the subsequent activity assay and as a consequence the activity in fractions eluting after 12 mL could not be determined. When the active protein was eluted with phosphate, the total activity of the sample was reduced by 30.34% when compared to the other elution salts tested. This result suggests that, although phosphates are the second strongest eluting salt evaluated, not all the active protein is eluted from the column when using a 500 mM phosphate solution as the elution salt. However, subsequent elution with 1M NaCl did not yield any additional active protein indicating that high phosphate concentrations may influence the activity assay. Finally, elution with NaCl, the most widely used elution salt, resulted in 5 well-defined peaks, compared to 4 in the other chromatograms. Since the contaminating proteins are resolved more efficiently from the active protein (Figure 3.17.A), the use of NaCl also reduces the amount of subsequent purification steps required.
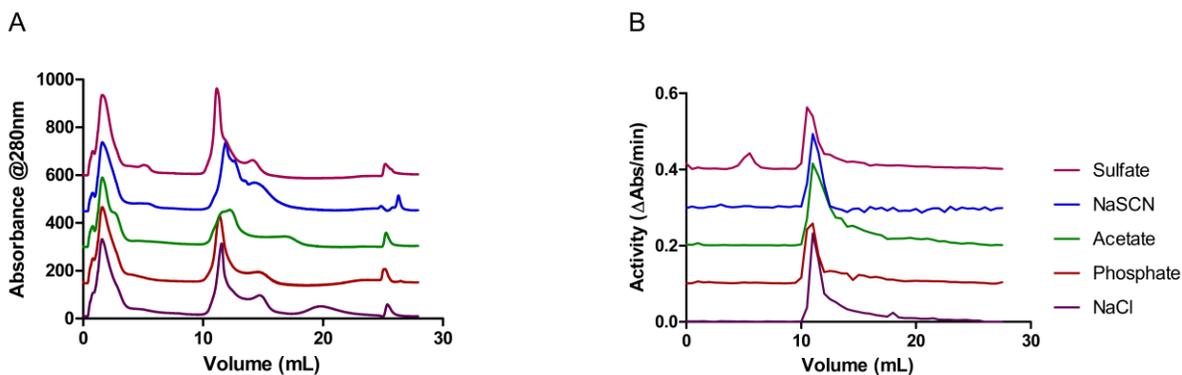
Figure 3.17 (A) Elution profiles of 0.1 mL lentil extract injected onto a 1 mL DE32 column and eluted with the salts as indicated. Throughout the run 0.5 mL fractions were collected. The activity of each fraction was subsequently determined and plotted against elution volume to produce figure B.

Based on the results presented, all subsequent anion exchange chromatography procedures were performed using Tris-HCl as the buffering salt and NaCl as the elution salt. This buffer system was also used for the second chromatography step. The remaining chromatography procedures were optimized in a similar manner with the use of 1 mL columns.

Purification of lentil LOX

In order to ensure efficient and reproducible purification, yielding the highest degree of purity, multi-step purification procedures are often used. In the first step, known as the protein capture step, the objective is to remove as many of the major contaminating proteins, pigments and lipid material as possible. In this step resolution is often sacrificed for capacity and speed. For this reason, the protein capture step should always be followed by subsequent purification steps to increase the final purity. In the intermediate purification step, focus is placed on capacity and resolution since the major contaminants have already been removed in the first chromatography step. As was the case in this study, intermediate purification is often performed using high performance resins in order to achieve higher selectivity. Finally, the protein of interest is purified to within acceptable purity using a high-resolution resin. Since most of the contaminating proteins have already been removed, the focus is on achieving the highest possible resolution in the final polishing step.

For optimal recovery of the protein of interest, techniques with complementary selectivity are often used. For example, following precipitation with ammonium sulfate, hydrophobic interaction chromatography can be used for protein capture. This not only results in the removal of contaminating proteins, but also results in a reduction in ammonium sulfate, thereby eliminating the need for desalting. In this study hydrophobic interaction chromatography was evaluated for protein capture. This technique, however, resulted in a significant reduction in protein yield. As discussed previously, LOX precipitates at relatively low levels of ammonium sulfate and anion exchange chromatography was therefore used for protein capture and intermediate purification.

In this study lentil LOX was purified to relative homogeneity using two anion exchange chromatography steps followed by cation exchange chromatography as polishing step. As mentioned previously, ammonium sulfate precipitation was used for initial extraction and concentration. Although it resulted in some loss in active protein (Table 3.2), ammonium sulfate precipitation resulted in an increase in the protein purity factor.

Protein capture

A difficulty often associated with the isolation of plant proteins is the presence of lipids and pigments, both of which can interfere with the subsequent activity assays. For optimal efficiency during the initial chromatography step, the protein of interest should ideally be resolved from as many contaminating proteins as possible. This could be achieved by increasing the length of the elution gradient. However, this

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would also result in peak broadening and increase the total run time which would not be practical when up scaling for larger volume production of the enzyme. A sharp, or stepped, elution gradient would reduce the volume in which the protein of interest elutes. However, this would also increase the presence of co-eluting proteins. The chromatogram presented in Figure 3.18, shows the elution profile obtained in this study. As can be seen in this figure, the active LOX could be separated from the major contaminating components present in the extract with the use of a linear elution gradient.

Interestingly, following elution, the fractions containing the non-bound proteins of the flow-through became cloudy during the remainder of the chromatography run at 4°C. Once these samples were brought to room temperature, the cloudiness dissipated. As discussed earlier, the extract also became cloudy when stored at 4°C following extraction. Furthermore, clarification by centrifugation coincided with a loss in enzyme activity. In a previous study it was shown that soybean LOX 2 associates with the lipid fraction of soybean seed[33]. Defatting of the soybean flour prior to extraction, however, resulted in a significant loss in LOX 2 activity. The results presented here suggest that the lentil LOX forms similar associations. The lentil extracts were therefore clarified using Triton® X-100 prior to injection. During loading and elution, the detergent was sufficiently diluted to result in the reappearance of cloudiness. However, since the active enzyme had been successfully separated from the lipid material, the active fractions remained clear, even after prolonged storage at 4°C.
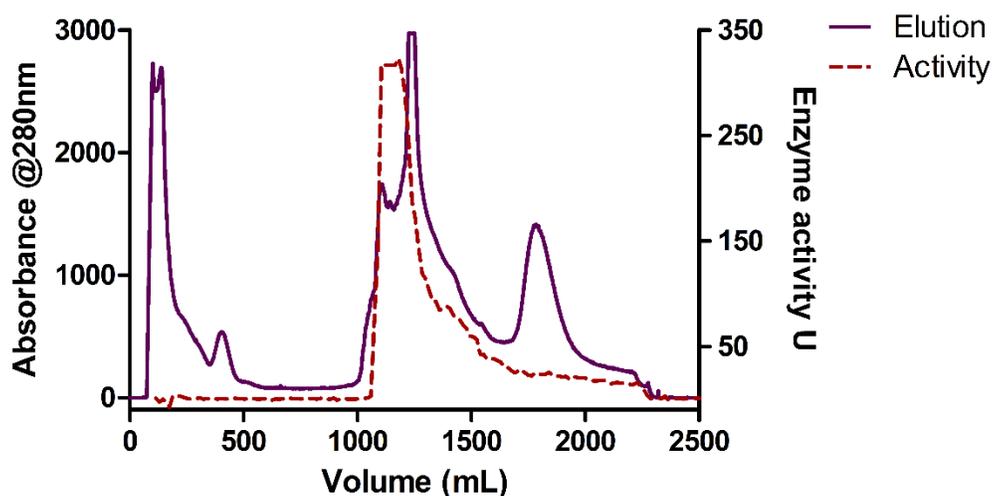
Figure 3.18 Elution profile for the first chromatography step. After the sample was loaded, fractions were collected throughout the remainder of the run. LOX activity of each fraction was determined, and plotted against fraction volume. The activity data was subsequently overlaid with the chromatogram for peak identification.

During this initial chromatography step, the active protein was also resolved from contaminating water-soluble pigments. These contaminants eluted in the last peak of the chromatogram presented in Figure 3.18. In this step the major contaminating species were resolved from the active protein, with only minor overlap with the active fractions. Although their presence did result in a high initial absorbance during subsequent activity assays, these contaminants had little to no effect on any of the subsequent analyses of active protein.

The results of SDS-PAGE analyses of fractions collected from each of the aforementioned fractionation steps are presented in Figure 3.19. This figure shows that although the protein of interest was by no means pure, each step did result in an increase in the relative concentration when gauged by band intensity. The results presented in this figure corroborate the results presented in Table 3.2 where it is shown that precipitation with ammonium sulfate resulted in an increase in protein purity. Here it can be seen that precipitation selectively increased the relative concentrations of, especially, the proteins present at ≈100 kDa. It is of interest to note that there were two bands present in lane 3 at a molecular weight corresponding to ≈100 kDa.
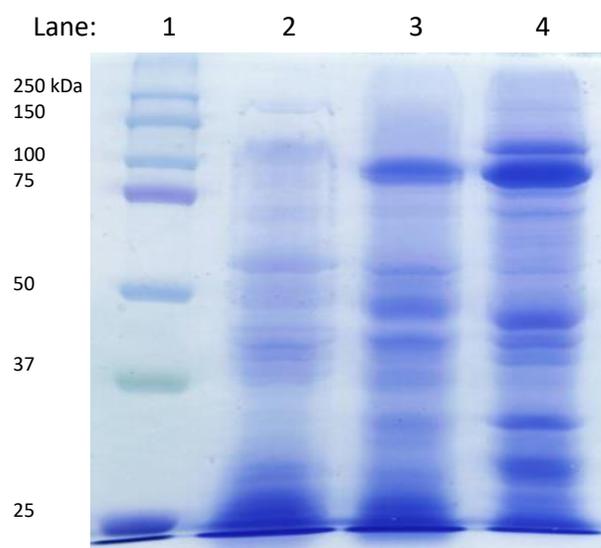
Figure 3.19 Image of a 12% SDS-PAGE gel indicating an increase in purity of the lentil extract following (lane 2) crude extraction of lentil flour, (lane 3) precipitation using ammonium sulfate and (Lane 4) the first chromatographic step. The first lane contains 7.5 µg of Kaleidoscope protein standard. Each of the remaining lanes were loaded with 20 µg of each fraction described.

The presence of these two bands, of which the relative concentration was selectively increased to a similar extent, suggests the presence of two closely related proteins. The relative molecular weights were subsequently determined, by comparison to the molecular weight standard, to be 111 and 100 kDa, respectively (Table 3.4). During earlier experiments with lentil LOX, Eskin and Hendeson[16] showed, using disk gel electrophoresis, that lentil LOX has a molecular mass of 110 kDa. On the other hand, Hilbers *et*

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al.,[17] described a lentil LOX with an Mr between 94-96 kDa. It is therefore possible that the bands present at ≈100 kDa in Figure 3.19, correspond to the two separate the lentil LOX isoforms described previously.

Table 3.4 Estimated molecular weight determination of each of the most intense bands present in Figure 3.19. The size of each band was calculated by comparison to the molecular weight standard in lane 1

Marker Mr	Marker R_f	Lane 3 R_f	Estimated Mr	Lane 4 R_f	Estimated Mr
250	0.070	0.191	111	0.040	243
150	0.111	0.211	100	0.156	133
100	0.181	0.352	48	0.206	103
75	0.236	0.427	33	0.432	32
50	0.392	0.477	25	0.603	13
37	0.543	0.528	19	0.678	9

As each lane was loaded with an equal amount of protein, an increase in protein purity following the first chromatography step resulted in slight overloading of the proteins present R_f = 0.206. As a result, the relative molecular weight could not be determined. In order to prevent overloading during subsequent SDS-PAGE analysis, the total amount of protein loaded was decreased from 20 µg to 10 µg per lane in order to compensate for the increase in protein purity.

Intermediate purification

During the intermediate purification phase, the goal was to remove as many of the remaining contaminants as possible. In contrast to the initial protein capture step, during intermediate purification the focus is on resolution. For optimal resolution, linear or stepped linear elution gradients are often employed. Such gradients allow for the separation of proteins with closely related pI's. As such, the aim of this step was to remove the remaining pigment and major contaminants which remained following protein capture. For intermediate purification three elution gradients were evaluated.

The chromatogram for the first elution gradient evaluated is presented in Figure 3.20. During the initial optimizations, using DE32 resin, it was found that the active protein eluted below a NaCl concentration of 250 mM. A stepped linear gradient was therefore applied during this phase of chromatography to reduce the total run-time without compromising resolution. Since protein capture was performed using anion exchange chromatography, relatively few proteins were expected to elute in the flow-through. The flow-through fraction did, however, exhibit high absorbance, due to the presence of Triton® X- 100 in the sample. Of the remaining peaks, only two poorly resolved peaks showed LOX activity. As discussed previously, following SDS-PAGE analysis of the samples collected during the fractionation and protein

capture steps, two protein bands could be observed at 100- and 110 kDa, respectively. The presence of a split chromatography and activity peaks at an elution volume of ~400 mL offers more support for the possibility of multiple LOX isoforms. Since these peaks were not well-resolved, the duration of the elution gradient was increased by 1.5 column volumes to increase the separation of the two components with LOX activity.
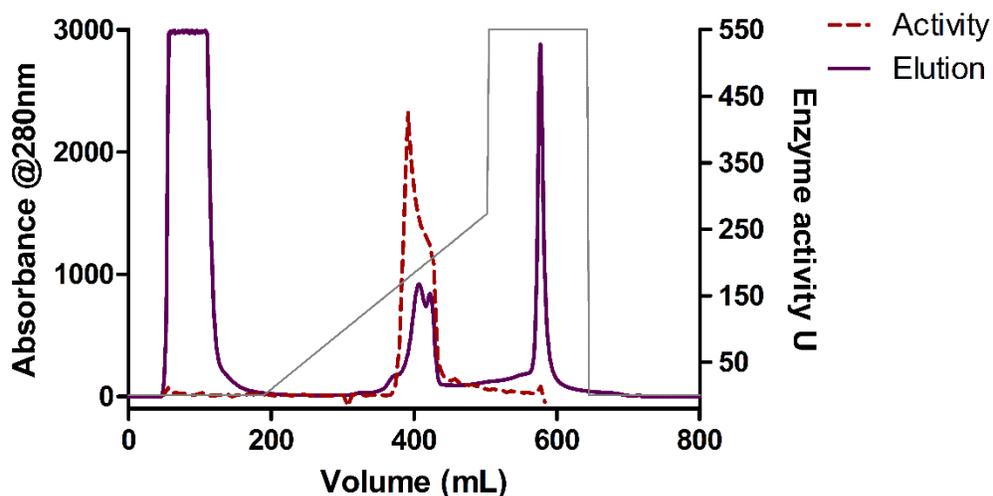
Figure 3.20 Chromatogram for the first elution gradient evaluated for intermediate purification. It is apparent that the active peak is well-resolved from the major contaminants present in the last peak.

As can be seen in Figure 3.21, increasing the length of the elution gradient resulted in an increase in peak separation. However, this also increased the total run-time by 45 minutes. Although run-time is often sacrificed for resolution during the intermediate purification stage, an increase in run time necessitates increased reagent usage which would significantly reduce the financial viability of the process when upscaled. Nevertheless, using this gradient did result in an increased peak resolution. The active peaks were subsequently collected and injected separately onto the final cation-exchange chromatography column.
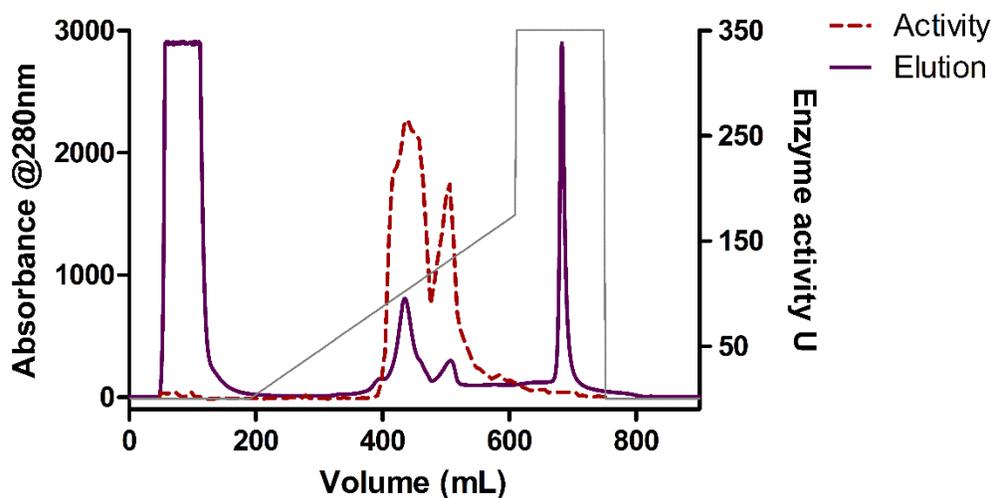
Figure 3.21 Chromatogram for the second elution gradient evaluated. In this figure two active peaks are present. The major contaminants were eluted by employing a stepped increase in the concentration of NaCl from 250 – 500 mM.

Figure 3.22 shows the results of SDS-PAGE analysis of the active fractions collected after fractionation, protein capture and intermediate chromatography using the second elution gradient. As can be seen in lanes 5 and 6, the fractionation steps performed have removed most of the contaminating proteins present in lanes 2-4. However, although the fractions obtained from the intermediate purification step were relatively pure, with only minor contaminating proteins, the gradient was adjusted in an attempt to elute the active fractions separately.
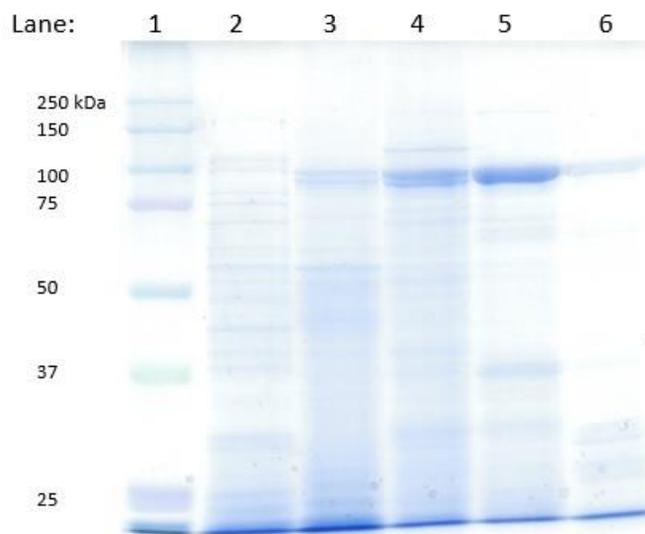
Figure 3.22 Samples collected from (lane 2) crude extraction of lentil flour protein, (lane 3) protein precipitation using ammonium sulfate, (lane 4) the active fraction collected from the protein capture step, (lane 5) the first active peak from the run presented in Figure 3.20 and (lane 6) the second active peak of the same run. Each lane was loaded with 15 µg protein. Lane 1 was loaded with 7.5 µg Kaleidoscope marker.

To improve separation of the active fractions presented in Figure 3.21, the elution gradient was adjusted to a stepped-linear elution gradient. The gradient depicted in Figure 3.23 was devised based on the concentration of elution buffer at which each active peak eluted using the gradient shown in Figure 3.21. To maintain the resolution attained in the previous run, the initial elution still involved a linear increase in the concentration of NaCl to a concentration of 125 mM. The remaining proteins were eluted by a step-wise increase in the concentration of elution buffer. However, although the aim of a stepped-linear gradient was to separate the active proteins, this goal was not achieved. As such, the second elution gradient evaluated was used for all subsequent purifications. The active fractions were pooled separately.
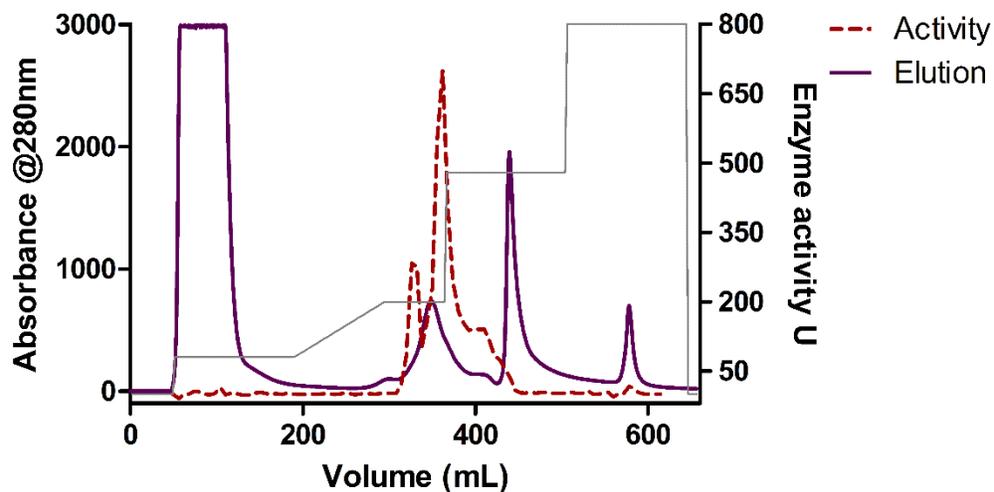
Figure 3.23 Chromatogram of the final elution gradient evaluated. Although two active fractions were obtained, baseline separation was not achieved. In this step the major contaminating proteins were again separated from the active protein.

Cation exchange chromatography

The purpose of the chromatographic procedures up to this point were to remove the major impurities, except for trace amounts, closely related proteins, and structural variants of the protein of interest. As such, the goal of the polishing step is to reduce the amount of the remaining contaminants to acceptable levels. In this step the focus was therefore placed on resolution.

During the initial purification of soybean LOX, isoforms 2 and 3 could only be separated using cation-exchange chromatography[25]. In this study the active fractions obtained from the intermediate purification step of lentil LOX were subsequently separated using cation exchange chromatography for polishing. The result for this separation is presented in Figure 3.24. As can be seen, the major remaining contaminant proteins were eluted during the loading, wash and regeneration stages of elution. As was the case during the previous purification steps, activity was associated with multiple chromatographic peaks. Although this result indicated the presence of multiple isoforms of LOX, it remained a possibility that lentil LOX formed complexes with other seed proteins in suspension. To determine if the results presented was to due multiple isoforms or complex formation, the molecular characteristics of each of the active fractions were determined. Fractions containing the highest specific activity were also analyzed using IEF. This data is presented and discussed in the following chapter.
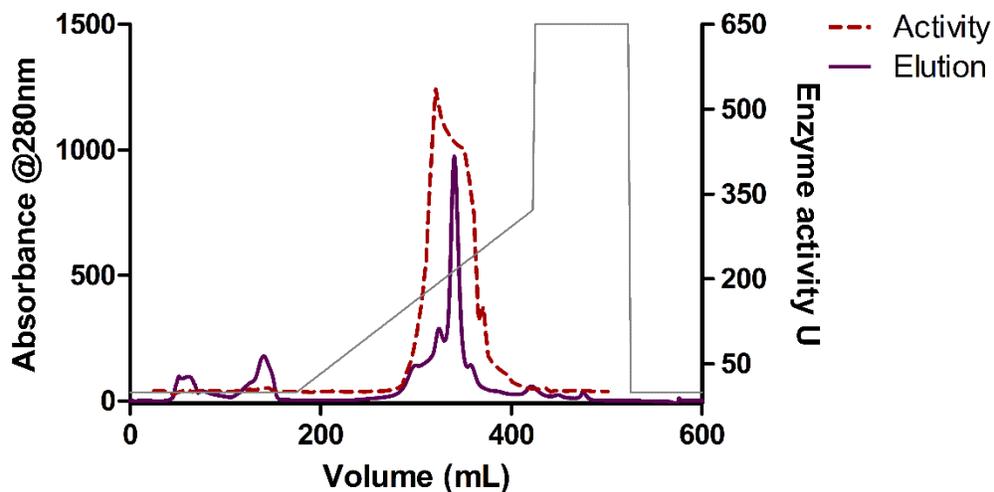
Figure 3.24 Chromatogram for the polishing step of the active samples obtained using the gradient depicted in Figure 3.22. Minor impurities were removed in this step and the activity was present in more than one peak.

Following chromatography, each of the active fractions were evaluated for purity using SDS-PAGE as presented in Figure 3.25. As can be seen, the initial samples still contained trace levels of contaminting proteins. However, fractions loaded in lanes 8-17 were comparatively pure. Although a maximum of 10 µg protein was loaded per well, due to the high level of purity, the band at ≈100 kDa was slightly overloaded. However, the bands at roughly 100 kDa in lanes 7-10 appear to be composed of two bands of similarly sized proteins.
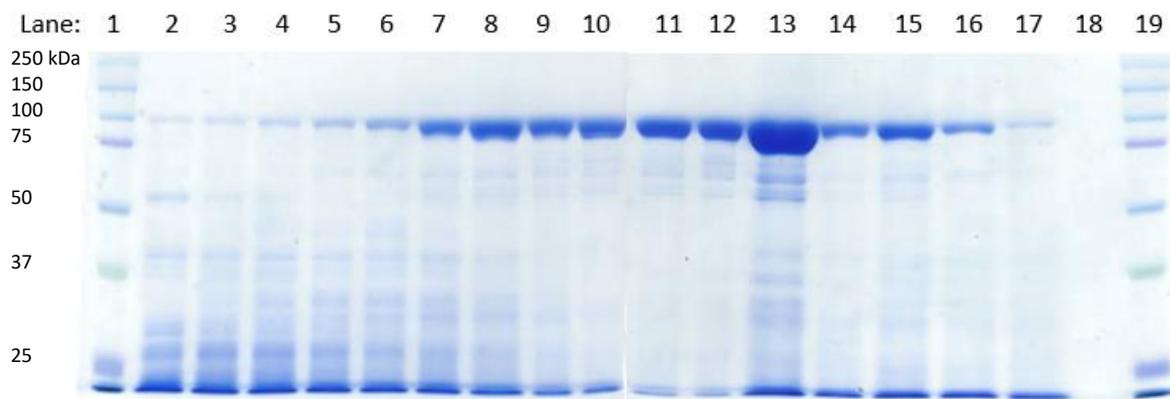
Figure 3.25 Image of a 12% SDS-PAGE gel of loaded with the active fractions obtained from the cation exchange chromatography presented in Figure 3.25. Two 10 well gels were run, and are presented side by side. Lanes 1 and 19 were loaded with 7.5 µg Kaleidoscope marker. Lanes 2-17 were loaded with 10 µg of each of the cation chromatography fractions 54-69.

The fractions were subsequently analyzed on 7% SDS-PAGE gels in order to enable separation of similarly sized proteins. As can be seen in Figure 3.26, the band at ≈100 kDa of fractions 5 – 17 were composed of two similarly-sized proteins. The molecular mass of each band was calculated to be 98 and 92 kDa, respectively, when compared to the molecular markers presented in lanes 1 and 19. These bands were subsequently analyzed for similarity using tryptic digest tandem Mass Spectroscopy. The results of these analyses is presented in Chapter 4. Since fractions 58-69 (lanes 6-17) were relatively pure, 100 µg protein aliquots were pooled. These pooled samples were subsequently used for the determination of the catalytic properties of lentil LOX, and will be discussed in Chapter 4.
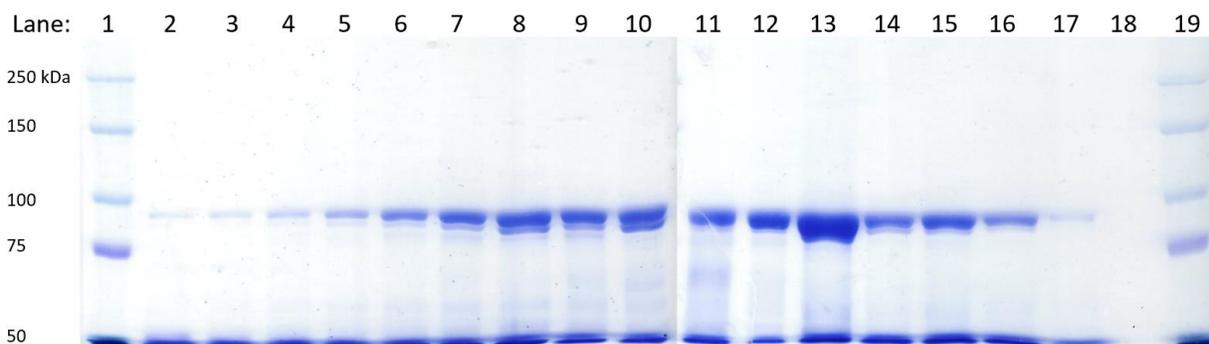
Figure 3.26 Image showing the result of a 7% SDS-PAGE gel loaded with the active fraction obtained from cation exchange chromatography. As with Figure 3.26, 7.5 µg Kaleidoscope marker was loaded in lanes 1 and 19 while the remaining wells, except for well 18, were loaded with 10 µg protein from each of the chromatography fractions 54-59.

Purification results

The results for the extraction, and purification of lentil seed LOX are presented in Table 3.5. As can be seen in this table, crude extraction resulted in a loss of up to 40% of the active protein. However, once the major contaminating proteins had been removed in the initial capture step, the average loss in active protein per purification step was significantly reduced. Extraction and purification of lentil LOX, using the methods described in this study, subsequently resulted in a 21.85-fold purification, with a final recovery of 40 %.

Table 3.5: Purification of lentil LOX from lentil flour. The data presented in this table is the mathematical mean of three separate extraction and purifications

Purification step	Activity/mL (U/mL)	Volume (mL)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Fold purification
Crude extraction	1331	230	306104	429	100.00%	1.00
Concentrated extract	3619	50	180944	394	59.11%	0.92
DE32 Active fraction	2815	50	154806	4289	50.57%	10.00
Q Speharose active fraction	2499	138	142144	7249	46.44%	16.90
Fractogel active fraction	6958	18	125238	9374	40.91%	21.85

The initial loss in active protein may be due to various factors. However, protein loss during purification of plant proteins is often due to the action of proteases. Although seeds are often considered to be dormant, they contain a host of defense mechanisms designed to cause structural modification in the proteins of their attacker. For this purpose, proteases are often present in very high levels[153]. Since the exact nature of these proteases is often not known, generally used protease inhibitors may not be effective during extraction. The action of proteases during extraction are therefore often responsible for large losses in the protein of interest. However, the results presented in Section 3.2.1 show that prolonged extraction (16 h Vs. 1 h) resulted in a relatively minor decrease in protein recovery. The reduction in protein recovery is therefore most likely not due to the action of proteases alone.

3.4 CONCLUSION

This chapter described the purification of lentil LOX from lentil seed flour. For peak identification during chromatography, high-throughput methods for the detection and quantification of LOX activity were required and the development of such an assay is described prior to the purification of lentil LOX.

As discussed in Chapter 2, the conjugated diene method is the current standard whereby LOX activity is determined. However, due to the low molar absorbance of the reaction products, previous authors have suggested that this assay is not suited for high-throughput screening of multiple samples. For this reason, assays which rely on the use of dyes for detection were evaluated. The data presented suggest that the methods evaluated could be used for characterization of the LOX enzyme. However, these methods were not sufficiently sensitive to allow detection in chromatography fractions. Based on the sensitivity associated with direct determination of the formed conjugated diene, the conjugated diene method was subsequently adapted for use in 96-well microtiter plate based activity assays. This newly developed high-throughput method was sufficiently sensitive to detect activity in samples containing less than 0.1 µg/mL LOX. This method also allowed the determination of LOX activity in multiple samples simultaneously. The results obtained with the microtiter plate assay were in good accordance with the full-scale conjugated diene activity assay. This modified assay could also be applied in kinetic characterization experiments.

Lentil LOX was subsequently purified 21.85-fold using a three-column purification procedure. However, when analyzed on SDS-PAGE, multiple similarly sized proteins were observed. Although it has been suggested that lentil seeds may contain multiple LOX isozymes, the molecular characteristics have not yet been determined. The purified fractions obtained in this study were therefore characterized as will be described in the following chapter. These enzymes were also applied in the baking trials discussed in Chapter 5.

Chapter 4

CHARACTERIZATION OF PURIFIED LENTIL LOX

4.1 INTRODUCTION

The use of enzymes in the baking industry is well established. During bread making, enzymes are routinely added to improve dough rheology, fermentation, stability, crumb color and grain texture. As discussed in Chapter 2, lipoxygenases (LOX), in the form of enzyme active soybean flour, is added up to 0.5 % ($^w/_v$) to improve the crumb color and overall dough rheology. However, soybean flour is considered a major food allergen limiting its use. In this study lentil seed LOX was evaluated as a possible replacement for soybean seed LOX due to the reduced allergenicity of lentils.

Although a great deal of information is available about the catalytic properties of soybean LOX isozymes, lentil seed LOX, in contrast, has not received as much attention. There are significant changes in temperature during different stages of the baking. White bread dough is furthermore mildly acidic with a pH of 5.5. A detailed knowledge of the optimum activity conditions under which the LOX isozyme(s), isolated in this study operate, would enable optimization of the process efficiency during baking. Such knowledge would also allow for better comparison with the soybean flour benchmark currently used during the production of white bread. The availability of kinetic information on lentil seed LOX could also be used for dosage optimization by enabling prediction of substrate interactions. This section therefore describes the characterization of the lentil seed LOX isolated, and purified as previously described in Chapter 3.

4.2 MATERIALS AND METHODS

Unless stated otherwise, all reagents and chemicals used in this study were of analytical grade and were obtained from reputable supply houses. Linoleic acid and glyceryl trilinoleate used in this study had a purity of at least 98% and was obtained from Sigma-Aldrich (St. Louis MO, USA). Type I-B Lipoxygenase from *Glycine max*, used as LOX standard, was also obtained from Sigma-Aldrich (St. Louis MO, USA). Greiner UV-Star® 96 well plates were purchased from Sigma-Aldrich (St. Louis MO, USA). The enzyme solutions used in this study were those purified in Chapter 3.

4.2.1 LOX activity determination

The microtiter plate conjugated diene activity determination method, described in Chapter 3, was used for all characterization experiments. In this assay 150 µL substrate solution, consisting of 1 mM linoleic acid in 200 mM Tris-HCl, pH 8.0, containing 0.25 % $^v/_v$ Tween® 20, was added to 50 µL of each test sample in the wells of a Greiner UV-Star® 96 well plate. Soybean seed LOX I-B was used as positive control while assay buffer was used as the blank control. Following addition of the substrate solution, the absorbance of each well was determined at 234 nm at 10 second intervals at 25°C for a total of 5 min using a Tecan Spark 10M microtiter plate reader. Unless stated otherwise, 1.25 µg protein was used for all determinations described in this chapter. Since the protein concentration remained constant, specific activity was calculated by using Equation 4.1.

$$\frac{U}{mg} = \Delta Abs_{234} \times 95909.73$$

Equation 4.1 ΔAbs_{234} refers to the change in absorbance for the linear section of the progress curve obtained using the method described above.

4.2.2 Characterization of purified lentil LOX

SDS-PAGE analysis

For evaluation of protein purity, SDS-PAGE analysis was performed on each of the fractions used in the experiments described in this section. SDS-PAGE analysis was performed according to the method of Laemmli[154]. Polyacrylamide gels (10%) were hand-cast and the samples were resolved at 200 Volt (V) and 4°C using a Bio-Rad mini-PROTEAN system until the indicator front was ± 0.5 cm from the bottom of the gel.

Samples were prepared for SDS-PAGE analysis by diluting each enzyme solution to a final protein concentration of 2 mg/mL prior to 1:1 treatment with 2x treatment buffer (120 mM Tris-HCl, pH 6.8, containing 4% $^w/_v$ SDS, 20% $^v/_v$ glycerol and 0.02% $^w/_v$ bromophenol blue). Each sample was briefly centrifuged and the proteins were denatured at 90°C for 10 min. Once the samples had cooled to room temperature, each sample was briefly centrifuged before the equivalent 5 µg protein was loaded on the polyacrylamide gels. Following electrophoresis, the proteins were stained with Coomassie Brilliant blue stain. The gel was destained using a methanol/acetic acid (50/10) mixture.

Samples were also denatured using urea instead of heat-denaturation. These samples were treated 1:1 with 2x urea treatment buffer containing 8 M urea, 0.375 M Tris-HCl, pH 8.8, 2 % $^w/_v$ SDS, 20 % glycerol, 2 % $^w/_v$ dithiothreitol (DTT) and 0.02% $^w/_v$ bromophenol blue. Samples were incubated for 10 min at room temperature before the equivalent of 5 µg protein was loaded onto the 10 % polyacrylamide gel.

pH-optima determination

To determine the pH-optima for the lentil LOX purified in this study, LOX activity was determined every 0.5 pH units over a pH range of 4.5 – 10, by measuring LOX activity with 2 mM linoleic acid prepared in the buffers described in Table 4.1, using the microplate conjugated diene method. Buffers were selected based on their effective buffering range. Once the optimal pH was established, all subsequent assays were performed in the appropriate buffer.

Table 4.1 Buffers and pH values used for the determination of optimal pH of the purified LOX enzyme(s). The effective pH range for each buffer is also presented

Buffer	Concentration	pH	Effective pH range
Sodium acetate	200	4.5	4.8-5.2
	200	5.0	
MES	200	5.5	
	200	6.0	5.5-6.7
	200	6.5	
Tris	200	7.0	
	200	7.5	
	200	8.0	7.0-9.0
	200	8.5	
	200	9.0	
Sodium carbonate	200	9.5	9.5-11.1
	200	10.0	

Temperature optimization

The optimal temperature for oxidation of linoleic acid was determined by assaying solutions containing 25 µg/mL protein at temperatures ranging between 25 and 45°C. Each assay was performed in triplicate with 2 mM linoleic acid in Tris-HCl buffer, pH 7.0. Following the assay, the temperature was increased by 5 °C, and the sample was re-assayed.

Kinetic characterization

Once the optimal assay conditions were established, enzyme activity was measured with varying concentrations of linoleic acid. Prior to these assays, enzyme samples were diluted to a final protein concentration of 25 µg/mL to yield a total of 1.25 µg protein per well during the assay. A total of 12 assays

were performed over a linoleic acid concentration range of 0.07 to 5 mM. The initial reaction rates, determined for the first minute of the reaction, for each concentration of linoleic acid was subsequently plotted against substrate concentration to construct a kinetic curve. Based on the shape of the curve, a non-linear regression line was fitted to the data and the catalytic constants; K_m and V_{max} were determined using GraphPad Prism 5 software.

Interaction with esterified substrates

The ability of lentil seed LOX to utilize esterified substrates was determined using small unilammelar vesicles (SUVs), consisting of glyceryl trilinoleate as substrate. The SUV solutions were prepared as described by Rautenbach[155]. Briefly, 2 mM glyceryl trilinoleate was prepared from a 10 mg/mL stock solution in chloroform, in chemically clean thin-walled glass test tubes. The chloroform was evaporated under a stream of nitrogen to leave a thin film of lipid on the glass. The lipid was subsequently suspended in a 200 mM Tris-HCl buffer, pH 7.0 flushed with nitrogen. This suspension was then sonicated at 30°C until the suspension became translucent. Typically, no more than 5 min of sonication was needed. During the assay, heat-inactivated enzyme suspensions were used as blanks.

Iso-electric focusing

To determine the isoelectric point (pI) of the bands observed at ≈ 100 kDa as described in Chapter 3, isoelectric focusing (IEF) was performed as follows:

Sample cleanup

Prior to IEF, of the active protein fractions 58, 62 and 66 were pooled (refer to section 3.3.3, Chapter 3). Sample cleanup was then performed using a Bio-Rad ReadyPrep™ 2-D Cleanup kit (Hercules, CA, USA) as per the manufacturer's instructions. For this method, a maximum of 500 µg protein was required. The protein concentration of the sample to be analyzed was first determined using the Bradford method with BSA as protein standard. Sample cleanup was subsequently performed in duplicate to obtain a total of 460 µg protein. For cleanup, 100 µL (2.3 µg/µL) sample was transferred to a 1.5 mL Eppendorf tube to which 300 µL precipitating reagent 1 was added. The samples were then incubated on ice for 15 min, followed by the addition of 300 µL precipitating agent 2. Each sample was subsequently centrifuged at 13,000 x g for 5 min, and the supernatant discarded. The pellet was then washed with 40 µL wash reagent 1 followed by centrifugation at 13000 x g for 5 min and the supernatant was discarded. The pellet was resuspended in 25 µL proteomic grade water. One mL pre-chilled (-20 °C) wash reagent 2 was

subsequently added to each sample followed by rigorous mixing for 1 min followed by incubation at -20 °C for 30 min. During incubation, the tubes were vortexed every 10 min for 30 s. The protein precipitate was subsequently collected by centrifugation at 13,000 x g for 5 min, and the resulting pellet was air-dried at room temperature for 5 min. The dried pellet of one tube was subsequently dissolved in 300 µL Bio-Rad 2-D rehydration buffer by sonication. Once dissolved, the 300 µL solution was quantitatively transferred to a second tube.

Isoelectric focusing

For IEF, 17 cm Bio-Rad ReadyStip™ IPG strips with a pH range of 3 – 10 were used. Prior to electrophoresis, the IPG strip was hydrated with the 460 µg protein solution prepared as described above. This was achieved by incubating the IPG strip, gel-side down, in the 300 µL solution in a disposable rehydration tray for 1h at room temperature. After 1h the IPG strip was carefully overlaid with 2 mL Bio-Rad biotechnology grade mineral oil. The rehydration tray was then covered and incubated for 16h on a level surface at room temperature. Following hydration, the mineral oil was removed by suspending the IPG strip vertically for 1 min. The remaining mineral oil was subsequently removed by blotting on a paper towel. The IPG strip was transferred to the focusing tray of a Bio-Rad PROTEAN® IEF cell system. The strip was again overlaid with 2 mL mineral oil as before. Focusing was performed at 20°C using the following program: 250 V for 15 min followed by a linear increase in voltage to 10,000 V, which was maintained for 3 h. The final focusing step was performed at 10,000 V for 60,000 V-hours.

2D-PAGE

Prior to separation in the second dimension, the IPG strip was prepared for SDS-PAGE by first incubating the strip for 10 min in Bio-Rad equilibration buffer 1 (6M urea, 0.375 M Tris-HCl, pH 8.8, 2% SDS, 20 % glycerol and 2 % $^v/_v$ DTT). After 10 min equilibration buffer I was replaced with equilibration buffer II which contained 2.5 % $^v/_v$ iodoacetamide instead of 2 % DTT. Once equilibrated, the protein on the IPG strip was separated by SDS-PAGE on a 20 x 20 cm 10 % acrylamide gel using a PROTEAN II XL cell (Bio-rad). The proteins were resolved at 16 mA for 30 min followed by 24 mA until the bromophenol blue front was roughly 1 cm from the bottom of the gel.

Tryptic digest mass spectrometry analysis

In-gel trypsin digest

The protein spots obtained after 2D-PAGE analysis of the sample described above were submitted to the Center for Proteomic and Genomic Research (CPGR), Cape Town, for analysis by mass spectrometry (MS). Each spot was excised from the SDS-PAGE gel and transferred to a 1.5 mL Eppendorf tube followed by destaining using 100 mM NH_4HCO_3:Acetonitrile (50:50) until all residual Coomassie blue stains were removed. The samples were then dehydrated, and the proteins were reduced for 15 min with 2 mM triscarboxyethyl phospine (TCEP) in 25 mM NH_4HCO_3 at room temperature. After reduction, the excess TCEP was removed, and the gel slices were again dehydrated. The cysteine residues were subsequently carbamidomethylated by incubating the samples for 30 min in 20 mM iodoacetamide in 25 mM NH_4HCO_3 in the dark. Following incubation, the gel slices were washed with 25 mM NH_4HCO_3 and dehydrated. The proteins were subsequently digested by incubating the samples overnight at 37 °C in a solution of sequencing grade modified trypsin (20 µg/mL, Promega PRV5111). Following tryptic digestion, the resulting peptides were recovered from the gel matrix by treating the gel slices once with 50 µL proteomic grade water, and once with 50% acetonitrile. The supernatant of each treatment was pooled, and dried under a thin stream of nitrogen. The resulting peptides were then resuspended in 30 µL 2% acetonitrile in water containing 0.1% trifluoracetic acid (TFA).

Residual treatment reagents were subsequently removed using C18 stage tips activated and equilibrated with 30 µL methanol, and 30 µL 2% acetonitrile in water containing 0.05 % TFA, respectively. The peptide solutions were loaded onto the tips, and washed using the equilibration solution followed by elution with 30 µL 50% acetonitrile in water containing 0.05 % TFA. The peptides were dried under a thin stream of nitrogen and resuspended in 2% acetonitrile in water containing 0.1% formic acid. This solution was then subjected to liquid chromatography - MS (LC-MS) analysis.

Liquid chromatography

For liquid chromatography the peptide solutions from above were separated on a Pepmap C_{18} column (25 cm x 75 µm) equipped with a C_{18} trap column (300 µm x 5 mm x 5 µm) using Dionex Ultimate 3000 nano-ultrahigh performance liquid chromatography (UHPLC) system coupled to a Q-Exactive quadrupole-Orbitrap MS. The solvents used consisted of (A) 2 % acetonitrile in water containing 0.1 % formic acid, and (B) 100 % acetonitrile containing 0.1 % formic acid. Each sample was injected onto the column at a flow rate of 5 µL/min and eluted using a stepped linear elution gradient at a constant flow rate of 250 nL/min

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and 50 °C. The gradient program used was as follows: 100 % solvent A for 10 min, solvent B was then increased to 30 % over 30 min followed by a linear increase from 30 – 50 %B over 15 min. Solvent B was then increased to 80 %B and this concentration was maintained for 5 min. The column was re-equilibrated with 100 % A for 10 min prior to injecting the next sample.

Mass Spectrometry

Data was acquired in positive mode with a capillary temperature of 320 °C. The electrospray voltage and temperature were set at 1.95 kVolt and 320°C, respectively. MS scans were performed over a m/z scan range of 350 – 1,650 with the collision energy set to 30 %. The mass analyzer resolution was set to 120,000.

Data analysis

The RAW files generated by the MS were imported into Byonic™ version 2.6.46 software (Protein Metrics Inc., USA), and processed using Proteome Discoverer™ algorithms. The data was subsequently interrogated against soybean, pea, and lentil seed proteomic databases (Uniprot) with trypsin cleavage allowing for 2 missed cleavages. N-terminal acetylation, deamination and oxidation were allowed as dynamic protein modifications while carbamidomethylation of cysteine residues was allowed as static modifications. The precursor mass tolerance was set to 5.0 Da and the fragment tolerance was set to 20.0 ppm.

4.3 RESULTS AND DISCUSSION

In Chapter 3, the purification of lentil seed LOX to relative homogeneity is described using a three-column purification procedure. However, when analyzed using SDS-PAGE each of the active fractions displayed two bands at approximately 100 kDa, which could not be separated by chromatography. Since literature suggests that lentil seeds may contain more than one LOX isoform[16,17], it is possible that each of the bands observed correspond to a different LOX isoform. Because these proteins could not be separated by chromatography, the characterization of active fractions, eluting at different times, were described in this chapter. The fractions chosen for characterization experiments were fractions 59 (F59), 62 (F62) and 66 (F66) eluting as shown in Figure 4.1.A. These fractions were selected based on the intensity of each band present on the SDS-PAGE gel presented in Figure 4.1.B. For instance, fraction 59 (lane 2) contained a major band at ≈ 100 kDa while fraction 62 (lane 3) contained 2 bands with similar intensity. Due to an increased relative abundance of protein in both bands at ≈ 100 kDa it was argued that different isoforms should yield different kinetic properties when compared to fractions 59 and 66 (lane 4) which show an abundance for only one protein.
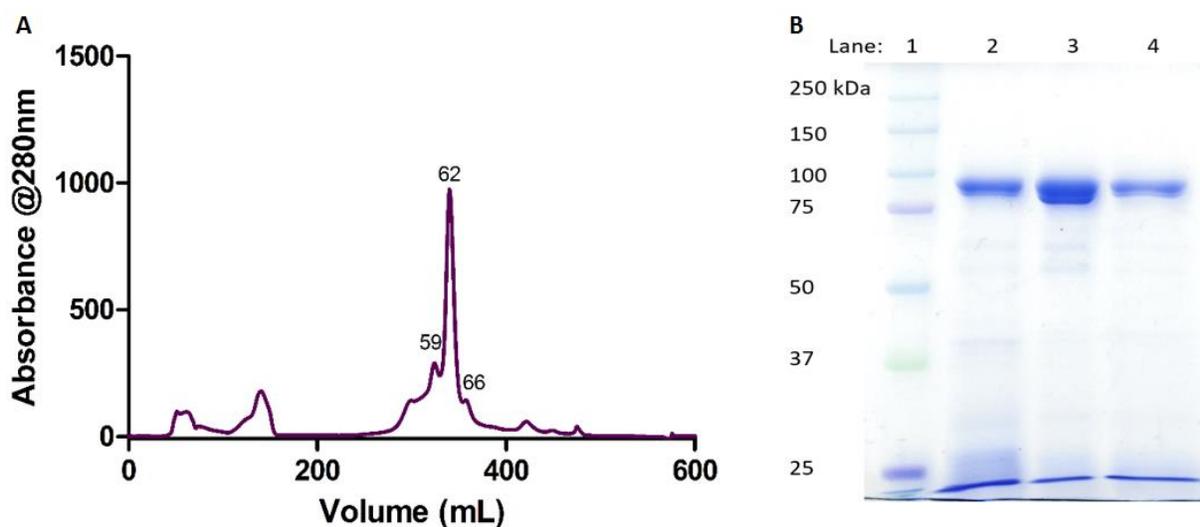
Figure 4.1 (A) Chromatogram of the final chromatography step for purification of lentil seed LOX. (B) The peaks indicated following separation on a 10% acrylamide SDS-PAGE gel. For the gel displayed in Figure B, lane 1 was loaded with 7 µg Kaleidoscope marker (Bio-Rad); lane 2, 5 µg of F59; lane 3, 5 µg of F62, and lane 4, 5 µg of F66 as indicated in Figure A.

The fractions indicated in Figure 4.1 were characterized in terms of temperature-optima, pH-optima, k_m, V_{max}, interaction with esterified substrates, and pI. Following each characterization step, the optimal conditions determined were used for subsequent analyses.

4.3.1 Characterization of purified lentil LOX

Temperature optimization

LOX isozymes often differ in terms of optimal temperature for activity. It has, for instance, been shown that soybean LOX 1 and 3 has optimal activity at 50°C, and further increases in temperature result in a rapid loss in activity[156]. Conversely, soybean LOX 2 exhibits optimal activity at temperatures between 20- and 40°C. Wheat LOX on the other hand, has optimal activity at 35°C[157]. During the bread making process, temperatures for each step in the process differ dramatically. During kneading the dough temperature is increased from ≈20 °C to 30°C. The dough is then proved at 55-60 °C for 60 min followed by baking at 220 °C for 20 min. For use in baking, enzymes with a low temperature-optima would therefore only deliver its effects during kneading and resting which typically last no more than 15 min. Enzymes with high temperature optima on the other hand, would only reach maximum activity during proving which last roughly around 60 min. Based on the incubation times for each step, enzymes with low temperature-optima would therefore have to be dosed at higher levels to yield similar results as their high-temperature-optima counterparts, in order for it to have a desired effect on the final product. Since the

lentil seed LOX isolated in this study is intended for use during the baking of white bread, the optimal temperature at which the enzyme is active was determined.

The data obtained when purified enzyme preparations were assayed at increasing temperatures, from 25 °C, is presented in Figure 4.2. A minimum temperature of 25 °C was chosen as the lowest temperature since the temperature of dough is roughly around 30 °C during mixing and resting. Of the temperatures tested, lentil LOX activity was optimal at 25 °C. Increasing the temperature to 40 °C did not result in a marked decrease in activity when compared to activity at 25 °C. However, once the assay temperature was increased to above 40°C, up to 38.8 % of the original activity, determined at 25°C, was lost. These results suggest that lentil LOX would only influence the baking properties of dough during the kneading and resting stages of the baking process, when temperatures are typically below 35 °C.
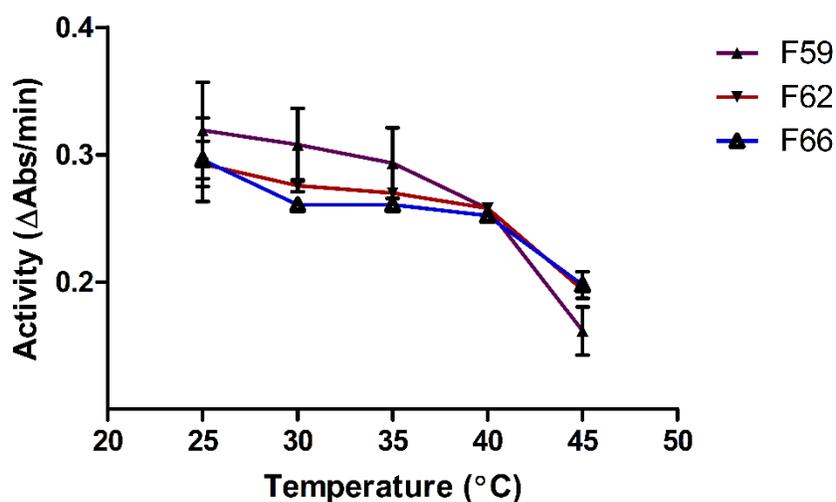
Figure 4.2 Effect of temperature on the activity of purified LOX fractions (n=3). Based on these results, all subsequent assays were performed at 25 °C. Temperatures below 25 °C were not evaluated since this is below the minimum temperature of dough during the manufacturing of bread.

pH optimization

LOX isoenzymes from different species, and even in the same species, differ considerably in terms of their optimal pH of catalysis. For example, soybean seed LOX 1 has a pH-optimum between 9.0 and 9.5 while LOX 2 and 3 has optima between pH 6 and 7. In a study by Hilbers *et al.,*[17] it was shown that during germination lentil seed LOX is active over a broad pH range between 5.7 and 9.0.

Figure 4.3 shows the pH-optima profile determined for fractions 59, 62, and 66 obtained from the final chromatography step described in Chapter 3. The data presented shows that, as indicated by Hilbers *et al.*[17]*,* lentil seed LOX has four pH-optima. However, in their study these authors ascribed the different pH-optima to LOX isoforms present in different prototypical tissue during germination. In the current study, three active peaks were eluted from the final cation exchange column, suggesting isozymes with varying pI values. However, only two bands could be seen after SDS-PAGE analysis of the selected fractions. This result in combination with the pH-optima presented here suggests the presence of at least two different LOX isoforms.

As discussed in Chapter 2, LOX enzymes are classified as either type I or type II enzymes based on the enzyme's pH-optima. Type I enzymes have pH-optima in the alkaline region, while type II enzymes are optimally active under neutral or acidic conditions. The results presented in Figure 4.3, however, indicate that the lentil seed LOX, isolated in this study, have pH-optima in both the alkaline, and neutral pH regions. As such, it is not possible to classify the enzyme(s) isolated in this study as either type I, or type II based on pH-optima alone. This result does, however, strengthen the case for the presence of multiple isoforms of LOX since LOX enzymes having pH-optima in both the acidic and alkaline regions have not yet been described elsewhere.

Since bread dough is mildly acidic with an pH in the range of 5.5[158], the efficiency of added LOX enzymes is dictated by their pH-optima. The results presented in Figure 4.3 show that the purified lentil LOX used in this study, although not as active as at pH 7.0, also has a pH-optima at pH 5.5, which correspond with the pH of bread dough. Based only on pH-optima, lentil seed LOX could therefore function as an ideal alternative for soybean LOX during the baking of white bread.
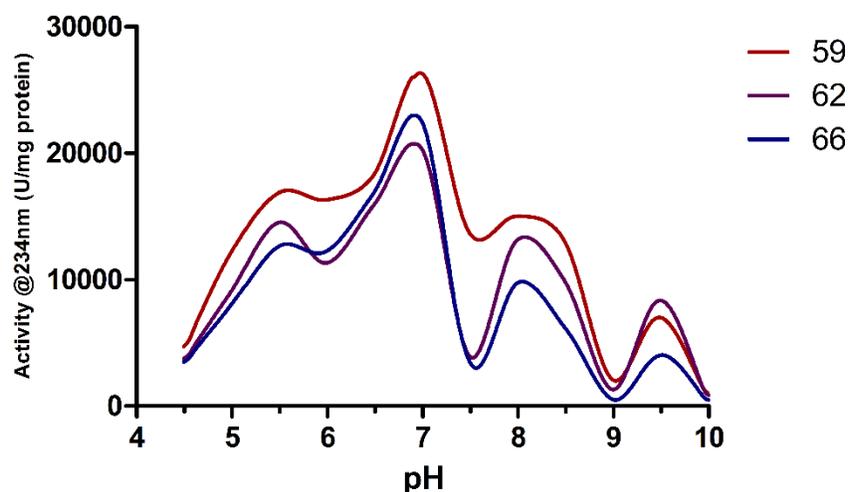
Figure 4.3 LOX pH-optima determined for fractions 59, 62 and 66 obtained from the final chromatography step described in Chapter 3. The purified enzyme fractions each had pH-optima at pH 5.5, 7.0, 8.0 and 9.5.

Kinetic characterization

Due to their importance in the baking industry, soybean seed LOX isozymes are of the most well-characterized plant proteins[9]. However, compared to soybean seed LOX, the kinetic characteristics of enzymes from lentil seed have only been partially described. In order to optimize the efficiency of LOX enzymes applied in an industrial setting, an in-depth understanding of the kinetic parameters governing substrate conversion is crucial. Knowledge of these parameters would not only enable a better comparison of the results obtained from trials using soybean seed LOX isozymes, which still serve as the benchmark in the baking industry, but also would allow the manipulation of processing parameters during bread manufacture in order to achieve the optimal or desired effect of the LOX in the final product.

Earlier it was shown that the lentil seed LOX isolated in this study has multiple pH-optimas, namely at pH of 5.5, 7.0, 8.0 and 9.5. However, since the pH of white bread dough is generally in the region of pH 5.5, only the kinetic analyses performed at pH-optima in the acidic and neutral pH regions will be discussed. Figure 4.4 shows the results of kinetic analysis performed at pH 5.5 (Figure 4.4.A) and pH 7.0 (Figure 4.4.B) of the selected chromatography fractions. At both pH values assayed in this study, the enzyme followed Michealis-Menten type kinetics suggesting that the enzyme is not allosterically regulated. The kinetic parameters K_m and V_{max} were subsequently determined from this data.
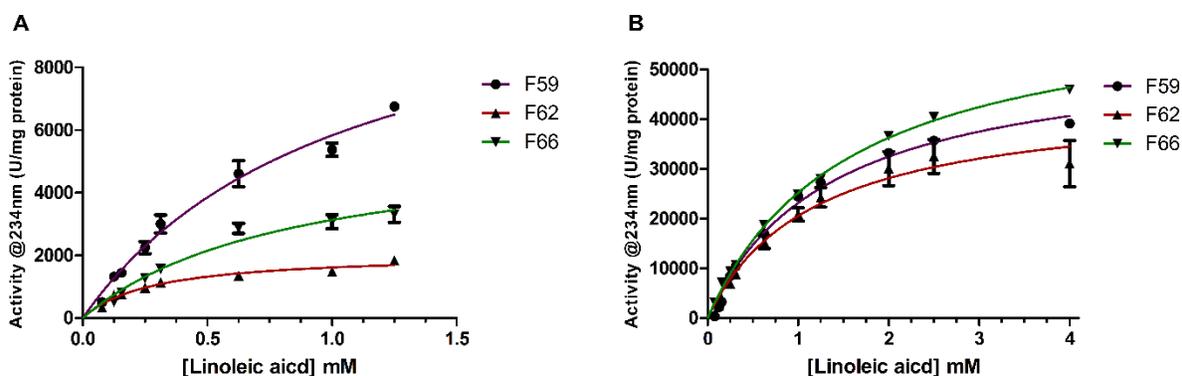
Figure 4.4 Kinetic curves for fractions 59, 62 and 66 obtained using the microplate conjugated diene method with varying concentrations of linoleic acid at (A) pH 5.5 and (B) 7.0 (n=3).

The calculated K_m and V_{max} values for each fraction when assayed at pH 5.5, and pH 7.0 is summarized in Table 4.2. The data presented in this table shows that, although the V_{max} was lower at pH 5.5 than at pH 7.0, the enzymes of each fraction had a higher affinity for linoleic acid at pH 5.5 than at pH 7.0, as indicated by lower K_m values. Fraction 62 had the highest affinity for linoleic acid at pH 5.5 with a K_m of 0.283 mM. The K_m determined for this fraction was similar to that determined for soybean LOX I-B (K_m = 0.196 mM, Chapter 3, section 3.3.2). However, the V_{max} values differed dramatically.

The data presented in Table 4.2 furthermore show that although the K_m values for each fraction are similar when assayed at pH 7.0, when assayed at pH 5.5 the K_m for fraction 62 was vastly different from that of fractions 59 and 66. This was also the case for the V_{max} values of each fraction. The data presented until now suggests that fraction 62 contains a LOX isoform with a pH-optima at pH 5.5 which is different from the enzymes present in fractions 59, and 66. However, since the peaks could not be fully separated, and the kinetic parameters for all fractions are similar when assayed at pH 7.0, it is likely that fraction 62 is contaminated with LOX isoforms from fractions 59, and 66, which overlap with that of fraction 62 during elution. Therefore, since these fractions eluted at different volumes, this data suggests the presence of three different LOX isoforms, which overlap during elution. In order for the proteins to elute at different concentrations of NaCl during exchange chromatography, each enzyme must have a unique pI. As such, the isoforms were subsequently separated based on pI by IEF followed by SDS-PAGE. The results are discussed in the next section.

Table 4.2: Kinetic parameters of lentil seed LOX isolated in Chapter 3. Activity was determined with linoleic acid concentrations between 0.07 - 5 mM at the pH indicated

Assay pH	Parameter	F59	F62	F66
5.5	Km (mM)	1.055 ± 0.185	0.283 ± 0.045	0.844 ± 0.179
7.0		1.347 ± 0.111	1.170 ± 0.203	1.558 ± 0.085
5.5	Vmax (U/mg)	11980 ± 1207	2070 ± 124	5778 ± 654
7.0		54360 ± 1823	44597 ± 3116	64491 ± 1493

As discussed in Chapter 2, LOX enzymes are product activated resulting in a lag period prior to full activation of the enzyme, resulting in a sigmoidal progress curve. Although no lag period was observed during the kinetic studies performed with 1.25 µg purified protein, in contrast, lag periods were observed during the initial purification steps. Figure 4.5 shows the activity of fractions eluted from a Whatman DE32 column during initial purification. In this figure the lag phase described earlier can be observed in fractions 23 and 24. However, once V_{max} was reached, fractions 23 and 24 showed a slight, but gradual decline in absorbance, which did not occur in fractions 25 and 26. Fitting a regression line through the points from 15 – 30 min showed that the rate of absorbance declined and was calculated at -0.006 and -0.0076 absorbance units per min for fractions 23 and 24, respectively. The maximal velocity of these fractions was also lower than that of fractions 25 and 26, although this could be enzyme concentration-related. Since, during kinetic studies using purified enzyme solutions, no lag period or reduction in absorbance was observed, these results suggest that the lipid hydroperoxide products are consumed by secondary reactions. Consumption of the lipid peroxide "activator" by secondary reactions could further contribute to the observed lag period. These results could explain the discrepancies between the conjugated diene and oxygen uptake activity determinations[88].
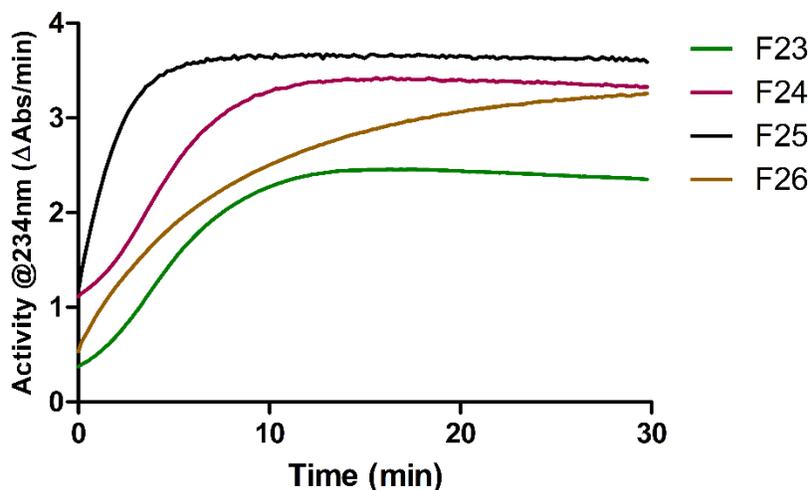
Figure 4.5 Activity, in ΔAbs/min, of chromatography fractions showing a sigmoidal progress curve.

Interaction with esterified substrates

Although most LOX have an absolute requirement for polyunsaturated free fatty acids as substrates, some isozymes, such as soybean LOX 2, have the unique ability to oxidize esterified unsaturated fatty acids in membranes[36,40]. This ability to utilize esterified fatty acids results in an increase in substrate availability, which could be beneficial for augmenting specific enzyme activities during baking. In this study, however, when incubated with SUVs consisting of glyceryl linoleate, none of the fractions tested showed any activity toward esterified substrates.

Iso-electric focusing

During the purification of lentil seed LOX, described in Chapter 3, LOX activity was eluted over three peaks. When each collected fraction was analyzed using SDS-PAGE, multiple bands were observed at ≈100 kDa. Molecular weights of lentil seed LOX have been reported to range from 94 – 110 kDa[53,16,17]. In this study SDA-PAGE analyses yielded two bands corresponding to molecular weights of approximately 98 and 92 kDa, respectively, in the majority of the fractions collected. In order to determine if these bands could be separated based on pI, IEF was performed on pooled fractions 58, 62, and 66. Figure 4.6 shows the result of 2-D page when the proteins were first separated over a pH range of 3 – 10 on a 17 cm IPG strip, followed by separation on a 10 % SDS-PAGE gel. Based on the data presented in Figure 4.6, the pI values for each spot was calculated to be 6.10, 6.45 and 7.25, respectively. These values correlate well with the pI values obtained by Hilbers *et al*[53,17]*.,* with the exception of the protein at pH 7.25. The results presented here therefore suggest that although only two bands are present during SDS- PAGE analysis, the major band at

≈ 98 kDa is most probably composed of two separate enzymes with similar molecular masses while the band at ≈ 92 kDa corresponds to a third isozyme. However, the calculated molecular masses for the proteins in spots 1, 2, and 3 were 42-, 46-, and 47 kDa, respectively, and no larger proteins were visible. This result suggests that lentil LOX is a dimeric protein, of which the individual monomers were not separated during preparation for SDS-PAGE analysis. This result may be due to the different sample preparation, and reducing methods used for SDS-PAGE and IEF, respectively. For SDS-PAGE the proteins are reduced using β-mercaptoethanol and heat, while the samples are reduced using iododiacetic acid and DTT for 2-D PAGE. It is therefore likely that these reducing agents affect the enzyme differently. However, since the possibility of a multimeric LOX could not be confirmed based only on the data presented here, each spot (1, 2 and 3) was excised and sent for mass fingerprinting by LC-MS/MS following tryptic digestion of the proteins.
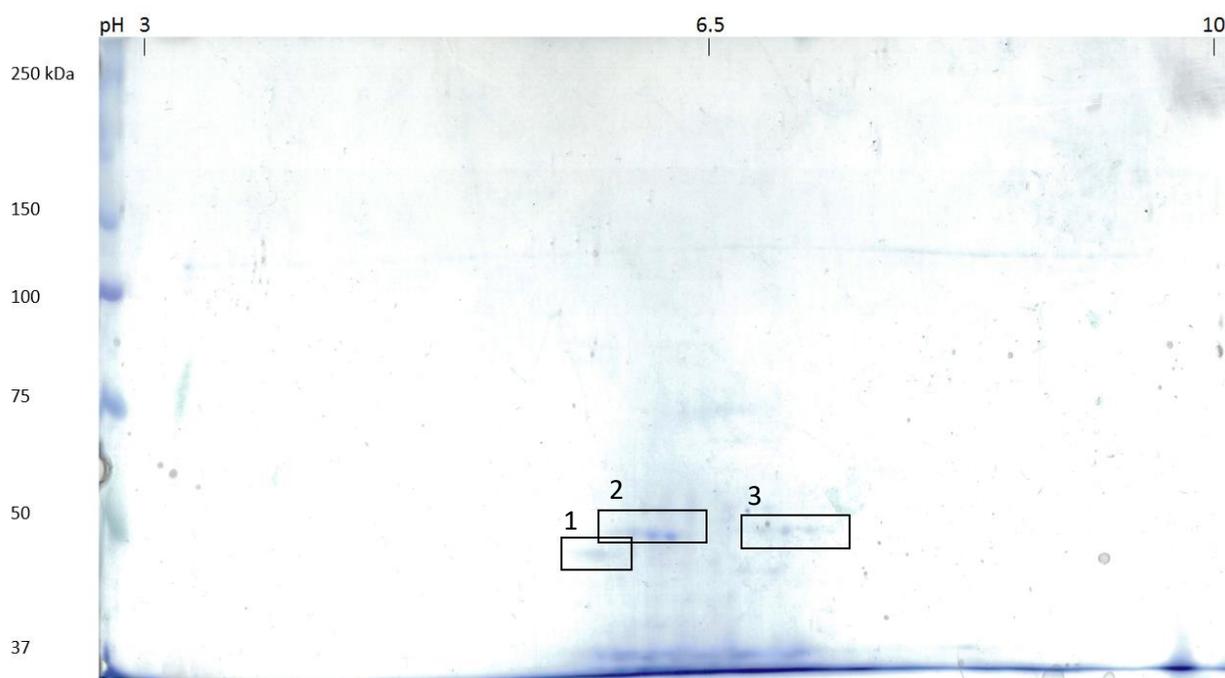
Figure 4.6 2-D PAGE of pooled fractions 58, 62, and 66 obtained from cation exchange chromatography.

Tryptic digest MS

Although an exact role for LOX in plants have not yet been discovered, it has been suggested that LOX plays an essential role in host defences[35]. As such, LOX isozymes contain various conserved regions in the protein primary structure[159,160]. As can be seen in Table 4.3, all three spots excised and analyzed using tryptic digest MS analysis showed the presence of conserved LOX peptides. The score reflects the absolute quality of the peptide-spectrum match on a scale of 0 – 1000, with 300 being a good fit while values larger

than 500 is considered to be a correct fit. Based on the scores presented in Tbale 4.3, the peptides detected showed good similarity to the different soybean- and pea seed LOX isozymes 2, 3 and 3b described in Chapter 2. These peptides furthermore shared various unique peptides with the reference sequences. Since the proteins were first separated based on pI, this result confirms the presence of multiple lentil seed LOX isoforms. This result also confirms that lentil seed LOX is a dimeric protein consisting of combinations of the protein monomers present in spots 1, 2 and 3 since the calculated size of each spot was roughly half that of the active protein. Although previous authors have analyzed lentil seed LOX using IEF[16], the presence of multimeric LOX have not yet been presented in literature. The results presented in this thesis therefore represent the first confirmed account for the presence of multiple lentil seed LOX isoforms, and the multimeric nature of these proteins.

Table 4.3 Results from a database search of the peptides obtained following tryptic digest MS analysis of the protein spots presented in Figure 4.6. The description shows the protein identified, following a database comparison of the peptide sequences obtained

Spot #	Description	Score	Unique peptides	Modified peptides	Coverage %	AA's in protein
1	Soybean seed LOX 2 |P09439|[*]	271.60	4	0	2.77	865
	Soybean seed LOX 3 |P09186|	298.80	2	0	1.63	857
	Pea seed LOX 3 |P09918|	298.80	8	0	7.78	861
2	Pea seed LOX 2 |P14856|	351.80	9	1	8.68	864
	Pea seed LOX 3 |P09918|	378.5	16	2	17.65	861
	Soybean seed LOX 4 (3b) |P38417|	351.80	5	1	3.63	853
3	Pea seed LOX 3 |P09918|	413.70	14	2	13.59	861
	Soybean seed LOX 4 (3b) |P38417|	287.00	5	1	4.22	853

[*] Accession number of the identified protein.

4.4 CONCLUSION

In modern times, the use of enzymes in the baking industry is common practice. However, the temperatures and conditions during the baking process often differ significantly. In order to ensure optimal performance, and product quality, an in-depth knowledge of the enzyme's catalytic parameters is vital. Although the optimal conditions for soybean seed LOX has been established, not much is known about the kinetic characteristics of lentil seed LOX. This chapter therefore described the kinetic- and molecular characterization of lentil seed LOX.

Results from 2D-PAGE analysis of the pooled fractions from Chapter 3 confirmed, for the first time, that lentil seeds contain multiple LOX isoforms. The results also showed that lentil seed LOX is a dimeric protein, composed of combinations of at least three different monomers. However, the combinations, which make up the active enzymes, were not determined in this study but should be the subject of investigation in future studies.

The results presented also show that the lentil seed LOX isolated in Chapter 3 has pH- and temperature optima similar to the conditions present in bread dough during mixing and resting. However, since a rapid loss in activity was observed at temperatures in excess of 40 °C, the improving effects of lentil seed LOX will only be observed during mixing and resting. Since soy LOX remains active at temperatures greater than 40°C, lentil seed flour may not be as efficient a dough improver as soybean flour. Furthermore, none of the purified fractions (F59, F62 and F66) were able to utilize esterified substrates, thereby limiting substrate availability when compared to soybean LOX-2. In order to test if lentil seed LOX could indeed function as an alternative to soybean flour, extracts and purified enzyme solutions were applied in baking trials, as described in Chapter 5, and the results of these trials are discussed in detail.

Chapter 5

EFFECTS OF LENTIL LOX ON THE BAKING QUALITY OF WHITE BREAD DOUGH

5.1 INTRODUCTION

Soybean seed flour has been used in the production of white bread since 1928[24], when it was discovered that addition of a small amount of soybean flour, to white bread flour, results in a whiter crumb. As discussed in Chapter 2, the bleaching capacity was ascribed to the presence of high concentrations of Lipoxygenases (LOX) present in soybean seeds[6,39]. Since then it has been shown that LOX also improves dough rheology[33]. As such, soybean flour is routinely added, up to 0.5 % $^w/_v$, to wheat flour dough during the commercial production of white bread.

Besides abundant use in the food industry, soybean products are also used in petrochemical and textile industries[14,15,161]. In order to supply a growing demand for soy, genetically modified soy is increasingly being used. It is estimated that up to 90 % of all soy produced in South Africa is genetically modified[162,163]. However, due to the allergenicity of soybean products, along with a growing public concern about the use of genetically manipulated products, other sources of LOX for use in the baking industry is required.

This chapter describes the application and evaluation of lentil seed flour, and flour extracts, as possible alternatives to soybean seed flour as source for LOX during the baking of white bread. The effect of purified lentil seed LOX on the baking properties of white bread dough was also investigated.

5.2 MATERIALS AND METHODS

All reagents and chemicals used during the preparation of soy, and lentil seed extracts were of analytical grade and were obtained from reputable chemical supply houses. Lentil flours were obtained from Alliance Grain Traders (AGT, North Dakota, USA). Full fat enzyme active soybean flour was provided by Impilo Products (Pretoria, South Africa). Protein concentrations were determined using the Piece BCA protein quantification kit (Pierce, Rockford III, USA) with bovine serum albumin as protein standard. White bread flour used in the baking trials was obtained from Sasko Mills, Malmesbury, South Africa. All baking trials were performed under supervision of head baker, Lélan de Jongh, at DuPont® Sub-Saharan Innovation center, Cape Town. Two independent panels of senior bakers evaluated the bread loaves, and the results are presented as the mathematical mean. All the recipes used in this study are presented in Addendum A at the end of this chapter.

5.2.1 Baking procedure

For all the trials performed, enough dough was prepared to bake two breads of 780 g each. The ingredients were mixed with water at 18 °C for 5 min using a Morton laboratory mixer. The resulting dough was then rested for 3 min and scaled to 780 g. The dough was then rested for an additional 5 min after which it was molded in oiled baking pans and proofed at 55 °C and 80 % relative humidity until the dough had risen to 130 mm. The breads were then baked at 230 °C for 20 minutes in a forced air rotating oven. Once baked, the bread height was measured, and the breads were allowed to cool for 1 h before packaging into standard plastic bags. The breads were cut and evaluated the next day.

5.2.2 Additive selection

Various lentil flours for use in baking are available. The flours used were full fat enzyme active soybean flour, lentil PulsePlus® V6000 flour, Laird lentil flour, red split lentil flour, green lentil flour, and brown lentil flour. To establish the effect of each of the individual lentil flours, breads containing 0.2 %, 0.3 %, 0.4 %, 0.6 % and 0.8 % of each flour were baked, respectively. These breads were then evaluated against breads baked with the same concentrations of full fat enzyme active soybean flour. The baking sheet for these trials is presented in Table 5.4 in Addendum A. All the lentil seed flours evaluated in this study were supplied by AGT, North Dakota, USA.

5.2.3 Activity determination

LOX activities of the fractions used in baking trials were determined using the microplate conjugated diene method described in Chapter 3. For this assay, 50 µL of each extracted sample, and a buffer blank, was added to the wells of a Greiner UV-Star® 96 well plate. To each sample 150 µL substrate solution (1 mM Linoleic acid in 200 mM Tris-HCl, pH 7.0, and 0.25 % $^v/_v$ Tween® 20) was added and the absorbance of each well was determined at 234 nm in 10 second intervals at 25 °C for a total of 5 min using a Tecan Spark 10M micro titer plate reader. The specific activity of each sample was subsequently calculated using Equation 5.1. In this equation ΔAbs_{234} refers to the slope for the linear section ($\approx$ 0 − 1.5 min) of each progress curve.

$$\frac{U}{mg} = \frac{\left(\left(\frac{\Delta Abs_{234}}{14180}\right) \times 1.7 \times 10^6\right)}{mg\ protein}$$

Equation 5.1 Equation for the calculation of LOX specific activity in U/mg protein.

As discussed in Chapter 2, due to a risk of volatile off-flavor formation, soybean seed flour is only used up to 0.5 % $^w/_w$ for bread making[10,33]. In this study, soybean, and lentil flour was used at a concentration of 0.4 % $^w/_w$. Since soybean, and lentil flours were extracted in a 1:10 $^w/_v$ ratio, 40 mL of the crude extracts were added during trials to yield LOX activities representative of 0.4 % flour. To compensate for the volume increase due to the addition of the extract, the volume extract was subtracted from the total amount of water added to the final recipe. The total LOX activity for 40 mL crude extract was subsequently determined and this value was used to calculate the amount of concentrated extract, purified fraction 1- and 2, needed for the same LOX activity present in 0.4 % flour.

5.2.4 Flour extraction

Crude extraction

For crude extraction of lentil seed flour, 100 g lentil PulsePlus™ V-6000 flour was stirred in 10 volumes 10 mM sodium phosphate buffer, pH 7.0, for 1 h at 4°C. The extract was subsequently collected by centrifugation at 17,000 x g at 4°C for 30 min. The supernatant was collected and filtered before being stored at -20°C until use. In the baking trials performed, these fractions were denoted as "Crude extract".

The crude extracts obtained as described above, were also freeze dried to determine the effect of lyophilization on LOX activity. For lyophilization, 250 mL crude extract was quantitatively transferred to a pre-weighed 1 L round bottom flask and freeze-dried. Once completely dry, the powder was weighed, and stored at 4°C until use.

Full fat enzyme active soybean flour was extracted in the same way, without lyophilization. However, during extraction a layer of lipid material formed on top of the supernatant after centrifugation. Following collection of the supernatant fraction, the lipid material could not be re-suspended and formed aggregates. The extract was subsequently divided into two aliquots, and the aggregated material of one fraction was removed by filtration through Whatman No.2 filter paper. Both fractions were used in baking trials.

Concentration of extracted protein

Crude extracts were also concentrated for use during baking using ammonium sulfate. For this, 100 g lentil PulsePlus™ V-6000 was extracted as described above. Following extraction, solid ammonium sulfate was added to a final saturation of 60 % $^w/_v$ (373 g/L). The protein was precipitated at 4°C for 1 h. The precipitated proteins were subsequently collected by centrifugation at 17,000 x g at 4°C for 30 min. The protein pellet was then dissolved in 200 mL distilled water. Once fully dissolved, the extracts were dialyzed overnight against 50 volumes distilled water at 4°C using Spectra/Por® dialysis tubing with a 14,000 kDa molecular weight cut-off. In subsequent baking trials, these fractions were labeled "Extracted protein". Extracts of soybean flour, for use as control, were also prepared in the same way.

Purification of lentil LOX

To determine the effect of purified LOX enzymes on baking quality of white bread flour, lentil seed LOX was purified to various degrees using anion exchange chromatography. Active fractions obtained from the first column were denoted Purified F1 in subsequent baking trials, while the active fractions from the second column were denoted Purified F2. All the extracts and purified fractions were prepared fresh prior to each trial. The protein concentration and LOX activity of each fraction was determined no less than 24 h before use.

Preparation of Purified F1

For purification, 50 g lentil PulsePlus™ V-6000 flour was extracted in 500 mL 10 mM sodium phosphate buffer, pH 7.0 as described above. The extracts were then concentrated as described above using ammonium sulfate. Following precipitation, the precipitate was dissolved in 80 mL 20 mM Tris-HCl buffer, pH 7.5 and dialyzed against 10 volumes of the same buffer. After dialysis, the extract was brought to a final volume of 100 mL using dialysis buffer.

The extract was subsequently divided into two 50 mL aliquots, which were injected separately onto a 32 x 190 mm column packed with Whatman DE32 resin. After loading the sample, the column was washed with 5 column volumes equilibration buffer and eluted over 6 column volumes with a linear gradient of 0 – 500 mM NaCl, as described for protein capture in Section 3.2.3. The active fractions obtained from this chromatography step were subsequently pooled and concentrated using ammonium sulfate. The protein precipitate was then dissolved in 40 mL distilled water and dialyzed against 20 volumes deionized water. Following dialysis, the sample was brought to a final volume of 50 mL with deionized water. This fraction, denoted purified F1, was used in subsequent baking trials.

Preparation of Purified F2

The concentrated active fraction from above was purified further on an 18 x 200 mM Q Sepharose™ fast flow column equilibrated with 20 mM Tris-HCl, pH 7.5, containing 1 mM EDTA. After the sample was loaded, the column was eluted as described in Intermediate purification, Section 3.2.3, using a stepped-linear gradient of 0 – 500 mM NaCl in equilibration buffer. The active fractions obtained were pooled and concentrated using ammonium sulfate as described above. The protein concentrate was subsequently dissolved in 40 mL distilled water and dialyzed against 5 L deionized water. Following dialysis, the sample was brought to a final volume of 50 mL with deionized water. In subsequent baking trials this fraction was denoted purified F2.

5.3 RESULTS AND DISCUSSION

Unless stated otherwise, the breads from all baking trials were evaluated by Senior Baking Application Specialists at DuPont® SSA Innovation center pilot bakery, Cape Town. All trials were performed in at least duplicate, and the data is presented as the mathematical mean. Since the major role for LOX is carotenoid bleaching, the standard recipes used were selected to yield consistent loaves with optimal volume. For this reason small amounts of amylases, xylanases and lipases were added to improve loaf volume, dough workability, and strength[2,93,164]. Any additional benefits gained over the controls were due to the test compounds added.

5.3.1 Baking trial - Additive selection

To determine if lentil seed flour could be used as alternative to soybean seeds as a source for LOX, the effect of various lentil seed flours on dough quality were evaluated in baking trials. Table 5.1 lists the proximate composition of each cultivar evaluated in relation to soybean seed. As can be seen in this table, soybean seeds contain 2 times more protein than any of the lentil seed cultivars, and up to 20 times more fat which contain up to 47 % linoleic acid[165]. It has furthermore been reported that the LOX constitute about 2 % of the total protein makeup of soybean seeds[8]. However, according to Chang and McCurdy[12], lentil seeds contain LOX activities comparable to that soybean seeds[12]. Furthermore, as was shown in Chapter 4, lentil seed LOX, unlike soybean seed LOX, has a pH-optima at pH 5.5 which is similar to the pH of bread dough. Therefore, although lentil seeds contain less protein, and fats which include the substrates for LOX, lentil seed flour should yield similar effect to soybean seed flour based on LOX activity.

Table 5.1 Proximate composition of the lentil seed flours tested in this study. Data obtained from AGT, USA

Nutrient	PulsePlus V6000	Split red lentil	Queen green lentil	Laird lentil	Brown lentil	Full fat soy
Moisture (%)	11.5	11.8	10.4	10.4	10.4	11.0
Protein (%)	11.0	25.0	25.8	25.8	25.8	51.0
Carbohydrates (%)	75.0	59.0	60.0	60.0	60.0	35.0
Fat (%)	1.6	2.1	1.1	1.1	1.1	21.0
Crude fiber (%)	1.4	10.8	30.5	30.5	30.5	10.0

To determine if lentil seed flour has a similar effect on the baking characteristics of wheat dough as soybean flour, white bread dough was fortified with varying levels of flour from the different cultivars of *Lens culinaris* listed in Table 5.1. A protein- and fat extracted lentil seed flour namely, PulsePlus™ V6000 was also evaluated. The results obtained were subsequently compared to breads baked with similar amounts of soybean flour. Since soybean flour is mainly added for its bleaching capacity, the breads baked in this trial were only evaluated based on crumb color. It was found that when dosed at 0.3 % $^m/_m$ lentil PulsePlus™ (AGT) yielded similar results to that obtained using 0.4 % $^m/_m$ full fat enzyme active soybean flour.

PulsePlus™ V6000 lentil is a mechanically milled and processed portion of dehulled split yellow lentil cotyledons. As such, the fat and up to > 50 % of the total protein content has been removed during its preparation. With this, the specific activity is increased resulting in higher LOX activity per gram flour than the full seed flours. These results emphasize the benefit of value added products since a high-quality product was obtained, while the extracted fats and proteins could still be used in other applications. This ability to reap multiple benefits from a single source constitutes the term value added product.

As was shown in Chapter 4, lentil seed LOX has optimal activity below a temperature of 40 °C. Further increases in temperature results in a rapid loss of LOX activity. Based on this, the baking characteristics of the dough would therefore only be affected by lentil seed LOX during mixing, and resting when the temperatures are below 40°C. Soybean seed LOX on the other hand, remains active at proving temperatures. As such, due to the increased contact time, soybean seed flour should yield an improved dough when compared to that prepared using lentil seed flour. However, the results presented here show that breads baked using 0.1 % $^m/_m$ less lentil seed flour had a whiter crumb when compared to breads baked using soybean flour. These results suggest that, even when using soybean seed flour, no additional benefit, in terms of crumb color and dough rheology, is gained past the mixing and resting steps. The improving function of LOX is therefore limited to the first ≈ 20 min of the baking process. Based on these results, only lentil V6000 flour and enzyme-active soybean flour was used for the studies in this thesis.

5.3.2 Baking trial – Evaluation of extract performance

To determine whether extraction had any effect on the dough improvement capacity of each flour, the initial trials were performed using only crude, and concentrated flour extracts. The effect of added linoleic acid was also evaluated. In order to establish clear effects, low quality flour with a falling number below 250 seconds (high amylase activity) was used in these trials to exaggerate the effects of each additive. The results for these trials are presented in Table 5.2. Interestingly, as can be seen in Figure 5.1, although the dough was normal during mixing, during proofing large bubbles formed on the surface of the bread, which contained extracted soybean protein (bread # 7). More bubbles formed once the bread was placed in the oven. Bubble formation did not occur on the breads baked using lentil flour extracts prepared in the same manner, nor did bubbles form on any of the other breads.
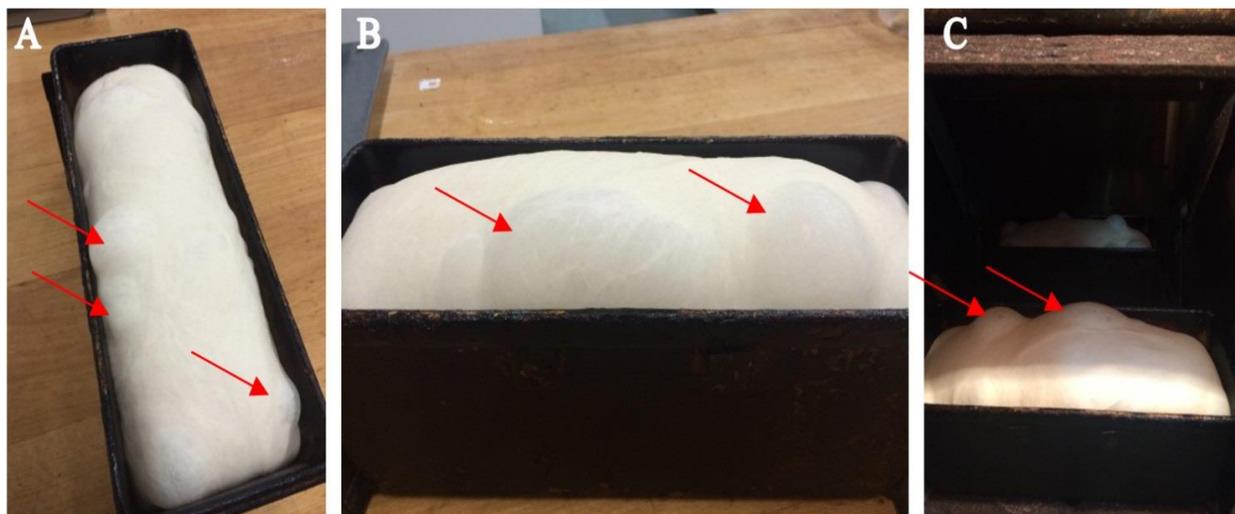
Figure 5.1 Bubbles formed in breads baked using extracted soy protein. The arrows indicate the major bubbles formed (A) during proofing, (B) after proofing, and (C) in the oven.

Furthermore, once baked, the volume of bread #7 was reduced, and the crust color was considerably darker than any of the other breads baked for the same time (Figure 5.2). This result suggests that, in contrast to the dough-strengthening effect, which was expected, the dough was weakened upon addition of the extract containing soybeans seed protein. The dough was subsequently not able to maintain its shape during inflation while proofing. The data presented in Table 5.2 shows that addition of concentrated soybean extract also results in significant flaws in terms of appearance and crumb structure. Curiously, these results were not observed in any of the breads baked with concentrated lentil protein, which were prepared in the same way.
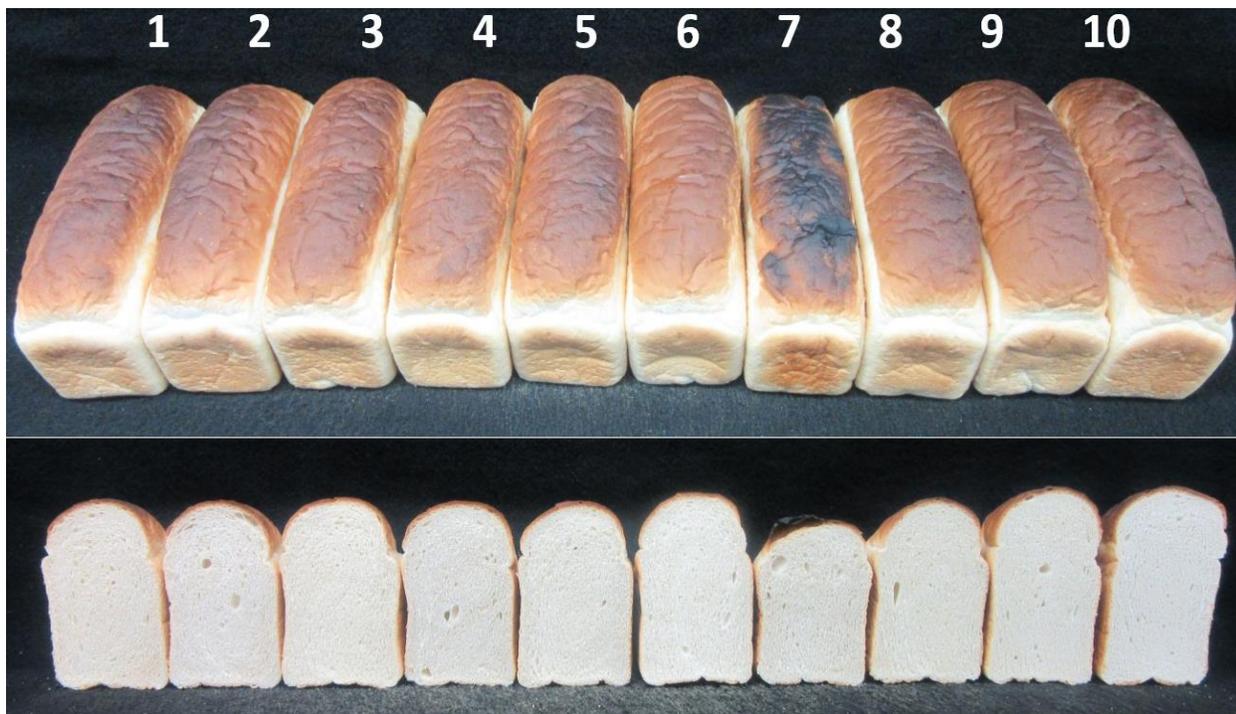
Figure 5.2 Breads baked using the additives described in Table 5.5. The breads were each baked for 20 min at 220 °C in a forced-air rotating oven. Bread number 1; no additive control, 2; soybean flour control, 3; lentil flour control, 4; crude unfiltered soybean extract, 5; crude unfiltered soybean extract + 300 mg linoleic acid, 6; crude filtered soybean extract, 7; extracted soybean protein, 8; crude unfiltered lentil extract, 9; crude unfiltered lentil extract + 300 mg linoleic acid and 10; extracted lentil protein.

Since addition of extracted soybean protein resulted in both reduced dough-strength, and Maillard browning, this result is most likely due to the action of proteases present in soybean seeds. Soybean seeds contain at least 11 different proteases which is required for mobilization of the seed storage proteins during germination[166]. In Chapter 3 it was shown that following precipitation, up to 40 % of the original LOX activity in the crude extract is lost. As such, more of the concentrated protein solution is required to obtain LOX activities similar to that of the crude extract. Therefore, since soybean seeds also contain large amounts of proteases, the relative concentration of proteases is inadvertently increased during protein concentration. During proofing proteases hydrolyze proteins, which results in weakening of the gluten structure. Furthermore, protein hydrolysis also increases the amount of free amino groups, which can subsequently interact with reducing sugars during baking. This interaction then leads to Maillard browning, and, in this case, blackening of the crust. Since these reactions did not occur when using lentil seed extracts, the results would suggest that lentil contains less, or less active, proteases.

Previous authors have suggested that addition of purified LOX to flour results in a reduction in bleaching capacity, and rheological improvement[10,31]. Since up to 48 % of the free fatty acid content of soybean seeds is linoleic acid, the preferred LOX substrate, extraction could result in a reduction in substrate

availability[10,165,167,168]. To determine the effect of dough supplementation, pure linoleic acid was added to the flours containing crude soybean, and lentil extracts. As can be seen in Table 5.2, the addition of linoleic acid did not deliver any benefit when compared to the non-supplemented crude extracts. However, these loaves still obtained an overall higher baking score than any of the controls, validating the efficiency of lentil seed extracts. This results suggest that wheat flour contains a sufficient amount of linoleic acid to deliver the positive effects associated with the addition of LOX.

During the extraction of soybean flour, a layer of lipid material formed on top of the supernatant following centrifugation. To determine the effect of these native soybean lipids on the baking characteristics of white bread dough, the extracted sample was divided, and the lipid material of one aliquot was removed by filtration. As can be seen in Table 5.2, when compared to the control baked with soybean flour, and the other soybean seed extracts, filtration resulted in an increase in the crumb structure, squeeze, and softness. The crumb color, grain texture and resilience were increased by 6 % when compared to soy flour control. Fats are often added to bread dough in order to increase loaf volume by increasing gas retention. However, adding too much fat reduces gluten formation resulting in a reduced loaf volume[10,168]. Carotenoid bleaching capacity could also be reduced by association of the hydrophobic carotenoids and added fats, protecting the carotenoids from attack by LOX derived hydroperoxides. Filtration removed most of the undissolved lipid material, increasing both loaf volume and crumb color. Since a relatively large volume of extract was added to maintain constant LOX activities, a large amount of lipid was also added with the extract. Based on the results presented, the amount of lipid added most likely exceeded the maximum amount allowed to obtain positive effects resulting in a reduction in product quality. These results are in agreement with previously published data where it was shown that addition of a high percentage of linoleic acid had a negative impact on the final bread quality[31,33].

When comparing the overall scores presented in Table 5.2, it can be seen that the breads baked using concentrated lentil seed protein had the best overall score. These loaves had the highest volume, whitest crumb, best resilience, and was also among the softest loaves. Furthermore, besides the filtered soybean crude extract ranking second best, breads baked using lentil seed extracts ranked 1st, 3rd, and 4th best of all the breads baked. Moreover, breads baked using lentil seed extracts scored on average 1.92 % better than the lentil flour control, and 7.15 % better than the soybean extracts. On the other hand, breads baked using concentrated soybean protein had the lowest overall score.

The results presented here suggest that the bleaching capacity, and rheological improvements facilitated by lentil seed LOX is not lost during extraction, and concentration. Furthermore, it was also shown that

lentil seed extracts are as effective at dough improvement as soybean flour indicating that lentil seed flour is a valid alternative to soybean flour. These experiments were subsequently repeated in trials with the addition of purified lentil seed enzyme extracts.

Table 5.2: Evaluation score-sheet for the trials conducted using extracted soybean and lentil flour as additives. L.A refers to the 0.03 % linoleic acid which was added during mixing

	TRIAL 2.		Control	Control soy	Control lentil	Soybean				Lentil		
	Bread #		1	2	3	4	5	6	7	8	9	10
Evaluation	Parameter	Score - total	Control	+ FF Soy flour	+ Lentil flour	Crude extract - Unfiltered	Crude extract - Unfiltered + L.A	Crude extract - Filtered	Extracted protein	Crude extract - Unfiltered	Crude extract - Unfiltered + L.A	Extracted protein
Appearance	Volume	10	9	9	9	9	9	9	4	9	9.25	9.5
	Shape - Overall	10	9	9	9	9	9	9	4	9	9	9
	Shape - Top	10	9	9	9	9	9	9	2	9	9	9
	Shape - Sidewalls	10	9	9	9	9	9	9	9	9	9	9
	Break and shred	10	8.5	8.5	8.5	8.5	8.5	8.5	8.5	8.5	8.25	8.25
	Crust colour	10	9	9	9	9	9	9	1	9	9	9
	Max:	60	53.5	53.5	53.5	53.5	53.5	53.5	28.5	53.5	53.5	53.75
Crumb	Crumb colour	10	2	7.5	7	7.5	7.5	9	4	7.5	7.75	9.5
	Grain (texture)	10	7	7	7	7.5	7.5	8	2	8	8	8
	Resilience	10	8	8	8	8.25	8	8.5	8	8.25	8.25	8.5
	Recovery	10	9	9	9	9	9	9	9	9	9	9
	Moistness	10	9	9	9	9	9	9	9	9	9	9
	Max:	50	35	40.5	40	41.25	41	43.5	32	41.75	42	44
Squeeze	1/27/2016 **Max:**	10	7.5	7.5	7.5	7.5	7.45	7.6	7.25	7.5	7.25	7.25
Softness	1/27/2016 **Max:**	10	7.75	7.75	7.75	7.75	7.75	8	7.75	7.75	7.5	8
	Total score:	150	103.75	109.25	108.75	110	109.7	112.6	75.5	110.5	110.25	113
Score	Score	%	79.81%	84.04%	83.65%	84.62%	84.38%	86.62%	58.08%	85%	84.81%	86.92%
	Ranking	-	9	7	8	5	6	2	10	3	4	1

5.3.3 Baking trial – Evaluation of extract and purified enzymes performance

In the trials described in Section 5.3.2, low quality wheat flour was used in order to exaggerate the effects of the extracts tested. In these trials it was shown that lentil seed extracts had a positive effect on the baking characteristics of white bread dough. In the subsequent trials, however, high quality flour with a falling number larger than 350 seconds (low amylase activity) was used to simulate industrial conditions. As can be seen in Figure 5.3, the bread baked with concentrated soybean extract (bread 5) once again had the darkest crust although bubble formation was not as pronounced as in the previous trials.
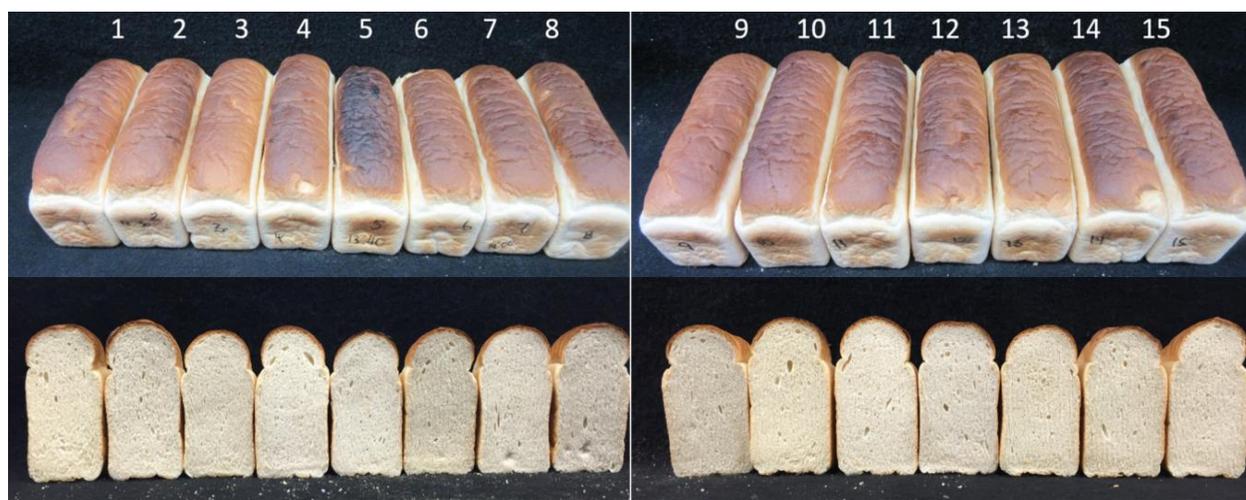
Figure 5.3 Breads baked using soybean, and lentil seed extracts described in Table 5.6. The additives used were: bread number 1; no additive control, 2; soybean flour control, 3; lentil flour control, 4, crude soybean extract, 5; extracted soybean protein, 6; crude lentil extract, 7; extracted lentil protein 1, 8; extracted lentil protein 2, 9; purified F1, 10; purified F2, 11; 1 g freeze dried crude extract, 12; 2 g freeze dried lentil extract, 13; purified F2 with 0.2 g linoleic acid, 14; purified F2 with 0.4 g linoleic acid, and 15; purified F1 with 0.4 g linoleic acid.

Table 5.3 compares the results obtained after evaluation of the loaves baked using the additives described in Table 5.6. As can be seen from the data presented, addition of the lentil extracts yielded a product with similar, or greater, quality than the soybean flour control. As was observed in previous trials, the data in this table show that concentration of lentil seed extracts resulted in an increase in the overall bread-baking quality of the flour used. The loaves baked using concentrated lentil protein ranked 5th and 6th for loaves 7 and 8, respectively, and had crumb colors similar to the soybean seed flour control.

The results presented for loaves 9 and 10 show that the overall positive effect on dough baking characteristics is affected by the degree of LOX purification. The bread baked using purified F1 yielded a loaf with improved quality when compared to the bread baked using purified F2. Furthermore, addition of linoleic acid to the loaf baked using purified F1 resulted in an increase in the overall baking score.

However, purification resulted in a decrease in bleaching capacity when compared to the loaves baked using extracted lentil protein (loaves 7 and 8). The positive effects observed were therefore only due to improvements in appearance and crumb structure. In contrast, addition of linoleic acid to breads baked using purified F2 fraction did not result in an increased appearance; however, it did result in a whiter crumb and an increase in grain texture.

Since the bleaching capacity of the purified F1 fraction was not considerably influenced by the addition of linoleic acid, but purified F2 was, the results suggests that the purified F1 fraction also catalyze secondary reactions, which reduce bleaching capacity. As was shown in Chapter 3, the initial purification step removes the bulk of the contaminating material. However, the active fraction obtained after the first chromatography step still contained multiple contaminating proteins, and pigments. As shown in Chapter 4, this fraction contained enzymes capable of reducing the formed lipid hydroperoxide, leading to a gradual reduction in absorbance at 234 nm once maximal velocity was reached. During subsequent chromatography, these contaminants are removed resulting in the increased bleaching capacity of purified F2 in the presence of linoleic acid. However, if this is the case, apart from reducing the bleaching capacity, the contaminating proteins also play a role during dough improvement since the improving capacity was reduced with purified F2.

Since PulsePlus® lentil V 6000 flour contain roughly 11 % protein (Table 5.1), the amount of freeze-dried extract added was calculated to correspond to 0.4 - and 0.8 % lentil flour, respectively. As can be seen in Table 5.3, addition of freeze-dried extracts (loaves 11 and 12) yielded higher baking-scores than the lentil flour control (loaf 3). However, the score for crumb color of loaf 11 was less than the control. Furthermore, although doubling the dosage resulted in an increase in the overall baking score, there was no considerable increase in loaf whiteness. Lyophilization therefore results in the loss of some factor, present in the lentil flour, which is required for bleaching capacity since LOX activity was not affected.

Table 5.3 Evaluation score-sheet for the trials conducted using soybean and lentil flour extract, as well as purified lentil seed LOX. In this table L.A refers to linoleic acid which was added at different levels during mixing

	TRIAL 4.		Control	Control soy	Control lentil	Soybean		Lentil									
	Bread#		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Evaluation	Parameter	Score - total	Control	+FF Soy Flour	+Lentil Flour	Crude extract	Extracted protein	Crude extract	Extracted protein 1	Extracted protein 2	Purified F1	Purified F2	Freeze dried	Freeze dried	Purified F2 + L.A 200	Purified F2 + L.A 400	Purified F1 + L.A 400
Appearance	Volume	10	9	9	9	9	8	9	9	9	8.75	8.5	8.75	8.75	8.5	8.75	9
	Shape - Overall	10	8	8.25	8.25	8	8	8	8.5	8.25	9	9	8.25	8.5	8.75	8.75	9
	Shape - Top	10	10	9.5	9.25	9.5	9.5	9.75	9.5	9.5	9.75	10	10	9.75	9.5	10	10
	Shape - Sidewalls	10	10	9.5	9.25	9.5	9.5	9.75	9.5	9.5	9.75	10	10	9.75	9.5	10	10
	Break and shred	10	9	9	8.75	8.5	8.75	8.5	8.75	8.5	9	9.25	8.75	9	8.75	9	8.75
	Crust colour	10	9	9	9	6	9	9	8.75	9	9	8.75	8.75	8.5	9	8.75	9
	Max:	**60**	**55**	**54.25**	**53.5**	**50.5**	**52.75**	**54**	**54**	**53.75**	**55.25**	**55.5**	**54.5**	**54.25**	**54**	**55.25**	**55.75**
Crumb	Crumb colour	10	6.5	8.5	7.875	8.375	8.5	7.25	8.375	8.5	7.375	6.75	7	8	7.25	7.25	7.5
	Grain (texture)	10	7.5	7.25	7.5	7.25	7.25	7.25	7.5	7.5	7.25	7	7.25	7.5	7	7.25	7.25
	Resilience	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
	Recovery	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
	Moistness	10	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7
	Max:	**50**	**41**	**42.75**	**42.375**	**42.625**	**42.75**	**41.5**	**42.875**	**43**	**41.625**	**40.75**	**41.25**	**42.5**	**41.25**	**41.5**	**41.75**
Squeeze	**Max:**	10	7	6.875	6.875	7.125	7	6.75	6.875	7	7.125	7	7.125	7.125	7	6.75	7
Softness	**Max:**	10	7	6.875	6.75	7	6.875	6.875	6.875	6.875	6.875	6.875	7.125	7.25	7	6.875	6.875
Score	Total score:	130	110	110.75	109.5	107.25	109.375	109.125	110.625	110.625	110.875	110.125	110	111.125	109.25	110.375	111.375
	Score	%	**84.62%**	**85.19%**	**84.23%**	**82.50%**	**84.13%**	**83.94%**	**85.10%**	**85.10%**	**85.29%**	**84.71%**	**84.62%**	**85.48%**	**84.04%**	**84.90%**	**85.67%**
	Ranking	-	10	4	11	15	12	14	5	6	3	8	9	2	13	7	1

5.4 CONCLUSION

During the production of white bread, soybean flour is routinely added during mixing to improve the crumb color, and, to a minor extent, the loaf volume of the final product. However, due to concerns regarding the allergenicity, and high-level use of GM soy, an alternative for soybean seed flour was investigated. In this chapter lentil seed flour was evaluated as an alternative source for LOX during the production of white bread.

The results presented in this chapter show that addition of lentil seed flour had a similar effect on dough rheology, and crumb color as soybean flour did. Extraction and concentration of lentil seed protein resulted in an increase in the bleaching capacity. Purification of lentil seed LOX resulted in an increase in dough rheology, however, the carotenoid bleaching capacity was reduced. These results are in line with those previously reported for purified soybean seed LOX[33]. In Chapter 4 it was shown that secondary reactions, which consume the lipid hydroperoxides formed by LOX, occur in crude lentil seed extracts. Since bleaching capacity was at its maximal prior to purification, the results presented suggest a role for these secondary reactions during carotenoid bleaching. However, the nature of these secondary reactions was not investigated further in this study. This conclusion is supported by the fact that lyophilization resulted in a decreased carotenoid bleaching capacity, while LOX activity was unaffected. The identification and role of the secondary reactions on bleaching capacity will be investigated in future studies.

ADDENDUM A – RECIPES USED

Table 5.4: Recipes used during the additive selection trials. The values in this table are presented in grams

Ingredient Name	Baking Trial 1 - Thursday 03/07/2014					
	Control	0.20%	0.30%	0.40%	0.60%	0.80%
White Bread Flour	1,000.00	1,000.00	1,000.00	1,000.00	1,000.00	1,000.00
Salt	18.00	18.00	18.00	18.00	18.00	18.00
Calcium Propionate	3.00	3.00	3.00	3.00	3.00	3.00
Ascorbic Acid	1.00	1.00	1.00	1.00	1.00	1.00
Amylase	0.50	0.50	0.50	0.50	0.50	0.50
Xylanase	1.00	1.00	1.00	1.00	1.00	1.00
Lipase	0.50	0.50	0.50	0.50	0.50	0.50
Additive[*]	-	2.00	3.00	4.00	6.00	8.00
Yeast	35.00	35.00	35.00	35.00	35.00	35.00
Water	620.00	620.00	620.00	620.00	620.00	620.00
Calculated batch size	**1,681**	**1,683**	**1,684**	**1,685**	**1,687**	**1,689**

[*] The additives used in this study was: full fat enzyme active soybean flour, lentil PulsePlus® V6000 flour, Laird lentil flour, red split lentil flour, green lentil flour, and brown lentil flour.

Table 5.5: The recipe used in trials evaluating the effect of extraction of dough-improving capacity of soybean and lentil seed extracts. The values in this table is presented in grams

TRIAL 2 Bread # Ingredient Name	CONTROL 1 Control	CONTROL SOY 2 + FF Soy Flour	CONTROL LENTIL 3 + Lentil Flour	Soy 4 Crude extract - Unfiltered	Soy 5 Crude extract - Unfiltered + L.A	Soy 6 Crude extract - Filtered	Soy 7 Extracted protein	Lentil 8 Crude extract - Unfiltered	Lentil 9 Crude extract - Unfiltered + L.A	Lentil 10 Extracted protein
White Bread Flour - Sasko Paarl	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00
Salt	18.00	18.00	18.00	18.00	18.00	18.00	18.00	18.00	18.00	18.00
Calcium Propionate: Orthocem	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Ascorbic Acid	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Amylase	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Xylanase	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Lipase	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
FF Soya flour	-	4.00	-	-	-	-	-	-	-	-
Lentil Flour - V6000	-	-	4.00	-	-	-	-	-	-	-
Crude soy extract - Unfiltered	-	-	-	40.000	40.000	-	-	-	-	-
Crude soy extract - Filtered	-	-	-	-	-	70.000	-	-	-	-
Extracted soy protein	-	-	-	-	-	-	70.000	-	-	-
Crude lentil extract - Unfiltered	-	-	-	-	-	-	-	40.000	40.000	-
Extracted lentil protein	-	-	-	-	-	-	-	-	-	72.000
Linoleic Acid	-	-	-	-	0.300	-	-	-	0.300	-
Yeast	35.000	35.000	35.000	35.000	35.000	35.000	35.000	35.000	35.000	35.000
Water	620.000	620.000	620.000	580.000	579.700	550.000	550.000	580.000	579.700	548.000
Calculated batch size	**1679.000**	**1683.000**	**1683.000**	**1679.000**	**1679.000**	**1679.000**	**1679.000**	**1679.000**	**1679.000**	**1679.000**

Table 5.6: The recipes used for the trials performed with the addition of flour extracts, freeze dried extracts, purified enzyme solutions. The effect of added linoleic acid was also investigated

TRIAL 4	CONTROL	CONTROL SOY	CONTROL LENTIL	Soybean		Lentil									
Bread #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Ingredient Name	Control	+ FF Soy Flour	+ Lentil Flour	Crude extract	Extracted protein	Crude extract	Extracted protein 1	Extracted protein 2	Purified F1	Purified F2	Freeze dried	Freeze dried	Purified F2 + L.A 200	Purified F2 + L.A 400	Purified F1 + L.A 400
White Bread Flour	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000
Salt	18,000	18,000	18,000	18,000	18,000	18,000	18,000	18,000	18,000	18,000	18,000	18,000	18,000	18,000	18,000
Calcium Propionate	2,000	2,000	2,000	2,000	2,000	2,000	2,000	2,000	2,000	2,000	2,000	2,000	2,000	2,000	2,000
Ascorbic Acid	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000
Amylase	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000
Xylanase	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000
Lipase	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000
FF Soya flour	-	4,000	-	-	-	-	-	-	-	-	-	-	-	-	-
Lentil Flour - V6000	-	-	4,000	-	-	-	-	-	-	-	-	-	-	-	-
Crude soy extract	-	-	-	40000	-	-	-	-	-	-	-	-	-	-	-
Extracted soy protein	-	-	-	-	50000	-	-	-	-	-	-	-	-	-	-
Lentil crude extract	-	-	-	-	-	40000	-	-	-	-	-	-	-	-	-
Extracted lentil protein 1	-	-	-	-	-	-	70000	-	-	-	-	-	-	-	-
Extracted lentil protein 2	-	-	-	-	-	-	-	70000	-	-	-	-	-	-	-
Purified F1	-	-	-	-	-	-	-	-	50000	-	-	-	-	-	50000
Purified F2	-	-	-	-	-	-	-	-	-	40000	-	-	40000	40000	-
Freeze dried extract	-	-	-	-	-	-	-	-	-	-	1000	2000	-	-	-
Linoleic acid	-	-	-	-	-	-	-	-	-	-	-	-	200	400	400
Yeast	35,000	35,000	35,000	35,000	35,000	35,000	35,000	35,000	35,000	35,000	35,000	35,000	35,000	35,000	35,000
Water	620,000	620,000	620,000	580,000	570,000	580,000	550,000	550,000	570,000	580,000	620,000	620,000	569,600	580,000	580,000
Calculated batch size	1,679,000	1,683,000	1,683,000	1,679,000	1,679,000	1,679,000	1,679,000	1,679,000	1,679,000	1,679,000	1,680,000	1,681,000	1,679,200	1,679,000	1,679,000

Chapter 6

GENERAL DISCUSSION AND CONCLUSION

"*If thou tastest a crust of bread, thou tastest all the stars and all the heavens*" - Robert Browning

Bread is one of the most common staple foods of the world, and has been for centuries. Records from ancient Egypt show that molded breads have been baked from early as 2,400 B.C[169]. Although the processes used to produce bread in ancient Egypt were vastly different form the highly mechanized methods used today, the basic principles of bread manufacture remain unchanged. For breadmaking wheat flour, water and yeast are mixed to form a visco-elastic dough which is then fermented and baked. However, although seemingly straight-forward, the process of bread making involves a series of complex physical, biochemical, and chemical transformations in the product[2,170]. The constituents of the wheat flour dictate the extent of the individual transformations. For example, since amylases are responsible for the conversion of non-fermentable starch to fermentable maltose, loaf volume is directly influenced by the activity of amylases[170]. However, amylase activity of unmalted flours are low, resulting in low loaf volumes and reduced product quality[2]. Due to the variability in the levels of the flour constituents, it is difficult to produce breads of similar quality using different batches of wheat flour. However, the modern consumer demands both quality and consistency. As a result, various additives are added to compensate for flour variability, and to improve control over the baking process[2,170].

In the past, these additives were mainly of chemical nature, and included chemical compounds such as potassium bromate, azodicarbonamide, chlorine dioxide, calcium peroxides and acetone peroxide[89,90]. However, due to a growing public concern about the effects on human health, chemical additives have been gradually replaced with enzymes[2,89]. Enzyme catalysis is highly specific, and does not result in the formation of non-specific by-products. As a result, enzyme technology have received much attention leading to the availability of various proteases, lipases, amylases, xylanases, glucose oxidases etc. for use in baking[2,170].

Although it is common practice to add enzymes for flour standardization, enzymes are also added to modify dough rheology, crumb color, and crumb softness[2]. Enzymes can be added in purified form, or as part of a complex mixture. An example of such a complex mixture is soybean seed flour. As described in Chapter 2, soybean flour is often added to white bread dough for its pigment bleaching capacity. This

capacity has been ascribed to the activity of lipoxygenases (LOX), which are especially abundant in soybean seeds[2,6,35,118]. Although soybean flour is mainly added for its dough-whitening capacity, LOX enzymes have also been shown to improve dough rheology by enhancing cross-linking of flour proteins, resulting in stronger doughs[6,10,33]. However, since soybean products are widely used from the food- to petrochemical industries, a large portion of the current commercial soybean crop is genetically modified (GM)[14,15,163]. It is estimated that GM soybean constitutes up to 50 % of the annually produced GM crop[163]. Due to a growing public concern about the possible negative effects of GM products on biodiversity and human health, there has been a call for an "organic" alternative[171,172]. These difficulties could be overcome by purification of the enzyme from its native source.

The ability to purify enzymes from natural sources would enable the production of multiple products from the same source, thereby increasing its economic value. Enzyme activity in the natural source is furthermore highly variable, reducing the ability to reproduce the same product when using straight flour as additive. The use of purified enzymes also allows for better control of the product quality. However, for successful application of purified enzymes, various quality control measures need to be in place to ensure batch-to-batch consistency. In this study the standard conjugated diene method was adapted to 96 well microtiter plate format in order to enable high-throughput screening of large amounts of samples. It was shown that this assay could also be automated allowing for use in industrial settings, such as enzyme blending-, and plant breeding facilities.

Methods for the purification of soybean LOX have been described. However, soybean seeds contain multiple isoforms of LOX which differ in catalytic, and molecular properties[6]. Studies have shown that soybean LOX isozymes also display synergism, and all three isoforms are required to improve bread quality[10,11,33]. Large-scale purification of all three isoforms would be difficult, and labor-intensive which would reduce the financial viability of producing a purified product[21,41,47]. Ideally, a single LOX enzyme, with activity comparable to that of soy, capable of both carotenoid bleaching and rheological improvement is required.

In a study by Chang and McCurdy[12], it was shown that lentil seeds contain LOX activity comparable to soybean seeds, when assayed at neutral pH. Moreover, although it has been suggested that lentil seeds may contain multiple isoforms of LOX, this possibility has not yet been conclusively proven[53,16]. Therefore, the aim of this thesis was to investigate lentil seed flour as a possible alternative to soybean flour as a source for LOX in the bread-baking industry. Lentils are often used in the breadmaking process. However, they are not added for their contribution to loaf whiteness or dough rheology, but for nutritional

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value[173,174]. Lentils are a readily available source for protein, fiber, potassium and B-vitamins while having low levels of fat, cholesterol and sodium[174]. In Chapter 4 it was shown that lentil seed LOX is particularly well suited for use in breadmaking since this enzyme has pH, and temperature optima which correspond to the pH, and temperature of dough during mixing and resting. In trials performed early in 2014, it was shown that inclusion of lentil seed flour yields loaves with comparable crumb color to those baked using a soybean seed flour control. Since then lentil seed flour has been identified as one of the possible sources for LOX in the South African baking industry (DuPont®, South Africa).

Since 2014, however, the prices for lentils have increased dramatically due to consecutive poor harvests. In an ttempt to add value to the lentil flour used, the possibility of producing a purified lentil LOX enzyme for use in the baking industry was also investigated. During initial trials it was shown that PulsePlus™ V6000 lentil flour scored the best in baking trials, when compared to flour from different lentil seed cultivars, and soybean seed flour. During the preparation of PulsePlus™ V6000 lentil flour, various proteins are removed from yellow lentil seeds for use in other applications. In this way, the specific LOX activity was increased when compared to whole lentil seed flours, resulting in an increased bleaching efficiency. PulsePlus™ V6000 lentil flour is therefore already considered a value-added product since multiple marketable products, i.e. protein and flour, can be produced from the same starting material.

In order to determine the effect of purified lentil seed LOX on the baking characteristics of white bread dough, LOX was subsequently purified from PulsePlus™ V6000 lentil flour, and applied in baking trials. It was found that although purified lentil LOX results in an increase in dough rheology, crumb color was only influenced to a minor extent. The extent to which the crumb color was positively impacted was also dependent on the degree of LOX purification and the concentration of linoleic acid. These results are in agreement with previous studies performed using purified soybean LOX isozymes[31]. Together these results show that, although it has been suggested that both rheological improvement and pigment destruction occurs via the same mechanism[10], it is possible that these effects occur via different but related mechanisms. In Chapter 4 it was shown that secondary reactions, which consume lipid hydroperoxides, occur in crude lentil seed extracts. However, these reactions were not present in purified LOX preparations, suggesting a role for secondary reactions during pigment destruction.

To gain a better understanding into the mechanism of reactions catalyzed by lentil seed LOX, the catalytic parameters of lentil seed LOX were determined. In combination with results reported in Chapter 3, the results presented in Chapter 4 suggest that lentil seeds contain at least three different LOX isoforms, although these isozymes could not be separated chromatographically. However, three separate spots

were observed when active chromatography fractions were pooled and analyzed by isoelectric focusing and SDS-PAGE. Following tryptic digest MS analysis, it was shown that each spot corresponded to a separate LOX isoform. Furthermore, these analyses showed, for the first time, that lentil seed LOX is a dimeric protein.

The K_m values were subsequently determined to be 1.055, 0.283 and 0.844 mM, respectively, at pH 5.5 using partially purified fractions. It is known that the different isoforms of soybean seed LOX play different roles during rheological improvement, and carotenoid bleaching[33]. Since the different lentil LOX isozymes could not be separated chromatographically, it remains possible that activity of one of the LOX isozymes is lost during purification, resulting in a reduction in bleaching capacity. However, it may also be that other seed enzymes, which are removed during purification, play a role during dough bleaching.

Future recommendations:

In this thesis lentil seed LOX was purified to relative homology using anion- and cation exchange chromatography. However, although different isoforms could not be separated from one another by chromatography, evidence for the presence of at least three different isoforms of lentil seed LOX were presented. Future studies would therefore endeavor to fully separate these isoforms. The effect of each isoform on dough rheology and carotenoid bleaching would then be investigated. Following purification of each isozyme, each enzyme will be fully characterized, as was done in this thesis; however, this characterization will extend to in-dough enzyme characterization. With the use of this information, dosages could be tailored in order to prepare a product with predetermined characteristics.

It is well known that soybean seed LOX-2 is responsible for the production of various off-flavors. However, the flavor profile for lentil seed LOX has not yet been established. The substrate, and product specificity of lentil seed LOX has also not been investigated. In future studies the flavor profile for the products produced by lentil seed LOX catalysis will be investigated and characterized.

The current purification process allows for the collection of lentil oil, and meal during the preparation for chromatography, while various proteins and lipids are collected in the flow-through during chromatography. As such, this purification procedure could be incorporated into existing processing lines to utilize the waste-stream products of these processes. In this way, multiple lentil seed products could be produced from the same processing line, limiting waste. Therefore, future research would endeavor to optimize the purification process, and to incorporate purification as a downstream process in existing

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processing lines. For example, the process would be designed to first collect the lentil seed oil for application in the food industry. Following crude extraction, the insoluble material will be processed into animal feed, or fertilizer. During each round of purification, the contaminating proteins will be collected, and prepared for use as supplements in other food products. Finally, lentil seed LOX isozymes will be purified, and blended with other enzymes for use in improver blends. In this way, a single process line could yield multiple products thereby increasing the commercial value of the raw starting material. This would not only increase the value of the initial starting material, but also the profitability of the entire process. Since multiple products are produced in the same process, this would also reduce the carbon footprint of the products produced.

As discussed earlier, it is common practice to add various purified enzymes to wheat flour in order to improve the baking characteristics of the resulting doughs. The current trend is to prepare mixtures of enzymes which are synergistic in their effects, thereby amplifying the effect of each individual enzyme[2]. However, for the preparation of such synergistic mixtures, an in-depth knowledge about the catalytic characteristics, and the interactions between each of the components is required. Although LOX have been used in the baking industry for almost a decade[24], not much is known about the possible synergies of LOX with native wheat-, and added enzymes. Therefore, possible synergies with native wheat proteins and enzyme additives will be investigated once lentil seed LOX isoforms have been purified to homogeneity. For instance, lipases are already used in bread making to increase the surface activity of endogenous lipids, resulting in an increased loaf volume[175]. Since lentil seed LOX was not able to utilize esterified substrates (Chapter 4), combination with lipases should increase substrate availability by liberation of esterified linoleic acid, augmenting the effects of LOX. However, for this synergy to yield optimal results, the levels and activities of each enzyme must be well balanced. A greater understanding of these interactions would therefore enable the preparation of high-quality enzyme blends.

During the baking trials performed it was found that the greater the degree of purification of lentil seed LOX, the less effect the enzyme had on carotenoid bleaching. However, there was still an improvement on dough rheology. Since it was shown that addition of crude extracts resulted in both a whiter crumb as well as improved loaf volume, the results presented suggest that reactions other than that catalyzed by LOX occurs in bread dough. Proof for secondary reactions, which consume the lipid hydroperoxides resulting from the interaction between LOX and linoleic acid was presented in Chapter 4. However, these reactions were not investigated further. In future the enzymes responsible for such reactions need to be

isolated and characterized. The effect of such enzymes, in isolation and in combination with LOX, on dough rheology and bleaching capacity during the manufacturing of bread, should be further elucidated.

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Comprehensive REVIEWS in Food Science and Food Safety

Lipoxygenases: From Isolation to Application

Stefan Hayward, Tertius Cilliers, and Pieter Swart

Abstract: The positive effect of lipoxygenase, added as an enzyme-active soy flour, during the production of white bread is well established. In addition to increasing the mixing tolerance and overall dough rheology, lipoxygenase is also an effective bleaching agent. It is known that these effects are mediated by enzyme-coupled cooxidation of gluten proteins and carotenoids. However, the mechanism whereby these effects are achieved is not yet fully understood. In order to gain a better understanding into the reactions governing the beneficial effects of lipoxygenases in bread dough, an in-depth knowledge of the lipoxygenase catalytic mechanism is required. Until now no single review combining the molecular enzymology of lipoxygenase enzymes and their application in the baking industry has been presented. This review, therefore, focuses on the extraction and molecular characterization of lipoxygenases in addition to the work done on the application of lipoxygenases in the baking industry.

Keywords: food additives, food chemistry, lipid peroxidation, lipoxygenase, rheological properties

Introduction

The existence of an enzyme catalyzing the oxidative destruction of carotene was 1st reported by Bohn and Haas (1928). These authors discovered that the inclusion of small quantities of soybean flour in wheat dough resulted in the bleaching of wheat flour pigments. Because it was thought that color loss was solely due to oxidation of carotene, the enzyme was subsequently named carotene oxidase (Bohn and Haas 1928; Axelrod 1973). Around the same time period, Andre and Hou (1932) showed that soybeans contained a "lipoxidase" enzyme which catalyzes the peroxidation of various unsaturated fatty acids (Axelrod 1973; Chedea and Jisaka 2013). Craig (1936) subsequently showed that suspensions of *Lupinus albus* consumed large quantities of oxygen, compared to the amount of CO_2 produced, in the presence of lipids. This author designated the name unsaturated fat oxidase to the responsible enzyme. It was only 4 y later when Sumner (1940) and Tauber (1940), respectively, recognized that both reactions are catalyzed by the same class of enzyme. Following this, the names carotene oxidase, unsaturated fat oxidase, and lipoxidase were consolidated and the responsible enzyme officially became known as lipoxygenase.

Lipoxygenase (linoleate: oxidoreductase, EC 1.13.11.12, LOX) is a group of non-heme metal-containing dioxygenases which catalyze the regio- and stereo-specific dioxygenation of polyunsaturated fatty acids to conjugated unsaturated fatty acid hydroperoxides (Faubion and Hoseney 1981; Addo and others 1993; Robinson and others 1995; Brash 1999; Liavonchanka and Feussner 2006; Baysal and Demirdoven 2007; Permyakova and Trufanov 2011). Although these enzymes are widely distributed in the animal and plant kingdoms, they are particularly abundant in grain legume seeds and potato tubers (Axelrod 1973; Faubion and

Hoseney 1981; Robinson and others 1995; Casey and others 1999; Baysal and Demirdoven 2007; Miguel and others 2013). Plants furthermore contain multiple isoforms of the LOX enzyme which differs in terms of its substrate preference, optimal pH, product formation, and stability (Faubion and Hoseney 1981; Luning and others 1991; Brash 1999; Baysal and Demirdoven 2007; Chedea and Jisaka 2011, 2013). Because of the high level of soybean LOX expression, most of this knowledge on the enzymology and structural biology of lipoxygenase enzymes are derived from studies on soybean LOX isoforms (Andre and Hou 1932; Skrzypczak-Jankun and others 1997; Kuhn and Borchert 2002; Chedea and Jisaka 2011, 2013).

The use of enzyme-active soy flour to fortify wheat flour, intended for the production of white bread, is well documented (Frazier and others 1977; Faubion and Hoseney 1981; Gardner 1988; Luning and others 1991; Shiiba and others 1991; Addo and others 1993). Apart from increases in protein content and nutritional value, enzyme-active soy flour enhances the baking properties and color of wheat flours fortified with it (Addo and others 1993). The latter of these effects are mediated by a complex interaction of LOX with native wheat flour lipids and carotenoids. In order to gain a better understanding of the mechanism whereby these effects are achieved, an in-depth knowledge on the reaction mechanism of LOX catalyzes is required. However, to the best of our knowledge, a review combining the molecular enzymology of LOX enzymes, and the application of LOX enzymes in the baking industry, has not yet been presented. This paper therefore aims to resolve this by reviewing the current knowledge on the molecular enzymology of the LOX family of enzymes. This will be followed by a discussion on the mechanism whereby exogenous LOX improves the baking properties of wheat flour dough. However, although LOX from many sources has been studied, throughout this work emphasis is on soybean LOX as this is this commercial source for LOX in the baking industry.

Molecular Enzymology of LOX
Classification and nomenclature

LOX isozymes were historically named based on their ease of purification, stability, and optimal pH of catalysis. However, owing to the diversity of the LOX enzymes currently known, this system is no longer practical. Higher plants produce multiple isoforms of the LOX enzyme. These isozymes differ in terms of optimal pH of catalysis, substrate preference, regiospecificity, and ability to bleach carotenoid pigments (Veldink and others 1977; Brash 1999; Casey and others 1999; Baysal and Demirdoven 2007). The natural substrate for plant LOX are the C18-polyunsaturated fatty acids linoleic and α-linoleic acid (Brash 1999; Chedea and Jisaka 2011, 2013). The reaction of these substrates with LOX yields either 9S- or 13S-hydroperoxides depending on the LOX isozyme catalyzing the oxygenation (Gardner 1988; Brash 1999; Liavonchanka and Feussner 2006; Baysal and Demirdoven 2007; Ivanov and others 2010; Chedea and Jisaka 2013). This nomenclature for LOX enzymes is therefore based on the specificity of the enzyme acting on its substrate (Brash 1999). Soybean seed LOX-1 catalyzes the oxygenation of α-linoleic acid to, almost exclusively, 13S-hydroperoxy-octadecadienoic acid (HPODE; Brash 1999; Chedea and Jisaka 2013). The LOX-1 enzyme is therefore designated 13-LOX. However, soybean seed LOX-2, a 9/13-LOX, produces equal amounts of both 9S-HPODE and 13S–HPODE (Skrzypczak-Jankun and others 1997; Chedea and Jisaka 2011). This isozyme is furthermore unique in its ability to utilize esterified unsaturated fatty acids in membranes, compared to LOX-1 which has an absolute requirement for free fatty acids (Gardner 1988; Chedea and Jisaka 2011). LOX-3, on the other hand, has a moderate preference for the production of 9S-HPODE resulting in the classification as a 9-LOX. When comparing plant LOX with its animal counterparts, the chain lengths of the natural substrates (linoleic acid vs. arachidonate) result in the plant 13-LOX corresponding to a 15-LOX in animals (Brash 1999). However, both enzymes act on the ω-6 position of the fatty acid chain.

This system of nomenclature has, however, become somewhat confusing with growing family diversity. The major reason for this confusion is that the current nomenclature does not take evolutionary and functional relatedness into account (Kuhn and Borchert 2002; Ivanov and others 2010). This becomes especially apparent when comparing plant and animal LOX enzymes, since they do not use the same substrates. Further complications arise when multiple isoforms of, for example, mammalian 12-LOX, are present in the same organism (Brash 1999; Ivanov and others 2010). Ivanov and others (2010) therefore proposed a classification procedure which is based on phylogenetic relatedness. However, no unifying LOX nomenclature, which could overcome these difficulties, has been introduced. Currently, different LOX isozymes, catalyzing the same reaction within the same organism, is named after the prototypical tissue of their occurrence with reference to their regiospecificity (Brash 1999; Liavonchanka and Feussner 2006). For example, there are 3 isoforms of mammalian 12-LOX. These enzymes are therefore designated platelet, leukocyte, or epidermal 12-LOX (Brash 1999).

Extraction and purification

Soybean seeds are the richest known source of LOX contributing up to 2% of the total protein content (Rapoport and others 1978; Brash 1999; Permyakova and Trufanov 2011; Miguel and others 2013). As such, most of the current knowledge on LOX-catalyzed oxygenation of polyunsaturated fatty acids is based on studies using soybean LOX isoform 1. Since the time this enzyme was 1st described in the 1st half of the 20th century, the family diversity has expanded to include both the animal and plant kingdoms (Axelrod 1973; Faubion and Hoseney 1981). Interest was sparked in this enzyme for its biotechnological application in baking (Bohn and Haas 1928), and later flavor production (Gigot and others 2010). However, in order to study and optimize its application in industrialized settings, pure LOX isozymes are required.

Aqueous extraction of soybean flour yields a complex mixture of protein, peptides, carbohydrates, oligosaccharides, pigments, and other low-molecular-mass compounds (Lakshmi and others 2009). Purification of LOX from this complex mixture is further complicated by the presence of multiple isoforms with similar molecular mass and pI values (Axelrod 1973; Diel and Stan 1978; Faubion and Hoseney 1981; Shiiba and others 1991; Chedea and Jisaka 2013). This close resemblance can lead to the misidentification of a specific LOX isoform. When the purified enzyme is intended for mechanistic studies, misidentification would cause errors during the interpretation of the results (Faubion and Hoseney 1981). Two main types of LOX have been described. Type I LOX enzymes, such as LOX-1, have an optimal activity between pH 9 and 10, although type II LOX, which includes isoforms 2 and 3, are most active between pH 6.5 and 7 (Robinson and others 1995). Care should therefore be taken during identification of the purified enzyme.

Soybean LOX was 1st purified to considerable purity by Balls and others (1943). The enzyme was subsequently crystallized by Theorell and others (1947). At that time, however, it was thought that a single LOX isozyme (LOX-1) was responsible for the carotene-bleaching ability of soybean flour. The 2nd (LOX-2) and 3rd isoforms (LOX-3), along with an isozyme designated "LOX-b," were subsequently purified by the groups of Christopher and others (1970, 1972) and Yamamoto and others (1970). In these studies it was shown that LOX enzymes can be purified by conventional techniques of protein isolation, including ammonium sulfate precipitation, ion-exchange, and size-exclusion chromatography (Balls and others 1943; Axelrod 1973; Diel and Stan 1978). Each LOX isoform can be extracted from ground soybean flour, with varying yields, using different buffers. In a study by Diel and Stan (1978) the authors showed that LOX-1 is optimally extracted using 0.1 M sodium acetate, pH 4.5, although LOX-2 is most efficiently extracted using 0.05 M sodium phosphate, pH 6.0. However, others have reported using buffers in the pH range between 4.5 and 6.8 for extraction (Yamamoto and others 1970). Active LOX enzymes can subsequently be precipitated using ammonium sulfate in the saturation range of 30% to 60% (Diel and Stan 1978). Following precipitation, LOX enzymes are recovered using successive chromatographic procedures. Each soybean seed isozyme has a distinct isoelectric point ranging between 5.7 and 6.4 (Yamamoto and others 1970; Christopher and others 1970, 1972; Diel and Stan 1978). Based on these differences in pI, the enzymes can be purified from ammonium sulfate-concentrated flour extracts using successive anion and cation exchange chromatography steps (Christopher and others 1972; Axelrod 1973). Furthermore, soybean LOX isozymes are large monomeric proteins with a molecular mass ranging between 94 and 100 kDa (Diel and Stan 1978; Faubion and Hoseney 1981; Brash 1999; Chedea and Jisaka 2011, 2013). Due to this relatively high molecular mass the enzyme can be resolved from smaller proteins and other low-molecular-mass compounds by gel permeation chromatography (Shiiba and others 1991; Yang and others 1993; Hilbers and others 1996). However, gel permeation chromatography has only a
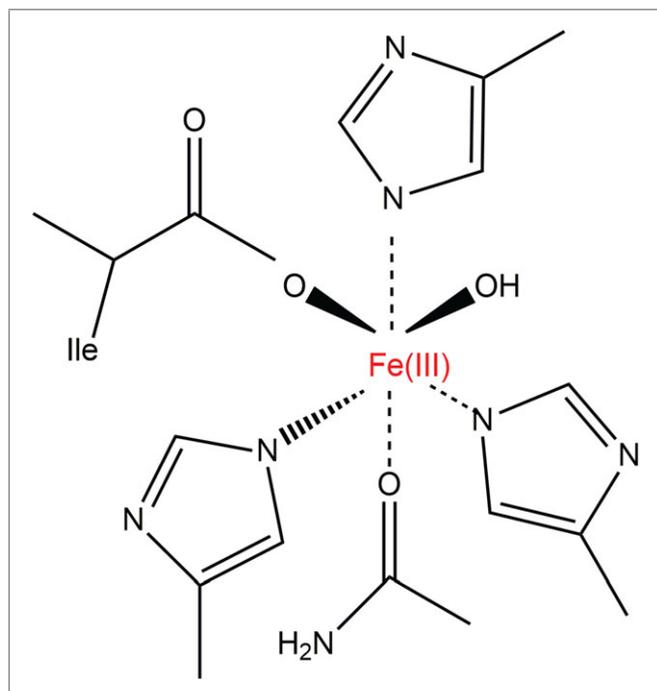
Figure 1–Octahedral coordination of the non-heme iron in the LOX active site. The figure was adapted from Brodhun and Feussner (2011).

limited scope separating large samples of similarly-sized proteins. This technique is therefore employed during the final clean-up of samples separated using ion exchange chromatography.

LOX Reaction Mechanism
Enzyme primary structure

LOX enzymes are non-heme iron-containing enzymes folded into a 2-domain secondary structure (Brash 1999; Ivanov and others 2010; Chedea and Jisaka 2013). The *N*-terminal region of 25 to 30 kDa consists of a *β*-barrel domain which shares significant homology with the C-terminal domain of mammalian lipases (Gillmor and others 1997; Brash 1999; Liavonchanka and Feussner 2006; Ivanov and others 2010). Based on this homology, it is thought that this domain plays a role in membrane-binding and the acquisition of lipid substrates during catalysis (Brash 1999; Ivanov and others 2010). The 2nd larger *α*-helical catalytic domain contains a single non-heme iron molecule octahedrally coordinated by 3 histidine residues, the C- terminal isoleucine, an asparagine residue, and a hydroxide as shown in Figure 1 (Gillmor and others 1997; Brash 1999; Lehnert and Solomon 2003; Liavonchanka and Feussner 2006; Ivanov and others 2010). This non-heme iron exists in 1 of 2 oxidation states, namely, Fe(II) or Fe(III) (Zoia and others 2011). According to this working mechanism, in the absence of substrate and autoxidized lipid species, the native enzyme remains inactive and the iron is in the low-spin Fe(II) state (Berry and others 1998; Zoia and others 2011). The enzyme is subsequently activated by oxidation of the active-site iron to the ferric form by hydroxylipids. Once activated, the enzyme cycles between the inactive (Fe[II]) and active (Fe[III]) states by product activation. In the active form, the enzyme catalyzes stereospecific hydrogen abstraction and oxygen insertion in polyunsaturated lipid species (Berry and others 1998; Ivanov and others 2010; Zoia and others 2011).

The active site of soybean LOX consists of an inter-domain-crevice containing 2 major cavities (cavities I and II) which intersect in close proximity of the non-heme iron (Boyington and others 1993; Ivanov and others 2010). Cavity II is furthermore subdivided into 2 subcavities, namely, cavity IIa and IIb by the side chains of Val354 and Arg707 (Ivanov and others 2010). Cavity IIa is thought to function as the substrate-binding pocket because this cavity is intersected by a side channel, between Ile553 and Trp500, which is thought to direct oxygen to the active site (Ivanov and others 2010). Ile553 has been implicated in modulating the alignment of fatty acids entering the active site (Meyer and others 2008; Ivanov and others 2010). Once aligned inside the substrate binding site, a hydrogen is abstracted and oxygen is introduced at either the [+2] or [−2] position from the original site of abstraction (Liavonchanka and Feussner 2006; Ivanov and others 2010). Because different LOX isozymes produce either 9- or 13- HPODE, an orientation-related hypothesis for positional specificity, as depicted in Figure 2, has been established. In this mechanism, a fatty acid substrate penetrates the active site, methyl end 1st in the case of 13-LOX enzymes; whereas in the case of 9-LOX enzymes, the substrate is forced into the binding pocket with its carboxyl group 1st (Gardner 1991; Liavonchanka and Feussner 2006). In this way radical rearrangement may be facilitated by the same mechanism in both cases. This theory is supported by the observation that the position of oxygen insertion may be governed by pH (Gardner 1991; Robinson and others 1995). LOX-1 exclusively produces 13-HPODE at a pH between 9 and 10. However, with a decrease in pH the formation of 9-HPODE is favored (Bild and others 1977; Gardner 1989). Formation of the product is therefore thought to be mediated by pH-dependent deprotonation of the carboxylic acid to form a more polar carboxylate which is not able to enter the hydrophobic active site (Gardner 1991; Robinson and others 1995). The substrate subsequently enters the active site with the less polar methyl group 1st. This theory suggests that the regiospecificity of oxygen insertion is controlled by the carboxylate anion/carboxylic acid ratio of the substrate (Gardner 1989; Robinson and others 1995).

Reaction mechanism

Fatty acid oxygenation occurs in 4 consecutive steps: (i) hydrogen abstraction, (ii) radical rearrangement, (iii) oxygen insertion, and (iv) peroxyradical reduction as indicated in Figure 3 (Brash 1999; Rickert and Klinman 1999; Kuhn and Borchert 2002; Segraves and Holman 2003; Ivanov and others 2010; Chedea and Jisaka 2013). Hydrogen abstraction and oxygen insertion occurs in antarafacial sides in relation to the 1Z,4Z-pentadiene unit (Chedea and Jisaka 2011, 2013). During catalysis hydrogen abstraction constitutes the rate-limiting step and corresponds to proton-coupled electron transfer (Rickert and Klinman 1999; Segraves and Holman 2003; Ivanov and others 2010). In this reaction, the electron is directly tunneled from the substrate to the ferric state iron (Glickman and Klinman 1995; Lehnert and Solomon 2003; Hatcher and others 2004). In this way, the enzyme is cycled between its active and inactive state by its substrate and products, as illustrated in Figure 3 (Brash 1999). As such, the enzyme is product-activated and, in the absence of fatty acid hydroperoxides, the enzyme remains in an inactive state. It has furthermore been shown that the type of hydroperoxide, such as 9- or 13-HPODE, plays an important role during activation of different LOX isoforms (Ramadoss and others 1978). These studies will be discussed in more detail below. Nevertheless, in kinetic studies, this "activation" step is observed as a lag phase that could
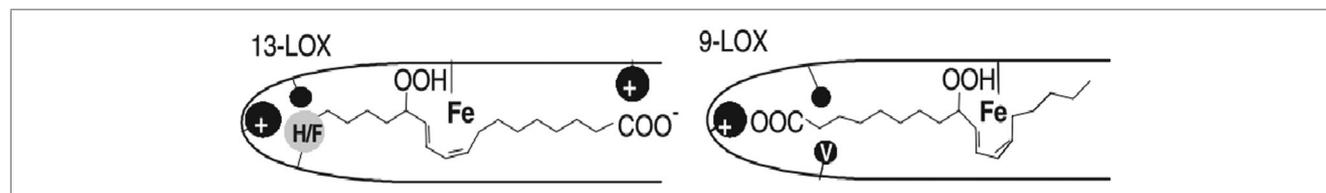
Figure 2–Depiction of the orientation-related hypothesis for the production of both 13- and 9-HPODE by a single LOX enzyme. In this figure the fatty acid substrate enters the active site methyl end 1st to produce 13-HPODE and carboxyl end 1st to produce 9-HOPTE. Based on observations from soy LOX-1 this mechanism is thought to be governed by a pH-dependent formation of a carboxylate which, based on its polarity, cannot enter the active site (Liavonchanka and Feussner 2006).
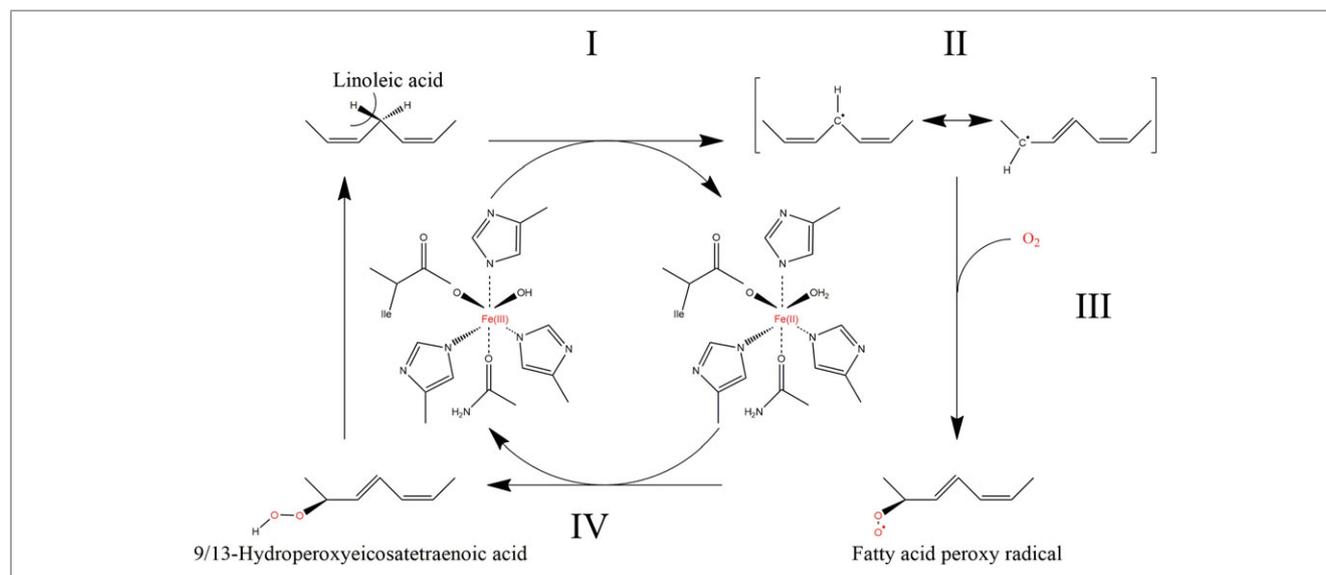

Figure 3–Mechanism of LOX catalysis. As depicted here, the LOX enzyme is cycled between the active Fe (III) and inactive Fe (II) states by the products and substrates of the reaction, respectively. Hydrogen abstraction results in the formation of a carbon radical which is subsequently stabilized by electron rearrangement. This is followed by oxygen insertion and reduction to form a hydroperoxide product. Figure adapted from Brodhun and Feussner (2011).

be abolished by the addition of small quantities of fatty acid hydroperoxides (Gardner 1988; Rickert and Klinman 1999; Baysal and Demirdoven 2007). In wheat flour dough, initial activation, by conversion of Fe(II) to Fe(III), is thought to occur via a small pool of autoxidized substrates leading to a chain reaction culminating in activation of the remaining inactive enzyme (Rickert and Klinman 1999; Pokorny and others 2001; Baysal and Demirdoven 2007; Chedea and others 2008).

Following hydrogen abstraction, a carbon radical is formed which is stabilized by a Z,E-double bond conjugation (Kuhn and Borchert 2002; Ivanov and others 2010; Chedea and Jisaka 2013). This is followed by the stereospecific insertion of molecular oxygen at the +2 or -2 position with relation to the original radical carbon to form a peroxy radical as well as a conjugated *trans, cis*-diene chromophore (Brash 1999; Kuhn and Borchert 2002; Liavonchanka and Feussner 2006; Ivanov and others 2010; Chedea and Jisaka 2011). This peroxyradical intermediate is subsequently reduced to form hydroperoxide in the 4th step of catalysis.

Under normal conditions, this reaction may not be considered an effective source for free radicals because the intermediates remain enzyme-bound (Ivanov and others 2010; Zoia and others 2011). However, it has been shown that, under certain conditions, a considerable proportion of the reactive oxygen species may be prematurely released leaving the enzyme an inactive Fe(II) state (Berry and others 1998; Casey and others 1999; Zoia and

others 2011). Berry and others (1998) showed that regiospecificity and radical release, as measured by oxidation of β-carotene, relies on the amount of dioxygen present. Based on these observations, Zoia and others (2011) subsequently set up experiments using ^{31}P NMR spin trapping to obtain direct evidence for the radical-escaping mechanism in relation to dioxygen concentration. In this study, the authors could define 3 distinct phases in terms of radical generation.

(1) An initial dioxygen consumption phase during which the dioxygen concentration is not limiting. In this phase, the regiospecificity of the LOX reaction is maintained, and low levels of oxygen-centered radical species are released. The formation of oxygen-centered species relates to regiospecificity, as described by Berry and others (1998).

(2) In the 2nd phase, where the dioxygen concentration becomes limiting, an increased rate of carbon-centered radical release is observed. Carbon-centered radicals are correlated to a reduction in the regiospecificity of the LOX reaction. These results are in agreement with those in the earlier literature (Berry and others 1998).

(3) In the 3rd phase, where the dioxygen concentration is essentially zero, the enzyme generates radicals up to complete deactivation due to an inability to reactivate via hydroperoxides (Reis and others 2006; Baysal and Demirdoven 2007).
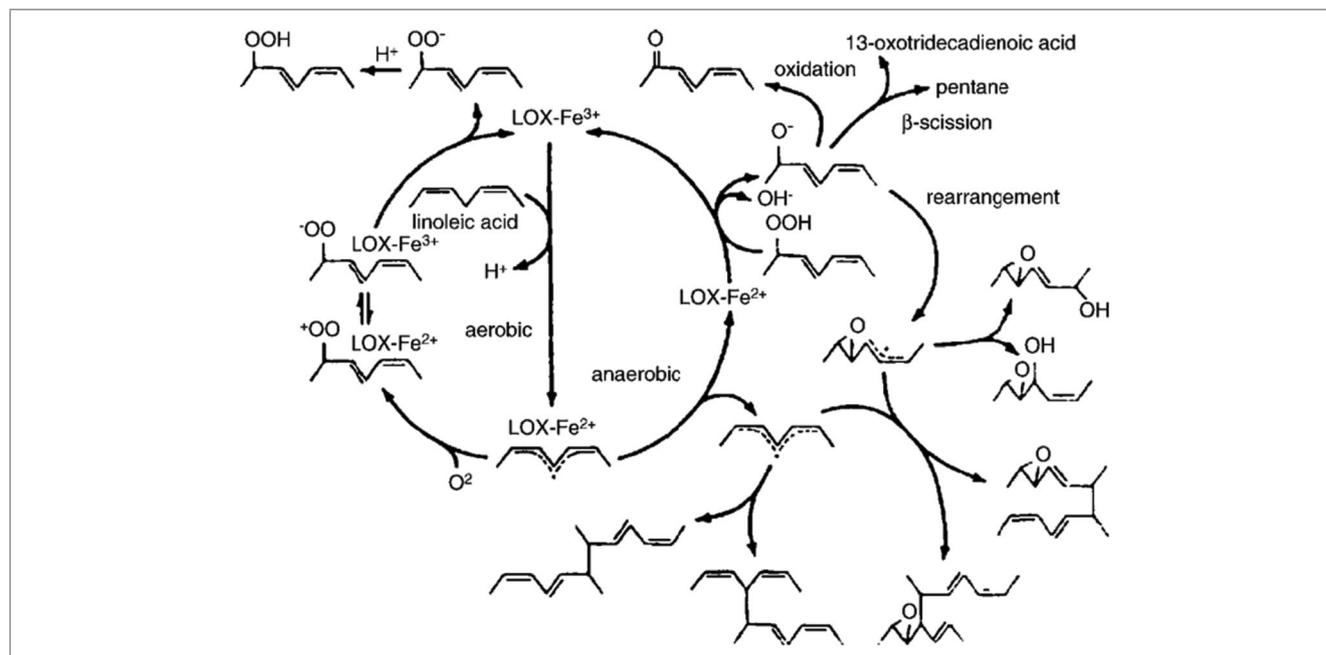
Figure 4–LOX-catalyzed product formation during aerobic and anaerobic reactions with polyunsaturated lipid substrates as suggested by Gardner (1988).

Once released from the enzyme active site, the reactive oxygen species interact with, and oxidize, sensitive molecules, such as thiol-containing proteins, antioxidants, and pigments (Casey and others 1999). These interactions have important functions in dough rheology and bleaching.

Oxygen requirement of LOX catalysis

LOX also functions under anaerobic conditions, on condition that both polyunsaturated fatty acids and hydroperoxide products are present (Garssen and others 1971, 1972; de Groot and others 1973). The anaerobic reaction, depicted in Figure 4, is initiated in a similar manner to the aerobic mechanism wherein a radical is formed due to hydrogen abstraction from the linoleic acid substrate (Garssen and others 1971). However, because no oxygen is available for oxygenation, this reaction results in the formation of various carbonyl compounds. In order to maintain activity under oxygen-limiting conditions, the active-site iron is oxidized to Fe(III) by hydroperoxides instead of O_2 (de Groot and others 1973; Gardner 1988; Berry and others 1998). In this reaction, the hydroperoxide product is reductively cleaved into a hydroxide ion and an alkoxyl radical (Garssen and others 1971, 1972; Gardner 1988).

The radicals produced have various downstream implications in the baking industry. Although thorough mixing is associated with aeration of the dough, oxygen concentrations may be reduced during fermentation by yeast metabolic processes. As such, radical formation may be augmented during the proving of the dough. It has been shown that these radicals have major implications in carotenoid bleaching and flavor development (Grosch and others 1977; Ramadoss and others 1978). A reduction in oxygen concentrations have furthermore been associated with a decrease in the specificity of LOX–catalyzed oxygen insertion. In a study by Berry and others (1998) it was shown that the products 13-HPODE and 9-HPODE are produced by LOX–1 in a 1:1 ratio under anaerobic conditions. This is in stark contrast to the high–level regiospecificity (13-HPODE: 9-HPODE 95:5) observed from LOX-1 catal-

ysis under aerobic condition. As will become clear in the ensuing section, carotenoid bleaching, flavor development, and rheological improvement all rely on combinations of the activity of the LOX isozymes. However, the activities of these enzymes are modulated by their reaction products. These effects will be discussed in more detail in the following sections.

Chiral-specificity of oxygenation

In most cases, especially in plants, the reaction of LOX with polyunsaturated fatty acids produces hydroperoxy products which are in the S configuration, irrespective of the carbon which is oxygenated (Robinson and others 1995; Chedea and Jisaka 2013). However, LOX enzymes catalyzing the formation of HPODE products in the R configuration have been found among invertebrates, plants, and mammals (Brash 1999). The reason for this chiral specificity is still unknown. It has, however, been reported that the antarafacial relationship between the abstracted hydrogen and the inserted oxygen determines the chirality of the resulting product (Robinson and others 1995). This relation is thought to be due to the orientation of the oxygen connected to the iron atom in the active site.

LOX Activity Determination

For the successful industrial application of enzymes, details of their activity and reaction mechanism is required. For this purpose, a wide array of activity assays has been developed. In the ensuing section, the major assays, and some of their drawbacks, will briefly be discussed.

Spectrophotometric assays

As discussed previously, the presence of LOX in the seeds of legumes was based on the ability to react with pigments, mainly xanthophylls, in the presence of unsaturated fatty acids (Bohn and Haas 1928; Axelrod 1973). As such, one of the 1st methods for the detection of LOX activity was based on the cooxidation of carotene in the presence of polyunsaturated fatty acids (Balls and

others 1943). In this method, the rate of carotenoid-bleaching is determined in timed intervals. However, determination of bleaching efficiency is not an accurate activity measure, because this assay has a limited range over which activity is proportional to enzyme concentration. The substrate mixture is furthermore inherently unstable, and large variations between carotene oxidase of different LOX enzymes, compared to their peroxidizing activities, are common (Axelrod 1973; Lilius and Laakso 1982).

In 2 separate studies the groups of Holman (1945) and Theorell and others (1946), observed an increase in the absorbance at 234 nm of LOX-oxidized fats. These authors independently showed that the increase in absorbance was due to the formation of a conjugated diene in linoleic acid. As such this method has become known as the diene conjugation method (Theorell and others 1946; Axelrod 1973). The diene conjugation method is more sensitive than the carotene cooxidation assay, since it measures product formation directly. This assay could furthermore be applied for kinetic studies when a recording spectrophotometer is used (Surrey 1963; Axelrod 1973). However, although this method is superior in sensitivity, it has the disadvantage of a tendency towards turbidity due to a limited solubility of linoleic acid in aqueous suspensions (Lilius and Laakso 1982). Surrey (1963) subsequently optimized this method with the introduction of "Surrey's" substrate mixture wherein linoleic acid is dissolved and clarified using 0.25% Tween® 20 and 1 M NaOH, respectively. To date, adaptations of this substrate mixture are still the most commonly used substrate. The conjugate diene method, performed with Surrey's substrate mixture, is this standard method for LOX activity determination based on sensitivity and reproducibility. However, due to the low molar absorbance of the reaction products, this assay remains susceptible to interference by protein absorption when preparations of low specific activity are assayed (Axelrod 1973).

Based on the above-mentioned drawbacks, it was subsequently shown that interference could be overcome by determination of downstream products of the formed hydroperoxides. One of the oldest of these methods relies on the interaction of hydroperoxide products with ferrous thiocyanate (Frankel and others 1990). During this reaction the formed hydroperoxides oxidize Fe (II) to Fe (III), which subsequently reacts with thiocyanate to form a colored product which can be determined spectrophotometrically (Frankel and others 1990; Anthon and Barrett 2001). This colored product is, however, unstable which limits its use. Waslidge and Hayes (1995) subsequently substituted thiocyanate with xylenol orange, a quantitative cation indicator (Gay and others 1999). The use of xylenol orange, in the ferrous oxidation–xylenol orange (FOX) method, yields a stable colored product which has a high molar absorption, eliminating the need for specialized equipment (Waslidge and others 1995; Gay and others 1999; Anthon and Barrett 2001). This assay is furthermore highly reproducible, rapid, and not sensitive to oxygen (Gay and others 1999). However, due to the requirement of an acidic environment for color development, this assay can only be performed in end-point styled assays. As such, it is not suited for kinetic studies and can only account for the presence of LOX, and is not an accurate representation for the relative amount of LOX unless compared to a known standard. False positives are also possible when the sample can chelate iron (Whent and others 2010).

Spectroscopic assays

The hydroperoxides formed during LOX catalysis are also able to oxidize a variety of electron-donating probes in reactions catalyzed by heme-containing compounds such as hematin, hemoglobin, and cytochrome C (Anthon and Barrett 2001). Such coupled assays provide colorimetric (Sivaram and others 2003), fluorometric (Esterbauer and others 1993), and chemiluminescent (Lilius and Laakso 1982) methods for the detection of hydroperoxides formed by LOX (Anthon and Barrett 2001). For this purpose, various probes and dyes have successfully been applied. Ohkawa and others (1979) has shown that thiobarbituric acid reacts with linoleic acid hydroperoxides to form malondialdehyde, a red pigment which can be determined spectrophotometrically. However, this assay has the drawback that color development is optimal at pH 4.0, well below the optimum pH for LOX, and the assay is sensitive to variations in pH, thus limiting its use for kinetic characterization of the enzyme. The use of fluorescent probes, which can be oxidized by fatty acid hydroperoxides, can overcome the difficulties associated with background interferences in spectrophotometric assays. Whent and others (2010) showed that addition of fluorescein to solutions containing linoleic acid and LOX results in high fluorescence. Fluorescein has previously been used as a probe in free radical-scavenging assays, as this probe is degraded by peroxy radicals to yield a fluorescent product (Moore and others 2006; Whent and others 2010). However, this assay is susceptible to interferences by Tween® 20 and secondary lipid oxidation products which can contribute to fluorescence. Therefore, although this assay is highly sensitive, care must be taken when the assay is performed, because higher LOX concentrations may influence the total assay time. The data from large amounts of samples, with varying concentrations of LOX, are therefore not readily comparable. This method could, however, be developed into a high-throughput assay (Whent and others 2010). Chemiluminescent assays have also been developed. Lilius and Laakso (1982) showed that free radical processes during lipid peroxidation result in the emission of low-level chemiluminescent light. These authors showed that this low-level chemiluminescent light could be amplified by the addition of luminol. It was furthermore shown that, when performed under properly selected conditions, this method is comparable in sensitivity to the spectrophotometric assay. Luminescence could furthermore be enhanced by the addition of cytochrome C (Radi and others 1991; Nakayama and others 1995).

Oxygen consumption

Based on the requirement for oxygen during the LOX-catalyzed oxygenation of polyunsaturated fatty acids, activity can be determined by monitoring O_2 usage in an attempt to overcome the difficulties associated with spectrophotometric assays. This method is as sensitive as the conjugated diene method, however, the results from these assays do not always agree. Holman (1947) noted that when secondary reactions occur, as is the case with crude soybean extracts, spectrophotometric assays fall short on theoretical yield based on oxygen uptake. This observation is due to reactions which consume the hydroxylipids during catalysis, and cannot be accounted for when using the spectrophotometric assays. Activity assays which are based on O_2 consumption are, therefore, superior in accuracy. However, based on equipment requirements, this method has limited value as a high-throughput activity assay for large numbers of samples.

LOX in the Baking Industry

Various chemicals are frequently added to flour in order to improve its bread-making performance. The benefits for the use of chemical additives, such as potassium bromate and azodicarbonamide, on dough rheology is well established (Thewlis 1974;

Popper and others 2006). However, due to doubts about the effect on health such additives have been gradually replaced with additives generally regarded as safe (Popper and others 2006). As such, the use of enzymes as replacements for chemical improvers have recently received substantial attention due to restrictions on the use of chemical additives (Cauvain and Young 2006; Lundkvist and Serj Olse 2007; Miguel and others 2013). Enzyme catalysis is furthermore highly specific, and limits the production of non-specific by-products. Efficient application could therefore enable the production of products with predetermined qualities. Various enzymes are currently used in order to improve the quality, and shelf-life, of the final product. For a review of these enzymes, and their purposes, please see references shown by Martínez-Anaya (1996), Miguel and others (2013), and Popper and others (2006). As discussed earlier, enzyme-active soy flour has been used in the baking industry for almost 100 y (Bohn and Haas 1928). The ensuing section will discuss the role of LOX during dough rheological improvement and bleaching in more detail.

Gluten

The production of bread requires dough to be elastic, so as to enable inflation during fermentation. The ability to retain CO_2, resulting from fermentation, relies on the viscoelastic properties of dough, a property which is reliant on the protein content of the wheat flour. The mixing characteristics of wheat flour and the rheological properties of the resulting doughs are largely determined by the properties of the major storage proteins of wheat germ, the prolamins (Bekes and others 1994). When mixed with water, these proteins interact to form a cohesive protein network known as gluten (Shewry and others 1986). The term gluten therefore does not describe a single protein, but the proteinaceous network formed upon interaction of the wheat storage proteins described below.

The proteins comprising the prolamins can be broadly classified based on their extractability in aqueous alcohols into either the extractable gliadins or unextractable glutenins (Shewry and others 1995, 2002, Shewry and Tatham 1997). The extractability of these proteins relies largely on their ability to form inter-chain disulfide bonds, with glutenins consisting of high-molecular-weight polymers entirely stabilized by disulfide bonds (Shewry and others 1995; Shewry and Tatham 1997). Reduction of the glutenin polymer results in the liberation of, predominantly, low-molecular-weight glutenin subunits (Wieser 2007). These low-molecular-weight glutenins contain 8 cysteines, 6 of which partake in intrachain disulfide bonds (Grosch and Wieser 1999; Lutz and others 2012). Because of steric hindrance, the remaining 2 cysteine residues are not able to form intrachain disulfide bonds and, consequently, only form interchain disulfide bonds with other glutenin proteins (Wieser 2007). This intra- and interchain disulfide bonding results in the formation of the high-molecular-weight glutenin polymers (Lutz and others 2012). Based on the formation of such disulfide bond stabilized polymers, glutenins contribute to the elasticity of dough. In contrast to glutenins, gliadins are monomeric proteins which contain either no cysteine (ω5- and ω1,2-gliadins) or only intramolecular disulfide bonds (α- and γ-gliadins), and they contribute towards viscosity (Lutz and others 2012). Because of these properties the prolamins confer the property of viscoelasticity, an important factor in determining the suitability of flour for its end use (Bache and Donald 1998; Shewry and others 2002; Cauvain and Young 2006). For instance, bread-making requires highly elastic "stronger" doughs containing more glutenins, although doughs for cakes and biscuits should be

more extensible, consisting of more gliadins (Shewry and others 1995; Shewry and Tatham 1997; Bache and Donald 1998).

The proteins comprising gluten can be separated by SDS-PAGE analysis into high- and low-molecular-weight fractions which differ in terms of gene composition (Shewry and others 1986; Costa and others 2013). The high-molecular-weight proteins are encoded by genes located at the Glu-A1, Glu-B1, and Glu-D1 loci on the long arms of chromosomes A1, B1, and D1, respectively (Payne and others 1984; Costa and others 2013). The short arms of the same chromosomes contain the loci Gli-A1, Gli-B1, and Gli-D1 which, respectively, encode for ω-gliadins, γ-gliadins, and low-molecular-weight glutenins (Payne and others 1984). The remaining low-molecular-weight gliadins are encoded by genes located the Glu-A3, Glu-B3, and Glu-D3 loci (Costa and others 2013). Because both glutenins and gliadins contain low-molecular-weight proteins, which differ in in terms of gene composition, confusion can arise when ascribing low-molecular-weight proteins to either the glutenins or gliadins. Recent research therefore caused a gradual move from the terminology glutenin and gliadin to high- and low-molecular weight proteins, though the terms glutenin and gliadin remains valid. Although both the high- and low-molecular-weight fractions are important in determining the functional properties of wheat dough, the high-molecular-weight fraction of glutenins are the key determinants of wheat dough quality (Butow and others 2003; Anjum and others 2007; Costa and others 2013). Furthermore, the composition and size distribution of this group of proteins play a major role in the functional properties of the resulting doughs (Bekes and others 1994; Shewry and Tatham 1997; Shewry and others 2002; Bache and Donald 1998). Early experiments have shown that the addition of reducing agents such as mercaptoethanol or dithiothreitol results in a weaker dough with a decreased tolerance to overmixing (Bekes and others 1994; Shewry and others 1997). Conversely, addition of oxidizing agents is associated with an increase in the average molecular weight of dough proteins and an increased tolerance to overmixing (Bekes and others 1994; Dunnewind and others 2002; Permyakova and Trufanov 2011). As such, addition of compounds broadly known as improving agents to flour is common practice during the production of breads and rolls of all types (Faubion and Hoseney 1981). These additives not only play an important role in flour maturation, but also accelerate dough development, improve dough strength and workability, and increase reproducibility of the final product (Shewry and Tatham 1997; Dunnewind and others 2002). These effects are mainly facilitated by the oxidation of cysteine residues and the concurring formation of intramolecular disulfide bonds between glutenin proteins during gluten formation (Popper and others 2006; Permyakova and Trufanov 2011). Disulfide bond formation is regarded as the major force binding wheat storage proteins incorporated into the integrated gluten network (Permyakova and Trufanov 2011).

LOX is considered an oxidative improving agent, because the products formed during the oxidation of flour fatty acids have a cross-linking effect on the flour proteins by oxidation of the glutenin thiol groups (Faubion and Hoseney 1981; Addo and others 1993; Permyakova and Trufanov 2011). As such, the use of LOX, in the form of enzyme-active soybean flour, as dough improver is commonplace during the production of white bread (Faubion and Hoseney 1981; Luning and others 1991). In addition to improving mixing tolerance and dough rheology, inclusion of enzyme-active soybean flour also bleaches the wheat flour carotenoid pigment to yield a whiter crumb (Faubion and Hoseney 1981; Shiiba and others 1991; Cumbee and others 1997;

Permyakova and Trufanov 2011; Miguel and others 2013). However, although it is now generally accepted that rheological improvement and dough bleaching is mediated by processes of cooxidation, the mechanism of cooxidation is complex and remains obscure (Frazier and others 1977; Faubion and Hoseney 1981). The major reason for this is that lipid oxidation and carotenoid bleaching and/or sulfhydryl oxidation, although linked, are not the same activities (Faubion and Hoseney 1981; Robinson and others 1995). Various groups have shown, using enzyme extracts, that pigment destruction could be inhibited by the addition of antioxidants, such as ascorbic acid, without a reduction in diene formation (Walsh and others 1970; Mc Donald 1979). Likewise, Frazier and others (1977) showed that inhibiting the appearance of lipid peroxidation products, by addition of nordihydroguaiaretic acid (NDGA), also did not have an effect on dough rheology. In these studies, ascorbic acid and NDGA, respectively, removed the intermediate and the product of the LOX reaction with lipids. These results suggested that it is a reactive intermediate in the LOX reaction that is involved in cooxidation, and not the final hydroperoxide product. This theory is strengthened by the observation that addition of exogenous hydroperoxides have no effect on dough rheology or pigment destruction (Frazier and others 1977; Faubion and Hoseney 1981).

Rheological improvement

Historically wheat flour was matured by exposure to oxygen in order to improve its baking properties. Dahle and Sullivan (1963) suggested that the efficiency of natural aging was, at least in part, due to the destruction of antioxidants. Among these, the –SH groups of glutenins may also be considered as an antioxidant, although it is not as efficient as tocopherols and pigments which are also present. The improving effect of natural aging has also been associated with an increase in the free lipid content and a concurrent decrease in the –SH content (Dahle and Sullivan 1963; Yoneyama and others 1970). Hlynka and Tsen (1962) argued that the –SH groups are oxidized by fatty acid peroxides, which are formed during LOX-catalyzed peroxidation of free lipids (Dahle and Sullivan 1963). However, Frazier and others (1977) showed that replacing the flour-free lipids with peroxidized lipids had no effect on the rheological properties of the dough when baked. Based on these results, it was suggested that the radical intermediates, formed during LOX-catalyzed lipid oxygenation, were responsible for oxidation of –SH groups in dough during mixing. According to Graveland and others (1976), normal wheat contains between 5 and 7 μmol thiol per gram flour, of which 0.5 to 1.5 μmol is oxidized during mixing (Gardner 1988). Bloksma (1964) consequently concluded that a conversion of a relatively few sulfhydryls to disulfide bonds could significantly increase a dough's resistance to deformation and overmixing.

In addition to the formation of disulfide bonds, the oxidized thiyl moieties can also cross-link with water-soluble pentosans, via ferulic acid esterified to arabinoxylans, to form viscous gels in a process known as oxidative gelation (Lineback and Rasper 1964; Sidhu and others 1980; Izydorczyk and Biliaderis 2006). During oxidative gelation the double bond of ferulic acid is activated by free radicals, enabling oxidative phenolic coupling of arabinoxylan molecules with adjacent arabinoxylans, protein tyrosine residues, and thiyl residues (Lineback and Rasper 1964; Sidhu and others 1980; Hoseney and Faubion 1981; Izydorczyk and Biliaderis 2006). This cross-linking results in the formation of a high-molecular-weight entity with many cross-linkages, which, in aqueous suspensions, translates to increases in viscosity (Hoseney and Faubion 1981). Cross-linking only occurs in the presence of oxidizing agents capable of producing free radicals, and not in the presence of the common oxidants potassium iodate, potassium bromate, or ascorbic acid (Lineback and Rasper 1964; Sidhu and others 1980; Hoseney and Faubion 1981). Oxidative gelation is therefore an alternative mechanism whereby LOX improves dough rheology based on the formation of reactive intermediates during the oxygenation of linoleic acid (Izydorczyk and Biliaderis 2006; Ren and others 2015).

Pigment destruction

As discussed earlier, the LOX family of enzymes was 1st discovered based on the catalysis of wheat flour pigment destruction (Bohn and Haas 1928; Christopher and others 1970; Axelrod 1973). Carotenoids are among the most common naturally occurring pigments, with over 600 molecular species being fully characterized (Stahl and Sies 2003; Chedea and Jisaka 2013). In photosynthetic organisms these compounds play an important role in the protection against photo-oxidative stresses and are essential structural components of the photosynthetic antenna (Bartley and Scolnik 1995). Here these compounds are efficient antioxidants which are able to scavenge singlet molecular oxygen and peroxy radicals (Stahl and Sies 2003). Protection against molecular oxygen depends largely on physical quenching and involves energy transfer between both molecules resulting in ground-state oxygen and a triplet excited carotene (Conn and others 1991; Stahl and Sies 2003). The carotenoid subsequently dissipates the energy by an interaction with the surrounding solvent. However, this reaction is of minor importance in terms of pigment destruction because the carotenoid remains intact following return to ground-state energy.

Of the known carotenoids, β-carotene is the most prominent (Sies and Stahl 1995). It has been shown that β-carotene is capable of inhibiting various free-radical reactions (Kennedy and Liebler 1991; Kubicka and Troszynska 2003). As such, it has been hypothesized that β-carotene, as well as other carotenoids, contribute toward protection of membranes from lipid peroxidation products (Sies and Stahl 1995; Stahl and Sies 2003). In wheat flour dough, fortified with LOX, this antioxidant activity relates to pigment destruction. The mechanism of peroxyl radical inactivation is based on the formation of resonance-stabilized carbon-centered radical adducts (Kennedy and Liebler 1991; Sies and Stahl 1995; Stahl and Sies 2003). In this reaction, peroxy radical attack on carotenoids usually occurs at the terminal double bond, resulting in epoxide formation at the site of addition (Kennedy and Liebler 1991). This is followed by homolytic cleavage of the peroxide bond resulting in the formation of an epoxide and release of an alkoxyl radical (Kennedy and Liebler 1991). This reaction may also occur via abstraction of an electron directly from the polyene by a peroxy radical resulting in the formation of an intermediate ion pair consisting of a peroxide ion and a radical cation of β-carotene (Kennedy and Liebler 1991). Irrespective of the route of formation, these products subsequently combine to form the epoxide radical addition product. As such, interaction of β-carotene with peroxy radicals results in the formation of a β-carotene radical which subsequently reacts with oxygen to form carotenoid peroxyl radicals (Krinsky 1989; Handelman and others 1991; Robinson and others 1995). Following their formation, these radicals initiate attack on other β-carotenoids, resulting in a chain reaction culminating in pigment destruction and the formation of 5,6 epoxides, β-apo-13-carotenone, and conjugated polyene ketones (Friend 1958; Krinsky 1989; Handelman and others 1991).
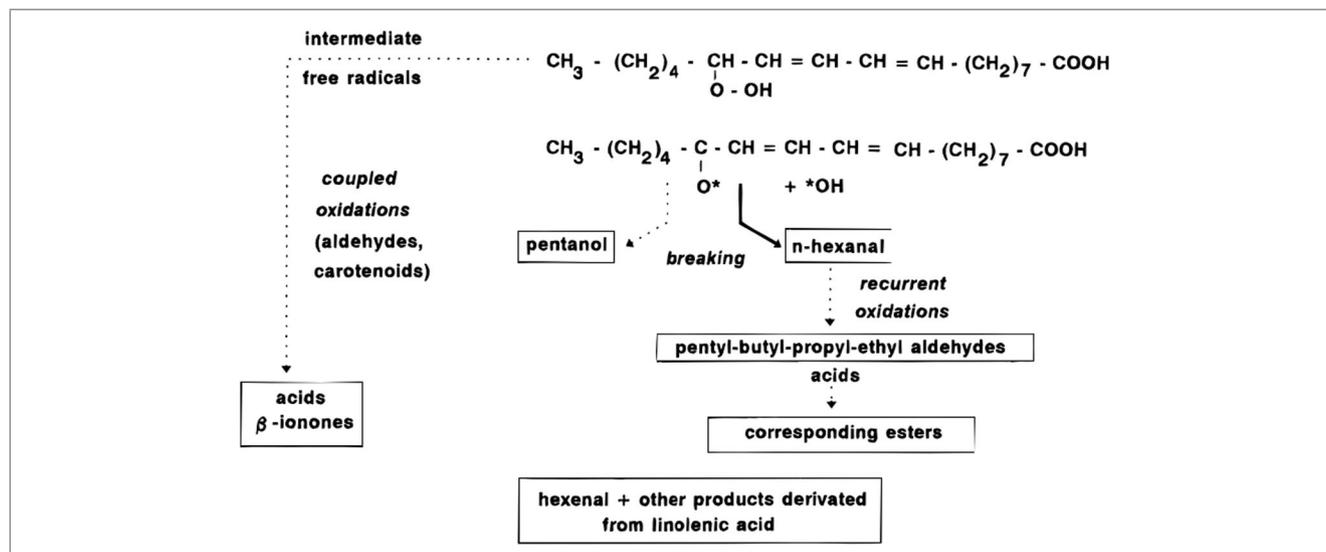
Figure 5–LOX catalyzed volatile formation originating from coupled oxidation reactions. Linoleic acid-derived products, such as hexanal, have various implications in the food industry (Martínez-Anaya 1996).

A simplified schematic for the formation of volatile flour compounds, as presented by Martínez-Anaya (1996), is presented in Figure 5.

LOX and flavor

Because flavor is one of the most appreciated sensory attributes of food products, the formation of volatile flavor compounds is of major importance to food scientists. However, although most volatile flavor compounds are perceived as having a positive contribution to the sensory experience, some compounds may have a deleterious effect, even at low levels. For example, short-chain C_6- or C_9-aldehydes and alcohols, formed by hydroperoxide lyases, isomerases, and dehydrogenases, are responsible for the natural aromas of fruits and vegetables (Robinson and others 1995; Casey and others 1999). However, in some cases, these products may be perceived as serious off-flavors. The use of soy is a good example of such a case. Although the use of soy in various food products is well established, if used in excess, the addition of soybean may result in the formation of volatile off-flavors. For use in bread, soy flour is therefore only used up to 0.5% to keep the adverse effect of volatile flavor down (Addo and others 1993; Cumbee and others 1997). Fujimaki and others (1966) showed that hexanal, derived from LOX-catalyzed hydroperoxidation of linoleic acid, is primarily responsible for flavors perceived as grassy or beany (Wolf 1975; Linko and others 1997; Gigot and others 2010). The flavor produced by hexanal furthermore has an extremely low taste threshold. The use of soybean in other food products has also been associated with the formation of off-flavors during storage (Luning and others 1991). On the other hand, in some applications these compounds, known as green leaf volatiles, are perceived as fresh natural flavors (Gigot and others 2010). Depending on the application, care should therefore be taken when using soybean flour, for its LOX function, in order to limit off-flavor production.

Cooxidation potential of LOX isoforms

Studies on the cooxidation of β–carotene have shown that the efficiency of cooxidation is dependent on the LOX isoform catalyzing the reaction. During early experiments Kies (1947) showed that heat treatment of partially purified preparations of soybean LOX resulted in a loss of the ability to cooxidize β-carotene without having an effect on the peroxidation of linoleic acid. However, at that time it was not yet known that soybean has multiple LOX isoforms. This author subsequently concluded that catalysis of the coupled oxidation reaction relies on a heat-sensitive entity which is distinct from the one purified by Theorell and others (1947). Although various LOX isoforms were subsequently discovered, these results indicated that each of the different isoforms plays a significant role during catalysis of β-carotene cooxidation. Moreover, these results indicate that the enzymes display synergism with relation to cooxidation of β- carotene.

Evidence for a synergistic mechanism of the LOX isozymes was presented by Ramadoss and others (1978) when they showed, using purified LOX isozymes, that none of the LOX isozymes are individually efficient catalysts for cooxidation of β-carotene. Bleaching capacity was only regained using combinations of LOX-1 or LOX-2 and LOX-3, respectively. However, bleaching capability was only observed for isoforms 2 and 3. LOX isoform 1 is not an efficient catalyst for enzyme-coupled oxidation of carotenoids. These results suggested that LOX-1 and 2, respectively, produce a product which is able to facilitate carotene-bleaching by LOX-3. In an analysis of this process, it was discovered that addition of 13-HPODE, produced by LOX-1 and to some extent LOX-2, to inactive LOX-3, recovered bleaching ability (Ramadoss and others 1978; Krinsky 1989). Conversely, addition of 9-HPODE was much less efficient in promoting carotenoid-bleaching (Veldink and Vliegenthart 1984; Robinson and others 1995). As discussed earlier, depending on the incubation conditions, LOX-2 produces mixtures containing different ratios of 13- and 9-HPODE. Based on these results it can be assumed that activation of LOX isozymes relies strongly on activation by 13-HPODE. Similar work by Weber and others (1974) showed that crocin was only bleached in the presence of either LOX-2 or 3, but not LOX-1, suggesting the formation of especially active radicals in the presence of LOX-2 and 3.

Studies evaluating the effect of LOX isozymes on baking

Multiple attempts have been made to isolate the various soy LOX isozymes and to investigate the rheological and baking properties of doughs fortified with them (Addo and others 1993;

Cumbee and others 1997; Permyakova and Trufanov 2011). Van Ruth and others (1992) studied the effect of stored soybean preparations on the baking properties of wheat flour dough. During these experiments it was shown that LOX isoforms 2 and/or 3 were primarily responsible for bleaching and the formation of volatile flavor compounds. Studies by Addo and others (1993) subsequently showed that soybean LOX-2 is also the major isoform responsible for the production of the undesirable *n*-hexanal aroma compound (Robinson and others 1995). However, these studies did not elaborate on the effect of individual LOX isozymes on other baking properties of wheat flour dough. Cumbee and others (1997) subsequently used soy mutant isolines and purified LOX enzyme preparations to investigate the effect on wheat flour dough rheological and bread-baking properties. In this study addition of a null mutant containing only isoforms 1 and 2 resulted in an increase in dough tenacity (resistance to extension), consistent with the addition of control soy flour containing all 3 isoforms. Furthermore, dough extensibility decreased when null mutants for isoforms 1 and 3 were added. Contrary to the increase in dough strength observed when soy flour containing all 3 isoforms were added, addition of the isoform 3 null mutant did not have any effect, although a decrease was observed upon addition of null mutants for isoforms 1 and 2. As a result, loaf volume of bread loaves baked using mutants containing only isoforms 2 and 3 and the mutant containing only isoforms 1 and 2 were increased over the control. Among these, the isoform 1 null mutant had the greatest effect on loaf volume. In contrast to the results obtained using null mutants, addition of purified LOX isozymes resulted in an increase in dough extensibility and strength. These results were in agreement with that obtained by Shiiba and others (1991) using wheat flour LOX suggesting that LOX-1 plays an accessory role rather than an active role in dough improvement.

Taken together, these results indicate that, in terms of dough rheology, isoforms 1 and 2 are required for dough-strengthening. However, of these isoform 2 is the most important since omission of this isoform results in a reduction in tenacity, extensibility, and dough strength. In terms of carotenoid bleaching ability, Isoforms 2 and 3 are most essential, because isoform 1 does not display significant bleaching capacity. However, LOX isoform 2 is also responsible for the formation of undesirable volatile flavor compounds, and isoform 3 relies on the products of isoform 1 for bleaching capacity. Based on the data presented it is safe to assume that, when using soy bean LOX, the ratio of each isoform present plays a significant role in product quality. However, apart from catalyzing the oxygenation of polyunsaturated fatty acids, LOX-2 is also capable of using ester lipids bound to membranes and lipoproteins (Kuhn and Borchert 2002). It is therefore possible that the overall positive effects of LOX-2 are due to an ability to use substrates which are inaccessible to LOX-1 and 3.

Recombinant LOX as improver

In most cases it is possible to use enzyme-active soy flour as an improvers. However, soybean seeds contain multiple enzymes which reduce polyunsaturated substrate availability, or metabolize the hydroperoxide products. The benefit of added LOX can therefore be modulated by the presence of these enzymes and/or the products of their catalysis (Casey and others 1999). In addition to volatile off-flavor production, and possible reaction modulation by secondary reactions, soybeans are considered a major food allergen warranting labeling of its use (Astwood and others 1996; Taylor and Hefle 2006; Ballmer-Weber and others 2007). These limitations could be circumvented with the use of recombinant enzyme

technology. The use of recombinant enzymes could overcome the allergenicity of soy, by production of LOX in the absence of soybean flour. Among others, heterologous expression of soybean LOX-1 (Steczko and others 1991), soybean LOX-3 (Kramer and others 1994), rice LOX-2 (Shirano and Shibata 1990), *Arabidopsis thaliana* LOX-1 (Kaye Peterman and others 1994), pea seed LOX-2 and 3 (Hughes and others 1998), barley seed LOX-2 (Vörös and others 1998), and lentil LOX-1 (Hilbers and others 1996), along with stress-induced LOX from potato (Royo and others 1996) and rice (Peng and others 1994) have been successfully achieved (Hughes and others 1998). However, many of these proteins have been expressed as fusion or truncated proteins for use in mechanistic studies. Furthermore, application of recombinant soy LOX proteins to baking may be difficult since synergism exists between these LOX isozymes. The positive effects conveyed by enzyme-active soybean flour are therefore reliant on the presence of all the LOX isozymes. LOX isozymes, which are capable of carotenoid bleaching and dough strengthening, from other sources should therefore be evaluated. The group of Zhang (2013) succeeded in expression and purification of LOX from *Anabaena* sp. PCC 7120 in *Bacillus subtilis*. Following on these results, the authors showed that a single recombinant enzyme could be used for the improvement in both whiteness and loaf volume, as compared to benzoyl peroxide and potassium bromate. This study illustrated the potential of recombinant enzyme technology. However, this study did not compare their results against enzyme-active soy flour as an improver but against benzoyl peroxide. No conclusions for its effectiveness when compared against this standard, could therefore be reached. LOX isozymes from other sources may therefore be better suited to the baking industry.

Conclusion

The LOX enzyme family is diverse and isozymes often differ in terms of activity, substrate specificity, and product formation, even in the same organism. Although enzyme-active soybean flour has been used for its improving effect on dough rheology and crumb color for about a 100 y, its use remains limited to 0.5% due to off-flavor production and allergenicity. Recombinant enzyme technology offers a solution to these problems. However, due to synergy of soybean LOX isozymes a single recombinant soybean LOX may not be feasible as an improving agent. A single recombinant LOX enzyme capable of bleaching and rheological improvement is therefore required. Various authors have succeeded in the isolation and purification of both native and recombinant LOX enzymes. However, of these only a few have been evaluated as active ingredients in baking, and very few studies resulted in significant improvement. Therefore, although much work has been done to gain a better understanding on the mechanism whereby LOX acts as an improver, much research remains to be done to elucidate the complex role LOX plays as an improver during baking.

Authors' Contributions

Stefan Hayward is the main author responsible for critical review of the relevant literature and preparation of review manuscript.

Dr. Tertius Cilliers played an active role during the literature review phase of this paper as well as during the preparation of the manuscript.

Prof. Pieter Swart initiated the project, guided it along the way, and contributed to the preparation of the manuscript.

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