Synthesis of anti-inflamamtory lipid mediators using soybean flour lipoxygenase

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Enzymatic synthesis of anti-inflammatory lipid mediators using soybean flour lipoxygenase

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List of Abbreviations

SPM	specialised pro-resolving mediator
Rv	resolvin
PD	protectin
LX	lipoxin
Ma	maresin
LOX	lipoxygenase
COX	cyclooxygenase
P450	cytochrome P450
sfLOX	soybean flour lipoxygenase
SBF	soybean flour
SBM	soybean meal
LC-PUFA	long chain polyunsaturated fatty acid
FFA	free fatty acid
EFA	esterified fatty acid
FAME	fatty acid methyl ester
TAG	triacylglycerol
TL	trilinolein
DHA TAG	tridocosahexaenoin
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
AA/ETE	arachidonic acid/eicosatetraenoic acid
DPAn-3	omega-3 docosapentaenoic acid
DPAn-6	omega-6 docosapentaenoic acid
LA	linoleic acid
LA ME	linoleic acid methyl ester
ALA	alpha linolenic acid

GLA	gamma linolenic acid
PL	phospholipid
PC	phosphatidylcholine
17-HDHA	17-hydroxy docosahexaenoic acid
18-HEPE	18-hydroxy eicosapentaenoic acid
17-H(p)DHA	17-hydro(pero)xy docosahexaenoic acid
7S,17S-diH(p)DHA	7S,17S-dihydro(pero)xy docosahexaenoic acid
10S,17S-diH(p)DHA	10S,17S-dihydro(pero)xy docosahexaenoic acid
13-HLA	13-hydroxy linoleic acid
MGDG	monogalactosyl diacylglycerol
IL-1β	interleukin 1 beta
TNF-α	tumor necrosis factor-alpha
NP-HPLC	normal phase-high performance liquid chromatography
RP-HPLC	reversed phase-high performance liquid chromatography
DAD	diode array detector
GC-MS	gas chromatography-mass spectrometry
UV-vis	ultra violet-visible spectrophotometry
NMR	nuclear magnetic resonance

ABSTRACT

Specialised pro-resolving mediators (SPMs), especially resolvins and protectins, have potential to be used as pharmaceuticals and supplements for the treatment and prevention of numerous chronic diseases linked to inflammation, as they are found naturally in the body and are involved in the active resolution of inflammation. My research investigates for the first time, a simple and inexpensive method to produce bioactive resolvin and protectin analogues through the direct use of full fat enzyme-active soybean flour (SBF) as an enzyme source with a variety of long chain polyunsaturated fatty acid (LC-PUFA) substrates. This simple method can be used as an alternative to expensive commercial lipoxygenases. A range of substrates including free LC-PUFAs, triacylglycerols (TAGs) and fish oils (a complex lipid mixture of TAGs) were investigated.

The enzymatic synthesis of resolvin and protectin analogues from omega-3 (n-3) and omega-6 (n-6) free fatty acids using SBF lipoxygenase (sfLOX) was studied, using docosahexaenoic acid (DHA) as a model compound. The sfLOX-catalysed DHA reaction generates bioactive resolvin D5 (RvD5) and protectin DX (PDX). Trilinolein (TL) was used as a model triacylglycerol and tuna oil was tested as a complex lipid mixture, in reactions catalysed by sfLOX. The reactions were optimised to obtain the maximum amount of mono- and di-hydroxylated compounds (DHA and tuna oil as substrates) and tri-hydroxylated molecules (TL as a substrate). The products formed under optimised conditions were characterised using a variety of analytical techniques such as NP-HPLC, RP-HPLC, chiral RP-HPLC, GC-MS, UV-visible and NMR spectroscopy.

The optimised conditions found for the sfLOX-catalysed DHA reaction were applied to other n-3 and n-6 LC-PUFAs. A comparison between commercial 15-sLOX-1 and

sfLOX-catalysed reactions with DHA for making resolvin and protectin analogues was included. sfLOX shows the same efficiency per milligram substrate in synthesising anti-inflammatory and/or pro-resolving lipid mediator analogues from LC-PUFAs as commercially available 15-sLOX-1. These results have offered a potential method for the production of bioactive compounds from the direct use of a cheap raw enzyme source.

The optimised reaction conditions for the sfLOX-catalysed tuna oil reaction were also applied to tridocosahexaenoin (DHA TAG) and anchovy oil substrates. It was found that sfLOX could act directly on these complex esterified n-3 LC-PUFAs substrates (tuna oil, DHA TAG and anchovy oil), without prior hydrolysis (enzymatic or chemical), which cannot be achieved using commercial 15-sLOX-1. This would be useful for biotechnology applications in separating components of complex lipid mixtures after selective oxygenated of PUFAs by sfLOX. TAGs containing hydroperoxylated EPA and DHA could be easily separated from the remaining mixture of unreacted saturated and mono unsaturated fatty acids based on differences in polarity. The hydroperoxylated compounds could then be used for pharmaceutical and nutraceutical applications, whilst the remaining saturated and monounsaturated fatty acids could be used for the production of biofuel.

This work is the first time sfLOX was used to catalyse a reaction with biologically important C20-22 LC-PUFAs (DHA, EPA, AA, DPAn-3, and DPAn-6) and to synthesise di-hydroxylated compounds as SPM analogues. The bioactivity of the synthesised compounds was tested *in vitro* in human monocytic cells (THP-1) and mouse brain cells (C8-B4). Most of these compounds have shown potential anti-inflammatory properties by reducing IL-1β and TNF-α levels in LPS stimulated cells. Using SBF as an inexpensive source of lipoxygenase has the potential for application

to large-scale SPM production for commercial nutraceuticals and pharmaceuticals. SBF has been demonstrated to be more versatile than a commercial enzyme in producing the same amount of RvD5 and PDX per milligram of DHA and being able to react with esterified fatty acids (pure substrates and natural oils).

PUBLICATION

Hoang-Anh T. Tu, Eleanor P. Dobson, Luke C. Henderson, Colin J. Barrow and Jacqui L. Adcock 'Soy flour as an alternative to purified lipoxygenase for the enzymatic synthesis of resolvin analogues', *New BIOTECHNOLOGY*, 41 (2018) 25-33.

Manuscripts in preparation

Hoang-Anh T. Tu, Tim D. Nalder, Colin J. Barrow and Jacqui L. Adcock 'Investigating soy flour lipoxygenase activity on esterified fatty acids and fish oils without prior hydrolysis', *Manuscript in preparation*

Hoang-Anh T. Tu, Chiara C. Bortolasci, Ken R. Walder, Colin J. Barrow and Jacqui L. Adcock 'Bioassays of potential anti-inflammatory lipid mediators from n-3 and n-6 LC-PUFAs enzymatically synthesised by soybean flour lipoxygenase', *Manuscript in preparation*

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CHAPTER I

INTRODUCTION

According to the World Health Organisation, cardiovascular diseases are the leading cause of death worldwide with 17.9 million deaths in 2016 [1]. Cardiovascular diseases are also the cause of 27% of all deaths in Australia, as reported by the Heart Foundation Australia in 2017, with one Australian dying due to cardiovascular diseases every 12 minutes [2]. Cardiovascular diseases are a group of diseases related to heart and blood vessels. The initiation and development of cardiovascular diseases are proved to be strongly linked to inflammation. A number of pro-inflammatory cytokines including TNF-α and IL-6 have been found to be associated with an increase of cardiovascular disease risk. C-reactive protein can be used as an effective downstream inflammatory biomarker to detect cardiovascular diseases [3-5]. Many other chronic diseases such as diabetes, Alzheimer's disease and cancer are also known to be associated with inflammation. Resolving inflammation could be the key factor in preventing cardiovascular diseases and other inflammatory diseases. Anti-inflammatory drugs using natural agents with minimal side effects are promising for the treatment of inflammatory diseases like cardiovascular diseases. Specialised pro-resolving mediators (SPMs) are anti-inflammatory and pro-resolving lipid mediators, produced endogenously in the body in the active resolution of inflammation [6]. Synthesised SPMs could be used as anti-inflammatory drugs and supplements for the potential treatment and prevention of a range of chronic diseases linked to inflammation. Development of an economical and effective method for the synthesis of SPMs will enable the large-scale industrial production of SPMs for pharmaceuticals, nutraceuticals, and functional foods to fight inflammation.

1. Resolution of inflammation and specialised pro-resolving lipid mediators

Inflammation is a protective response which is controlled primarily by the innate immune system of the human body. It is an essential biological process that protects the host from attack by harmful stimuli, which may be exogenous or endogenous, and ideally results in elimination of the pathogens and healing of the injured tissues [7]. As such, inflammation is a necessary process for wound healing. One of the first steps in the inflammatory response to microbial invasion and tissue injury is the production of pro-inflammatory lipid mediators, known as leukotrienes and prostaglandins, along with other chemical mediators such as cytokines and chemokines [8]. The release of these mediators results in acute inflammation, with specific symptoms including heat, redness, swelling, pain, and loss of tissue function [9, 10]. Acute inflammation is followed by other signalling cellular processes that are initiated in order to protect the host from damaged tissue and infection [6]. This phase should last for a few hours or days [7]. Specifically, white blood cells – mainly neutrophils – are recruited to the inflamed tissues to cope with the invaders. They release chemical compounds of reactive oxygen species such as hydroxyl radicals and hydrogen peroxides to destroy and digest harmful stimuli [11]. After the acute inflammatory phase, resolution of inflammation, abscess formation, scar formation, and chronic inflammation are all possible outcomes [9, 10, 12]. Resolution of inflammation is the ideal outcome of acute inflammation, resulting in a return to a non-inflammatory state for host cells, with benefits of tissue recovery and stimulus elimination [7, 9, 12]. In contrast, uncontrolled or prolonged acute inflammation develops into an unresolved condition called chronic inflammation, an undesirable outcome of inflammation, with an increase in pro-inflammatory mediator generation [9]. Chronic inflammation is associated with a wide range of diseases [6, 12] such as

diabetes, asthma, cardiovascular diseases, inflammatory bowel disease as well as neurological disorders (Alzheimer's disease, etc.) [6] and the progression of cancer [7, 13]. Therefore, anti-inflammatory drugs have received tremendous consideration in clinical medicine [7]. Research into new anti-inflammatory compounds is needed as many current drugs have a range of side-effects and some such as Vioxx have even been removed from the market due to serious side effects [14, 15].

The current treatment for inflammation with nonsteroidal anti-inflammatory drugs is based on blocking pro-inflammatory mediator pathways, but it is not entirely effective as many of the enzymes involved triggering inflammation are also involved in the resolution of inflammation, and therefore resolving pathways are also inhibited. This causes side effects related to inflammation such as increased risk of cardiovascular symptoms [7]. Another approach for the treatment of chronic inflammation is to target compounds which initiate the resolution pathways [6, 7, 16]. The resolution of inflammation is not simply a passive process where production of pro-inflammatory mediators decreases over time when the harmful stimuli are removed. Resolution of inflammation has been demonstrated to involve the active biosynthesis of lipid mediators which can switch off the acute inflammatory state. These bioactive compounds are anti-inflammatory and/or pro-resolving compounds, which can (among other things) stimulate the clearance of neutrophils (polymorphonuclear leukocytes) by apoptosis and then recruit monocytes for phagocytosis of apoptotic cells and microbes [9, 12]. They are a family of compounds termed the specialised pro-resolving mediators (SPMs) and are divided into different groups including lipoxins, resolvins, protectins, and maresins. [6, 9, 17-19]. The active biosynthetic process of resolution is currently studied by investigating self-limited inflammatory exudates and tissues enriched with docosahexaenoic acid (DHA) [9, 19]. The SPMs derived from the omega-3 long

chain polyunsaturated fatty acids (n-3 LC-PUFAs), eicosapentaenoic acid (EPA) and DHA, were first discovered in resolving inflammatory exudates and DHA-enriched tissues in the early 2000s [20-22]. Since then, a range of new SPMs have been continuously discovered. As a result, these SPM compounds have emerged as new fascinating drug candidates with therapeutic potential for inflammatory diseases [23, 24].

2. Endogenous metabolism of fatty acids into SPMs

Linoleic acid (LA) and alpha-linolenic acid (ALA) are the precursors of omega-6 and omega-3 LC-PUFAs (n-6 and n-3 LC-PUFAs), respectively, and are essential for good health. However, they cannot be synthesised in the body and must be obtained from the diet [25]. These fatty acids are synthesised in plants and marine algae containing delta 12- and delta 15-desaturase enzymes, which are necessary for the synthesis of n-6 and n-3 LC-PUFAs and do not exist in animals [26]. From LA and ALA, other n-6 and n-3 LC-PUFAs can be synthesised with more double bonds and carbons through desaturation and elongation processes catalysed by desaturase and elongase enzymes (Figure 1.1) [25]. Fatty acids can be labelled using a numbering system that indicates the number of carbons, the number of double bonds, and the unsaturation subclass (eg. omega-3 or omega-6) which is the position of the first double bond counted from the methyl end of a fatty acid molecule. For example, linoleic acid is labelled as 18:2n-6, indicating that it contains 18 carbons and 2 double bonds, and is an omega-6 fatty acid (the last double bond is located six carbons from the methyl end).

Omega 6

18:2n-6
Linoleic acid (LA)
↓

18:3n-6
Gamma-Linolenic acid (GLA)
↓

20:3n-6
Dihomogammalinolenic acid
↓

20:4n-6
Arachidonic acid (AA)
↓

22:4n-6
Adrenic acid
↓

22:5n-6
Docosapentaenoic acid (DPAn-6)

Delta-6
Desaturase

Elongase

Delta-5
Desaturase

Elongase

Delta-4
Desaturase

Omega 3

18:3n-3
Alpha-Linolenic acid (ALA)
↓

18:4n-3
Stearidonic acid
↓

20:4n-3
Eicosatetraenoic acid
↓

20:5n-3
Eicosapentaenoic acid (EPA)
↓

22:5n-3
Docosapentaenoic acid (DPAn-3)
↓

22:6n-3
Docosahexaenoic acid (DHA)

Figure 1.1: Synthesis of n-6 and n-3 LC-PUFAs from LA and ALA precursors, respectively [25].

EPA (20:5n-3) and DHA (22:6n-3), downstream products of ALA bio-metabolism, are important to the maintenance of good health for both foetal development and healthy aging. EPA and DHA are also beneficial to the prevention of many diseases such as Alzheimer's disease, cardiovascular disease and cancer [27, 28]. Although EPA and DHA are beneficial, the mechanism of action is still unclear. The Serhan research group was the first to propose that their role as precursors for the biosynthesis of potent SPMs was likely explain their benefits in human health [6].

In the human body, the conversion of ALA to EPA and DHA is not efficient, therefore EPA and DHA need to be obtained from the diet; the best source is oily fish [26]. There are various recommendations for omega-3 intake from different countries and organisations, ranging from 300 to 4000 mg per day depending on health status [29]. For example, it has been recommended that the minimum amount of n-3 LC-PUFAs (EPA and DHA) consumption per day should be approximately

500 mg in human nutrition in order to prevent clinical symptoms of deficiency [29, 30]. However, 1 g of DHA and EPA should be consumed by patients with coronary disease [29]. Many people do not have an adequate intake of omega-3 PUFAs naturally in their diets, so supplementation (typically with fish oils) is required to reach the recommended daily intake. Fish oil supplements with up to 90% EPA and DHA can be produced from natural fish oils which usually contain approximately 30% EPA and DHA [31]. Therefore, the global omega-3 market has continued to develop, and is now a multi-billion dollar industry, estimated to be valued at USD $9.94 billion in 2015. It is predicted to grow at an annual rate of 13.8% from 2015 to 2020 [32].

The parent n-6 and n-3 LC-PUFAs compete for the same enzymes (desaturases and elongases) to synthesise the downstream fatty acids. Therefore, the synthesis of n-6 LC-PUFAs depends not only on the consumption of LA but also on ALA, and similarly, the synthesis of n-3 LC-PUFAs depends on the consumption of both LA and ALA. The downstream products of n-6 LC-PUFAs (mainly from AA) are pro-inflammatory, whereas n-3 LC-PUFA metabolites, particularly from EPA and DHA, shows primarily anti-inflammatory and pro-resolving properties [25]. Appropriate n-6 and n-3 LC-PUFA consumption could benefit the body by balancing between acute inflammation and resolution of inflammation, and preventing the onset of chronic inflammation. Therefore, the dietary ratio between omega-6 and omega-3 fatty acids is important in maintaining good health. There are many recommendations for this ratio from different studies and for various health conditions [29]. It has been generally suggested that a ratio of approximately 4:1 n-6 to n-3 LC-PUFAs may be ideal for good health, instead of the 10:1 ratio common in modern western societies [29, 30].

As the biologically produced SPMs are active *in vivo* at low concentrations, typically in the pico to nanomolar range [6], supplementation with very small amounts of SPMs could be an alternative to n-3 fish oil supplementation, which requires much higher doses due to lower levels of activity. SPMs could be consumed directly for the prevention and/or treatment of inflammatory diseases. As these compounds have potential pharmaceutical and nutritional utility, effective, simple and inexpensive methods to produce these bioactive molecules would be commercially useful.

3. Lipoxygenase as a biocatalyst for the synthesis of SPM analogues

Typically, pharmaceuticals are synthesised using complicated and time-consuming chemical reactions, often using harmful processes and chemicals [33, 34]. Similarly, SPMs have been synthesised in the same way. For example, resolvins D2, D5 and D6 have been produced by total organic synthesis using lengthy protocols (approximately 20 steps), with low total reaction yields (less than 10%), and utilising toxic substances such as aluminium lithium hydride, lithium hydroxide, benzene and hexane [35-37]. Neuroprotectin D1 analogues were produced by total organic synthesis of more than 10 steps with each step requiring 1 – 22 hour reaction times [38]. Other resolvins, protectins, and maresins have also been chemically synthesised [39-41]. In contrast, using enzymes as biocatalysts has a number of advantages, including operating at mild pH, temperature and pressure; high biodegradability; and low production of by-products requiring heavy downstream processing [42]. Enzyme reactions can also synthesise products with higher purity and higher yields using simple techniques compared to organic synthesis, and can often be applied to a wide range of substrates without re-optimisation of reaction conditions [43]. Highly stereo- and regio-specific molecules can be readily obtained from enzyme-catalysed reactions, which is important for producing useful pharmaceuticals where chirality is

important for bioactivity [44]. Enzyme technologies have been used in biotechnology for pharmaceutical synthesis as an alternative to traditional chemical synthesis [45, 46]. Enzymes are currently well-established products in biotechnology with the global market valued at ~ USD $7 billion in 2017 and expected to increase annually by 5.7% from 2018 to 2024 [47].

Enzymatic synthesis using soybean lipoxygenase as a biocatalyst is a potential method to produce various known and novel bioactive lipid mediators in one step for pharmaceuticals, nutraceuticals, and functional foods. A commercially purified soybean lipoxygenase isoenzyme 1 which oxygenates arachidonic acid at carbon 15 (15-sLOX-1) has been widely investigated as a biocatalyst to produce hydro(pero)xy fatty acids [48-52], and in some studies, SPMs [43, 53, 54]. However, the problem with the commercial enzyme 15-sLOX-1 is the high cost of acquiring the enzyme. Purified lipoxygenase isoenzymes from soybean have been used as a lipoxygenase source but LOX-2 is unstable when purified, and the purification process can be time consuming [55, 56]. If the soybean lipoxygenase enzymes can be sourced cheaply and simply from natural materials, then low cost methods may be developed for their industrial use.

4. Research questions and project aims

Soybean flour is a commercial product used as an alternative to wheat flour in a variety of products and is available as defatted or full-fat flours [57], which could be used directly as a source of lipoxygenase. Soybean flour has been used as an alternative to the commercial enzyme in reactions with hydrolysed oils containing mainly C18 LC-PUFAs to make hydroperoxides for various purposes, such as substrates for green note compound synthesis [58-60]. Soybean flour has been shown to contain lipoxygenases able to catalyse reactions with both free fatty acids (such as

LA and ALA) and triacylglycerols (such as trilinolein) [56, 61]. However, no work has been done on C20-22 LC-PUFAs for the synthesis of resolvin analogues, despite the biological relevance of these compounds and their potential high value; and complex lipid mixtures, such as fish oils, have not been investigated as esterified fatty acid substrates with soybean flour as a biocatalyst, despite their wide availability. Soybean flour could be used as a cheap and simple alternative to the commercial 15-sLOX-1 for the synthesis of resolvin analogues from a range of biologically important LC-PUFAs for pharmaceuticals, nutraceuticals and functional foods.

This project aimed to produce bioactive specialised pro-resolving mediator analogues from a range of pure LC-PUFAs and natural oils such as fish oils using soybean flour as an inexpensive source of lipoxygenase enzymes. The project addressed the following questions:

- Can soybean flour be used directly as a natural and inexpensive source of lipoxygenase enzymes to produce a variety of potentially bioactive resolvin and protectin analogues from a range of substrates, including free and esterified LC-PUFA?

- What are the characteristics (product formation efficiency and suitability to catalyse a range of substrates) of soybean flour lipoxygenase and is it more versatile than the commercially available 15-sLOX-1?

To answer these questions, the project aims were:

1) To characterise the reaction catalysed by soybean flour lipoxygenase with DHA as a free n-3 LC-PUFA substrate model for the synthesis of resolvin and protectin analogues, with full product characterisation. Then, to apply the optimised conditions from the soybean flour lipoxygenase-catalysed DHA

reaction to a range of biologically important free LC-PUFA substrates (EPA, ARA, DPAn-3, and DPAn-6).

2) To characterise the reaction catalysed by soybean flour lipoxygenase with trilinolein as a pure esterified fatty acid model substrate without prior hydrolysis, including full product characterisation.

3) To characterise the reaction catalysed by soybean flour lipoxygenase with tuna oil as a complex lipid system, without prior hydrolysis for the synthesis of resolvin and protectin analogues.

4) To identify structure-activity relationships for the bioactivity of the synthesised resolvin and protectin analogues, using inflammatory bioassays.

5) To compare the activities of a commercial 15-sLOX-1 enzyme and soybean flour lipoxygenase.

CHAPTER II

LITERATURE REVIEW

Resolvins and protectins are biologically important anti-inflammatory and pro-resolving lipid mediators for the potential treatment and prevention of a range of inflammatory diseases including cardiovascular disease, diabetes and cancer [6, 19]. These compounds are naturally found in the resolution of inflammation in the body [6, 19]. An up to date summary of specialised pro-resolving mediators, including their classification, biosynthesis, and bioactivity is presented and their applications as supplements and pharmaceuticals are highlighted. Characteristic properties of lipoxygenases in general, and soybean lipoxygenase in particular are summarised in relation to oxidation reactions with long chain polyunsaturated fatty acids. Soybean flour (SBF) with simple preparation has been investigated in previous studies as an inexpensive alternative to commercial lipoxygenases to produce mono-hydroxylated compounds from C18 fatty acids for volatile compound synthesis and other purposes. The reactivity of different sources of plant lipoxygenases with esterified fatty acids are also discussed.

1. Classification, biosynthesis, and bioactivity of lipid mediators

Lipid mediators are hydroxylated metabolites of omega-3 and -6 long chain polyunsaturated fatty acids (n-3 and n-6 LC-PUFAs). They are endogenously synthesised by different types of enzymes – lipoxygenases (LOX), cyclooxygenases (COX) in the absence or presence of aspirin, cytochrome P450 (P450), and other enzymes. Lipid mediators are classified into two groups: pro-inflammatory lipid mediators, and anti-inflammatory and pro-resolving lipid mediators. Each lipid mediator within the two groups has distinctive functions in different stages of inflammation [6, 62].

1.1. Pro-inflammatory lipid mediators

Pro-inflammatory lipid mediators are divided into two groups of compounds – leukotrienes and prostaglandins – each with a variety of derivatives. They are mainly biosynthesised from arachidonic acid (AA, C20:4n-6) by 5-LOX and COX-2 enzymes, although other enzymes may also be involved, and they are also synthesised from eicosapentaenoic acid (EPA, C20:5n-3; Figure 2.1). Leukotrienes and prostaglandins have specific roles in the initiation of inflammation: leukotrienes encourage neutrophil (a type of white blood cells) recruitment to the injured site to engulf and destroy the foreigner agents; whilst prostaglandins accelerate the inflammatory response. Pro-inflammatory lipid mediators help protect the host from attack by outside invaders. However, when acute inflammation is left unchecked these lipid mediators can lead to the development of chronic inflammation [6, 18, 63].
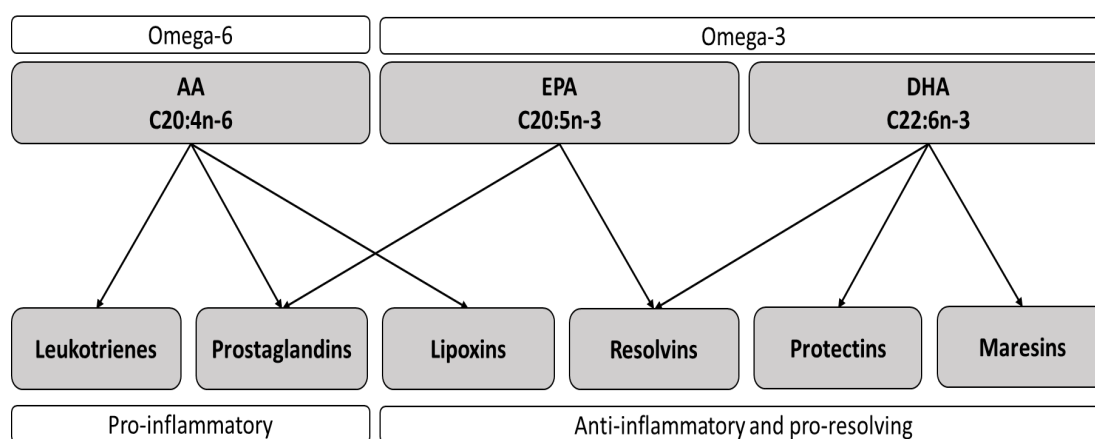
Figure 2.1: Important families of lipid mediators of inflammation and resolution, and their precursor fatty acids [43].

1.2. Anti-inflammatory and pro-resolving lipid mediators

Anti-inflammatory and pro-resolving lipid mediators are di- or tri-hydroxylated compounds synthesised from n-3 and n-6 LC-PUFAs, and contain a system of conjugated double bonds. They are known as specialised pro-resolving mediators

(SPMs) and consist of 4 subclasses: lipoxins (lipoxygenase interaction products), resolvins (resolution phase interaction products), protectins, and maresins (macrophage mediators in resolving inflammation). Lipoxins are derived from AA; resolvins can be derived from both EPA and docosahexaenoic acid (DHA, C22:6n-3); and DHA is the main precursor of protectins and maresins (Figure 2.1) [43]. Omega-3 and omega-6 docosapentaenoic acids (DPAn-3, C22:5n-3 and DPAn-6, C22:5n-6) have been recently identified as precursors of some resolvins, protectins, and maresins [64-67].

Serhan and his group are the leading research group investigating the endogenous biosynthesis and potent actions of SPMs from LC-PUFAs, with numerous studies and reviews in the field [6, 9, 16, 18, 19, 62]. They were the first to demonstrate that resolution of inflammation involves the production of SPMs and is an active process [68]. All of the known SPM compounds are summarised in Table 2.1 and categorised into their PUFA precursors and families (subclasses and intermediates). The chemical structures (stereospecificity and double bond configurations) are included. Key enzymes such as 15-LOX, 12-LOX, 5-LOX, COX-2 and P450 are known to be involved in their biosynthesis pathway [6, 17, 64, 66]. These molecules have potent actions in resolving acute inflammation and preventing the development of chronic inflammation to enable a return to homeostasis [16]. Their anti-inflammatory and pro-resolution biological activities have been shown at nano and picomolar concentrations both *in vitro* and *in vivo* [6]. At a cellular level, they can stop neutrophil infiltration (anti-inflammatory property), and accelerate monocyte recruitment and activation, followed by enhancing phagocytosis and clearance of apoptotic neutrophils by activated macrophages (pro-resolving activity) [6, 9, 18]. The biological activities of the compounds are dependent upon the chirality of hydroxy groups (*S* or *R*), geometry of double bonds (*cis* or *trans*), and their

conjugation system (conjugated diene, double conjugated dienes, conjugated triene, etc.). SPMs and aspirin-triggered SPMs have similar bioactivities, although they are synthesised through different biosynthetic pathways and have altered hydroxy group chirality. SPMs are synthesised by various LOXs and have *S* chirality, whereas aspirin-triggered SPMs are synthesised by COX-2 in the presence of aspirin or P450 and have *R* chirality [6].

Table 2.1: Classification of SPMs from AA, EPA, DHA, DPAn-3 and DPAn-6 into distinct families with stereochemical structures and double bond configurations [6, 10, 17, 35, 37, 64-67, 69, 70].

Precursor	Specialised pro-resolving mediators			
	Families		**Stereochemical structures**	**Double bond configurations**
AA	**Lipoxin**	**15S**	5*S*,6*R*,15*S*-trihydroxy-AA (LXA4) 5*S*,14*R*,15*S*-trihydroxy-AA (LXB4)	7*E*,9*E*,11*Z*,13*E* 6*E*,8*Z*,10*E*,12*E*
		15R	Aspirin-triggered LXA4 and B4 (AT-LXA4 and B4)	As above
EPA	**Resolvin E series**	**18S**	5*S*,12*R*,18*S*-trihydroxy-EPA (RvE1) 5*S*,18*S*-dihydroxy-EPA (RvE2) 17R,18*S*-dihydroxy-EPA (RvE3)	6*Z*,8*E*,10*E*,14*Z*,16*E* 6*E*,8*Z*,11*Z*,14*Z*,16*E* 5*Z*,8*Z*,11*Z*,13*E*,15*E*
		18R	Aspirin-triggered RvE1-3 (AT-RvE1 → AT-RvE3)	As above
DHA/DPAn-3/DPAn-6	**Resolvin D series**	**17S**	7*S*,8*R*,17*S*-trihydroxy-DHA (RvD1$_{DHA}$) 7*S*,8*R*,17*S*-trihydroxy-DPAn-3 (RvD1$_{DPAn-3}$) 7*S*,16*R*,17*S*-trihydroxy-DHA (RvD2$_{DHA}$) 7*S*,16*R*,17*S*-trihydroxy-DPAn-3 (RvD2$_{DPAn-3}$) 4*R*,11*R*,17*S*-trihydroxy-DHA (RvD3$_{DHA}$) 4*R*,5*S*,17*S*-trihydroxy-DHA (RvD4$_{DHA}$) 7*S*,17*S*-dihydroxy-DHA (RvD5$_{DHA}$) 7*S*,17*S*-dihydroxy-DPAn-3 (RvD5$_{DPAn-3}$) 4*S*,17*S*-dihydroxy-DHA (RvD6$_{DHA}$)	4*Z*,9*E*,11*E*,13*Z*,15*E*,19*Z* As above 4*Z*,8*E*,10*Z*,12*E*,14*E*,19*Z* As above 4*Z*,8*E*,10*Z*,13*Z*,15*E*,19*Z* 6*E*,8*E*,10*Z*,13*Z*,15*E*,19*Z* 4*Z*,8*E*,10*Z*,13*Z*,15*E*,19*Z* As above 5*E*,7*Z*,10*Z*,13*Z*,15*E*,19*Z*
		17R	Aspirin-triggered RvD1-D6 (AT-RvD1 → AT-RvD6)	As above
	Resolvin T series	**13R**	7,13*R*,20-trihydroxy-DPAn-3 (RvT1) 7,12,13*R*-trihydroxy-DPAn-3 (RvT2) 7,8,13*R*-trihydroxy-DPAn-3 (RvT3) 7,13*R*-dihydroxy-DPAn-3 (RvT4)	
	Protectin	**17S**	10*R*,17*S*-dihydroxy DHA (PD1$_{DHA}$) 10*R*,17*S*-dihydroxy DPAn-3 (PD1$_{DPAn-3}$) 16,17-dihydroxy DPAn-3 (PD2$_{DPAn-3}$)	4*Z*,7*Z*,11*E*,13*E*,15*Z*,19*Z* As above 7*Z*,10*Z*,12*E*,14*E*,19*Z*
			10*S*,17*S*-dihydroxy DHA (PDX$_{DHA}$) 10*S*,17*S*-dihydroxy DPAn-6 (PDX$_{DPAn-6}$)	4*Z*,7*Z*,11*E*,13*Z*,15*E*,19*Z* As above
	Maresin	**14S**	7*R*,14*S*-dihydroxy-DHA (MaR1$_{DHA}$) 7*R*,14*S*-dihydroxy-DPAn-3 (MaR1$_{DPAn-3}$) 13*R*,14*S*-dihydroxy-DHA (MaR2$_{DHA}$) 13*R*,14*S*-dihydroxy-DPAn-3 (MaR2$_{DPAn-3}$) 14,21-dihydroxy-DPAn-3 (MaR3$_{DPAn-3}$)	4*Z*,8*E*,10*E*,12*Z*,16*Z*,19*Z* As above 4*Z*,7*Z*,9*E*,11*E*,16*Z*,19*Z* As above 7*Z*,10*Z*,12*E*,16*Z*,19*Z*

Specific potent actions and further information on several well-studied SPMs in each group are also described below.

1.2.1. Lipoxins

Lipoxins, trihydroxytetraene fatty acids synthesised from AA, were the first mediators discovered with dual actions of anti-inflammation and pro-resolution confirmed both *in vitro* and *in vivo* [17]. For example, lipoxin A4 (LXA4) helps reduce organ fibrosis, and has a direct action on both vascular and smooth muscle and in reducing pain [17, 63].

1.2.2. Resolvins

There are three types of resolvins termed E-series, D-series, and T-series. E-series (RvE) and T-series (RvT) resolvins are derived from EPA and DPAn-3 respectively, whereas D-series resolvins are from either DHA or DPAn-3 (Table 2.1). There are three resolvins (RvE1, RvE2 and RvE3) in the resolvin E-series. RvE1 (Figure 2.2) containing one conjugated diene and one conjugated triene can specifically support tissue healing [24]. RvE2 and RvE3 show potent anti-inflammatory actions, by blocking or limiting neutrophil infiltration in zymosan-induced peritonitis both *in vitro* and *in vivo* at nanomolar concentrations, with much lower activity expresses by the unnatural stereoisomers [69, 71]. The newest E-series resolvin (RvE3) is anti-inflammatory but has not been confirmed to be pro-resolving like RvE1 and RvE2 [68].

D-series resolvins derived mainly from DHA receive considerable interest because DHA is enriched in the brain, synapses, and retina. These compounds can block the production of the pro-inflammatory cytokine interleukin 1 beta (IL-1β) as a rapid response to neural injury in microglial cells [17], and can also reduce pain and

release depression [68]. RvD1 from precursor DHA (RvD1$_{DHA}$) has a conjugated

tetraene (Figure 2.2) and especially helps promote tissue repair [24].

The T-series resolvins synthesised from DPAn-3 were discovered recently [65].

Their chemical structures have been proposed without full characterisation [64, 65].

These compounds are synthesised by COX-2 and the enzyme is stimulated by

atorvastatin via S-nitrosylation [64, 66]. They were found to suppress neutrophil

infiltration and expression of IL-1β, stimulate bacterial phagocytosis and increase

survival of *E. coli*-infected mice [64, 66].

Resolvin E1   **Resolvin D1**
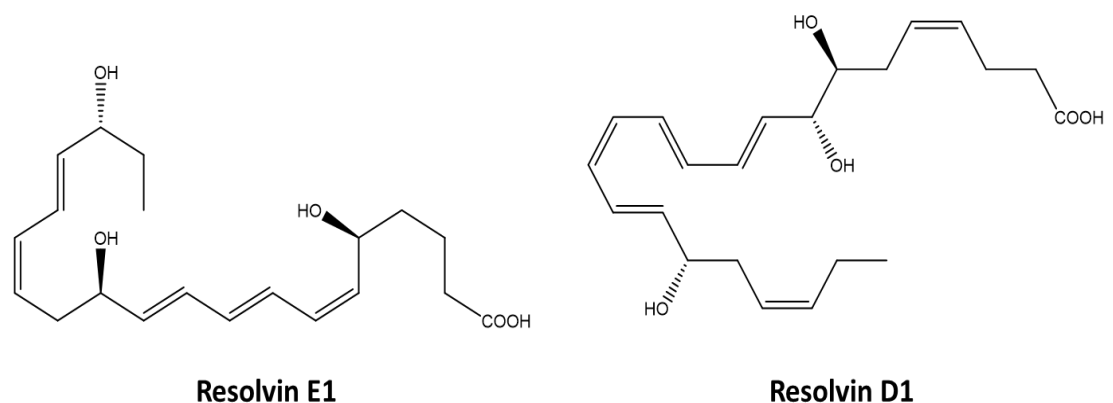
Figure 2.2: EPA-derived RvE1 and DHA-derived RvD1 [17].

1.2.3. Protectins and maresins

Protectins can be derived from DHA, DPAn-3 or DPAn-6 (Table 2.1). Protectin D1

(PD1$_{DHA}$, also known as neuroprotectin D1$_{DHA}$ (NPD1$_{DHA}$) when found in the brain)

can protect brain and retina tissues from oxidative stress-induced apoptosis by

promoting the expression of anti-apoptosis proteins and suppressing pro-apoptosis

proteins [72]. In Alzheimer's disease, PD1$_{DHA}$ promotes the survival and function of

brain cells [73]. PDX$_{DHA}$ is an isomer of PD1$_{DHA}$, with S stereo-specificity at carbon

C10 instead of R and double bond geometry between carbons C10 and C17 of *E,Z,E*

(PDX$_{DHA}$) instead of *E,E,Z* (PD1$_{DHA}$). PDX$_{DHA}$ was found to be less potent than

PD1$_{DHA}$ in resolving inflammation, but able to inhibit the aggregation of blood

platelets [74].

Maresins derived from DHA are produced by macrophages in the resolution of inflammation and have a direct action on phagocytes in enhancing efferocytosis [24]. Maresin R1 (MaR1) has higher bioactivity than MaR2. MaR1 in particular can stimulate tissue regeneration and reduce regeneration time [10].

D-series resolvins, protectins and maresin are also derived partly from DPAn-3 and DPAn-6. They are newer members of the SPM super-family. These compounds (separately or in mixtures) have also been shown to have anti-inflammatory and/or pro-resolving activities in several disease models [66, 67, 75]. For example, a 3:1 mixture of RvD1 and RvD2 from the DPAn-3 precursor can decrease neutrophil infiltration and enhance phagocytosis of zymosan and apoptotic neutrophils in the zymosan peritonitis model [66]. PDX_{DPAn-6} (DPAn-6 precursor) was found to suppress leukocyte migration significantly in the inflammatory response induced by tumor necrosis factor-alpha (TNF-α) in an air pouch model [67].

2. Resolvin analogues as supplements and pharmaceuticals

2.1. Supplements

Recently, resolvin analogues have been used in a commercial dietary supplement. Metagenics is the first company to commercialise SPMs as a nutraceutical product in collaboration with the Serhan group [76]. Metagenics launched OmegaGenics SPM Active in October 2015 as a dietary supplement for immune health to protect the body from environmental conditions such as unhealthy diet, stress, exercise, irritants, and aging. The product is served as softgels with active ingredients listed as: SPM compounds (250 – 500 mg) including 17-hydroxy-docosahexaenoic acid (17-HDHA) and 18-hydroxy-eicosapentaenoic acid (18-HEPE) fractionated from fish oils (anchovy and sardine). Metagenics is holding a patent-pending technology for advanced fractionation, termed Active IR to isolate, measure and verify the active

SPMs (17-HDHA and 18-HEPE) from fish oils to obtain a standardised material for the global healthcare practitioner market [76]. In March 2017, a clinical trial (Identifier: NCT03095157), sponsored by Brigham and Women's Hospital, was planned to investigate the role of the supplement in preventing chronic pain development up to 3 months after thoracic surgery. The study was estimated to be completed in July 2019, but no further updates have been reported since it was first posted [77].

2.2. Potential anti-inflammatory drugs

Resolvin analogues have been tested in humans for a wide range of inflammatory diseases. Resolvins were tested in clinical trials by Resolvyx Pharmaceuticals as a new class of medicines for the treatment of inflammatory diseases such as dry eye, retinal disease, asthma, inflammatory bowel diseases, rheumatic arthritis, cardiovascular diseases and lung inflammation [78]. RvE1 (RX-10001) was found to have positive Phase I clinical trial results in asthma, inflammatory bowel diseases, rheumatic arthritis and cardiovascular diseases. Phase II results showed that RX-10001 significantly improved signs and symptoms of corneal inflammation [78, 79].

RX-10045, a synthetic resolvin analogue of RvE1, successfully completed a Phase II study in treating dry eye to improve signs and symptoms and was under a Phase III clinical trial [78-80]. RX-10045 is still being improved for continuous Phase III testing in dry eye clinical trials due to having non-equivalent efficacy in Phase II results compared to that *in vivo* [81, 82]. This compound was also tested in various Phase II clinical trials for ocular conditions (Identifiers: NCT01639846 and NCT02329743) [83] but no further results have been released after completion. This product is developed by Resolvyx Pharmarceuticals with Celtic Therapeutics as a partner [78] and has been given the name navamepent [84].

PD1 (RX-20001) was under pre-clinical testing, for the treatment of multiple diseases, including dry eye, macular degeneration, lung inflammation and diabetic retinopathy. Other resolvin analogues for multiple indications were also under pre-clinial trial studies [78]. NPD1/PD1 was also tested in a clinical development program of Anida Pharma Inc., licensed by Brigham and Women's Hospital/Partners Health Care for neurodegenerative diseases and hearing loss [10, 18]. LXA4 was shown to have positive results on infants with eczema without adverse effects and on asthmatic patients [80, 83, 85]. Further results or present status for all these compounds have not been released. No SPMs have been made available in the pharmaceutical market.

As SPM compounds in general, and resolvin and protectin analogues in particular, have the potential to treat and prevent chronic inflammatory conditions, there is a need to develop methods for the large-scale synthesis of these compounds for the pharmaceutical, nutraceutical and functional food industries, as these methods are currently not well established. A one step lipoxygenase-catalysed reaction using SBF as an inexpensive enzyme source is a promising way to approach industrial-scale production of these bioactive molecules.

3. Lipoxygenase enzymes

LOXs are valuable enzymes that act as biocatalysts in forming hydroperoxy fatty acids which are used as precursors for many applications. These hydroperoxy fatty acids are substrates for further metabolism in the presence of other enzymes to produce diverse signalling molecules such as jasmonic acid and volatile compounds in plants [86]. Jasmonic acid and volatile aldehydes contribute to the defence response and wound healing in plants [87, 88]. The volatile aldehydes such as hexanal and hexenal can be used as fresh green flavours of fruits and vegetables for

food products [89]. The enzymes have commercial application in the food industry for bleaching activities on wheat flour, noodles and rice by co-oxidation of carotenoids and PUFAs [88, 90]. However, these enzymes also result in some negative effects such as degradation of pigments, off-flavour, odour production and fatty acid oxidation which leads to the deterioration of food quality [88].

LOXs are also involved in the biosynthetic pathways of a variety of lipid mediators in animals (eicosanoids and docosanoids), which are anti-inflammatory and pro-resolving compounds [91]. These compounds are emerging as a new class of anti-inflammatory drugs because they may have insignificant side effects as previously shown no adverse effect for several compounds in clinical trials [78, 85]. In contrast, a range of side effects were reported with the current anti-inflammatory drugs [14, 15]. Therefore, synthesis of these bioactive compounds for further clinical research and potential pharmaceuticals has received considerable interest [23, 41, 67].

LOXs, an enzyme family containing a non-heme iron active site, catalyse the single and double dioxygenation reaction of PUFAs containing one or more *cis,cis*-1,4-pentadiene sites (Figure 2.3A) by adding one or more oxygen molecules to create highly regio and stereo specific hydroperoxides with one or more conjugated *cis,trans* double bonds (Figure 2.3B) [86, 90, 91]. The regio and stereo specificity of products depend on the specific substrate, LOX isomer and reaction conditions.
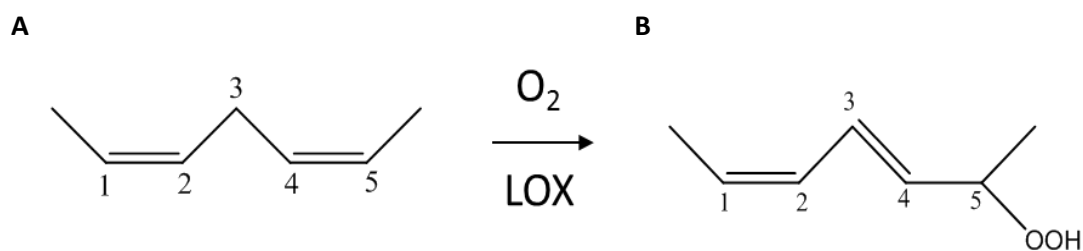
Figure 2.3: LOX catalysis of A) *cis,cis*-1,4-pentadiene unit into B) *cis,tran*-1,3-pentadienyl hydroperoxide with a conjugated diene.

3.1. Classification

LOXs are found in a variety of organisms including animals (mammals and fishes), plants and microorganisms [56, 87]. Soybean, peas, potato, cucumber, and rice have been identified as containing a diversity of LOX isoenzymes [92]. Grain legume seeds such as beans and peas, and potato tubers contain a large amount of LOX [88]. There are many ways of classifying LOX isozymes including pH optimum, positional specificity of substrate fatty acid oxygenation on linoleic acid (LA, C18:2n-6) or AA and product specificity [87, 88]. Type-1 LOX have pH optimum in an alkaline environment, whereas type-2 LOX isoforms have optimum activity at neutral pH [90]. In plants, linoleic acid is used commonly as a substrate, and 9-LOX and 13-LOX introduce molecular oxygen onto carbon atom 9 and 13 of LA substrate molecules, respectively [93] (Figure 2.4).
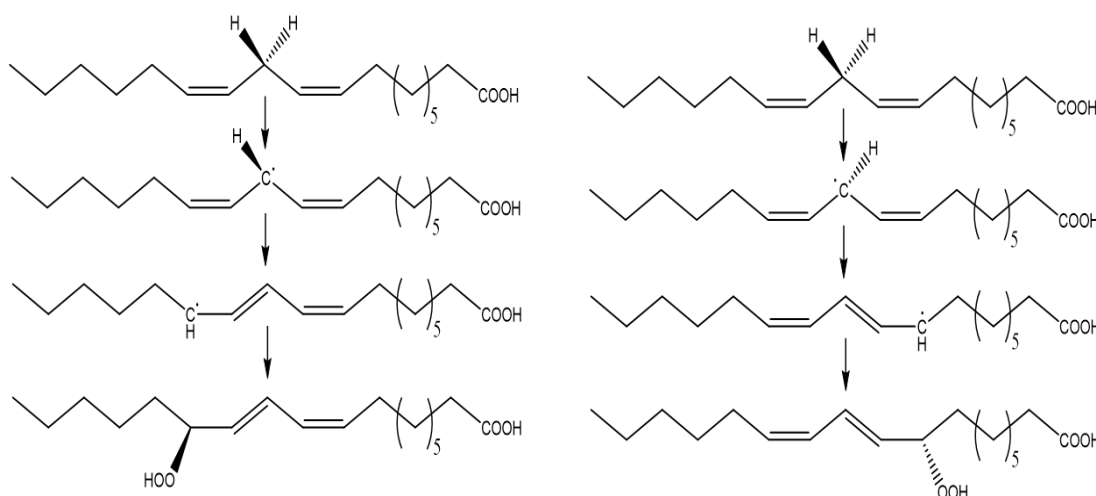
Figure 2.4: Comparison of the reaction mechanism of 13- and 9-LOX on LA (left and right, respectively) to produce 13S- and 9S-hydroperoxy products. Reproduced from [87] with modification.

In mammals, LOXs are labelled based on the specific position of oxygen insertion on the common substrate AA, such as C-5 (5-LOX), and there are six types of LOXs commonly found: 5-LOX, 8-LOX, 9-LOX, 11-LOX, 12-LOX and 15-LOX [94]. Soybean LOX, although technically classified as a 13-LOX (introduces molecular oxygen onto carbon 13 of LA), is often referred to as a 15-LOX, because it will also

accept AA as a substrate to form 15-hydroxylated AA. Soybean LOX has been used as a model LOX enzyme to study both plant and animal systems, and the naming of the enzyme reflects this. In both plants and animals, LOXs can be additionally classified by the stereo-specificity of the product (*S*/*R*) along with the positional specificity, such as 13*S*-LOX or 12*R*-LOX [87].

3.2. Mechanism

The mechanism of LOX catalysis on PUFAs to make hydroperoxides proceeds in four main steps within the catalytic pocket of the enzyme: 1) hydrogen abstraction; 2) radical rearrangement; 3) oxygen insertion; and 4) re-activation of the enzyme and proton addition (Figure 2.5) [87].
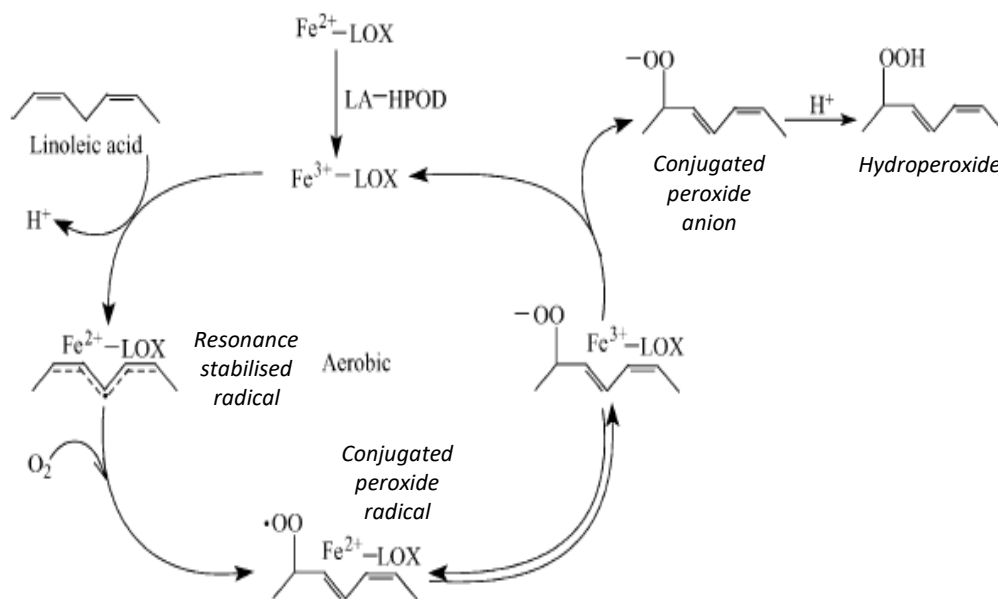
Figure 2.5: Catalytic mechanism of LOX-linoleic acid reaction under aerobic condition, following arrows starting from the middle top. Fe^{2+}-LOX: inactive state of LOX enzyme, Fe^{3+}-LOX: active state of LOX enzyme, LA-HPOD: LA hydroperoxides. The linoleic acid structure is expressed only from C8-C14. Reproduced from [95] with modification.

The iron in the active site of LOX plays an important role in the catalysed reaction. Under aerobic condition, the enzyme is in an inactive state as LOX-Fe^{2+} and is

activated by an oxidising reagent such as a lipid hydroperoxide to oxidise LOX-Fe^{2+} into LOX-Fe^{3+}. LOX-Fe^{3+} oxidises a fatty acid into a radical by hydrogen abstraction, re-forming LOX-Fe^{2+}. The oxidised fatty acid radical is resonance stabilised through radical rearrangement, and subsequently reacts with molecular oxygen at a position [+2] or [-2] from the location of hydrogen abstraction. LOX-Fe^{2+} donates an electron to the PUFA peroxide radical to form the anion and becomes the active LOX-Fe^{3+} for further catalysis. The peroxide anion is then protonated to form a hydroperoxy fatty acid containing a conjugated diene (Figure 2.5) [95, 96]. The hydrogen abstraction and oxygen insertion steps result in the specific regio- and stereo-chemistry of the products [87, 90].

3.3. Substrates

Fatty acids are mono-carboxylic acids with the aliphatic hydrocarbon chain numbered from the carboxyl end. The natural fatty acids usually have an even number of carbons (between 4 and 22) with C18 being the most common, and contain cis double bonds in their straight chains [97]. In humans and other organisms, fatty acids are a significant component of lipids, which is one of the three major constituents of biological matters along with carbohydrates and proteins. They exist in two forms: free fatty acids (FFA, Figure 2.6A) and esterified fatty acids (EFAs; eg. Figure 2.6B-D), and their main functions are energy storage and membrane structure [26]. EFAs include fatty acid methyl/ethyl esters (Figure 2.6B), triacylglycerols (TAG; Figure 2.6C), phospholipids (PL; including glycerophospholipids [Figure 2.6D] and sphingolipids) and glycolipids [98]. A fatty acid ester is formed from a carboxylic acid and an alcohol, and a TAG contains three fatty acids esterified to a glycerol backbone [98]. PLs consist of a glycerol backbone with two fatty acids and a polar head group containing phosphate [99]. The glycerol carbons of TAGs and PLs are labelled in the order of sn-1, sn-2, and sn-3 (Figure

2.6C-D). There is a stereocentre at the glycerol carbon sn-2 when R1 and R3 groups are different for TAGs [100] and R1 and R2 groups are different for PLs [99]. R1, R2 and R3 refer to aliphatic hydrocarbon chains with different length and number of double bonds.
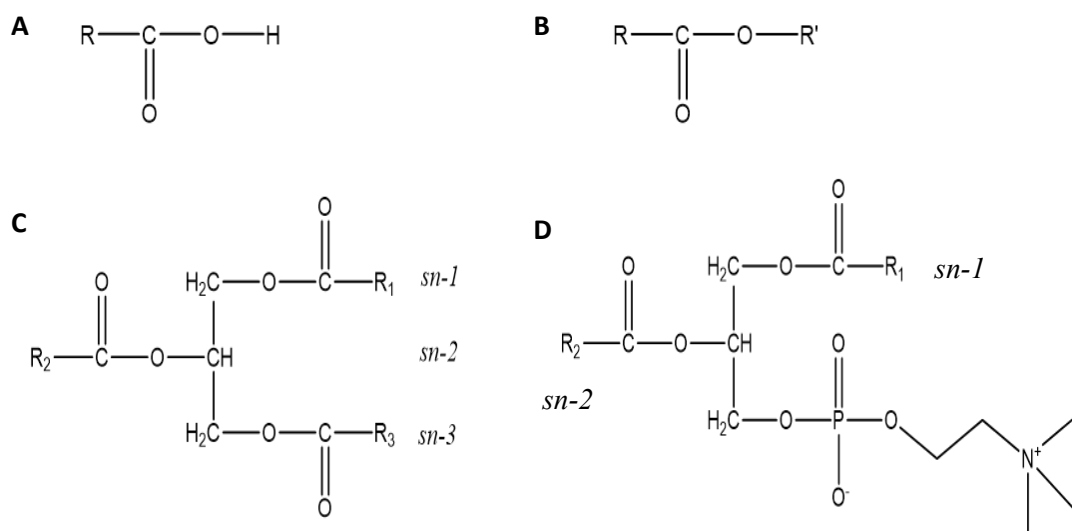
Figure 2.6: Chemical structures of A) free fatty acid (R is an aliphatic hydrocarbon chain); B) fatty acid methyl/ether ester (R': methyl/ethyl group); C) TAG with fatty acids R_1, R_2 and R_3 at the *sn-1*, *sn-2* and *sn-3* positions; and D) PL with fatty acids R_1 and R_2 at the *sn-1* and *sn-2* positions.

There are two types of fatty acids – saturated (containing no double bonds) and unsaturated (containing at least one double bond). Unsaturated fatty acids can be monounsaturated or polyunsaturated, depending on the number of double bonds (one or at least two double bonds, respectively) [98]. Polyunsaturated fatty acids are divided into two important subclasses: omega-3 and omega-6 polyunsaturated fatty acids (n-3 and n-6 PUFAs) [25]. The number 3 or 6 in their names expresses the position of the first double bond counted from the methyl end group of the fatty acid molecule [26].

TAGs and PLs (types of EFAs) are of interest as substrates for SPM formation as they are common fatty acids in natural oils. Both EPA and DHA (SPM precursors) are incorporated in TAG and PL at a significant percentage in fish and krill oils,

respectively. About 18% EPA and 12% DHA, the two major n-3 LC-PUFAs in fish oils, are present as TAGs at the *sn-2* position [31]. In krill oil, there are high concentration of n-3 LC-PUFAs (EPA and DHA) in the form of PL, which are present in a substantial amount compared to FFAs, monoacylglycerols (fatty acid monoesters of glycerol), diacylglycerols (fatty acid diesters of glycerol) and triacylglycerols [31].

4. Soybean lipoxygenases

LOX was first reported in soybean in 1932 as an enzyme for lipid peroxidation termed lipoxidase [86]. Soybean is an abundant source of LOXs and soybean LOX is well studied compared to other plant LOXs [88, 90]. There are two types of soybean lipoxygenases found in soybean seeds – type-1 LOX (LOX-1) and type-2 LOXs (LOX-2 and LOX-3) [88]. Type-1 LOX is commercially available and is used as a model LOX for mechanism studies, whereas type-2 LOX is not commercially available, due to lower activity and increased lability [56]. LOX-1 has optimum pH at 9 and only catalyses the dioxygenation of free PUFA substrates, forming 13-hydroperoxide as the major product from LA, whereas type-2 LOX isozymes have optimum pH around 7, and accept both free PUFA and TAG substrates, forming 9- and 13-hydroperoxides in a ratio of approximately 1:1 with LA as a substrate [88, 90]. As shown in a study regarding substrate specificity, LOX-2 preferred to catalyse AA at pH 6.1, whereas LOX-1 equally catalysed both LA and AA at pH 9 and the catalysis of LA by LOX-3 was more active at pH 6.5 [101]. Generally, soybean LOX-1 is best known to have exclusive *S* stereo-specificity, regardless of product regio-specificity [102-104], whereas a mixture of *S* and *R* products are formed by type-2 LOXs [105-107].

4.1. Product specificity hypotheses and mechanisms

There are two main hypotheses to explain the regio-specificity of the products (positions of hydroxy groups on products) of LOX enzymes: the active site space-filling theory and the substrate orientation in the catalytic pocket theory [87, 93]. In the space-filling theory, based on mammalian LOX research, it is proposed that either large histidine or phenylalanine residues, or small valine residues in the active site determine the depth the substrate can enter into the catalyst pocket. This results in positioning different parts of the substrate adjacent to the active site for radical rearrangement and oxygen accessibility, and therefore creating products with a different positional specificity. 13-LOX which has histidine or phenylalanine residues, and 9-LOX which has valine residues, are responsible for LA oxygenation at C-13 and C-9, respectively (Figure 2.7).
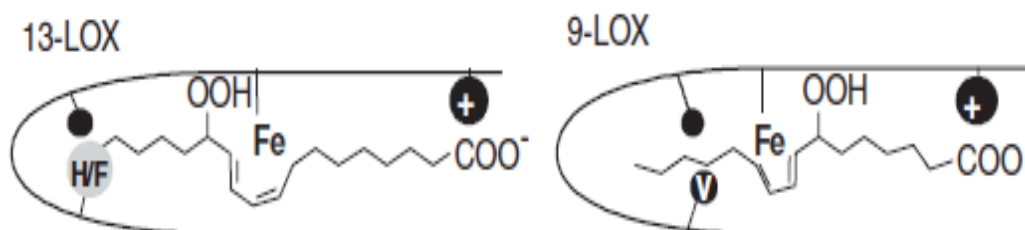
Figure 2.7: Substrate alignment in the active site of 13-LOX and 9-LOX for the space-filling theory. H/F: Histidine or phenylalanine residue, V: Valine residue. Reproduced from [93].

Alternatively, different orientation of carboxyl or methyl end of the substrate into the active site of plant LOXs may result in distinct regio-specificity of oxygenation products [87, 93, 108]. 9-LOX was proposed to orient the substrate carboxylate end first (inverse-substrate orientation) for 9-hydroperoxy LA formation, whereas 13-LOX is postulated to recognise the methyl end of the substrate (straight-substrate orientation) in the catalytic pocket for 13-hydroperoxy LA formation (Figure 2.8). This suggests only 13-LOX may be able to oxygenate EFAs as the large glycerol

backbone may be unable to penetrate the catalytic pocket, therefore esterified substrates can only be orientated with the methyl end first [108].
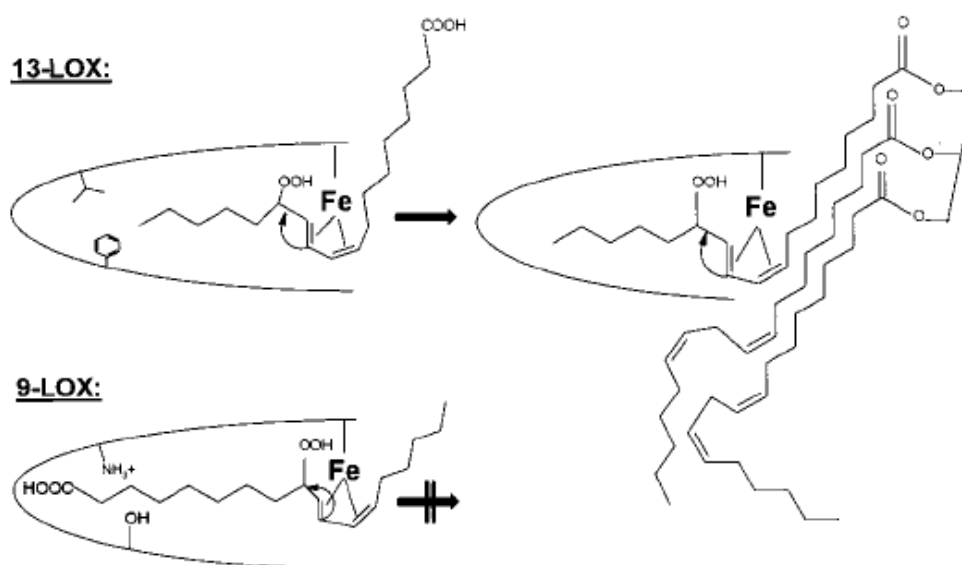
Figure 2.8: Lipoxygenase substrate orientation as methyl or carboxyl end first in the active site proposed for 13-LOX and 9-LOX. Shown with linoleic acid and trilinolein as substrate. Reproduced from [108].

In barley, 9-LOX is a type-1 LOX and 13-LOX is a type-2 LOX, giving 9- and 13-hydroperoxides, respectively. Only the type-2 LOX (13-LOX) is able to oxygenate esterified PUFAs [92], supporting the substrate orientation theory. However, in soybean 13-LOX is a type-1 LOX and only oxygenates free fatty acid substrates [88, 90], which contradicts the orientation hypothesis. Further research on substrate orientation using soybean LOX-1 (13-LOX) with mutated active site residues showed that both LA and AA substrates approach the active site of catalytic pocket using the carboxyl end first, not methyl end first [109]. This could explain why EFAs are not oxygenated by soybean LOX-1 (13-LOX), because the large glycerol backbone cannot be inserted into the catalytic pocket. As these studies are contradictive, the exact mechanism of substrate orientation for regio-specific LOX-catalysed PUFA reaction products is still not clear.

With regards to stereo-specificity, there has been some research into elucidating the mechanism using wild-type and mutant forms (where the residue alanine in the active site is mutated into glycine) of soybean LOX-1 with LA and AA substrates [48, 110]. The hydrogen with *S* stereo chemistry on C-11 of LA was identified to be involved in the step of hydrogen abstraction. *S* hydrogen abstraction is followed by oxygen insertion from the opposite side to the abstracted hydrogen to form *S* products using wild type soybean LOX-1. In contrast, both *S* and *R* products are formed with mutant soybean LOX-1 for both methyl end orientation (the first dioxygenation) and carboxylate end orientation (the second dioxygenation with AA as a substrate, and a second oxygen molecule was inserted onto the PUFAs) into the catalytic pocket [48]. For human 15*S*-LOX-2 and 12*R*-LOX, mouse 8*S*-LOX, and coral 8*R*-LOX, only one amino acid (alanine or glycine) in the active site is responsible for the stereo-specificity of hydroperoxides [110]. Alanine and glycine are conserved residues in LOX showing *S*- and *R*-activity with LA as a substrate, respectively. This is explained by both substrate orientation and the effect of shielding of the amino acid group on substrate alignment in the catalytic pocket (Figure 2.9). For *S*-LOX enzymes (Figures 2.9B and C), the methyl group of the alanine residue in the active site of the catalytic pocket shields either the C-9 or C-13 position depending on substrate orientation, so oxygen molecule is unable to access these positions, and as a result, oxygen insertion at the other side of 1,4-pentadiene unit leads to the formation of 13*S*-LOX or 9*S*-LOX products, respectively. On the other hand, without the shield effect of the alanine group (Figures 2.9A and D), 9*R*-LOX and 13*R*-LOX activities are preferentially exhibited in both substrate orientations of methyl end or carboxyl end first [87].
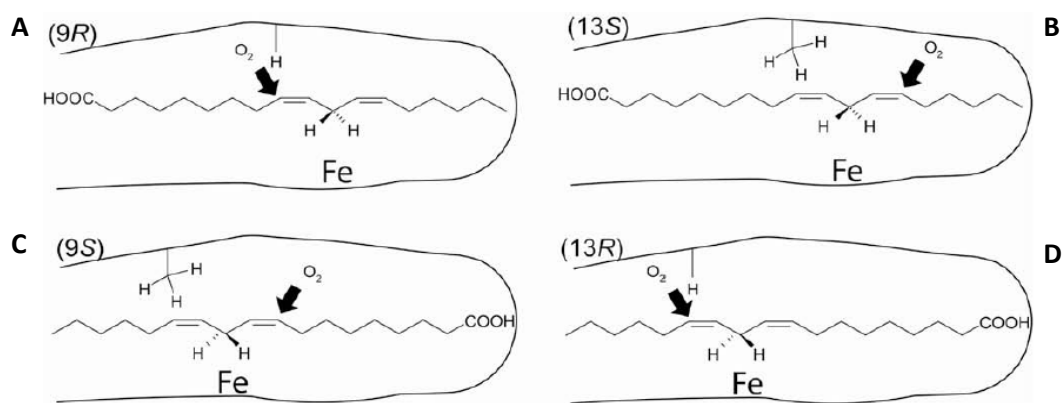
Figure 2.9: Alanine and glycine in the active site direct *S* or *R* stereo-specificity of products, respectively. B and C: for *S*-LOXs and both substrate orientation, oxygen inserts at the other side as alanine residue shields one side. A and D: for *R*-LOXs and both substrate orientation, oxygen inserts directly to the preferable side. Reproduced from [87].

4.2. Reaction conditions

LOX enzymes can be used as catalysts for the biosynthesis of lipid hydroperoxides, and a few studies have fully optimised the reaction of soybean LOX using a commercial LOX, crude SBF extract or soymeal with different substrates [43, 59, 111, 112]. pH, temperature, oxygen concentration, substrate and enzyme concentrations and ratio, and reaction time are all important variables in LOX-catalysed PUFA reactions. pH 10, air saturation and 5°C were found to be the optimised conditions for the reaction of LOX-1 from a crude SBF extract with hydrolysed safflower oil to maximise LOX-1 activity and improve product specificity [59]. The optimised conditions for a 10 mL reaction of DHA and a commercial 15-sLOX-1 were pH 9 for di-hydroxy DHA products, room temperature, no oxygenation, 15 min reaction time, 50 mM borate buffer, 0.1 mM DHA and 5 mg 15-sLOX-1. pH 12 was used to inhibit the double dioxygenation reaction in the presence of less enzyme (2 mg 15-sLOX-1) for the maximum formation of the mono-hydroxy DHA product [43]. The optimised conditions of the soymeal-catalysed reaction with soybean oil were pH 6, 1.5 g soybean oil, 2 g soymeal, 10% v/v ethanol, 2 – 3 hrs, 20°C [111]. LOX activity was found to reach a maximum

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at 1 hour and then decrease, possibly because the enzyme can be inhibited by the increase of hydroperoxides or inactivated by co-oxidation of the enzyme [111]. The optimum conditions for maximum enzyme activity of the purified type-1 on LA were ternary micellar systems with 50 µM Tween 80 and 3.5% v/v octane, or 10 µM Tween 40 and 4% v/v iso-octane, in 0.1 M Tris-HCl buffer pH 9 at 15°C and 40°C, respectively [112]. However, the reaction in an aqueous medium was found to have the greatest enzymatic catalytic efficiency [112].

Purified lipoxidase, previously known as a lipoxygenase enzyme without any other isozymes, was isolated from defatted soymeal using a lengthy method with many steps of precipitation, fractionation, dialysis and electrophoresis [113]. In a study by Holman, the purified lipoxidase-catalysed reaction was optimised using sodium linoleate as a substrate and the optimum pH was found to be at 9.4 and temperature was optimum at 30°C. In the studied temperature range of 0 to 37°C, higher temperatures gave higher initial rates but the rate slowed down over time compared to lower temperatures where it remained consistent [114]. Surrey found that purified soybean lipoxidase activity was optimum at pH 7.5 in the absence of non-ionic surfactant Tween 20, whereas the optimum shifted to lower pH at 7 in the presence of Tween 20. Enzyme activity decreased with increasing Tween 20 concentration and the optimum pH continued to shift to pH 6.5. In the presence of Tween 20, enzyme activity showed less variation than without surfactant in the pH range 6 – 7.5, with the optimum ratio of linoleate to Tween 20 of 1:1. In contrast with the purified enzyme, crude lipoxidase extract had optimum conditions at a lower pH of 6 in the presence of Tween 20 [115].

Non-ionic polyoxyethylene surfactants such as Tween 20, Triton X-100 and Lubrol were found to inhibit the activity of purified LOX-1 and LOX-2, except at linoleate concentrations above 100 µM where LOX activity was significantly inhibited by

substrate concentration and substrate aggregation [116]. Under these conditions, the reaction rate increased in the presence of surfactant with the optimum surfactant concentration at 0.2 g/L. It was proposed that the LOX isoenzymes catalyse the reaction with free linoleate as a monomeric substrate in the reaction medium, but not linoleate incorporated into surfactant micelles, and the enzyme is not stimulated directly by surfactant [116]. Tween 20 was also found to enhance the thermal stability of purified LOX-1. LOX-1 was inactive completely at 54°C at pH 9 and the midpoint of the thermal inactivation temperature increased from approximately 44°C to 48°C in the presence of 10 mM Tween 20 [117].

Other additives such as solvents, mild reducing reagents and exogenous iron ions were also used to enhance the enzyme activity in some studies. Exogenous iron (Fe^{2+} and Fe^{3+}) concentrations were optimised in the LA reaction with commercial soybean LOX, where it was shown that 0.5 µM of Fe^{3+} or 5 nM of Fe^{2+} could accelerate the initial and overall reaction rate. Their effects on the reaction could be due to Fe^{3+} rapidly converting inactive Fe^{2+}-LOX back to the active Fe^{3+}-LOX form in the aerobic catalytic cycle, or Fe^{2+} promoting oxidation of Fe^{2+}-LOX to Fe^{3+}-LOX [95]. In the presence of cysteine (at 1.5 – 2 times substrate concentration), the mild reducing agent could convert fatty acid hydroperoxides to hydroxides, eliminating product inhibition and increasing the reaction yield from 69% to 90% using a crude LOX extract from defatted SBF as the enzyme source and LA from hydrolysed safflower oil as a substrate [118].

The product specificity of LOX isozymes has been found to be affected by reaction conditions. Soybean LOX-1 is known to have 13*S*-hydroperoxy LA as the major product from LA, but product specificity decreased to form mixtures of different regio- and/or stereo-isomers at high temperature [59], low oxygen concentration [119] or at low pH [49]. Under these conditions, it was suggested that the

pentadienyl radicals could be released from the active site to react unspecifically with oxygen [49, 119]. Product specificity of LOX-2 varied significantly from about 30:70 13-hydroperoxy LA:9-hydroperoxy LA to 80:20 depending on the reaction conditions used [106, 107, 120], whereas the product ratio for LOX-3 was found to be consistent at approximately 35:65 [55, 106].

The physical state of the substrate can also affect regio- and stereo-specificity of LOXs. At high pH (> 8.5) and in the presence of Tween 20, only 13S-hydroperoxide was found to form from the reaction of soybean LOX-1 and LA [49]. This is because the carboxylate group is deprotonated and unable to insert into the hydrophobic catalytic pocket and so only methyl end substrate orientation approaches the enzyme. In contrast, at pH between 6 and 8.5, a mixture of 9S- and 13S-hydroperoxides were formed, as LOX-1 can catalyse reaction with protonated LA in both straight and inverse substrate orientations (methyl end first and head to tail end first, respectively). However, LA methyl ester cannot be dioxygenated at the C9 position, and only the 13S-hydroperoxide forms, regardless of these changes in pH [49]. The effect of substrate structure on the positional specificity of the enzyme was thoroughly investigated [119]. AA is known to be oxidised mainly at C15 by soybean LOX-1, through insertion of the methyl end at the active site. The methyl tail of AA free fatty acid and methyl ester were modified into a carboxylic acid, an alcohol, and a *tert*-butyl group to change the distance between the methyl end and the bisallylic C13 and also the polarity and bulk of the ligand to potentially induce inverse substrate binding. Iodine modification at the methyl end was also tested with the free fatty acid. These AA analogues were sonicated for 30 seconds to disperse the substrate homogeneously before starting the reaction at pH 9 by adding LOX-1. The modifications at the methyl end of the AA FFA did not change the positional specificity of 15-lipoxygenation of AA, but did reduce substrate affinity and decrease

reaction rate. Only AA methyl ester with an added hydroxy group caused formation of 5-AA (37%), 12-AA (46%) and 15-AA (17%), which suggested some inverse substrate orientation activity by LOX-1 [119].

In addition, regio-specificity of purified LOX-1 was investigated at pH 7.4 with LA and AA in phosphatidylcholine deoxycholate mixed (PDM) micelles as the substrate [121]. The result showed that pH optima for the reaction with LA and AA inserted into PDM micelles at low ionic strength were observed at both pH 7.4 and 10, whereas only one optimum (pH 9) was obtained with fatty acids in PDM micelles at high ionic strength. The reaction products of LA and AA in PDM micelles were 9- and 5-hydroperoxides respectively [121]. The reaction products from Tween 20 solubilised LA and AA with purified LOX-1 were 13- and 15-hydroperoxides at the optimum pH 9 with no dependence on ionic strength. The carboxylic acid group of the fatty acids exposed outside of the PDM micelles may be utilised as a recognition site for LOX-1 activity. LA methyl ester (LA ME) was compared as a substrate in the study to confirm this. Purified LOX-1 activity with LA ME at pH 9 in the presence of Tween 20 was approximately 11% of that with LA as a substrate under the same conditions. LA ME inserted into PDM micelles as a substrate did not show any activity with purified LOX-1 in the pH range studied (6 – 11). This confirmed that the carboxylic acid of the substrates had an important function in purified LOX-1 catalysed oxidation when the substrates were inserted into micelles [121].

The presence of different LOX isoenzymes in the reaction was also found to affect reaction conditions and product specificity significantly. Purified LOX-1 and LOX-2 in the following studies were prepared using the same Christopher, Pisiorius and Axelrod method [122]. LOX-2 was used to make di-hydroxy products from AA with the reaction rate displaying mono-phasic properties (similar reaction rate for both the first and second dioxygenation reactions) at pH 6.8 [123]. This differed to results

found for soybean LOX-1, where the first oxygenation at optimum pH 9 occurred quickly, whilst the second oxygenation with optimum pH at 7.5 – 8 was slow [124]. Similarly for the reaction of LOX-1 and AA at pH 6.8, the mono-hydroperoxide was produced fast within the first minute, whereas a conjugated triene (a dihydroxy product) formed slowly in the following 20 minutes [125]. For LOX-1 and LA prepared in water containing 0.35% v/v Tween 20, under conditions using an oxygen saturated solution at 0°C, the 13-isomer was the dominant product at all pHs tested with the maximum ratio of 13-isomer to 9-isomer (90:10) at pH 9. In contrast, under conditions at 25°C, without oxygen saturation, a racemic mixture of 13- and 9-isomers was observed at pH 7 – 9. With LOX-2 and substrate prepared as above, the 9-isomer was favoured at pH 7 – 9 regardless of oxygen and temperature conditions [120].

5. Soybean flour as a source of lipoxygenases

Commercial soybean LOX has been widely investigated as a biocatalyst compared to LOX extracts or raw sources from soybean. Studies with commercial soybean LOX have commonly used the following substrates LA, alpha linolenic acid (ALA, C18:3n-3), gamma linolenic acid (GLA, C18:3n-6), and AA, for single dioxygenation reactions to produce mono-hydroxylated compounds [48-52], rather than other LC-PUFA substrates such as DHA, EPA and DPA. Double dioxygenation reactions for the production of di-hydroxylated compounds, such as SPM analogues, has been conducted less intensively but on a wide range of n-3 and n-6 LC-PUFAs containing 1,4-pentadiene structures with at least three double bonds, including DHA [53, 54, 70], EPA [126], DPAn-6 [67], AA [48, 103, 125], GLA and ALA [127, 128], and DPA [67, 102].

Dobson et al. synthesised a variety of mono and di-hydroxy products including 13 lipoxin, resolvin and protectin analogues using the commercial type-1 soybean 15-LOX (15-sLOX-1) and various n-3 and n-6 LC-PUFA substrates in a simple and reproducible method [43]. However, the usefulness of these enzyme reactions with the commercial 15-sLOX-1 is limited by the high cost of acquiring the enzyme and maintaining activity. If an enzyme can be sourced cheaply from natural materials, then low cost methods may be developed for their industrial use. Another issue is that the soybean LOX-1 isozyme is unable to catalyse (or only reacts at a very low rate) with various EFAs compared to FFAs [129, 130], whereas other soybean LOX isozymes (LOX-2 and LOX-3) are more able to catalyse EFAs [122, 131], but are not commercially available.

Some research has worked on isolating individual LOX isozymes. The method of Axelrod et al. [55] has been used to isolate the LOX isozymes from soybean and defatted SBF [56, 132]. These isozymes could then be used to catalyse different types of substrates such as free and esterified fatty acids to generate various oxygenated products (mono- and di-hydroxy fatty acids, etc.). However, the process of isozyme isolation is generally time-consuming and complex. For example, the purification of LOX-1 from defatted SBF includes crude extract preparation with one hour of stirring, followed by filtration and centrifugation, then saturation with 30% and 60% ammonium sulphate with one hour of stirring and centrifugation, overnight dialysis and column chromatography, a further 15 hour of dialysis and a second column re-chromatography [55]. Recently, the extraction method was improved to increase the recovery of enzyme activity but this required a further purification step using an aqueous two-phase system [133]. The extracted enzyme should be used immediately or stored in a freezer for further use in order to avoid enzyme degradation.

A natural LOX source containing a variety of LOX types that is inexpensive and can be used directly in the reaction without extraction or purification, is highly desirable for industrial production of a range of compounds. SBF is a commercial product used as an alternative to wheat flour in a variety of products and is available as defatted or full-fat flours [57] which could be used directly as a source of LOX. In fact, SBF has long been used as an active LOX enzyme source in breadmaking to bleach wheat flour by co-oxidising LC-PUFAs and β-carotene [134]. SBF is also added to wheat flour to improve rheological and breadmaking properties [135]. SBF is not only an inexpensive raw material but it can also be used directly for the LOX-PUFA reaction without the need for purification. SBF is easy to store for long-term usage at 4°C, instead of storage at -80°C (15-sLOX-1) and for short-term usage at room temperature, instead of keeping on ice (15-sLOX-1). This is because the stability of lipoxygenase in SBF (especially type-2 LOX) was found to be significantly higher (50 times) compared to the isolated enzyme [56]. This could be because the enzyme is stabilised by natural immobilisation in a native microenvironment of the protein-starch matrix in the SBF [56].

Although SBF has many benefits as a LOX source, it also shows several disadvantages. SBF contains multiple types of LOX enzymes, which contributes to its tremendous potential for making a variety of products and reacting with various substrates including free and esterified fatty acids. However, it also means the reactions could be less specific. As SBF is not a pure LOX source, it contains various compounds and enzymes [136] which could interfere with the reaction and product extraction, and it generally needs to be used in higher volumes in the reaction (compared to purified enzymes). Hydroperoxide lyase, hydroperoxide peroxygenase, hydroperoxide isomerase enzymes, and other components (proteins, phospholipids, and vitamins) were recorded to be present in defatted SBF [137, 138]. LA and ALA

are the major LC-PUFA components of soybean oil. Defatted SBF still contains a small amount of oil. Therefore, it is possible that soybean LOX may catalyse the available LA and ALA in full fat or defatted SBF, and then 13-hydroperoxides may be metabolised into shorter chain compounds by available hydroperoxide lyase. Volatile compounds have been detected in SBF and crude soybean extracts [139, 140]. One study showed that significantly higher amounts of volatile compounds and higher sensory off-flavour scores in SBF formed under light compared to in the dark at 30°C from 0 to 6 days. The formation of volatile compounds in SBF increased with increasing storage time for both light and dark conditions. Volatile compounds were isolated by SPME with CAR/PDMS fiber in a 30°C water bath for 40 min and analysed by GC-MS. A chemical mechanism involving singlet oxygen was proposed for the formation of volatile compounds in SBF stored under light [139].

Another study showed that the crude soybean extract from grinding whole soybeans with phosphate buffer at pH 6.9 was able to significantly degrade 13-hydroperoxy LA, or oxidise LA and then convert the resulting hydroperoxide into various oxo, oxo-epoxy, and aldehyde compounds. The reaction was conducted at pH 6.9 and the substrates were emulsified in the presence of 0.5% Tween 20 [140]. In another study, volatile compound formation using a simple SBF extract was applied to increase green and fruity aromas in an olive oil extraction process [141]. Green and fruity notes (hexenals and hexenols) are formed in the industrial extraction of virgin olive oil and are regarded as indicators of fresh quality oil. Crude SBF extract (10% w/v in distilled water with centrifugation) was added to olive paste during the malaxation process to test if exogenous LOX could enhance olive oil aroma by increasing the formation of C6 volatile compounds. The results showed that in the presence of exogenous soybean LOX from SBF, the quality parameters of olive oil including peroxide value and acidity did not change significantly, but higher aroma content of

some volatile compounds was observed [141]. This demonstrated that the reaction products (PUFA hydroperoxides) could be used by hydroperoxide lyase in SBF to form volatile compounds.

Soybean LOXs have been intensively studied in the literature, especially LOX-1, as it is commercially available, whereas much less work has been done on the use of SBF (either directly or as a crude extract) as a source of lipoxygenase. In the following sections, studies using SBF or soybean meal (SBM) as a LOX source will be considered. Common reaction conditions and major applications with specific findings on SBF characteristics will be discussed. SBF and SBM are treated separately in the following sections as they are slightly different. SBF and SBM are both processed from soybeans but differ in particle size, and SBM is a cruder material compared to SBF. Soybeans are cracked, de-hulled and pressed into flakes (0.25 – 0.3 mm thickness) which is called SBM. Flakes are grinded into smaller size (< 149 μm) thickness (> 97% pass through a 100 mesh sieve) and the resulting powder is called SBF. The finer grades of SBF are passed through 150, 200 and 325 mesh sieves. Both SBM and SBF can be defatted to remove soybean oil by solvent extraction, or they can be left as full-fat products [57, 136, 142].

5.1. Preparation conditions

Both SBF and SBM (defatted and full fat) have been used as an active enzyme source of LOXs, either directly or with simple extraction (see Applications section, 5.2, and references therein). In this section, the common methods for SBF or SBM preparation as a LOX source either directly or with simple extraction are described (all references in this section).

5.1.1. Direct use

When being used as a LOX source, SBF and SBM have either been added directly into the reaction medium, or prepared in water or reaction buffer, with or without centrifugation and filtering. The SBF/SBM suspension or solution can then be mixed with the fatty acid substrate to initiate the reaction (see Applications section, 5.2 for examples). All LOX isoenzymes may be present in the reaction mixture and involved in the LOX-catalysed reactions. The presence of multiple isoenzymes could affect substrate specificity and product formation.

5.1.2. Simple extraction

Lipoxygenases from SBF and SBM have been extracted through simple protocols, and used as a crude LOX source. The crude extract contains specific LOX isoenzyme activity (mainly LOX-1 or LOX-2), which depends on the extraction pH. Activity of the major LOX isoenzyme (LOX-1 or LOX-2) in the crude extract may be significantly higher than the other LOXs, which may still be present in a smaller amount. No work on crude LOX-3 extraction has been found. LOX-3 has been removed along with LOX-2 at high temperatures in LOX-1 extractions to maximise the LOX-1/(LOX-2 + LOX-3) ratio [138, 143]. SBF and SBM are commonly prepared (without optimisation) in ~ 5 – 20 volumes of water or buffer. Typically, acidic pH is used to extract LOX-1, whilst neutral pH is used for LOX-2. Extractions are generally performed at low temperature (0 – 6°C) with stirring (up to 2 hrs) or using a homogeniser. The suspensions can be centrifuged and/or filtered to obtain a crude LOX solution (refer to Applications section, 5.2, for specific references). Only two studies, from the same research group, investigated the extraction conditions for LOX-1 and LOX-2 from defatted SBF in ~ 100 mL stirred tank reactors [137, 138]. Optimised extraction conditions are described below.

In the first study, the optimised conditions for LOX-1 extraction from defatted SBF were found to be water at pH 5.2 (adjusted using acetic acid), 40°C, stirring at ~ 5 rps for 10 min [138]. In the same study, further heat treatment at 70°C for 5 min was applied to decrease LOX-2 and LOX-3 activity, giving enhancement of the LOX-1/(LOX-2 + LOX-3) ratio [138]. This heat treatment was optimised in a previous study with different temperature, time, pH and ionic strength conditions using SBM. LOX-2 and LOX-3 have significantly lower thermal stability than LOX-1 [143]. In the second study, LOX-2 extraction from defatted SBF was found to be maximised in 0.05 M phosphate buffer at pH 6.5, 25°C, with stirring at ~ 5 rps for 10 min [137]. This extract may also contain LOX-1, but it is likely not as highly concentrated as in the acidic pH extraction. LOX-3 could also be present in the extraction. In scaling up to ~ 500 mL stirred tank reactors for industrial applications, both LOX-1 and LOX-2 extractions were maximised at a slower agitation speed (3 rps instead of 5 rps) [137, 138].

5.2. Screening lipoxygenase activity

A number of studies have used both SBF and SBM (defatted or full fat) either directly or after simple extraction to screen for LOX activity. These papers will be discussed in this section. Generally, LOX-1 activity was measured between pH 9 and 10, while LOX-2 + LOX-3 activity was measure between pH 6.5 and 6.8, and sodium linoleate or linoleic acid were used as substrates. UV-vis spectrophotometry at 234 nm was commonly used to monitor the formation of hydroperoxides or alternatively oxygen consumption was used to monitor the LOX-catalysed reaction. Reaction temperatures between 15 and 25°C were used.

Direct use

Luquet et al. demonstrated the potential benefits of directly using SBF as an inexpensive LOX source compared to commercial LOX to produce oxygenated

products from both free and esterified LC-PUFAs [56]. In this research, full-fat SBF prepared in water (pH 6.8) was used as a crude LOX source. It was possible to generate oxygenated products from not only linoleic acid as a free fatty acid (reaction in borate buffer at pH 9.22 or without solvent), but also from EFAs – trilinolein (TL) and sunflower oil (without solvent). The crude preparation was found to have much higher activity with TL compared to a commercial LOX. The authors proposed that the type-2 LOXs present in the flour were active for the oxygenation of the intact TAGs. In the same study, lipoxygenases were also intensively extracted from the SBF following a lengthy procedure [144] requiring many time-consuming steps. This purified enzyme mixture was found to be significantly less stable than the crude SBF preparation [56].

Simple extraction

Only one study has used an acidic buffer to extract LOX from defatted SBM [145]; all other studies have used neutral pH. In this study, LOX activities and LOX-1/(LOX-2 + LOX-3) activity ratios in 10 soybean cultivars, grown in Hungary over 3 continuous years were investigated. Defatted SBM was extracted in sodium acetate buffer at pH 4.5, before adjusting to pH 6.8 and the crude extract was used as a LOX source for LOX activity screening. The activities of the LOX isozymes were found to be affected more significantly by the changes in weather conditions for a cultivar type over the years, than by differences in cultivar type within the same year, even though LOX isoenzyme biosynthesis in soybeans is genetically controlled [145].

More work has been done using LOX-2 extracts (neutral extraction) compared to LOX-1 extracts and these will be discussed below. LOX activity from various soybean and plant species has been identified and compared. In one study, three LOX isozymes from soybean seed powder extracted in buffer at pH 6.8 were separated using HPLC ion-exchange chromatography [101]. The retention time was

matched with pure LOX isoenzyme standards and the mixture of all three pure isoenzymes. LOX-3 activity was specifically measured at 280 nm through the formation of ketodiene, whereas other LOXs were monitored at 234 nm. Two soybean mutants (PI-408251 and Wasenatsu) were tested using this method to determine the presence of different LOX isozymes and they were found to be absent of LOX-1 and LOX-2 + LOX-3 respectively [101]. In another study, crude extracts of SBF from 51 Indian soybean varieties and strains were tested to compare the LOX-1 and LOX-2 + LOX-3 activities [146]. SBF was homogenised in 100 volumes of phosphate buffer (0.2 M, pH 6.8) as a crude LOX source. Two varieties (Shilajeet and KhSb 2) were found to have lower activities from all LOX isozymes, and are therefore desirable for use in the food industry to produce less beany and off flavours (known to be caused by LOX products). Large variations between cultivars were found: ~ 150-3000 units/g SBF for LOX-1 and ~ 250-1500 units/g SBF for combined LOX-2 + LOX-3 activity [146]. In a study by Fauconnier and co-workers, the LOX activity of 35 plant species belonging to 5 different families was measured at pH 7 using crude extracts, to find an effective LOX source for the synthesis of green note precursors [147]. The ground seed extracts were prepared from 1 g flour in 100 mL phosphate buffer (pH 7). LA substrate was emulsified with Tween 40 (0.25% w/v) at a 1:1 ratio and adjusted to pH 7. The 105 mL-scale reaction was conducted under oxygen saturation, and after 45 min the reaction mixture was diluted in ethanol to measure LOX activity at 234 nm with a UV-vis spectrophotometer. Species of the three genuses (*Phaseolus*, *Trifolium*, and *Vigna*) belonging to Fabaceae family were found to have as high LOX activity as the species *Glycine max* (soybean) which is also derived from the same family. *Glycine max* had the highest LOX activity per gram flour whereas *Trifolium hybridum*, *Vigna angularis* and *Vigna unguiculata* had higher LOX activities per gram protein than soybean. LOX activity of the four

species (*Glycine max*, *Phaseolus coccineus*, *Vigna radiata* and *Vigna unguiculata*) were also investigated in the range pH 4 – 12 for the optimum. Soybean activity was found to have 2 optimum pHs, at pH 10 (corresponding to LOX-1 activity) and pH 6.5 (related to LOX-2 and LOX-3 activity). The other 3 species had only one optimum at pH 6. At pH 7, 13- and 9-hydroperoxy LA formed equally for all four species, whereas at pH 9, soybean (the only species active at this pH) was found to have higher regio-specificity, producing 80% 13-hydroperoxy LA (20% 9-hydroperoxy LA) [147].

Similar results regarding pH and LOX activity have been found in the following studies. Surrey showed that crude SBM extract had maximum activity at pH 6 in the range studied (pH 5 – 9) with sodium linoleate and Tween 20 (0.25% v/v), and continuous oxygen supply. The SBM (1 g) was extracted in 200 mL phosphate buffer at the reaction pH being tested [115]. In a study by Koch and colleagues, different types of substrates (free and esterified fatty acids) were used with crude defatted SBM extract as different LOX isoenzymes are present in the extract [131]. LA and TL were prepared in solvent (ethanol, and acetone: ethanol (1:1), respectively) to be used as substrates for the crude extract. The extract was prepared by suspending defatted SBM in water, and then the soluble fraction was further precipitated with calcium chloride to obtain the crude extract after centrifugation. LOX-1 (acid LOX) in the crude extract showed maximum activity with LA at pH 8.3, whereas type-2 LOXs (triglyceride LOX) oxygenated TL at both pH 5.5 and 8.3. Enzyme amount used was 4 times higher for type-2 LOX compared to LOX-1 [131]. In another study, LOX-2 activity from defatted SBF extracted in phosphate buffer at pH 6.5 was measured using pure LA as substrate, for comparison between two different methods (a spectrophotometric method at 234 nm and an isotachophoretic method, to measure the increase in conjugated dienes and the decrease in LA concentration respectively)

[148]. The assay was conducted using phosphate buffer at pH 6.5 in the presence of Tween 80 (1%). Product concentration increased linearly over 30 min, while substrate concentration decreased over the first 15 min faster than the period after 15 min [148].

Different types of SBM or SBF can affect LOX activity. In a study, crude soybean extracts from full fat or defatted SBM were found to have different LOX isozyme activities [149]. Pure LOX-1 (commercial), full-fat and defatted SBM extract activities were investigated with sodium linoleate through kinetic patterns. A kinetic curve was obtained by monitoring the increase of absorbance at 234 nm or 280 nm with a UV-vis spectrophotometer over 300 – 500 seconds for LOX-1 and LOX-2, or LOX-3 respectively. LOXs in the crude soybean extract from full-fat SBM at pH 6.1 were identified to have mainly LOX-1 and LOX-3 activity (ratio of 3:1) but low LOX-2 activity. In contrast, the defatted extract of SBM at pH 4.5, 6.1 and 7.1 contained LOX-3 as the major LOX and was low in LOX-1 and LOX-2. This study also showed that LOX-1 activity could be modified by enzyme/substrate ratio and buffer pH. Low pH and high enzyme substrate ratios were found to inhibit LOX-1 activity [149]. In another study, the activities of LOXs from three defatted SBF (described as fully toasted, white and enzyme active) extracted in water (50 mg flour/mL) were measured for comparison [150]. The white SBF has minimal heat treatment as described by the manufacturer. A more dilute solution of the enzyme active SBF in water (1 mg/mL) was required to achieve readable results to monitor enzyme stability of defatted SBF over time (up to 180 days) at 23°C. The enzyme active SBF was found to have the highest LOX activity, compared to no activity detected from the fully toasted SBF, and close to zero activity of the white SBF. The activity of the enzyme active SBF decreased at an increasing rate over the storage period studied [150].

5.3. Applications and LOX activity

SBF and SBM have been used (directly or after extraction) as crude LOX sources for many applications. The two major applications investigated in this section are the bleaching of beta-carotene and the synthesis of lipid hydroperoxides for various purposes. LOX activity is also discussed in relation to these applications.

5.3.1. Bleaching beta-carotene

LOX was originally known as carotene oxidase, and was first identified in 1928 in soybean through its ability to bleach carotene (as cited in [151]). In 1932, an enzyme in soybean was identified and termed lipoxidase which oxidised unsaturated fats, and in 1940 carotene oxidase and lipoxidase were confirmed to be the same enzyme [151]. As a result, lipoxidase is also referred to be LOX. SBM has been used directly and after extraction in water as a LOX source to bleach beta-carotene and other carotenoids in the presence of oil. The bleaching activity is caused by LOX catalysis through the co-oxidation of beta-carotene and PUFAs. Hydroperoxides from LOX-catalysed PUFA reactions are responsible for the oxidation of beta-carotene [152, 153].

In one study, an aqueous extract of fat-free SBM was found to bleach carotene in the presence of highly unsaturated hemp-seed oil without saponification in a much shorter time than bleaching without hemp-seed oil or with excessive amounts of the oil [154]. The optimum temperature for the bleaching of carotene in the presence of water by the crude SBM extract was found to be 40 – 45°C [154]. Summer and Smith found that finely ground defatted SBM extracted with acidic water (acidified by acetic acid) was able to decolourise bixin, a more stable carotenoid than carotene, in the presence of neutralised fatty acids (saponified soybean oil) and gum arabic [151]. At pH 6.5, the highest activity of carotene oxidase was identified to be at 10°C (studied range 10 – 35°C) by measuring bleaching time in seconds [151]. This

optimum temperature was lower than the optimised temperature of the bleaching with hemp-seed oil (40 – 45°C) found in the study above [154]. In a different study, SBM in water was used to bleach the yellow colour of the carotene in cottonseed oil and other unsaturated fats [155]. The enzyme activity was measured by iodine-thiosulfate titration. The optimum pH for the cottonseed oil bleaching reaction was found to be at pH 6.5 (phosphate buffer 0.1 N). The carotene oxidase activity decreased with increasing temperature in the studied range (15 – 30°C). The highest activity was found to be at 15°C, which is similar to the bleaching temperature in the saponified soybean oil reaction at 10°C [151]. When monitoring the reaction at 19°C, the rate was found to decrease over time [155].

Different results have been reported for LOX isozyme activity on bleaching beta-carotene [152, 153, 156]. The presence of LOX-1 was essential for bleaching beta-carotene either with fatty acid hydroperoxides or fatty acids. LOX-1 bleached beta-carotene at a higher rate in the presence of fatty acids compared to intermediate fatty acid hydroperoxides. The bleaching activity was measured spectrophotometrically at 452 nm for the decrease of beta-carotene absorbance. [152]. Weber and co-workers found that type-2 LOXs were more active than type-1 LOX in bleaching beta-carotene [156]. However, as LOX-1 is the major isozyme in SBF and more stable than other LOXs, it still may be the isozyme in SBF responsible for catalysing the cooxidation of beta-carotene and PUFAs [152]. A combination of LOX-1 + LOX-2 or LOX-2 + LOX-3 was found to bleach beta-carotene more effectively than individual LOX isozymes [153].

5.3.2. Synthesising lipid hydroperoxides

Both full fat and defatted SBF or SBM have been used directly as a readily available and inexpensive LOX source, without complex extraction or purification, in many studies to produce fatty acid hydroperoxides in milligram to gram scale reactions.

SBF but not SBM has been used by adding directly into the reaction without prior preparation, whereas both SBF and SBM have been used with simple extraction in water or buffer (acidic, neutral pH buffer or pH used in the reaction). Fatty acid hydroperoxides have been mainly used as precursors for the synthesis of green note flavour compounds (Table 2.2). The conversion of hydroperoxides into flavour compounds is catalysed by different hydroperoxide lyase enzymes. Green note flavour compounds are volatile C6-C10 aldehydes and alcohols such as hexanal and hexenal, and are naturally produced in plants by LOX and hydroperoxide lyase as part of their defence system in response to wounds and pathogen attacks [60, 105]. These fresh green odour molecules are widely used in recovering vegetable and fruit flavours lost over time. Methods for the production of these compounds on an industrial scale for the food industry using inexpensive natural materials are in demand [60, 61, 157]. The major substrates used for hydroperoxide synthesis are C18 fatty acids (LA and ALA) as either pure substrates or from hydrolysed vegetable oils (sunflower, safflower, and linseed), as well as natural oils such as soybean oil and butterolein as EFAs (Table 2.2). An advantage of using SBF and SBM, is the potential to catalyse not only FFAs and hydrolysed natural oils but also EFAs (TL, soybean oil, and butterolein) due to the presence of stable type-2 LOX. SBF and SBM have been used to synthesise hydroperoxides at different pHs which are optimised for type-1 LOX (pH 8 – 10) or type-2 LOX (pH 6 – 7). Most studies have used temperatures between 0 and 25°C, and oxygen saturation before initiating the reaction or oxygen/air flow over the course of the reaction, for the synthesis (Table 2.2). The conditions are dependent on the purpose of the study, and which isozyme is needed for the reaction as only type-2 LOXs can catalyse the oxidation of EFAs.

Table 2.2. Reports of simple soy flour preparations used for the synthesis of lipid hydroperoxides.

No.	Substrate	pH	Purpose	Year	Ref.
1	Safflower and soybean oil soap	9 – 10	Synthesis of hydroxy conjugated octadecadienoic acids	1971	[158]
2	Linoleic and linolenic acid	9.5	Production of natural green note flavour compounds	1995	[157]
3	Linoleic acid, linolenic acid	9.0 – 9.5	Green note compound synthesis (aliphatic alcohols and aldehydes)	1995	[159]
4	Hydrolysed safflower oil	10	Large scale production of 13-HPOD from hydrolysed safflower oil	1996	[59]
5	Linoleic acid in hydrolysed safflower oil	10	Synthesis of lipid hydroperoxides, for conversion to hydroxides for fine chemicals	1998	[118]
6	Hydrolysed safflower and linseed oils	8.25	Green note compound synthesis (hexanal and hexenal)	2002	[60]
7	Linoleic acid, hydrolysed sunflower oil (66% w/w LA)	9	Synthesis of hexanal	2002	[160]
8	Hydrolysed linseed oil	9	2(E)-hexenal synthesis from 13-HPOT	2004	[161]
9	Linolenic acid	6 or 8.7	Green note compound synthesis	2005	[105]
10	Butterolein	7.0	Enzymatic synthesis of C6-C10 flavour aldehydes	2006	[61]
11	Hydrolysed linseed oil	9.5	Development of a method for real time reaction monitoring	2007	[162]
12	Sodium linoleate	10	In comparison with hydroperoxide yield of mung bean lipoxygenase	2007	[163]
13	Hydrolysed linseed oil	9.0	Synthesis of cis-3-hexenal	2008	[164]
14	Linseed oil fatty acids	9.5	Synthesis of hexanal	2009	[165]
15	Hydrolysed linseed oil	9	Enzymatic synthesis of green notes and alcohols	2009	[58]
16	Soybean oil	6	Gram scale production of hydroperoxides	2011	[111]
17	Hydrolysed linseed oil	9.3	Preparing lipid hydroperoxide substrate for hydroperoxide lyase	2013	[166]

In some studies, characteristics of the LOX reaction used for hydroperoxide synthesis have been investigated and will be discussed below. These studies have been categorised into either direct use of SBF or prepared as a crude SBF extract.

Direct use of SBF

In a study by Emken and Dutton, sfLOX activity was compared between defatted SBF, defatted SBF extract (extracted at pH 10, as was also used in the reaction) and commercial purified LOX [158]. There was no difference in LOX activity between the crude SBF extract and the same SBF extract with further purification using $CaCl_2$. Defatted SBF and the aqueous SBF extract were found to have significantly higher activity than a commercial purified LOX when using the same enzyme concentration under the two following reaction conditions: 1) 4 mg/mL substrate, 10% ethanol, substrate/enzyme ratio of 80; and 2) 100 mg/mL substrate, 20% DMSO, substrate/enzyme ratio of 500. The commercial purified LOX was inactive and inhibited at high substrate/enzyme ratios (> 80), whilst at low substrate/enzyme ratios of 10 to 20, the enzyme had the same activity as the aqueous SBF extract. The reaction rate for defatted SBF and the percent oxidation of substrate decreased with increasing substrate concentration from 4 to 100 mg/mL with a constant substrate/enzyme ratio of 200 in the presence of 10% ethanol. Therefore, high substrate concentrations inhibited the enzyme. Under the conditions of 4 mg/mL substrate, 10% ethanol, substrate/enzyme ratio of 80, the aqueous SBF extract had dramatically higher activity than defatted SBF. However, increasing substrate concentration and substrate/enzyme ratio, the activities of both defatted SBF and the aqueous SBF extract were found to be comparable. High concentrations of solvent (more than 10% ethanol or from 30% DMSO) could denature the enzyme, which led to a decrease in reaction rate and total oxidation. 0.75% BHT was found to improve the reaction yield when high substrate concentrations were used. This work showed

the possibility for the economical and effective large-scale production of hydroperoxides using inexpensive fatty acids from soap stock and SBF as a direct LOX source without purification [158].

Haefliger and Sulzer found that for pure ALA substrate, the optimum reaction times at pH 9.5 and 8.5 were at approximately 20 min and 45 min respectively and longer reaction times caused a loss of hydroperoxide yield as hydroperoxides were reduced into corresponding hydroxides [162]. The reaction at pH 9.5 was about 2.5 times faster than at pH 8.5, but did not increase the reaction yield. The reaction yield with ALA from hydrolysed linseed oil as a substrate ($\sim$ 75%) was much higher compared to the reaction yield with pure ALA ($\sim$ 45%). The research suggested that the presence of other non-oxidised fatty acids such as oleic acid and steric acid in the hydrolysed linseed oil may help the emulsification process for better interaction between the substrate and water-soluble enzyme [162]. In a study by Kohlen and co-workers SBF LOX (sfLOX) and commercial purified LOX-1 activities on LA and TL were also compared [61]. The results showed that in the studied range (pH 5 – 10) sfLOX had significantly higher activity with TL at pH 7 than other pHs, whereas there was no activity for the reaction between purified LOX-1 and TL. With LA as a substrate, both sfLOX and purified LOX-1 had the same optimum at pH 9. Moreover, sfLOX activity was expressed over a broad pH range (pH 5 – 10), whereas LOX-1 activity significantly decreased at lower pHs [61].

In a milligram scale study, Fukushige and Hildebrand reacted ALA (2 mg) with defatted SBF (10 mg) in 2 mL phosphate buffer. LOX-1 and LOX-2 present in the SBF were targeted to produce hydroperoxides at pH 8.7 and 6, respectively [105]. LOX-2 was responsible for the highest production of hexenals in a one step reaction of hydroperoxide synthesis and hexanal conversion at pH 6 as this is the optimum pH

for both LOX-2 and hydroperoxide lyase. The hydroperoxide lyase was from watermelon overexpressing the enzyme in leaf tissues. LOX-1 also gave a high hexanal yield but the conversion required two steps as LOX-1 and hydroperoxide lyase have different optimum pH, at 8.7 and 6 respectively. LOX-3 gave a lower yield of 13-hydroperoxides compared to the other LOXs and further converted products into ketodienes, therefore the use of SBF lacking LOX-3 was found to signficantly increase hexanal formation [105].

Crude SBF extract

Two studies by Elshof et al. applied a crude extraction method using defatted SBF extracted in sodium acetate buffer pH 4.5 to oxygenate LA in hydrolysed safflower oil [59, 118]. In the first study, the optimum conditions were found to be pH 10, 5°C and air saturation with 80% yield and high product regio-specificity of > 95% 13-hydroxy LA and stereo-specificity of > 95% *S*. Stability of the LOX-1 extract kept at 4°C was tested over 65 days. The activity decreased significantly over the first few days and then the degradation rate slowed down [59]. In the second study, the LOX reaction was conducted at pH 10 under constant air flow. Cysteine, a mild reducing agent, was used to investigate the direct reduction of hydroperoxides into hydroxides during the reaction to increase the reaction yield as hydroperoxides are known to inhibit the LOX enzyme. The results showed that a molar cysteine:substrate ratio of 1.5 – 2:1 gave a higher yield of approximately 90% compared to the yield of without cysteine of almost 69% 13-hydroxy LA [118].

Other studies have applied the optimal conditions from the above study by Elshof et al. without cysteine to produce 13-hydroperoxides with minor modifications [160, 161, 165]. In one such study by the Nemeth group, defatted SBM was extracted in 0.05 M acetic acid (pH 5.2) and then heated at 70°C for 5 min to obtain specific LOX-1 activity. The synthesis of 13-hydroperoxy ALA (62% yield) as a precursor

for the production of 2(*E*)-hexenal was conducted at pH 9, 0 – 5°C and continuous air flow using linolenic acid (100 mM) from hydrolysed linseed oil as a substrate [161]. Santiago-Gomez and co-workers used SBF extracted in acidified water (pH 4.5) to oxidise linseed oil fatty acids (ALA and LA), in the presence of Tween 20, pH 9.5 (LOX-1 optimal pH for 13-hydroperoxide selectivity), oxygen saturation, 20°C, 30 min reaction time. The resulting hydroperoxides were then used to synthesise hexanal, and the reaction was optimised [165]. In a study by Marczy and colleagues, SBM was extracted in 0.05 M acetic acid and followed by heat treatment at 70°C for 5 min to minimise type-2 LOXs [160]. The reaction was conducted at pH 9 (corresponding to LOX-1 activity), 0 – 5°C to increase oxygen solubility and reduce side reactions and 2 bar oxygen pressure using LA as a substrate. The yield of the reaction (500 mL scale) was 72% 13-hydroperoxy LA [160].

Cai and co-workers found that SBM could be used to synthesise TAG hydroperoxides from soybean oil, under the optimum conditions at pH 6 (lower than the optimum pH for free fatty acids), 10% ethanol, 2 – 3 hours, 20°C. Crude defatted SBM extract in phosphate buffer (pH 6) gave a much lower hydroperoxide yield (< 30%) compared to the direct use of SBM (61%) [111]. Chow and colleagues found that the pH of crude extracts did not affect soybean LOX activity significantly with sodium linoleate as a substrate in the first step to form hexanal using hydroperoxide lyase from bell pepper fruits [163]. Three buffers (pH 4.5 acetate buffer, pH 6.5 phosphate buffer, pH 9 borate buffer) were used to extract LOX from freshly crushed soybeans. For extraction at pH 4.5, LOX had much less total protein content but higher specific LOX activity (unit/mg protein) compared to the extracts at pH 6.5 and pH 9. Therefore, the research suggested that high amount of proteins at high pHs may be unspecific and could inhibit the reaction. As for LOX reaction pH, there were two optima at pH 6 – 6.5 and pH 9.5 – 10 corresponding to LOX-2 + LOX-3 and

LOX-1 activity respectively. Extraction at pH 4.5 and reaction at pH 10 were used to form LOX products for hexanal production. These optimal reaction pHs were only observed for LOX extracted at pH 4.5 and 6.5, whereas the alkaline optimum was not clearly detected for LOX extracted at pH 9. In this work, fresh crushed soybean was homogenised with a high speed homogeniser at 10,000 rpm for 4 min [163].

6. Esterified fatty acids as substrates for hydro(pero)xylated compound synthesis

LOX reactions with FFAs as substrates have been well-studied in comparison to LOX reactions with EFA substrates (such as fatty acid methyl esters, triacylglycerols and phospholipids). LOX enzymes are known to catalyse FFAs as preferred substrates compared to EFA substrates [167]. However, soybean, barley, cucumber and other plant LOXs have been shown to have activities with EFAs in a number of studies (all references below). In this section, the use of soybean LOX and other plant LOXs to catalyse reactions with EFA substrates is described. The activities of different LOX isozymes on various EFAs and substrate preparations are investigated in these studies.

6.1. Soybean lipoxygenases

LOX-1 generally catalyses FFAs such as LA and ALA with high enzyme activity, whilst type-2 LOXs catalyse both FFAs and EFAs [88, 90]. However, in a number of studies, LOX-1 has been found to catalyse various EFAs at a low rate under specific conditions after optimisation [129, 168, 169]. In two studies, the optimum pH for the reaction of TL and 1,3-dilinolein with LOX-1 was identified to be at pH 8 – 9 in the studied range of pH 5 to 11 [129, 169]. This pH is the same as the optimum pH for LOX-1 catalysed reactions with FFAs. Three studies showed that the presence of a bile salt, deoxycholate (5 – 10 mM), acting as an emulsifier, was essential for the

reaction to occur, and could not be replaced by other surfactants such as Tween 20, Tween 80 and Triton X-100 [129, 168, 169]. The substrates used in these studies were egg yolk L-α-phosphatidylcholine (a phospholipid species) [168], TL [129] and 1,3-dilinolein [169]. Piazza et al. found that the oxidation rate for LOX-1 and 1,3-dilinolein decreased 2 – 3 fold when the reaction medium was changed from a multicomponent buffer (a mixture of four buffers) to a one component buffer [169].

Piazza and co-workers also investigated the relative oxidation rate of a range of EFAs with LOX-1 after normalising to the LA and LOX-1 reaction rate as 100% [129, 130, 169]. The reactions were conducted under optimum conditions at pH 8 – 9 with a multicomponent buffer and in the presence of 5 – 10 mM deoxycholate. Most EFAs reacted with LOX-1 at lower rates than LA [129, 130, 169]. The rate of 1,3-dilinolein oxidation was 40% of LA [129, 130]. No significant activity with 1,3-dilinolein was observed when deoxycholate was replaced with the surfactant, Tween 20, or in the absence of all surfactants [169]. TAGs containing moieties of LA, oleic acid and steric acid had an oxidation rate of 15% compared to LA [129]. In contrast, LA ME and 1-monolinolein were found to be oxygenated at a similar rate or slightly higher than LA respectively. However, the rate of LA ME and 1-monolinolein oxidation was much lower in the presence of Tween 20 or without any surfactant. LOX-1 showed approximately double the reaction rate with 1-monolinolein compared to its reaction with 1,3-dilinolein, whereas the oxidation rate of TL with LOX-1 (3% of the LA rate) was significantly lower than the rate of 1,3-dilinolein with LOX-1 (55% compared to LA rate). The results demonstrated that adding more linoleoyl residues to the glycerol backbone decreased LOX activity [169].

In a study by Nakashima et al., monogalactosyl diacylglycerol (MGDG, sonicated in the presence of deoxycholic acid) was also found to be a substrate for purified LOX-

1 catalysis at pH 9, whereas no activity was observed in the absence of the detergent [170]. MGDG containing mainly linolenate acyl moieties was extracted and purified from clover leaves. The optimum concentration of detergent for maximum enzyme activity was 6.9 mM, and at this concentration possible formation of mixed micellar aggregates recognised by the enzyme was suggested to be the mechanism for the catalysis of EFAs. The reaction had high product specificity for 13-hydroperoxy-ALA as the primary acyl moiety. Both mono- and di-hydroperoxy MGDGs were detected, and no preference for the hydroperoxy group on mono-hydroperoxy MGDGs at either sn-1 or sn-2 position was observed [170].

Different reaction conditions in the absence of the bile salt have been used in other studies. Kermasha, Dioum and Bisakowski found that LOX-1 had low activity with monolinolein, dilinolein and TL (less than 10% relative specific activity compared to LA) in aqueous media at pH 9 [112]. In addition, LOX-1 was found to have higher activity with monolinolein (up to 46% in comparison with LA) in ternary micellar organic solvent systems containing a buffer at pH 9, either octane or isooctane (3.5 – 4%) and a surfactant Tween 40 or Tween 80 (10 – 50 µM), whereas no significant increase was observed for dilinolein or TL [112]. Tayeb and co-workers used TL sonicated in buffer for 10 minutes to be catalysed by LOX-1 at pH 9 without surfactant [171]. Analysis of TL before and after LOX-1 treatment using ^{1}H-NMR indicated oxidation of TL. The study demonstrated that TL can be oxygenated by LOX-1, but characterisation of the products and calculation of the reaction yield were not performed [171]. Fukuzawa and colleagues reported that a commercial LOX-1 can catalyse phosphatidylethanolamine and egg yolk PC effectively in buffer at pH 9 in the presence of 20% methanol or 1% Triton X-100 to disturb the membrane structure of substrate vesicles [172]. This allowed an increase in substrate available to be accessed by the enzyme. The enzyme concentration required for the

reaction with EFAs was 1000 times higher than the reaction with LA free fatty acid. A longer reaction time was also observed for the LOX-1 catalysed EFA reactions [172].

In several studies, the activities of purified LOX-1 and LOX-2 (or type-2 LOX) have been compared for the oxygenation of EFAs. Christopher, Pisiorius and Axelrod fully isolated two types of LOX from defatted SBF to measure activities with LA, LA ME and TL (pH 4.5 – 10) [122]. Both pure enzymes were active on all substrates but had different optimum pH and relative activities. LOX-1 showed higher activity with LA than LOX-2 at all pHs, with a sharp optimum at pH 8. LOX-2 had optimum activity with LA at pH 7. LOX-2 catalysed the oxidation of LA ME and TL (prepared in acetone:ethanol (40:60)) more effective than LOX-1 at all pHs tested. LOX-1 showed a broad optimum pH between 8 and 10 for both LA ME and TL. LOX-2 activity on LA ME was optimum at pH 8, whereas a broad optimum pH (7 – 9) was observed for the LOX-2 catalysed TL reaction [122]. Koch, Stern and Ferrari found that 20°C was the optimum temperature for both purified LOX isozymes. In the absence of surfactant, LOX-1 was active with LA at pH ~ 8, whereas LOX-2 had two pH optima for the reaction with TL (prepared in acetone:ethanol (40:60)), at pH ~ 5.5 and 8 with the activity at pH ~ 5.5 greater than that at pH ~ 8 [131].

Christopher et al. used LA ME as substrate to be catalysed by purified LOX-1 and LOX-2, at both pH 7 and 9 with low yield (~ 10 – 18%). LOX-1 formed more 13-isomer than LOX-2 and LOX-2 produced almost the same ratio of 13- and 9-isomers at both pHs [120]. In a study by Roza and Francke, methyl esters of LA, ALA and GLA (50 mg) were emulsified in buffer (50 mL) containing 1 mg Tween 60 to be catalysed by purified LOX-1 and LOX-2 from soybean at pH 9 and ambient temperature, with oxygen saturation, and the results were compared to reactions with

their respective FFAs [173]. Product profiles were more complex with the methyl esters compared to their respective FFAs, but only methyl ester mono-hydroperoxides were investigated in this study. Similar product profiles between methyl esters and FFAs was observed for both LOX-1 and LOX-2. Only the 13-hydroperoxy methyl ester isomer was observed for LOX-1 catalysed reactions with LA ME and ALA ME, whereas more isomeric mono-hydroperoxides were produced from GLA ME. For LOX-2, a mixture of isomers was detected for all substrates. However at pH 7, a range of isomers was found for all substrates in both LOX-1 and LOX-2 reactions [173].

In a study by Bild and colleagues, the activities of the three purified soybean LOX isozymes were compared at pH 9 for LOX-1 and pH 6.8 for LOX-2 and LOX-3 for the catalysis of nonpolar substrates. LOX-2 showed much higher activity with LA ME, linolenyl acetate and linoleyl methane sulfonate compared to the other isozymes, but the activity was still much lower than LOX-1 activity on LA [174]. In a few studies, only LOX-2 or certain LOXs have been shown to catalyse EFAs, whereas no activity was observed for the other LOXs. Maccarrone and co-workers found that purified LOX-2 from soybean seedlings can catalyse the dioxygenation of biomembranes (neutral lipids and phospholipids), LA and ALA, and their methyl esters as suspensions in buffer when tested for 30 min at room temperature in the presence of oxygen and without surfactant [175]. LOX-1 was unable to oxygenate the esterified lipids. Different salts in buffer (10 mM Tris/HCl, 10 mM $MgCl_2$ and 1 M NaCl) at pH 7 were used as a medium for the LOX reactions. High regio- and stereo-specificity was observed in the reaction between LOX-2 and biomembranes compared to FFAs and their methyl esters. Membrane-embedded FFAs were not substrates for either LOX-1 or LOX-2 [175]. Muller et al. reported that two out of the five LOX isoenzymes present in soybean leaves were able to oxygenate all three LA

moieties in TL at pH 7.4 and the reaction products were found to be mainly 13S derivatives of LA. TL (10 mM) was prepared in ethanol and then sonicated to form micelles as the substrate. LOX isoenzymes from soybean leaves and soybean seeds are different [176].

6.2. Other sources of lipoxygenases

6.2.1. Barley

Compared to soybean LOX, barley LOX isoenzymes are known to have the opposite product specificity with LA as substrate; barley LOX-1 (9-LOX) produces mainly 9-hydroperoxides, whereas barley LOX-2 (13-LOX) primarily produces 13-hydroperoxides and has a higher affinity for EFAs than LOX-1 [32]. Yabuuchi investigated the activities of partially purified LOX-1 and LOX-2 from germinating barley embryos on EFAs [177]. LOX-1 is present in both germinating and ungerminated embryos, but LOX-2 was only found in germinating embryos. At pH 7.5, barley LOX-2 was found to have higher activity than barley LOX-1 with LA ME and TL (1 mM) as substrates. LOX-2 and LOX-1 activities with LA were normalised to be 100% and their activities with LA ME and TL were compared with LA. For LA ME, LOX-2 and LOX-1 had relative activity of 40% and 18%, respectively. For TL, LOX-2 had relative activity of 32%, whereas LOX-1 only showed trace activity [177]. Yang, Schwarz and Vick also found that barley LOX-2 catalysed the dioxygenation of LA ME and TL solubilised in aqueous solution by Tween 20 at a significantly higher rate than LOX-1 at pH 6.5. However, barley LOX-2 had low relative activities with LA ME and TL of approximately 10 – 20% when compared to barley LOX-2 activity on LA. Barley LOX-1 was found to have an optimum pH at ~ 6.3, whereas two pH optima were obtained for barley LOX-2, at pH 6 and 8 with the studied range between pH 5 – 9 [178].

Holtman and co-workers also showed that purified germinating barley LOX-2 was able to catalyse various EFAs – TL, dilinolein, LA ME and C16/LA-PC (1.1 mM) at pH 6.5, with a much higher reaction rate than LOX-1 (~ 2 – 3-fold) [92]. The barley LOX-2 reaction rates with these substrates were compared to its rate with LA. The relative reaction rates for LA ME, dilinolein, TL and C16/LA-PC with barley LOX-2 were 32, 49, 16 and 7%, respectively. Substrates (24 mM) were prepared as an emulsion by sonication in the presence of 15% (w/v) gum arabic in tris/HCl pH 7.5. Analysis of total lipid extracts from barley demonstrated that LOX-2 was responsible for the oxidation of EFAs in storage lipids as only 13-HLA derivatives were formed. It was suggested that oxidation of storage lipids may be involved in the onset of germination. Lipid hydroperoxides were suggested to act as substrates for beta-oxidation to produce acetyl-CoA, and to serve as a carbon and energy source for embryo growth [92].

In a study by Garbe and colleagues, pure LA ME, TL, dilinolein, monolinolein and C16/LA-PC, as well as polar and nonpolar lipid extracts (2.12 mM) were dioxygenated with catalysis by purified LOX-1 and LOX-2 from germinating barley or green malt at pH 6.5 [179]. Again, barley LOX-2 activity was much higher than barley LOX-1. Relative LOX activities with EFA substrates were calculated after normalising to LOX activities with LA. For barley LOX-2, LA ME and monolinolein had quite high relative activity of 39.5 and 46.9% respectively, whereas only 6.5 and 14.1% activity was observed with TL and dilinolein respectively. Substrates equivalent to 8 mM LA were prepared by emulsification with a sonifier in the presence of 1% Tween 20. Higher autoxidation products were reported for barley LOX-1 compared to barley LOX-2 with EFA substrates. Products from the LOX-2 catalysed reaction with TL had higher regiospecificity (13-hydroperoxy LA : 9-hydroperoxy LA = 94:6) compared to LOX-1 and LA reaction products (13-

hydroperoxy LA : 9-hydroperoxy LA = 87:13). Mainly *S*-chirality was detected for 13-hydroperoxy LA formed by the LOX-2 for all EFA substrates, but 9-hydroperoxy LA was observed to have predominantly *R*-chirality after LOX-2 biocatalysis due to autoxidation. LOX-1 had the highest regio- and stereo-specificity with C16/LA-PC compared to other substrates [179].

6.2.2. Cucumber

Cucumber LOX (cotyledons, roots and lipid body) including recombinant cucumber LOX has been found in several studies to catalyse TL and 1,3-dilinolein dioxygenation across a broad optimum pH range between 7 and 9. TL has been prepared as an emulsion in gum arabic solution using the method developed by Holtman et al. [92]. TL (20 – 25 mM) in tris/HCl buffer pH 7.5 or 8.5 containing 5% w/v or 15% w/v gum arabic was sonicated at highest power for 30 seconds under N_2 [180-183]. Feussner and co-workers proved that *in vitro* a purified lipid body LOX (LOX localised at the lipid storage organelles) from cucumber seedlings, could oxygenate TL and 1,3-dilinolein at pH 8.5 to form mono-, di- and tri-hydroperoxide derivatives by analysing the reaction products with HPLC and GC-MS after reduction and hydrolysis [183]. This suggested that oxygenated storage lipids detected in cucumber seedlings at different stages of germination *in vivo* were products of lipid body LOX catalysis. The structure of oxygenated storage lipids after extraction from cucumber seedlings was elucidated by HPLC, GC-MS and ^{1}H-NMR and the ratios of LA to LA hydroxides and LA hydroperoxides to LA hydroxides were included to confirm the action of lipid body LOX *in vivo* [183]. In another study by Feussner and co-workers, they showed that soybean LOX-1 formed 28% oxidation products from TL compared to the amount of oxidation products formed from recombinant lipid body LOX and TL at pH 9 (optimum pH for soybean LOX-1) [180]. Soybean LOX-1 only produced mono-hydroperoxide derivatives of

TL at the *sn-1* or *sn-3* position. Recombinant lipid body cucumber LOX oxidised TL at all three positions, and almost equal amounts of mono- and tri-hydroperoxy TLs were observed at pH 9. Di-hydroperoxy TL (~ 20% of total products in molar ratio) was also detected at this pH. At the optimum pH of 7.2, the main product was a TL trihydroperoxide derivative [180].

A low optimum pH of 5 – 6 has been found in two studies for the reactions of recombinant cucumber root LOX-1 and cucumber cotyledon LOX with FFAs, whereas pH 7 – 9 was the optimum pH for TL oxygenation. FFAs (50 mM) sonicated in the presence of 2 mg/mL Tween 20 were used, whilst TL was prepared as an emulsion in either gum arabic or Tween 20 [181, 182]. Matsui and Kajiwara found that cucumber cotyledon LOX was able to catalyse TL as an insoluble substrate at the lipid/water interface of emulsion droplets but not as a soluble substrate in an aqueous reaction medium [182]. The enzyme was pre-incubated with a triolein emulsion and afterwards was unable to catalyse TL. LOX activity decreased with increasing triolein emulsion concentration which may have been due to irreversible adsorption of the enzyme. No difference in LOX activity with TL emulsified with either gum arabic or Tween 20 was observed [182]. Matsui and colleagues also showed that recombinant cucumber root LOX-1 had maximum activity with TL over a broad pH range from pH 7 to pH 9, whereas the enzyme had highest activity with ALA at pH between 4.5 and 6. TL was prepared as an emulsion by sonication in the presence of gum arabic (50 mg/mL), and ALA was dispersed with Tween 20 (2 mg/mL) [181].

6.2.3. Other plants

Other plant LOXs have been shown to catalyse the dioxygenation of LA, LA ME and TL at different optimal pHs: pH 6 (broad beans), 9 – 10 (pea seeds, LOX-1) and 7.5 (canola seeds). Reynolds and Klein found that purified LOX-1 activity in pea seeds

with LA ME and TL as substrates was approximately 30% compared to its reaction with LA [184]. LOX-1 activity was much lower than type-2 LOX activity at the optimum pH of 6 – 7. LA was prepared either in ethanol or as a salt with Tween 20 and KOH, whereas LA ME and TL were in acetone:ethanol (40:60) [184]. Khalyfa, Kermasha and Alli showed that partially purified canola seed LOX extract was able to catalyse the dioxygenation of various EFAs with lower activity than LA in the following order LA>monolinolein>dilinolein>TL [185]. Substrates were prepared in buffer at pH 7.3 in the presence of Tween 20 and Na_2CO_3. Cyanide ions stimulated the enzyme activity with optimum concentration at 40 mM. The enzyme extract showed higher affinity to the canola lipid extract than LA and its esters. The optimum pH was at pH 7.5 which was close to the optimum pH of soybean LOX-2 (pH 6 – 7) but lower than LOX-1 optimum pH (pH 9 – 9.5) [185].

In another study, Abbas and co-workers used crude extracts and more purified LOX preparations from broad beans as a LOX source to test activity with different substrates in the presence and absence of calcium ions (Ca^{2+}) [186]. Substrates were prepared in aqueous solution and solubilised by Tween 20. The active LOX preparations, A, B and C, were obtained from crude extracts after further purification with ammonium sulphate fractionation, one step and two steps of chromatography, respectively. The active enzyme in the crude extracts and preparation C was activated by Ca^{2+} over a broad range of concentrations from 0.2 to 1.6 mM. At pH 7 the enzyme was found to be most affected by Ca^{2+}. Preparation C was found to oxidise both FFAs (LA) and EFAs (LA ME and TL) at an optimum pH of 6. Therefore, this study suggested that the isoenzyme in preparation C was LOX-2 [186].

7. Conclusion

As shown in this literature review, SBF can be used directly or prepared as a crude extract at either acidic or neutral pHs as a LOX source for the synthesis of hydroperoxides from hydrolysed vegetable oils (mainly C18 LC-PUFAs) to further produce volatile compounds. Overall, the best reaction conditions for maximum yield were found to be 5°C, air saturation, pH 10 and in the presence of cysteine at a ratio of 1.5 – 2 compared to the substrate concentration. The method could be applied to green note compound synthesis on an industrial scale for the food industry. Some work with purified and commercial LOXs on C20-22 LC-PUFAs has been reported but not directly with SBF. The commercial 15-sLOX-1 can be used to produce pure mono-hydroxy PUFAs (at pH 12 with a low enzyme concentration) and di-hydroxy PUFAs (at pH 9 using a high enzyme concentration) with high regio- and stereo-specificity.

In soybean seeds, EFAs are not preferred substrates for LOX-1, and LOX-1 is only able to catalyse EFAs under specific conditions at a much lower rate compared to reactions with LA. The presence of a bile salt (deoxycholate, 5 – 10 mM) as an emulsifier and a multicomponent buffer are essential to initiate the reaction at the optimum pH 9 of LOX-1. For both soybean and barley, LOX-2 is more effective than LOX-1 in catalysing EFAs at a large range of optimum pHs from acidic to neutral. In plant LOX-catalysed reactions, EFA substrates are commonly prepared as emulsions using a homogeniser or sonicator in the presence of an emulsifier. In comparison with LOX-catalysed FFA reaction, the dioxygenation reactions on EFAs catalysed by LOX were published in a limited number of studies and not thoroughly investigated with full product characterisation and optimum product formation. The physical state of substrates has an important role in LOX catalysed reactions in aqueous solution. For optimum enzyme activity, FFA substrates should be in

nonmicellar form, whereas EFA substrates are favoured in micellar forms in the presence of an emulsifier.

As C18 LC-PUFAs have already been demonstrated to be suitable substrates for SBF for the synthesis of hydroperoxides, it is likely that SBF can also be used without purification to catalyse dioxygenation of a wider range of substrates, including C20-22 LC-PUFAs and esterified LC-PUFAs. Furthermore, SBF may be more versatile than the commercially available 15-sLOX-1 in both product formation efficiency and ability to catalyse a range of substrates. The different LOX isozymes in SBF may be able to catalyse free and esterified FAs selectively under specific reaction conditions (pH, temperature, additives). In this thesis, sfLOX-catalysed reactions with DHA, TL and tuna oil will be investigated, with product characterisation and comparison with the commercial LOX enzyme. These results will be presented in Chapters 3 to 5. In Chapter 6, the bioactivity of the synthesised resolvin and protectin analogues from the sfLOX-catalysed C20-22 LC-PUFA reactions will be tested in two different cell lines to assess their anti-inflammatory activities and to identify structure-activity relationships.

CHAPTER III

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"Soy flour as an alternative to purified lipoxygenase for the enzymatic synthesis of resolvin analogues"

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ENZYMATIC SYNTHESIS OF RESOLVIN ANALOGUES FROM DHA USING SOYBEAN FLOUR LIPOXYGENASE

1. Introduction

Uncontrolled inflammation is an underlying factor contributing to a range of diseases such as cardiovascular disease, diabetes and cancer. Endogenously synthesised specialised pro-resolving mediators (SPMs) actively switch acute inflammation into resolution of inflammation, which was previously believed to be a passive process [17, 83]. SPMs act as immunoresolvents, whereas the current anti-inflammatory drugs are immunosuppressants causing undesirable side effects [68, 187]. Therefore, SPMs have emerged as a potential class of anti-inflammatory drugs following the natural pathway of resolution of inflammation. The new approach is promising as it may not show any side effects and could be the most effective solution to resolve inflammation. In fact, a range of SPMs such as lipoxin A4, resolvin E1, protectin D1 and some analogues have been tested in clinical trials and have shown positive results in various inflammatory-mediated diseases [10, 79, 83, 188]. SPM active, a product containing SPM analogues (17-HDHA and 18-HEPE), is being sold by Metagenics® as a supplement to support a healthy immune system [76].

The most common method to synthesise SPMs is total chemical synthesis. The procedures are normally lengthy and complex involving toxic chemicals to obtain specific double bond configuration, and stereo- and regio-specificity of the bioactive compounds [35-38]. In contrast, enzymatic synthesis is much simpler and operates under mild conditions to obtain products with high stereo- and regio-specificity. However, it is expensive to use purified enzymes for the efficient development of large scale SPM synthesis. Soybean flour (SBF) is a cheap lipoxygenase (LOX) source and has been used in a small number of studies to synthesise precursor

hydroperoxides on a gram scale for volatile compound synthesis [111, 157, 158, 164, 165]. However, no work has been done on SPM synthesis using SBF as a LOX source.

In this work, for the first time, SBF LOX (sfLOX) has been used to synthesise SPMs from a range of biologically important omega-3 and omega-6 long chain polyunsaturated fatty acids (n-3 and n-6 LC-PUFAs). DHA was used as a model substrate as it is the key precursor of various SPM families including resolvin D series, protectins and maresins. DHA is also found predominantly in brain and has important functions in brain health [189]. In addition, DHA (along with EPA) is present in significant amounts compared to other PUFAs in fish oils [31]. This work represents the first time SBF has been used to oxidise PUFAs with 20 and 22 carbons, and to produce di-hydroxy products from DHA.

The sfLOX-catalysed reaction of DHA was optimised to produce the maximum amount of di-hydroxylated DHA compounds. The reaction products were characterised with a variety of analytical techniques, including NP-HPLC, RP-HPLC and GC-MS. The reaction products were confirmed to be mainly 7,17-diHDHA (resolvin D5) and 10,17-diHDHA (protectin DX), and their chirality was identified to be specifically *S,S* (> 99.5%). The reaction yield was approximately 75%. Product formation per milligram substrate was comparable to that obtained with 15-sLOX-1 (a commercially available soybean LOX-1) in synthesising resolvin D5 and protectin DX from DHA. The optimised reaction conditions of DHA were applied to other biologically relevant LC-PUFAs (EPA, AA, DPAn-3 and DPAn-6). sfLOX and 15-sLOX-1 showed the same efficiency in synthesising the anti-inflammatory lipid mediator analogues from LC-PUFAs. These methods may be suitable for the large scale production of these bioactive molecules for pharmaceutical, nutraceutical and

functional food applications using SBF as an alternative cost-effective LOX source to commercial LOX.

2. Materials and methods

2.1. Materials

Soy flour (Kialla Pure Foods, certified organic) was purchased from Organic Wholefoods (Brunswick East, Vic, Australia). Soybean 15-lipoxygenase (15-sLOX-1, EC 1.13.11.33, P1, 9.42 Munits/mL, 15.2 mg of protein/mL, 0.62 Munits/mg of protein, Cayman Chemical), 7*S*,17*S*-dihydroxy DHA (Cayman Chemical), 10*S*,17*S*-dihydroxy DHA (Cayman Chemical) and prostaglandin B2 (PGB2; Cayman Chemical) were purchased from Sapphire Bioscience (Redfern, NSW, Australia). The fatty acids DHA, EPA, ARA, DPAn-3 and DPAn-6 (Nu-Chek Prep) were obtained from Adelab Scientific (Thebarton, SA, Australia). 2,2-Dimethoxypropane (Fluka), Dess-Martin periodinane (DMP), ferric chloride, glycine, L-cysteine, methylene blue, *N,O*-bis(trimethylsilyl)trifluoroacetamide (with 1% trimethylchlorosilane), platinum(IV) oxide, potassium phosphate monobasic, potassium phosphate dibasic, pyridine (>99%), sodium borohydride, sodium dodecyl sulphate (SDS), Trizma® base, Tween® 20, trifluoroacetic acid, (trimethylsilyl)diazomethane (2 M in diethyl ether), tris(2-carboxyethyl)phosphine hydrochloride (TCEP), *β*-carotene, Supelclean™ LC-Si solid phase extraction (SPE) tubes (6 mL, 1 g, Supelco Analytical) and SPE vacuum apparatus (Supelco Analytical) were purchased from Sigma Aldrich (Castle Hill, NSW, Australia). Agilent Bond Elut 500 mg, 3 mL SPE tubes and Vac Elut SPE vacuum apparatus were from Agilent Technologies (Mulgrave, VIC, Australia). Heptane (99 %, 0.2 micron filtered) and isopropanol were obtained from Fisher Scientific (Scoresby, VIC, Australia). Hydrochloric acid (32%) and sodium tetraborate were purchased

from APS Chemicals Ltd (Seven Hills, NSW, Australia). Ethanol, diethyl ether, dichloromethane, methanol and urea were purchased from Chem-Supply (Gilman, SA, Australia). Acetonitrile (Scharlau, Spain), acetic acid (BDH Lab Supplies, UK) and sodium hydroxide (Fisher Chemicals, UK) were purchased from Science Supply (Mitcham, VIC, Australia). Triton X-100^{TM} was obtained from Ajax Chemicals (Sydney, NSW, Australia). 4× Laemmli sample buffer, broad range SDS-PAGE standard, precision plus protein dual colour standard, dithiothreitol, and 7.5 and 12% SDS-PAGE gels (Mini-PROTEAN TGX Precast Protein gels) were purchased from Bio-Rad (Gladesville, NSW, Australia). Hydrogen, nitrogen and oxygen gases were obtained from Coregas Pty Ltd (Yennora, NSW, Australia). MilliQ water was obtained in lab by Milli-Q® Advantage A10 Ultrapure water purification system from Merck Millipore.

2.2. Lipoxygenase-catalysed reaction optimisation for maximum product formation

DHA (as a model free fatty acid substrate) was prepared at 0.1 mM (from 10 mM stock solution in ethanol) in 10 mL buffer (50 mM) with a range of pHs and buffer types (Table 3.1) [43]. 25 µL SBF extract was added to initiate the reaction [56]. The SBF extract was prepared by suspending 1 g SBF in 20 mL water [56], followed by shaking for 1 min and allowing to settle for at least 3 min. Aliquots of the crude SBF extract were used for the reactions without re-suspending the settled SBF. The extract should be used from 20 min up to 2 hours after preparation for the optimum LOX activity and stability. After addition of the SBF extract, the reaction mixture was stirred, and the reaction conducted at room temperature (23ºC) for 15 min and without oxygenation as the initial conditions. The reaction temperature was controlled by performing the reaction on ice, or in a water bath as required. Nitrogen, air or oxygen was used to sparge the buffer for 5 min, until saturation to alter the

dissolved oxygen concentration before adding substrate. The oxygen concentration of the buffer solution was measured by dissolved oxygen meter (Mettler Toledo). Surfactant and other additives were added as required prior to the addition of the SBF extract.

Table 3.1: Three types of buffer used and the pH ranges studied.

Buffer type	pH
Phosphate	$5-8$
Tris-HCl	$7-9$
Borate	$8-11$

The reaction was stopped by the addition of sodium borohydride (1 M solution in water, 200 µL for a 10 mL reaction) which reduced the hydroperoxides to hydroxy groups. This was stirred for at least 15 min. The mixture was then acidified with glacial acetic acid (50 µL for a 10 mL reaction), and further stirred for approximately 30 min. Prostaglandin B2 (PGB2) was used as an internal standard and was added (20 µL of 1 mg/mL solution in ethanol) into the reaction mixture prior to extraction as required.

The products were extracted by solid phase extraction (SPE) under vacuum. C18 SPE cartridges were conditioned with 1 column volume (3 mL) of methanol and 1 column volume of water. The sample was then loaded onto the column before washing with 1 column volume of water. The column was then dried under vacuum for $5-15$ min and the product eluted with 1 column volume of ethanol. The total solvent was evaporated under a stream of nitrogen or under vacuum [43, 56].

For reactions using higher volumes of SBF extract (≥ 300 µL), solvent extraction was used to extract the products, as an alternative to SPE as the SBF in the reaction

mixture blocked the SPE columns. The reaction mixture was adjusted to pH 3 with hydrochloric acid (4 M) and was extracted with diethyl ether (2×10 mL for a 10 mL reaction). The combined solvent after extraction was evaporated under vacuum.

The reaction was optimised to determine the conditions for the synthesis of the maximum amount of di-hydroxy products. Three variables, pH, buffer type and SBF extract volume (25, 100, 200, 300 and 400 µL), were examined at the same time. The best conditions were chosen as initial conditions for further optimisation, with the remaining variables tested one at a time (Table 3.2). The conditions with the best results were kept for further optimisation experiments and for the final optimised conditions.

The optimised sfLOX-catalysed DHA reaction was compared to the optimised 15-sLOX-1 and DHA reaction as described by Dobson et al. [43]. No detection of di-hydroxy DHA from autoxidation was observed in blank controls performed under the same reaction conditions without SBF or DHA.

Table 3.2: Reaction variables optimised in the sfLOX-catalysed reaction with DHA.

Variable	Range studied
DHA concentration (mM)	$0 - 1$
Surfactant concentration (% w/v)	$0 - 0.1$
Reaction time (min)	$1 - 180$
Oxygenation	Oxygen, Nitrogen, Air, None
Temperature (°C)	$0 - 40$
Cysteine concentration (mM)	$0 - 10$
Ferric chloride concentration (mM)	$0 - 0.2$

For optimisation of individual reaction variables, experiments were repeated between 1 and 3 times to ensure a clear trend and optimum was observed. Values from a

single representative experiment are shown in the results section for each variable. Results at different reaction conditions for each variable were considered to be different when the results varied by greater than ± 5%. The final optimised reaction conditions were repeated five times a day for three consecutive days. Average results were presented with error bars indicating (±) one standard deviation.

2.3. Monitoring reaction product formation by NP-HPLC

sfLOX-catalysed DHA reaction products were separated by NP-HPLC [43] using an Agilent Technologies 1200 series instrument, including a solvent degasser, quaternary pump, autosampler and diode array detector (DAD). An LC-diol column (Supelco Analytical, 25 cm × 3 mm, 5 μm particle size) was used. The composition of mobile phase was 95% v/v solvent A (heptane with 0.1% v/v acetic acid, 0.1% v/v 2,2-dimethoxypropane) and 5% v/v solvent B (isopropanol). Samples were separated under isocratic conditions within 35 min at 10°C with a flow rate of 0.5 mL/min. Elution of mono- and di-hydroxy products was monitored at 210 nm (substrate), 234 nm, 250 nm, 270 nm and 280 nm (PGB2). Samples were prepared in 1 mL mobile phase and the injection volume was 15 μL. Reproducibility was confirmed by repeat injections. Data was processed using Agilent Technologies ChemStation for LC 3D system B.04.01 software.

2.4. Determination of hydroxy group positions on reaction products by GC-MS

Positions of hydroxy groups on the sfLOX-catalysed DHA reaction products were determined by GC-MS [43] after hydrogenation and conversion to trimethylsilylated derivatives (see below). An Agilent Technologies 6890N Network GC system with a 5975 mass selective detector (electron impact) and a BPX5 column (30.0 m × 0.25 mm, 0.25 μm film thickness; SGE, Ringwood, VIC, Australia) was used. Injection volume was 2 μL, helium carrier gas, flow rate 1.2 mL/min (average velocity of

42 cm/second) with an inlet temperature of 250°C and a split ratio of 20:1. Initial

oven temperature was 150°C held for 2 min, increased to 300°C at a rate of 5°C/min

over 30 min and held for 13 min. Total ion chromatograms were collected and data

processed with Agilent Technologies MSD ChemStation D.02.00.275 software.

Hydrogenation

The optimised sfLOX-catalysed DHA reaction products after solvent extraction and

evaporation were re-dissolved in 2 mL ethanol. PtO_2 catalyst (10 mg) was added and

the reaction was stirred under a stream of H_2 for 25 min. The mixture was filtered

through a 0.45 µm filter and solvent evaporated under nitrogen.

Silylation

After hydrogenation, products were heated at 100°C for 1 hour in the presence of

200 µL of pyridine and 200 µL of *N,O*-bis(trimethylsilyl)trifluoroacetamide

(BSTFA). The samples were analysed by GC-MS.

2.5. Determination of stereochemistry of reaction products by chiral RP-HPLC

sfLOX-catalysed DHA reaction products after methylation (see below) were stereo-

specifically separated by chiral RP-HPLC. An Agilent Technologies 1260 Infinity

series instrument, including a solvent degasser, quaternary pump, autosampler and

diode array detector (DAD) was used. A Lux 5u Amylose-2 column (Phenomenex,

100 × 4.6 mm, 5 µm particle size) was used. The mobile phase contained solvent A

(acetonitrile) and solvent B (0.1% v/v trifluoroacetic acid). Different gradient

conditions were used to separate the four 7,17-diHDHA methyl ester stereoisomers

(40% to 47.5% solvent A over 45 min at 1 mL/min, 40ºC), and the four 10,17-

diHDHA methyl ester stereoisomers (35% to 50% solvent A over 30 min at

0.5 mL/min, 10ºC). Elution of the stereoisomers was monitored

spectrophotometrically at 210 nm, 234 nm, 250 nm and 270 nm. Samples were

prepared in acetonitrile and injection volumes of 1 to 10 µL were used.

Reproducibility was confirmed by repeat injections. Data was processed using

Agilent Technologies ChemStation for LC 3D system software (B.04.03).

Methylation

After extraction of reaction products by solvent extraction and evaporation of the

solvent, 200 µL methanol and 200 µL (trimethylsilyl)diazomethane were added and

the reaction mixture was kept at room temperature for one hour in a sealed vial. The

solvent was evaporated, and products dissolved in 1 – 3 mL heptane for extraction by

SPE. An LC-Si cartridge was conditioned with 3 column volumes of heptane (3 ×

6 mL) before the sample mixture was loaded. The SPE cartridge was washed with 1

column volume of heptane and dried under vacuum for 5 – 15 min. The methylated

products were eluted with 1 column volume of isopropanol. After evaporation of the

solvent under nitrogen gas or vacuum, the sample was diluted in 1 mL acetonitrile

and analysed by chiral RP-HPLC. The standards of 7*S*,17*S*-dihydroxy DHA and

10*S*,17*S*-dihydroxy DHA and synthesised racemic product mixture (see below) were

methylated using the same procedure prior to chiral RP-HPLC.

Synthesis of racemic dihydroxy DHA mixture

To develop the chiral RP-HPLC separations, a racemic mixture of 7,17-dihydroxy

DHA and 10,17-dihydroxy DHA isomers containing the four stereoisomers (*SS*, *RR*,

SR and *RS*) of each compound was synthesised. The racemic mixture was generated

from the enzymatic reaction product mixture, by oxidising the hydroxy groups to

ketones then reducing them back to hydroxy groups. Briefly, the extracted enzymatic

sfLOX-DHA reaction products (~ 0.7 mg of 7,17-diHDHA and 10,17-diHDHA)

were dissolved in 1 mL of dry dichloromethane in a round bottom flask and ~ 10 mg

of Dess-Martin periodinane (DMP) was added. Before use, the DMP was purified by

dissolving in dichloromethane and filtering to remove insoluble 2-iodoxybenzoic

acid (IBX). The reaction mixture was sealed and stirred for 20 min at room temperature, to form a cloudy solution. Completion of the reaction was monitored by TLC, using a solvent system of n-heptane/diethyl ether/acetic acid (40:60:1). Complete conversion of starting material (R_f 0.1) to oxidized ketone products (R_f 0.2) was observed. The solvent was evaporated under nitrogen and the products were dissolved in ethyl acetate (4 mL). The reaction products were washed with two volumes of sodium thiosulfate (100 g/L), stirring for 10 min each, followed by two volumes of water and dried over anhydrous sodium sulfate. The isolated oxidized product was yellow. The solvent was filtered and evaporated under nitrogen.

The oxidised products were then dissolved in 1 mL methanol and 10 mL water was added. Sodium borohydride in water (1 M, 200 µL) was added and the mixture stirred for 5 – 15 min to reduce the oxidized products. The yellow colour disappeared within 5 min. The reaction was stopped by adding 100 µL glacial acetic acid. The reduced products were extracted with 2 × 20 mL diethyl ether and the solvent was evaporated under vacuum. Completion of the reaction was confirmed by TLC using the same solvent system as above.

2.6. Assessment of enzyme kinetic parameters

The optimised reactions (3 mL scale) were performed in a quartz cuvette (4 mL, 10 mm path length) containing 2.5 mL buffer (50 mM, pH 8) saturated with oxygen, 50 µL DHA (10 mM) and 25 µL Triton X-100 (1% w/v) at room temperature. 200 µL of SBF extract (1 g in 20 mL water) was added to initiate the reaction. The enzyme kinetics were characterised by measuring the reaction rate (Abs/min) on a Cary 300 UV-Vis spectrophotometer (Agilent Technologies) with increasing DHA concentrations in the range (50 µM – 0.4 mM). The conjugated triene was monitored at 270 nm for 1 min with data averaging time of 0.1 second. A sample without SBF

extract was used as a reference. The activity was measured immediately after adding SBF. All measurements were conducted until three reproducible results were obtained. The Michaelis-Menten kinetic model, $r = V_{max}[S]/(K_m+[S])$ was used to calculate kinetic parameters [67].

2.7. Colorimetric test for LOX isozyme detection

Three visual screening tests developed previously by Suda et al. [190] were used to detect the individual LOX isozymes in the SBF and commercial enzyme preparations. The tests are based on the bleaching activities of the isozymes on methylene blue and β-carotene by co-oxidation with sodium linoleate. For each test 0.5 mL commercial enzyme solution or SBF suspension and 2 mL of dye-substrate solution were used. The dye-substrate compositions of the three tests are listed below:

- Test I for LOX-1 detection: sodium linoleate substrate (1.25 mM) and methylene blue (12.5 μM) in sodium borate buffer (50 mM) pH 9.
- Test II for LOX-2 detection: sodium linoleate substrate (1.25 mM), dithiothreitol (25 mM), methylene blue (12.5 μM) and 12.5% v/v acetone in sodium phosphate buffer (200 mM) pH 6.
- Test III for LOX-3 detection: sodium linoleate substrate (1.25 mM) and 12.5% w/v *β*-carotene in sodium phosphate buffer (200 mM) pH 6.6. 50% w/v *β*-carotene in acetone was used as the stock solution.

SBF suspension was prepared as 0.1 g (test I and III) or 0.2 g (test II) in 20 mL water. The commercial 15-sLOX-1 enzyme was diluted in water; 140 μg (test I and III) or 280 μg (test II) in 0.5 mL. The SBF suspension was settled for 3 – 10 min before adding the dye-substrate solution. The colour change was checked after 3 min for test I and III, 5 min for test II. One control and three samples were conducted for

every test. 0.5 mL water was used instead of enzyme solution or SBF suspension for the controls.

2.8. Protein separation of soybean flour and commercial lipoxygenase preparations by SDS-PAGE

Denaturing, reducing, discontinuous 1D SDS-PAGE was conducted according to Laemmli [191], with modifications. All SDS-PAGE analyses were performed using 7.5 and 12% acrylamide MiniPROTEAN® TGX™ precast gels (Bio-Rad) in a Mini-PROTEAN Tetra Vertical Electrophoresis cell (Bio-Rad) using a Tris-Glycine buffer system. SBF extract samples were prepared by adding 5 mg SBF to 250 µL sample loading buffer (475 µL 4× Laemmli sample buffer (Bio-Rad), 0.2 M dithiothreitol (DTT), 5 M urea) and incubated for 30 min. Urea was added to the loading buffer based on works carried out by Kitamura et al [192, 193]. The suspension was centrifuged at 13,300 rpm (Heraeus 17 Pico centrifuge, Thermo Scientific) for 3 min to remove insoluble material and the supernatant retained for further dilution. The commercial LOX preparations (unpurified and purified 15-sLOX-1) were diluted with sample loading buffer 10 and 5 times, respectively. Before loading samples on the gel, further dilutions from 1 in 2 to 1 in 8 as required were used to obtain adequate band intensity on the gel. Samples were incubated in a water bath at 80°C, 50°C or room temperature for 10 min. Aliquots (20 µL) of the samples were loaded onto the gel. A molecular weight standard was also loaded onto each gel as a mass reference, with either a Broad-Range SDS-PAGE standard (Bio-Rad, prepared by adding 2 µL of standard to 18 µL of sample loading buffer, 5 µL loaded) or Precision Plus Protein Dual Colour Standard (Bio-Rad, 3 µL loaded directly onto the gel) used. The 7.5% and 12% SDS-PAGE gels were electrophoresed for 30 min at 100 V, followed by 2 hr (7.5% gel) or 5 – 6 hr (12% gel) at 160 V at 4°C. After electrophoresis, each gel was stained with Bio-Safe Coomassie G-250 Stain with

agitation for 1 hr, followed by destaining in water with agitation overnight. Gels were imaged using a Molecular Imager® Gel Doc^TM XR+ Imaging System (Bio-Rad).

3. Results

3.1. Optimisation of reaction conditions for maximum product formation

Luquet et al. used SBF suspended in water as a crude LOX source without any complex preparation in the reaction with free fatty acids to produce oxygenated products. The reactions were performed with 0.3 mM LA, 50 µL suspended SBF solution (1 g per 20 mL water) in 20 mL borate buffer (pH 9.22) with 0.032 % Tween 20 [56]. DHA (Figure 3.1A) is the precursor of important biological compounds including RvD5 and PDX, which are anti-inflammatory and pro-resolving lipid mediators [19, 70]. These bioactive compounds have previously been synthesised successfully from DHA and commercial 15-sLOX-1 [43, 53, 54]. Dobson et al. used the following optimised conditions: 0.1 mM DHA, ~ 1.5 MUnits 15-sLOX-1, 10 mL borate buffer pH 9, without any surfactant in 15 min [43]. In this study, the three main products of DHA were synthesised and characterised. They were a mono-hydro(pero)xy product, 17*S*-hydro(pero)xydocosahexa-4*Z*,7*Z*,10*Z*,13*Z*,15*E*,19*Z*-enoic acid (17*S*-H(P)DHA; Figure 3.1B) with one conjugated diene; and two di-hydroxy products, 7*S*,17*S*-hydro(pero)xydocosahexa-4*Z*,8*E*,10*Z*,13*Z*,15*E*,19*Z*-enoic acid (7*S*,17*S*-diH(P)DHA; Figure 3.1C) known as RvD5 with two conjugated dienes, and 10*S*,17*S*-hydro(pero)xydocosahexa-4*Z*,7*Z*,11*E*,13*Z*,15*E*,19*Z*-enoic acid (10*S*,17*S*-diH(P)DHA; Figure 3.1D) also known as PDX which has a conjugated triene. One oxygen molecule per substrate molecule is required to form mono-hydroxy products, whereas two molecules of oxygen are needed for di-hydroxy product formation (Figure 3.1).
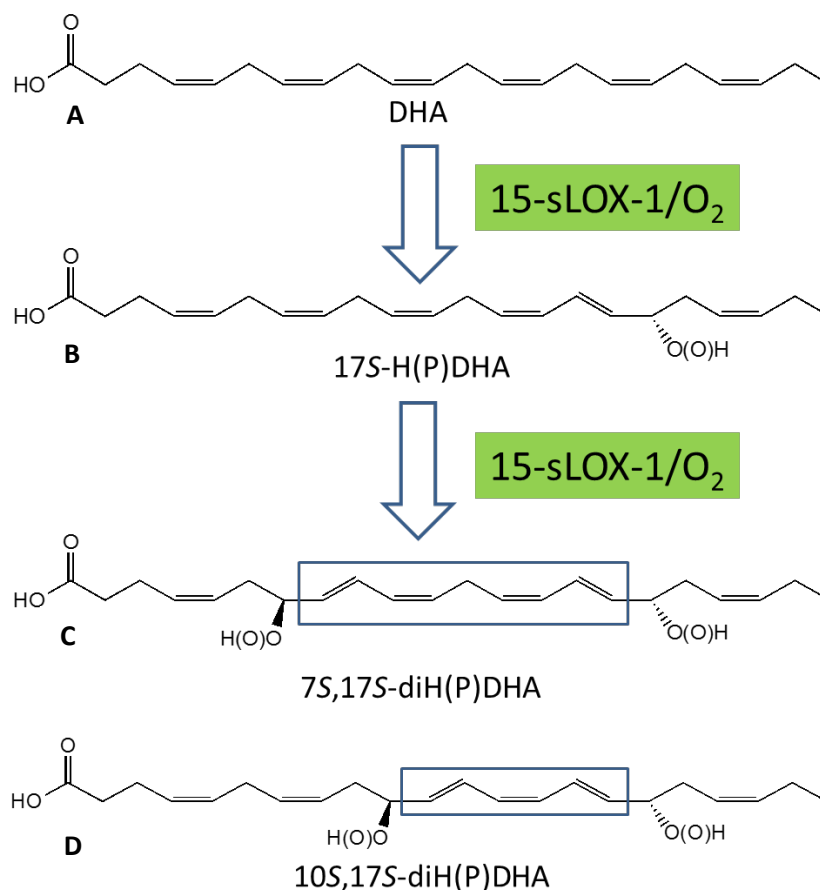
Figure 3.1: Chemical structures of hydroxylated products of DHA from the reaction with commercial 15-sLOX-1. A) DHA, B) 17S-H(P)DHA, C) 7S,17S-diH(P)DHA with the two conjugated dienes highlighted in box, D) 10S,17S-diH(P)DHA with the conjugated triene highlighted in box.

Based on these two references [43, 56], the initial reaction conditions chosen were: 0.1 mM DHA (as a model substrate for sfLOX and LC-PUFA reaction), 25 µL SBF extract (1 g SBF in 20 mL water), 10 mL buffer, ambient temperature, no surfactant, 15 min reaction time. The optimisation process aimed to identify the ideal conditions to produce the maximum amount of di-hydroxy DHA products. The formation of di-hydroxy and mono-hydroxy DHA products from the sfLOX-DHA reaction at different conditions was monitored by NP-HPLC with DAD. These compounds were separated and identified by their distinctive absorption spectra. The first di-hydroxy DHA product had two absorption maxima at 226 and 246 nm in the spectrum, consistent with the presence of two conjugated dienes (Figure 3.2 - orange trace). The second di-hydroxy DHA product had a maximum absorbance at 270 nm with

two shoulders at 260 nm and 280 nm in the spectrum, consistent with a conjugated triene structure (Figure 3.2 - green trace). Mono-hydroxy DHA products had maximum absorbance at 234 nm, consistent with a single conjugated diene (Figure 3.2 - blue trace). By comparison with the retention times and absorbance spectra of the products from the commercial 15-sLOX-1 catalysed reaction of DHA under the same NP-HPLC conditions [43], the two di-hydroxy DHA products and a major mono-hydroxy product from the sfLOX-catalysed DHA reaction were tentatively identified as 7,17-diHDHA, 10,17-diHDHA and 17-HDHA, respectively.
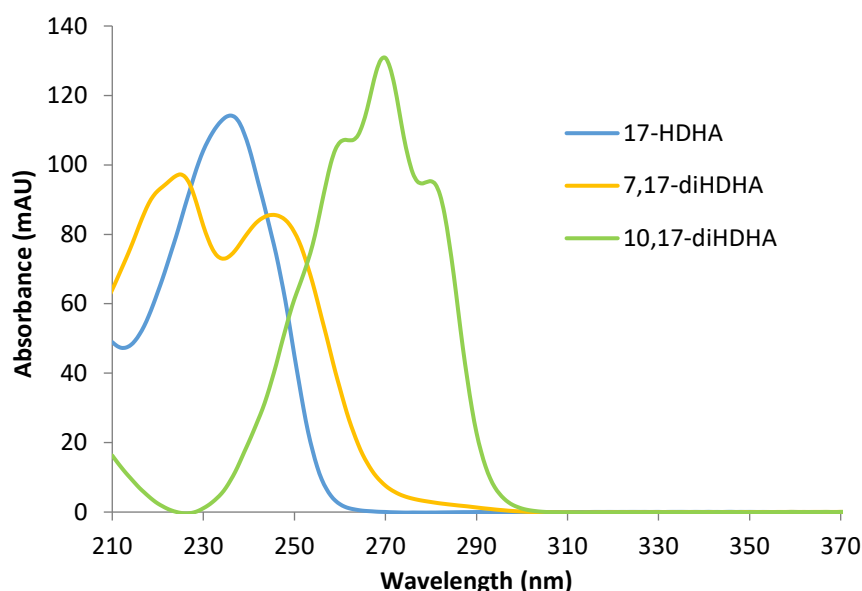
Figure 3.2: Absorption spectra recorded by NP-HPLC DAD for mono- (17-HDHA) and di-hydroxy DHA products (7,17- and 10,17-diHDHA) from the sfLOX-DHA reaction.

3.1.1. Effect of buffer type, pH and volume of SBF extract on product formation

It is important to optimise the pH of the sfLOX-catalysed DHA reaction, as the LOX reaction is known to be pH-dependent due to the different LOX isoenzymes present and physical state of the substrate [90, 91]. To determine the optimum pH of the sfLOX-catalysed DHA reaction, a large pH range (5 – 11) was studied using three different buffers (phosphate pH 5 – 8, Tris-HCl pH 7 – 9 and borate pH 8 – 11) and 25 µL SBF extract under the initial reaction conditions described in the previous

paragraph. At low pH (5.0 – 6.5), formation of all products was suppressed. The major mono-hydroxy DHA (17-HDHA) product increased significantly with increasing pH and a range of other mono-hydroxy products were also formed in small amounts. In contrast, di-hydroxy DHA formation was maximised at pH 7.5. To further optimise this, the optimisation was continued by increasing the volume of SBF extract, to increase the amount of LOX enzyme for the potential conversion of the remaining mono-hydroxy DHA into di-hydroxy DHA products. This was examined across a narrower pH range (7 – 10). At higher volumes of SBF extract ($\geq$ 100 µL), the optimum pH shifted to pH 8. The di-hydroxy products dramatically increased approximately 10 times for 10,17-diHDHA and 15 times for 7,17-diHDHA at pH 8 (phosphate buffer) when the volume of SBF extract was increased from 25 to 300 µL; whereas product formation began to decrease at 400 µL SBF extract (Figure 3.3). With 300 µL SBF extract, DHA was fully converted into di-hydroxy DHA products and a minimal amount of mono-hydroxy product remained. Mono product specificity was improved with increasing SBF extract volume until only two mono-hydroxy products were observed with 300 µL SBF extract.
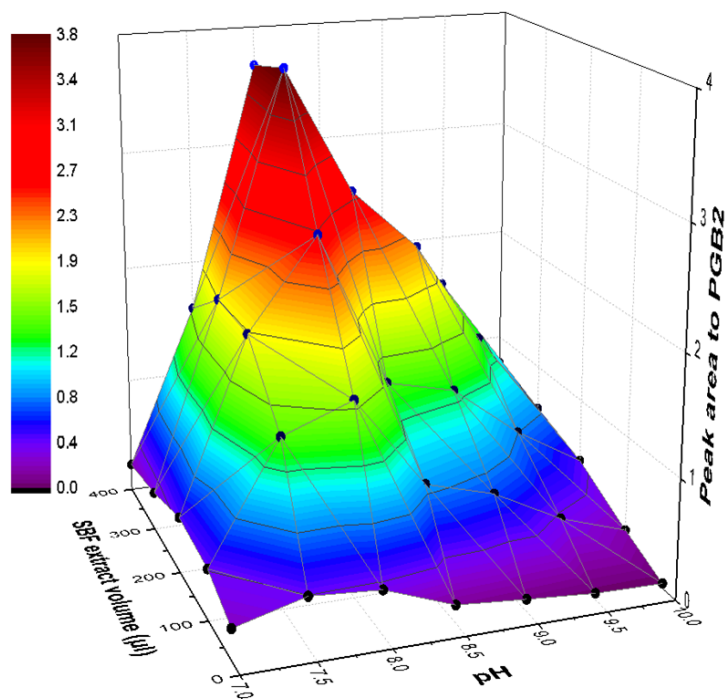
Figure 3.3: Effect of buffer pH and SBF extract volume on 10,17-diHDHA production from sfLOX-DHA reaction (25 – 400 µL SBF extract; pH 7 – 8: 50 mM phosphate buffer; pH 8.5 – 10.0: 50 mM borate buffer). Reaction conditions: 0.1 mM DHA, 10 mL buffer, ambient temperature, no surfactant, 15 min reaction time. The results are combined from multiple sets of experiments.

SBF extract volume was re-optimised in the range of 200 to 400 µL, and 300 µL remained the optimum value. Phosphate buffer showed higher product formation compared to borate and Tris-HCl buffers at high volumes of SBF extract (≥ 100 µL). The optimised conditions found through the simultaneous optimisation of the three variables (buffer type, pH and volume of SBF extract) were phosphate buffer (pH 8) and 300 µL SBF extract (Figure 3.3). A small amount of DHA (≤ 5 %) remained in the reactions using 25 µL SBF extract, whereas no DHA was observed under any conditions using higher volumes of SBF extract (> 25 µL).

Two unknown compounds eluted on NP-HPLC approximately 10 min after the two di-hydroxy products; unknown I had two absorption maxima at 226 nm and 246 nm, and unknown II had an absorption maximum at 270 nm with two shoulders at 260 nm and 280 nm in the absorbance spectrum, which indicated that they may have two

conjugated dienes and a conjugated triene, respectively. They appeared randomly in small amounts, under different reaction conditions, including at different pH and buffer types when using 100 µL SBF extract or higher. The unknown compounds have not been identified.

3.1.2. Effect of DHA concentration on product formation

At low substrate concentrations, substrate can be limiting in the reaction. In contrast, at high substrate concentrations, the reaction can be inhibited by substrate and/or products [194-197], and anaerobic reaction conditions can develop [43]. Therefore, it is important to optimise substrate concentration. DHA concentration was optimised in the range (0.025 – 1 mM), using 300 µL SBF extract and 10 mL phosphate buffer (pH 8, 50 mM). In the DHA concentration range studied, the formation of products increased significantly with increasing DHA concentration before reaching a maximum at 0.2 mM. At this DHA concentration, 7,17-diHDHA and 10,17-diHDHA increased by approximately 35% and 10% respectively, compared to at 0.1 mM DHA, or approximately 4-fold compared to at 0.025 mM DHA (Figure 3.4). However, the di-hydroxy DHA products decreased substantially to a minimal amount at 0.5 mM DHA, and then stayed constant up to 1 mM DHA. Instead, 17-HDHA increased significantly and two new products (unknown III and IV) were formed in large amounts. Unknown III eluted earlier than DHA by 2 min with λ_{max} = 240 nm and unknown IV (also detected for reactions using 0.3 mM DHA) eluted 3 min after 17-HDHA and before the di-hydroxy DHA products with λ_{max} = 235 nm. These compounds generated at high substrate concentrations may result from the development of anaerobic conditions. In a study by Dobson and colleagues, two similar compounds were also found in 15-sLOX-1 catalysed DHA reaction at DHA concentration > 0.1 mM, in which one compound was identified to be 17-oxoheptadeca-4Z,7Z,10Z,13Z,15E-pentaenoic acid and the other was suggested to be

a DHA dimer [43]. The optimum DHA concentration was determined to be 0.2 mM. At the new optimised DHA concentration, only a small amount of 17-HDHA remained. Volume of SBF extract was re-optimised across a larger range of 200 – 800 µL, but there was no change in the optimum SBF extract volume.
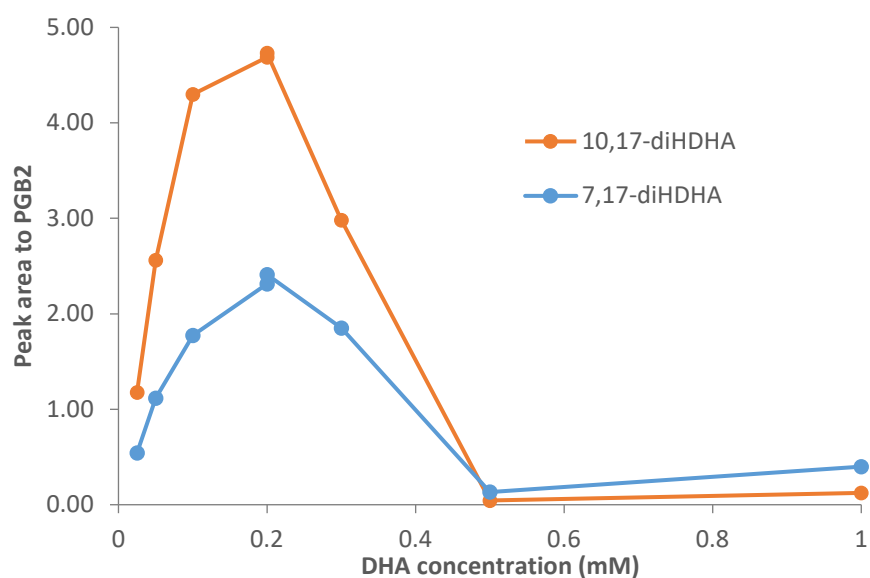
Figure 3.4: Effect of DHA concentration (0.025 - 1 mM) on sfLOX-DHA reaction under the conditions: 300 µL SBF extract, 10 mL phosphate buffer (pH 8, 50 mM), ambient temperature, no surfactant, 15 min reaction time. The results are combined from two sets of experiments (0.025 – 0.2 mM and 0.2 – 1 mM) presenting DHA concentration effect on product formation.

3.1.3. Effect of surfactant concentration on product formation

Non-ionic surfactants including Triton X-100 and/or Tween 20 have previously been demonstrated to increase the solubility of fatty acids in water, enhance LOX activity [116, 198, 199] and extract membrane-bound LOXs effectively from plant sources [200, 201]. As a result, Triton X-100 and Tween 20 were tested for their ability to increase the sfLOX-catalysed DHA reaction yield in making di-hydroxy DHA products. The concentration of both surfactants was optimised in the range 0.001 – 0.1% w/v. Increasing Triton X-100 concentration resulted in an increase in the production of both di-hydroxy DHA products up to an optimum in the range 0.01% w/v to 0.05% w/v within the error of the reaction, with an increase of ~ 40%

for 10,17-diHDHA compared to the reaction without Triton X-100. Then product formation of the two di-hydroxy DHA products tended to decrease at higher Triton X-100 concentrations (> 0.05% w/v) (Figure 3.5). The same patterns of surfactant effect were observed with Tween 20 but a lower overall yield of di-hydroxy DHA products was obtained compared to that with Triton X-100. Increasing surfactant concentration increased the formation of 17-HDHA. Triton X-100 was therefore the preferred surfactant for the reaction and the selected concentration for further experiments was 0.01% w/v.
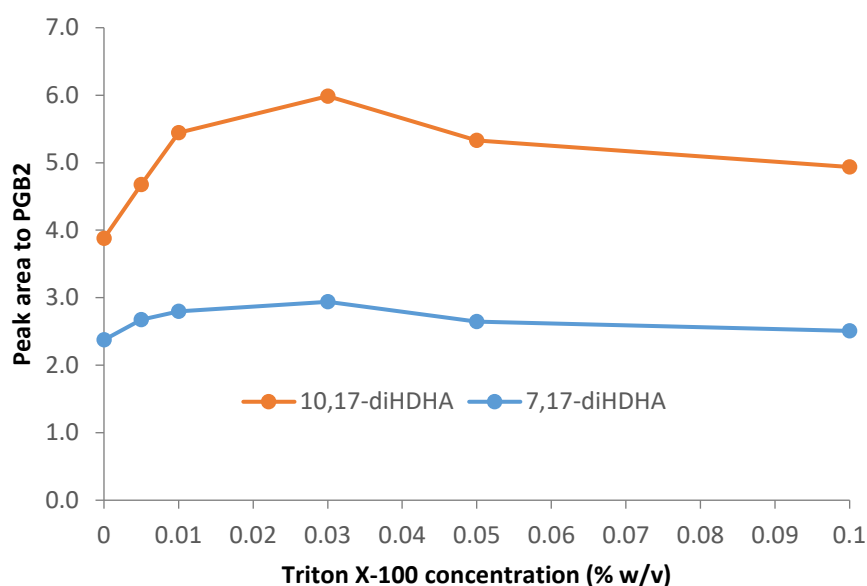
Figure 3.5: Effect of Triton X-100 concentration (0 – 0.1% w/v) on the sfLOX-catalysed DHA reaction under the conditions: 0.2 mM DHA, 300 µL SBF extract, 10 mL phosphate buffer (pH 8, 50 mM), ambient temperature, 15 min reaction time. The results from a single representative experiment present Triton X-100 effect on product formation.

3.1.4. Effect of reaction time on product formation

To monitor product formation over time for the maximum yield, large-scale reactions were performed (100 mL, 10 times the normal 10 mL reaction volume) and 10 mL aliquots were taken at specific time points (1 min to 180 min) after the reaction was initiated. The di-hydroxy DHA products were rapidly formed within the first minute and increased significantly over time to a maximum between 20 and 30 min; 10,17-

diHDHA increased by more than double, whilst 7,17-diHDHA increased by 1.5-fold, compared to product formation at 1 min. After 30 min, both products gradually decreased (Figure 3.6A), although the amount of 17-HDHA was still significant and available for conversion. Unknown I and II were also detected, and in contrast to the main di-hydroxy DHA products, reached a maximum concentration at 1 min then declined substantially over 20 – 30 min, and continued to decrease slowly over the remaining time (Figure 3.6B). The optimum reaction time was chosen to be 30 min.
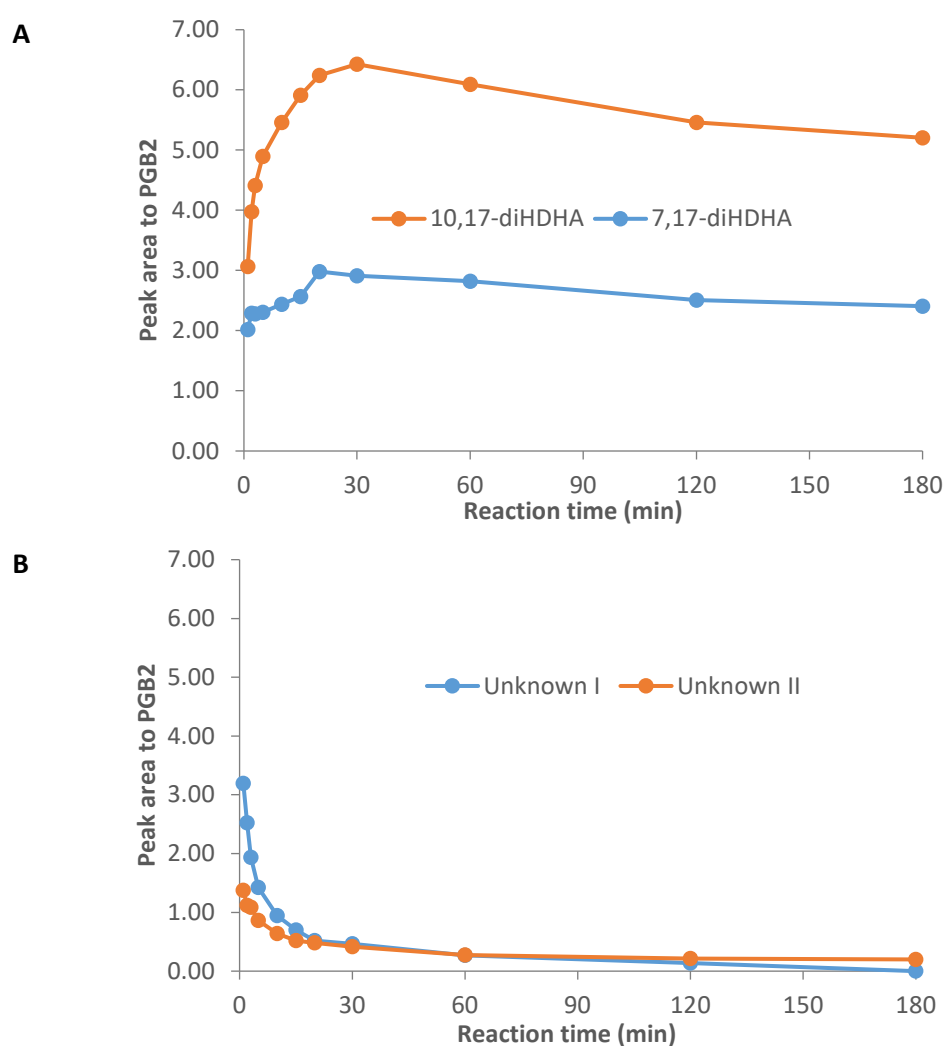
Figure 3.6: Product formation over time (1 – 180 min) in the sfLOX-catalysed DHA reaction; A) di-hydroxy DHA products, and B) unknown I and II. Reaction conditions: 0.2 mM DHA, 300 μL SBF extract, 0.01% v/v Triton X-100, 10 mL phosphate buffer (pH 8, 50 mM), ambient temperature. The results from a single representative experiment present reaction time effect on product formation.

3.1.5. Effect of oxygenation on product formation

Oxygen is an essential reagent, as it is also a substrate of the LOX-catalysed PUFA reaction, so oxygen concentration has a strong influence on the reaction. The sfLOX-catalysed DHA reaction using the conditions: 0.2 mM DHA, 300 µL SBF extract, 10 mL phosphate buffer (pH 8, 50 mM), Triton X-100 (0.01% w/v), ambient temperature, and 30 min reaction time, was optimised with different oxygen concentrations in the buffer. 10 mL buffer vials were sparged with pure oxygen, air, nitrogen for 5 min, or left untreated to obtain different oxygen concentrations from a maximum of 35 mg/L with oxygen sparging to a minimum of close to 0 mg/L with nitrogen sparging before the reactions were initiated. During the reaction time, these vials were sealed by balloons containing the same gas to help maintain the oxygen concentration over the reaction time. Saturation of the buffer with oxygen before adding the SBF extract resulted in an increase in mono- and di-hydroxy DHA product formation of approximately 16% and 30%, respectively. Purging air into the buffer solution did not significantly affect the generation of both mono and di-hydroxy DHA products, as expected. All product formation was suppressed when oxygen was removed from solution under nitrogen-sparged conditions (Figure 3.7). Instead, a major product, unknown IV (λ_{max} = 235 nm), previously observed at high DHA concentrations ($\geq$ 0.3 mM), was formed.

Figure 3.7: Effect of oxygen concentration on the sfLOX-catalysed DHA reaction. Reaction conditions: 0.2 mM DHA, 300 µL SBF extract, 0.01% v/v Triton X-100, 10 mL phosphate buffer (pH 8, 50 mM), ambient temperature, 30 min reaction time. The results from a single representative experiment present oxygen effect on product formation.

For the reactions performed under saturated oxygen conditions, no significant difference in product formation was observed between reaction vials covered with an oxygen-filled balloon and vials that were left open to the air (results not shown). Therefore, sealing the reaction with the relevant gas was not required for further experiments. Under oxygen-saturated conditions, 17-HDHA was present at a high amount and could potentially be converted into di-hydroxy DHA products. Therefore, the SBF extract volume was re-optimised over the range from 300 µL to 1000 µL. Increasing the SBF extract volume resulted in a significant increase in the amount of di-hydroxy DHA products and a decrease in mono-hydroxy DHA products remaining, reaching an optimum at 800 µL SBF extract (Figure 3.8). 10,17-diHDHA and 7,17-diHDHA increased by approximately 60% and 35%, respectively. The optimum conditions for the reaction after re-evaluation were oxygen saturated buffer prior to the reaction and 800 µL SBF extract.
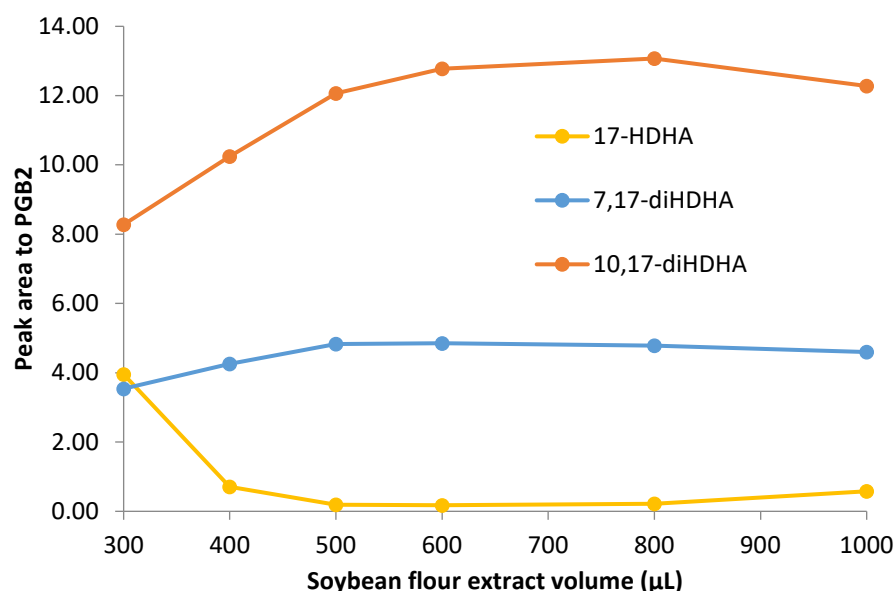
Figure 3.8: Effect of SBF extract volume re-optimisation (300 – 1000 µL) on the sfLOX-catalysed DHA reaction. Reaction conditions: 0.2 mM DHA, 0.01% v/v Triton X-100, 10 mL phosphate buffer (pH 8, 50 mM) saturated with oxygen, ambient temperature, 30 min reaction time. The results from a single representative experiment present the effect on product formation.

3.1.6. Effect of temperature on product formation

Temperature is an important variable to be optimised in the LOX-catalysed reaction. High temperatures could inactivate the enzyme [117], lower the dissolved oxygen concentration and promote non-specific oxidation [59]; while, low temperatures decrease the solubility of the fatty acid substrates and so could lower the interaction between substrate and the enzyme. The sfLOX-catalysed DHA reaction was optimised in the temperature range 0 – 40°C. The 30°C and 40°C reactions were heated in a water bath on a heating plate, the 0°C reaction was kept on ice, and the 10°C reaction was performed in an ice water bath with temperature monitored by a thermometer.

In the range studied, 10,17-diHDHA significantly increased from 0°C, reaching a maximum at ambient temperature 21 – 23°C (> 2-fold increase), and then decreased up to 40°C (roughly 3.5-fold compared to the maximum at ambient temperature). However, there was a less significant change in the amount of 7,17-diHDHA

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produced in the temperature range 0°C to 30°C (approximately 40% increase at ambient temperature compared to at 0°C), whereas there was an approximate 55% decrease at 40°C compared to at ambient temperature (Figure 3.9). Di-hydroxy DHA products were maximised at ambient temperature and suppressed at both low and high temperatures, and little 17-HDHA amount was formed across the temperature range studied. Two compounds, unknown I and II, appeared at the highest amounts at 0°C, compared to higher temperatures. They were formed at about 30% of 7,17-diHDHA and 10% of 10,17-diHDHA, respectively, compared to at ambient temperature. At 40°C, approximately 15% of the DHA remained unreacted at the end of the reaction. No increase in non-enzymatic products was observed when increasing temperature. The optimum temperature for the reaction was found to be ambient temperature (21 – 23°C).
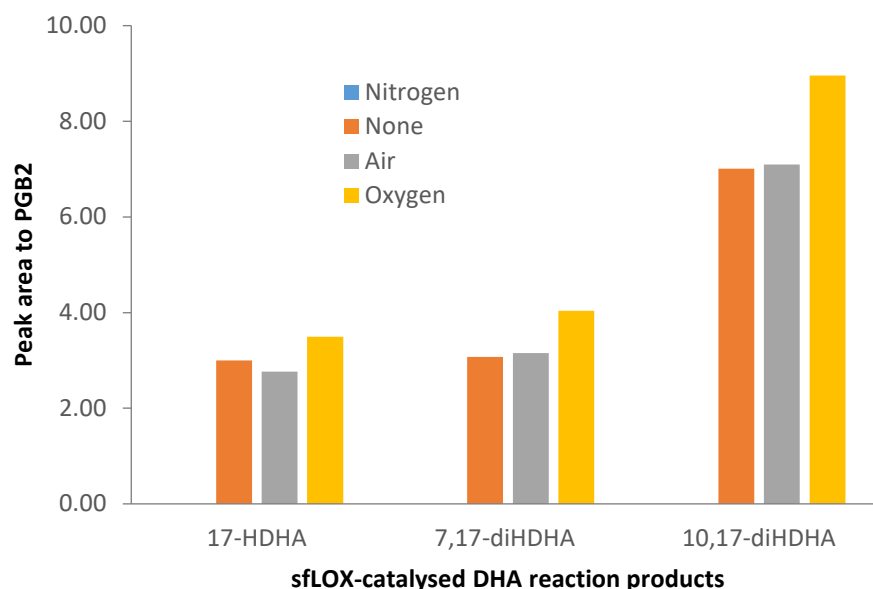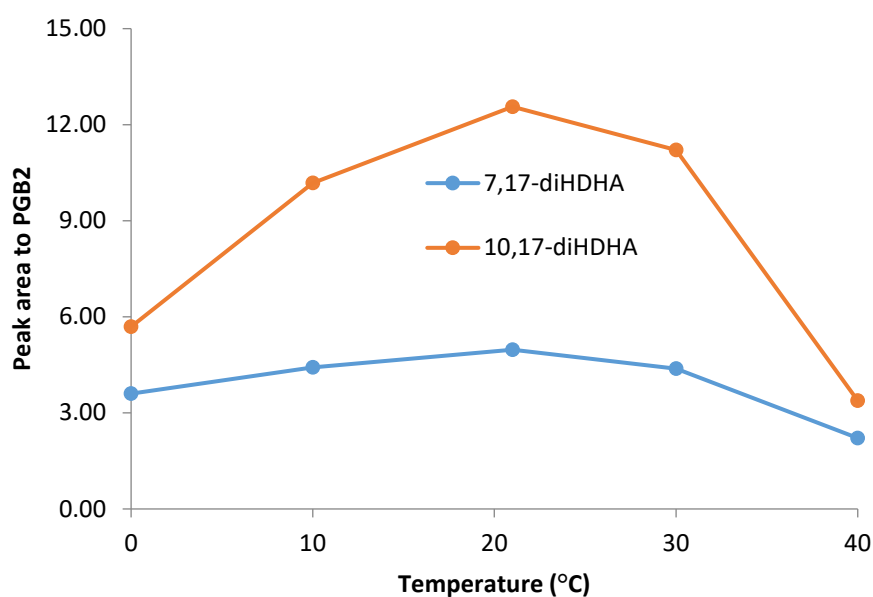

Figure 3.9: Effect of temperature (0 – 40°C) on the sfLOX-catalysed DHA reaction. Reaction conditions: 0.2 mM DHA, 800 µL SBF extract, 0.01% v/v Triton X-100, 10 mL phosphate buffer (pH 8, 50 mM) saturated with oxygen, 30 min reaction time. The results from a single representative experiment present temperature effect on product formation.

3.1.7. Effect of other additives on product formation

The sfLOX-catalysed DHA reaction was optimised in the presence of ferric chloride ($FeCl_3$, 0 – 0.2 mM) and L-cysteine (0 – 10 mM). These two additives have been previously shown to positively affect the sLOX-catalysed LA reaction to either improve the overall reaction rate or increase the reaction yield [95, 118], as discussed in detail in Chapter 2. However, in this work, the two additives did not show any positive effects on the sfLOX-catalysed DHA reaction for the formation of di-hydroxy DHA products.

TCEP, another water-soluble reducing agent, was previously shown to increase the 17-HDHA yield significantly in gram scale synthesis using the sLOX-1 catalysed DHA reaction. In the presence of TCEP, the reaction was reported to proceed slower but was able to continue oxidation for a longer time, and a higher amount of DHA was consumed [202]. TCEP can reduce PUFA hydroperoxides into PUFA hydroxides directly in the reaction, so a separate reduction step with sodium borohydride is not required. TCEP was applied in the above optimised sfLOX-catalysed DHA reaction using 0.2 mM DHA. TCEP concentrations of 0.4, 0.6 and 0.8 mM were tested. No significant difference in product formation between the TCEP concentrations tested was detected. TCEP did not increase total di-hydroxy DHA formation, however the ratio of the two dihydroxy products changed, with the generation of 10,17-diHDHA (containing a triene) decreasing and 7,17-diHDHA (containing two conjugated double bonds) increasing (Figure 3.10). In the presence of 0.6 mM TCEP, the reaction was further optimised with regards to reaction time (1 – 75 min) and DHA concentration (0.1 – 0.5 mM). Product formation did not improve. DHA (0.3 mM) was tested with increasing SBF extract volume (800 – 2000 µL). Again, no significant increase in reaction yield was observed.
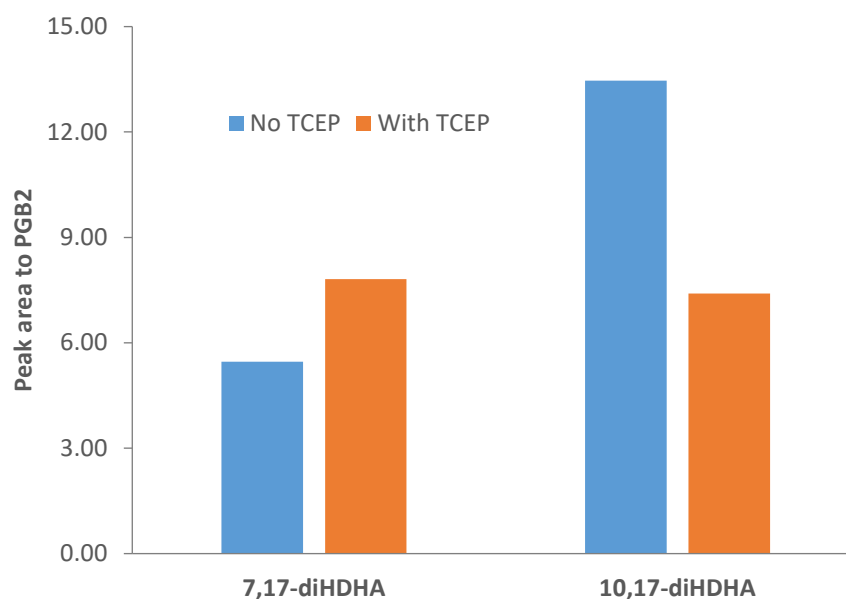
Figure 3.10: The sfLOX-catalysed DHA reaction products in the presence and absence of 0.6 mM TCEP. Reaction conditions: 0.2 mM DHA, 800 µL SBF extract, 0.01% v/v Triton X-100, 10 mL phosphate buffer (pH 8, 50 mM) saturated with oxygen, ambient temperature, 30 min reaction time. The results from a single representative experiment present TCEP effect on product formation.

3.1.8. Final optimised conditions for maximum product formation

The final optimised conditions were found to be: 800 µL SBF extract, 0.2 mM DHA, phosphate buffer (pH 8, 50 mM), oxygen saturation, Triton X-100 (0.01% w/v), ambient temperature, 30 min reaction time. Di-hydroxy DHA formation at optimised conditions increased approximately 40-fold compared to at the initial conditions (0.1 mM DHA, 25 µL SBF extract, 10 mL buffer, no surfactant, no oxygen saturation, 15 min reaction time). The reproducibility of the sfLOX-catalysed DHA reaction at optimised conditions in generating di-hydroxy DHA products was measured by repeating five reactions per day for three days. The averaged results are shown in Figure 3.11. The relative standard deviation (%RSD) of the inter-day results (15 reactions across the three days) was less than 5% for both compounds (7,17-diHDHA: 3.2% and 10,17-diHDHA: 4.8%). For the intraday results (each of the three days), there was more variation for 10,17-diHDHA (3.0–6.0%) than 7,17-diHDHA (2.5–3.7%).
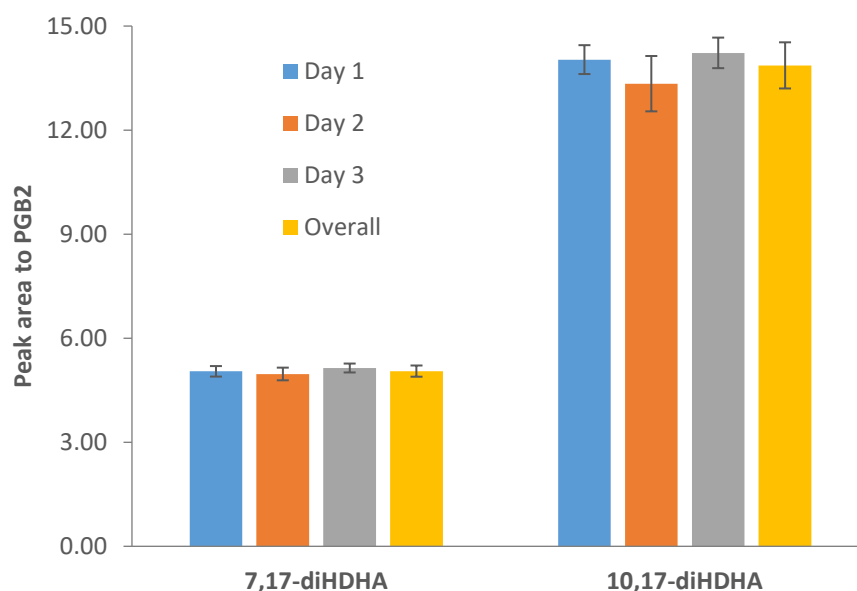
Figure 3.11: Reproducibility of the optimised sfLOX-catalysed DHA reaction in producing di-hydroxylated products (intraday and interday reproducibility shown, average results presented, error bars indicate (±) one standard deviation). Reaction conditions: 0.2 mM DHA, 800 µL SBF extract, 0.01% v/v Triton X-100, 10 mL phosphate buffer (pH 8, 50 mM) saturated with oxygen, ambient temperature, 30 min reaction time. Day 1 – 3: n=5; Overall: combined results from Day 1 – 3: n=15.

Yield

The yield of the reaction was estimated from the NP-HPLC peak area of di-hydroxy DHA products relative to the internal standard (PGB2). The yields of di-hydroxy DHA products were calculated by comparing the quantities produced in the optimised reaction with the maximum amount of di-hydroxy DHA products that could be produced (if all DHA was converted into di-hydroxy DHA products). The quantities of di-hydroxy DHA products were calculated through the Beer-Lambert equation based on the known concentration of PGB2 (0.02 mg PGB2 for 10 mL reaction) and their known peak area. Molar extinction coefficients were used to correct for differences in UV absorbance: PGB2 (280 nm) 30,000 M^{-1} cm^{-1} [203]; 7S,17S-diHDHA (234 nm) 33,500 M^{-1} cm^{-1} [103]; and 10S,17S-diHDHA (270 nm) 38,000 M^{-1} cm^{-1} [67]. The estimated yield for the optimised reaction was 25% 7S,17S-diHDHA and 48% 10S,17S-diHDHA.

3.2. Product characterisation

To confirm the identities of the major products of the sfLOX-catalysed DHA reaction (17-HDHA, 7,17-diHDHA and 10,17-diHDHA), GC-MS and chiral RP-HPLC were used.

3.2.1. Determination of hydroxy group positions on reaction products by GC-MS

The reduced reaction products were derivatised to compounds more volatile and thermally stable by saturation of double bonds and trimethylsilylation of hydroxy groups for GC analysis. The fully hydrogenated and trimethylsilylated (TMS) derivatives of the optimised sfLOX-catalysed DHA reaction products were analysed by GC-MS. The hydroxy positions of the mono- and di-hydroxy DHA products were identified by interpreting fragmentation patterns of derivatives. Two major di-hydroxy DHA products and two mono-hydroxy DHA products were detected while only one mono- and two di-hydroxy DHA products were observed by NP-HPLC. The two mono-hydroxy DHA products eluted between 26 and 27 min, and the two di-hydroxy DHA products eluted between 28 and 29 min (Table 3.3).

Table 3.3: Important *m/z* fragments of the hydrogenated, trimethylsilyl derivatives of mono and di-hydroxy products from sfLOX-catalysed DHA reaction under optimised conditions.

Retention time (min)	Product	Important *m/z* fragments
26.62	10-HDHA	73, 117, 271, 309, 331, 395, 485
26.94	17-HDHA	73, 117, 173, 309, 395, 429, 485
28.81	10,17-diHDHA	73, 117, 173, 331, 359, 427, 517, 573
28.85	7,17-diHDHA	73, 117, 173, 289, 402, 427, 517, 573

The four products were identified as fully hydrogenated, TMS derivatives of 10-HDHA and 17-HDHA (two mono-hydroxy DHA products), and 7,17-diHDHA and 10,17-diHDHA (two di-hydroxy DHA products). 10,17-diHDHA (Figure 3.12) and 7,17-diHDHA co-eluted partially, but separate fragmentation patterns could be distinguished. The results confirmed the presence of three compounds including one mono-hydroxy DHA product, 17-HDHA, and two di-hydroxy DHA products, 7,17-diHDHA and 10,17-diHDHA, which matched the NP-HPLC results. An additional mono-hydroxy DHA only identified by GC-MS was 10-HDHA.
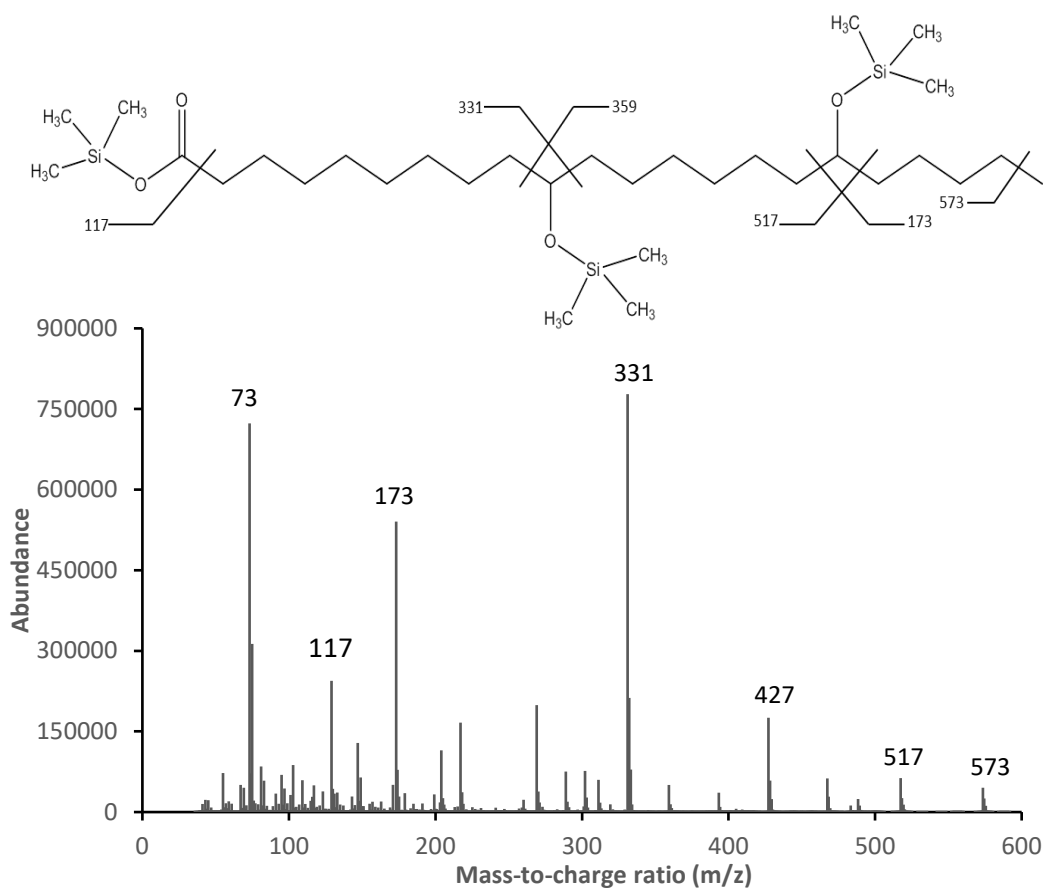
Figure 3.12: Structure showing assignment of important fragments, and mass spectrum of fully hydrogenated tri-TMS-10,17-diHDHA.

3.2.2. Determination of stereochemistry of reaction products by chiral RP-HPLC

Soybean lipoxygenase is commonly known to produce products with *S* chirality, although the specificity of this reaction can vary depending on reaction conditions

[49, 102]. To determine if the *S* specificity was preserved when using SBF as a LOX source, product chirality was identified by chiral RP-HPLC. The di-hydroxy DHA products of the sfLOX-catalysed reaction were methylated to produce less polar compounds to increase retention and improve peak shape prior to chiral RP-HPLC analysis. The racemic mixture of 10,17-diHDHA and 7,17-diHDHA stereoisomers (4 compounds for each di-hydroxy DHA product) synthesised from sfLOX-catalysed DHA reaction products could be distinguished by chiral RP-HPLC. 7,17-diHDHA from the sfLOX-catalysed DHA reaction (5 times dilution) was identified to be 7*S*,17*S*-diHDHA by matching its retention time with 7*S*,17*S*-diHDHA standard and one of the compounds of the racemic mixture (Figure 3.13A). 7,17-diHDHA was also tested undiluted by chiral RP-HPLC to confirm the chiral purity of the product (*S,S* ≥ 99.5%), and no other stereoisomers were detected (Figure 3.13B). 10,17-diHDHA from the DHA reaction catalysed by sfLOX was identified to be 10*S*,17*S*-diHDHA also with high purity (≥ 99.5%) in the same way as 7*S*,17*S*-diHDHA.
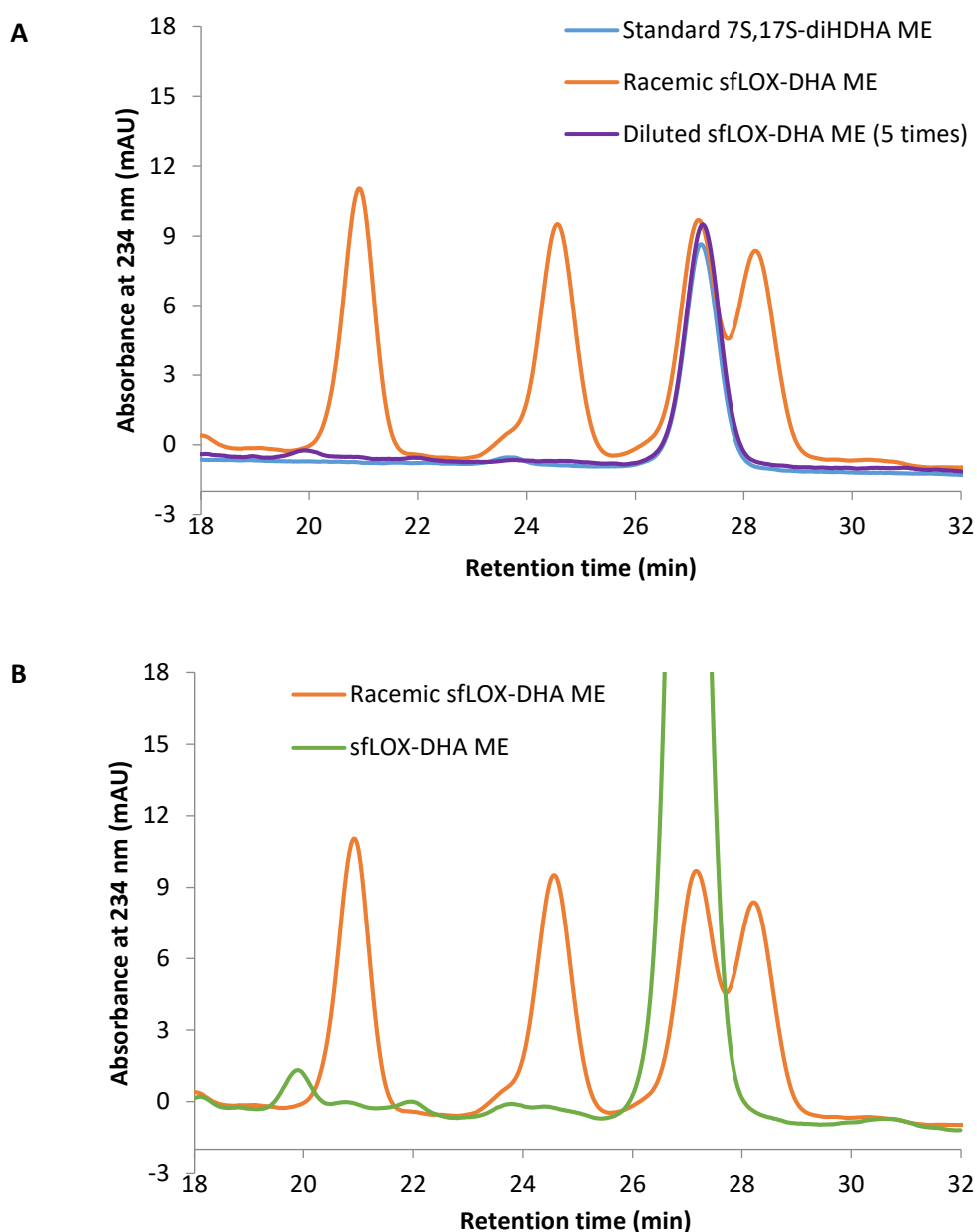
Figure 3.13: Chiral RP-HPLC chromatogram of A) standard 7*S*,17*S*-diHDHA, racemic mixture of sfLOX-catalysed DHA reaction product and 7,17-diHDHA from sfLOX-catalysed DHA reaction (diluted 1:5) after methylation; B) racemic mixture of sfLOX-catalysed DHA product ME and 7,17-diHDHA from sfLOX-catalysed DHA reaction after methylation.

3.3. Application of the optimised reaction conditions to other biologically relevant substrates

The optimised conditions of the sfLOX-catalysed DHA reaction were applied to other biologically relevant substrates (AA, EPA, DPAn-3 and DPAn-6). The results showed that all substrates were able to react with sfLOX to form di-hydroxy PUFA products as analogues of those synthesised from DHA. No substrate and very little

mono-hydroxy PUFA product remained at the end of the reactions (Figure 3.14). The ratio between the two di-hydroxy PUFA isomers was significantly different between substrates. For EPA, the reaction formed similar amounts of both isomers; for DHA and AA, the ratio was approximately 2.5:1 with the conjugated triene (the hydroxy groups separated by 7 carbons) as the preferred isomer; for DPAn-6, the ratio was approximately 7:1 in favour of this isomer. Only the DPAn-3 reaction produced significantly more of the double conjugate diene isomer (the hydroxy groups separated by 10 carbons) than the conjugated triene with a ratio of approximately 15:1. This was similar to previous results obtained from the commercial 15-sLOX-1 reaction [43].
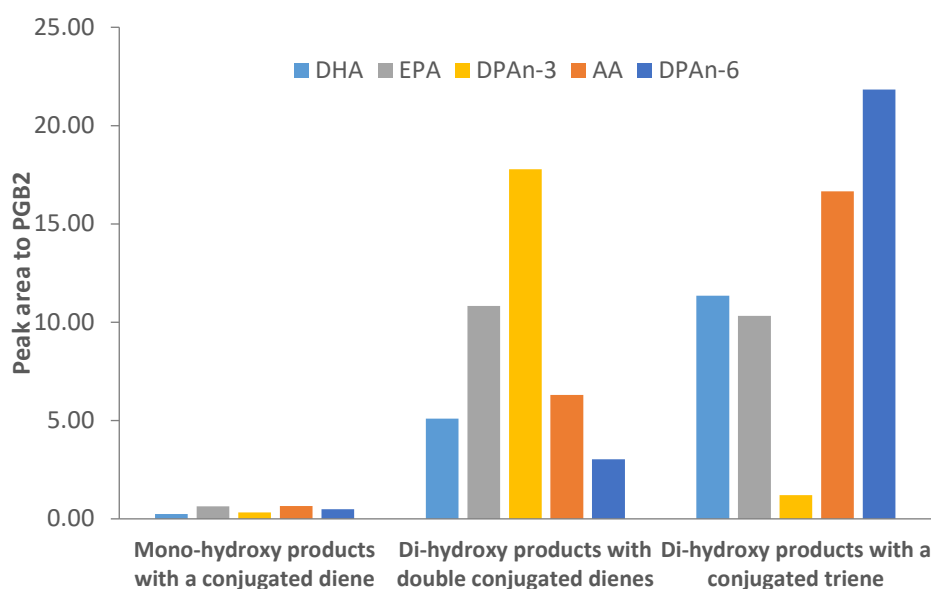
Figure 3.14: Application optimised sfLOX-catalysed DHA reaction conditions to other biologically relevant substrates (EPA, AA, DPAn-3 and DPAn-6). Reaction conditions: 0.2 mM PUFA, 800 μL SBF extract, 0.01% v/v Triton X-100, 10 mL phosphate buffer (pH 8, 50 mM) saturated with oxygen, ambient temperature, 30 min reaction time. The results from a single representative experiment present product formation from different substrates.

Due to the low formation of 7,17-diDPAn-6 compared to 10,17-diHDPAn-6 in the DPAn-6 reaction, TCEP 0.6 mM was applied to possibly increase the yield of 7,17-diHDPAn-6 as demonstrated above in Section 3.1.7 with DHA as substrate. As expected, the presence of TCEP in the sfLOX-catalysed DPAn-6 reaction increased

the yield of 7,17-diHDPAn-6 by ~ 40%, although 10,17-diHDPAn-6 yield slightly

decreased (~ 20%) (Figure 3.15).
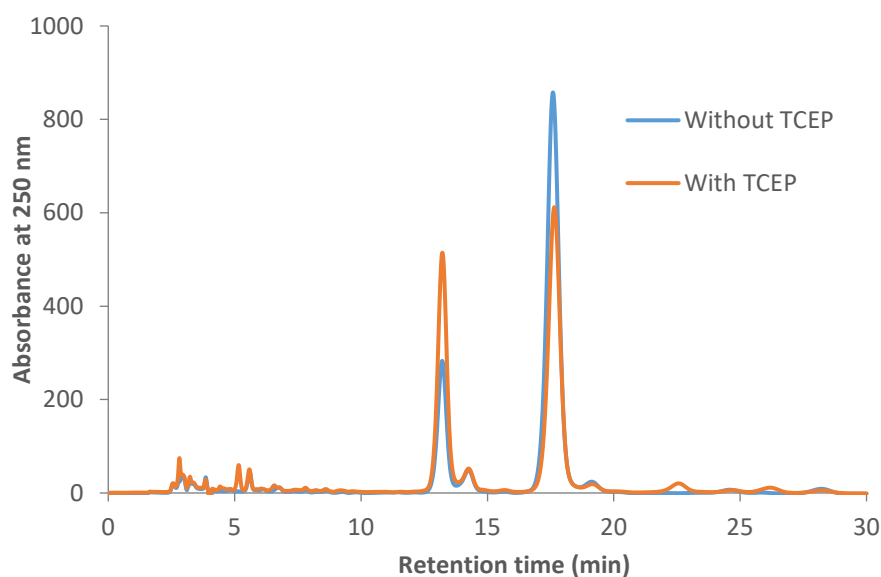
Figure 3.15: Chromatograms of sfLOX-catalysed DPAn-6 reaction products under the optimised
sfLOX-catalysed DHA reaction conditions with and without 0.6 mM TCEP. Reaction conditions:
0.2 mM DPAn-6, 800 μL SBF extract, 0.01% v/v Triton X-100, 10 mL phosphate buffer (pH 8,
50 mM) saturated with oxygen, ambient temperature, 30 min reaction time. The results from a single
representative experiment present TCEP effect on product formation.

3.4. Comparison between sfLOX and 15-sLOX-1

3.4.1. Product formation

Double the amount of DHA was used in the sfLOX-catalysed DHA reaction at

optimised conditions compared to the optimum conditions found for the 15-sLOX-1

catalysed DHA reaction in previous work [43]. Correspondingly, roughly double the

amount of di-hydroxy DHA products were produced by the sfLOX-catalysed

reaction compared to the 15-sLOX-1 catalysed reaction (Figure 3.16A). This means

that at the same reaction volume, sfLOX converted twice as much DHA into

products as 15-sLOX-1. By comparing the conversion per milligram of DHA

substrate, sfLOX showed approximately the same efficiency as 15-sLOX-1 in

making 7,17-diHDHA (5% lower) and 10,17-diHDHA (14% higher) (Figure 3.16B).

They also had similar relative standard deviation (< 5%) in producing di-hydroxy
DHA products.
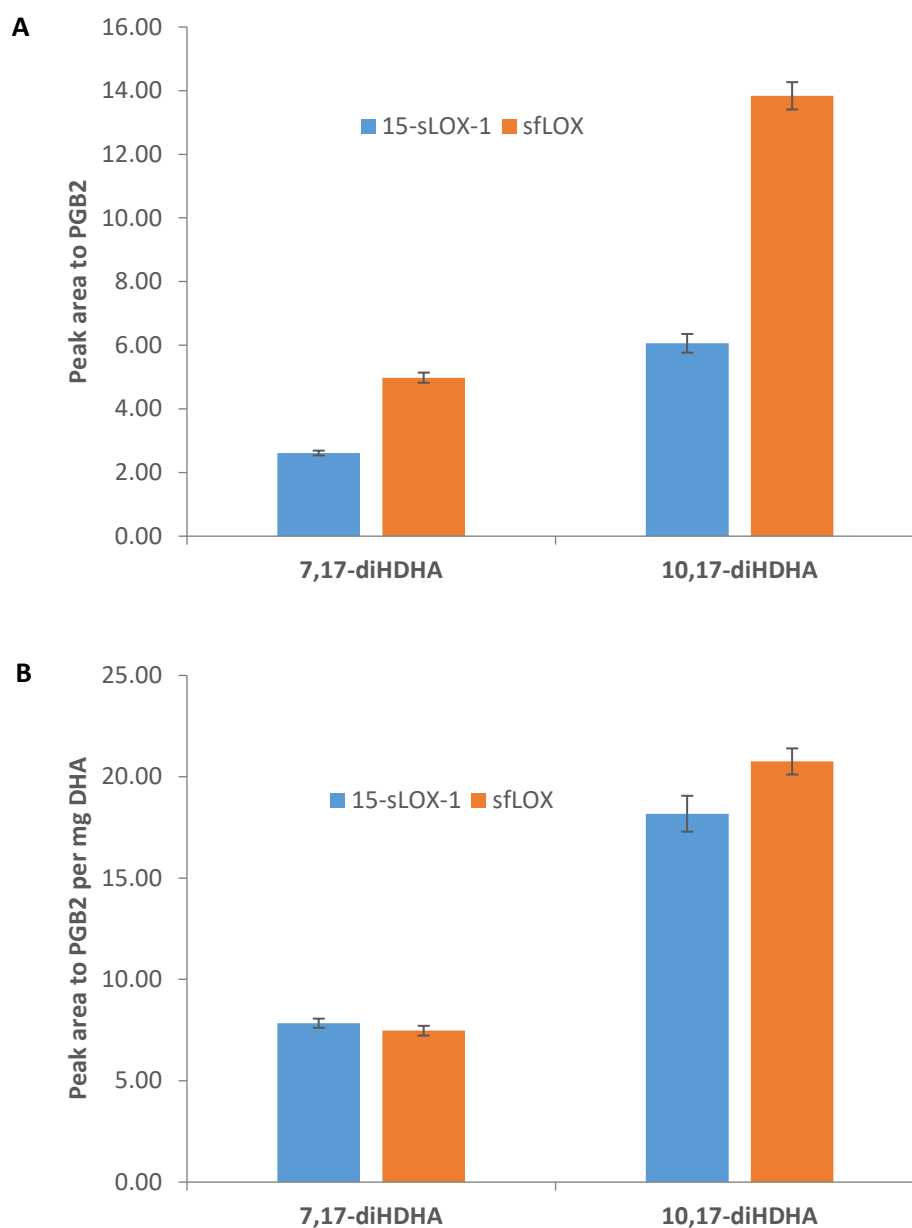
Figure 3.16: 15-sLOX-1 and sfLOX activity in producing di-hydroxylated products from DHA under
conditions optimised for each enzyme: A) Total amount produced (peak area to PGB2); B) Amount
produced per milligram DHA (peak area to PGB2 per mg DHA). Reproducibility shown, average
results presented and error bars indicate (±) one standard deviation (n=3).

3.4.2. Enzyme kinetic parameters

The reaction kinetics of the sfLOX-catalysed DHA reaction under the optimised
conditions were measured by UV-vis spectroscopy at 270 nm to monitor the

formation of the conjugated triene-containing product (10,17-diHDHA). The calculated values were compared to previous work (enzyme kinetics of 15-sLOX-1 catalysed DHA reaction under its optimised conditions) [43]. Comparison of results (Table 3.4) revealed that sfLOX had higher maximum rate, but required higher DHA concentration to reach 50% of the maximum reaction rate compared to 15-sLOX-1. Overall, the sfLOX-catalysed reaction proceeded much faster than the 15-sLOX-1 catalysed reaction with higher substrate capture (V_{max}/K_m). However, sfLOX was found to have less affinity with DHA compared to 15-sLOX-1 in making the di-hydroxy conjugated triene as the sfLOX-catalysed DHA reaction had a higher K_m value.

Table 3.4: Reaction kinetics for the formation of 10,17-diHDHA from DHA catalysed by sfLOX measured in this work, and by 15-sLOX-1 determined in previous work [43].

Parameter	V_{max} (µM/min)	K_m (µM)	V_{max}/K_m (min^{-1})
sfLOX-DHA	237.0	271.4	0.87
15-sLOX-1 DHA	26.6	49.5	0.54

3.4.3. Colorimetric test for LOX isozyme detection

Three different tests (I, II and III) developed by Suda et al. [190] were conducted to determine the presence of three LOX isozymes (LOX-1, LOX-2 and LOX-3) in SBF. In these tests, radicals formed in the reaction of sodium linoleate catalyzed by sfLOX isozymes are involved in redox reactions with methylene blue and β-carotene, resulting in bleaching of their colours. The results showed that in test I (LOX-1 activity, methylene blue, pH 9) the colour of methylene blue was bleached quickly (within 2 min) to colourless as expected, indicating the presence of LOX-1; whereas the blue colour of test II (LOX-2 activity, methylene blue and dithiothreitol, pH 6) completely lost colour much more slowly, taking up to 10 min. This indicates LOX-2

is present in SBF but at low concentration and/or activity as the bleaching time was longer than expected (within 5 min in the original paper). LOX-3 was detected by the colour change of β-carotene (from yellow to colourless) within 4 min in test III (LOX-3 activity, β-carotene, pH 6.6) which was similar to the expected time of 3 min, indicating the presence of LOX-3 (Table 3.5). As a result, the SBF used in this study for LC-PUFA oxidation was identified to contain three individual LOX isozymes: LOX-1, LOX-2 and LOX-3.

Table 3.5: Results of colorimetric tests for the detection of lipoxygenase isozymes. +: Positive, *: Inconclusive.

Test	Soy flour amount (mg)	Bleaching Start (min)	End (min)	Result	15-sLOX-1 amount (µg)	Bleaching Start (min)	End (min)	Result
I: LOX-1	2.5	0.5	1.5-2	+	140	0.5	0.75-1	+
II: LOX-2	5.0	3-5	9-10	+	280	10-15	20-25	*
III: LOX-3	2.5	0.5	3.5-4	+	140	0.5	2.5-3	+

Similarly, a commercial 15-sLOX-1 used for comparison of enzyme activities with sfLOX was also tested. Test I had a quick response (within 1 min) as expected, which confirmed the presence of LOX-1. The LOX-2 test was inconclusive as the bleaching time (20 - 25 min) was unclear and much longer than the original test. In test III, bleaching of the colour occurred within the expected time of 3 min, indicating the presence of LOX-3 in the commercial solution. It is possible to have multiple isozymes in the commercial preparation as it was only partially purified. A summary of the colorimetric test results for both LOX preparations is shown in Table 3.5.

3.4.4. Protein separation by SDS-PAGE

To further determine which LOX isozymes were present in the SBF, SBF and two commercial sLOX preparations (15-sLOX-1, unpurified and purified) were analysed by SDS-PAGE. To achieve better separation of the isozymes which have very similar molecular weights, electrophoresis was run for long durations, which meant that lower molecular weight species migrated off the gel. After running a gel for 2 hours (Figure 3.17), the molecular weights of LOXs in SBF and the commercial LOX preparations were confirmed to be in one major band at ~ 94 – 97 kD by comparison with the molecular weight standard. A thick band below the LOX band in SBF (lane 2 and 3), not observed with either of the commercial LOX preparations, was expected to be the α′ subunit of 7S globulin (β-conglycinin), as previously determined [192].
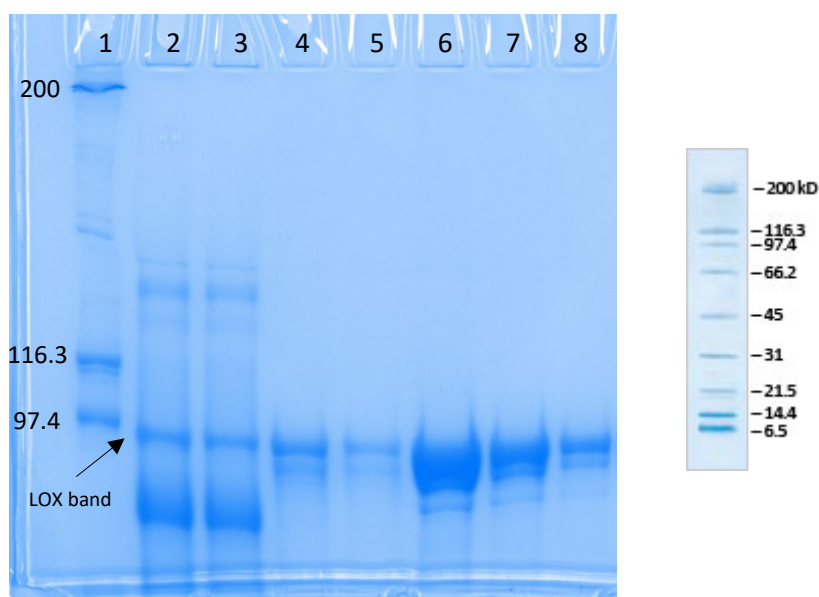
Figure 3.17: SDS-PAGE analysis of SBF and commercial lipoxygenase enzyme preparations (15-sLOX-1, unpurified and purified). 7.5% acrylamide gel was used and stained with Bio-Safe Coomassie G250 Stain. Samples were heated at 80°C before running SDS-PAGE. Lane 1: molecular weight standard, lane 2 and 3: SBF extract (diluted 1 in 8), lane 4: unpurified 15-sLOX-1 (diluted 1 in 4), lane 5: unpurified 15-sLOX-1 (diluted 1 in 8), lane 6: purified 15-sLOX-1 (diluted 1 in 2), lane 7: purified 15-sLOX-1 (diluted 1 in 4), lane 8: purified 15-sLOX-1 (diluted 1 in 8).

Previous studies indicated that the order of LOX isoenzymes on the gel should be LOX-3, LOX-2 then LOX-1, from highest to lowest molecular weight [204, 205]. However, on the gel, only one major band was observed at ~ 95 kD for all samples and there were no other bands accounting for LOX-2 and LOX-3 with higher molecular weight than this band (and lower than 97.4 kD). This suggests that the bands for the three isozymes were not separated under the running condition of this gel. There were also two minor bands with lower molecular weights underneath the major LOX band in commercial LOX samples (lane 4 – 8). These bands were unknown and not present in SBF.

In order to get separation of the isozyme bands, a 12% acrylamide gel was used and run for 5 – 6 hours. Heating of the sample at a lower temperature (50°C), and no heating before electrophoresis was tested, as LOX-2 is much less stable than LOX-1 at high temperature [122]. A better separation was observed with a 6 hour run time and without sample heating (lane 1 – 5, Figure 3.18). The LOX-1 band was expected to be the most intense as LOX-1 is the most abundant isozyme in soybean, and LOX-2 and LOX-3 should be minor bands. A major band was observed between the 75 and 100 kD molecular weight standard on the gel for both SBF and 15-sLOX-1 (lanes 1 – 5, Figure 3.18), confirming the presence of LOX-1. Above this band, SBF showed one minor band close to LOX-1 (lanes 2 – 4), whereas 15-sLOX-1 had two minor bands further from LOX-1 (lane 5). These three bands were not matched to each other and had significantly less intensity compared to the LOX-1 band.
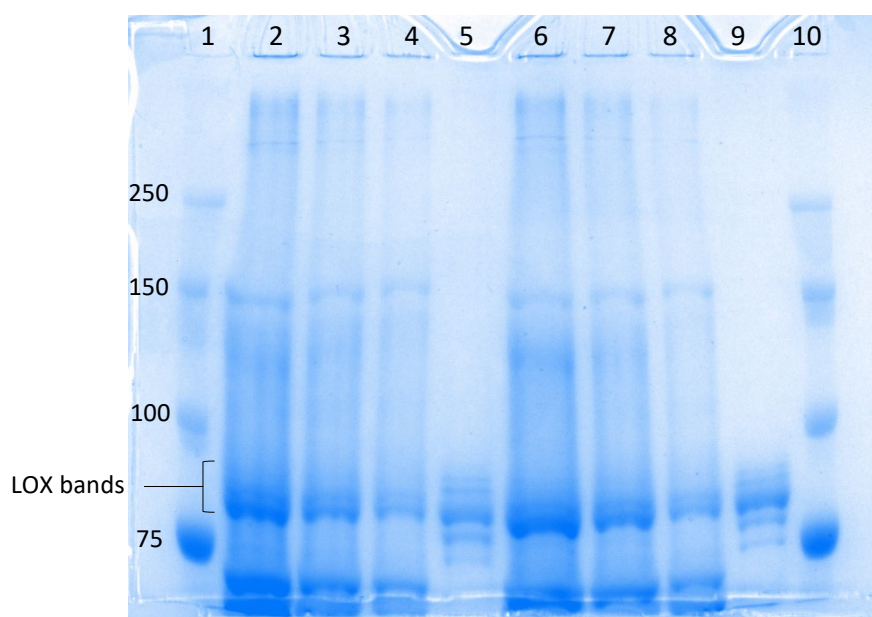
Figure 3.18: SDS-PAGE analysis of SBF and commercial lipoxygenase enzyme preparation of 15-sLOX-1 (unpurified). 12% acrylamide gel was used and stained with Bio-Safe Coomassie G250 Stain. Lanes 1 – 5 (samples without heating before running SDS-PAGE), lane 6 – 10 (samples heated at 50°C before running SDS-PAGE). Lane 1 and 10: molecular weight standard, lane 2 – 4 and 6 – 8: SBF extract (diluted 1 in 4, 1 in 8 and 1 in 16 respectively), lane 5 and 9: 15-sLOX-1 (diluted 1 in 8).

4. Discussion

Lipoxygenase-catalysed reaction optimisation

The three LOX isozymes (1, 2 and 3) were indicated to be present in SBF using a colorimetric screening method. LOX-2 in SBF appeared to be more active and/or higher in concentration than in a commercial 15-sLOX-1 preparation, as the commercial LOX required significantly longer time (20 – 25 min) to bleach the colour in test II than SBF (9 – 10 min). This supports previous findings that LOX-2 is unstable when purified, but the activity is more stable in the protein-starch matrix in flour, which acts as a natural immobiliser [56]. As for SDS-PAGE, LOX-1 was conclusively detected but less conclusive evidence was observed for LOX-2 and LOX-3 detection above LOX-1 in both SBF and commercial 15-sLOX-1, as these bands had significantly less intensity compared to the LOX-1 band. The type-2 LOXs in SBF and 15-sLOX-1 used in this work may not be present at high enough concentrations to be detected by SDS-PAGE. Due to the order and intensity of bands

on the gel, SBF may have more LOX-2, whereas 15-LOX-1 may contain more LOX-3 isozymes (LOX-3a and LOX-3b).

Previous work has shown that SBF can be used to catalyse reactions with C18 fatty acids for the formation of mono-hydroxy fatty acid products as precursors of volatile compounds (appropriate references in Table 2.2, Chapter 2). This is the first time SBF has been used directly to convert omega-3 and omega-6 LC-PUFAs (C20-22 fatty acids) into not only mono- but also di-hydroxy PUFAs for one step production of resolvin analogues. The results of the optimisation revealed many interesting characteristics of the sfLOX-catalysed reaction, which in part differed to the reaction catalysed by commercial 15-sLOX-1 in previous work [43], with both using DHA as the substrate.

The optimised pH of the sfLOX-catalysed DHA reaction for the synthesis of di-hydroxy products was pH 8. This pH is not the optimum pH for LOX-1, LOX-2 or LOX-3 (pH 9, 6.5 and 6, respectively) [88]. A previous study found that the optimum pH of LOX-1 was at pH 9 for the generation of di-hydroxy products from DHA [43]. A LOX-1 catalysed AA reaction was also performed at pH 9 to produce di-hydroxy AA products [125]. Purified LOX-2 was shown to oxygenate LA [120, 122] and AA [123] at neutral pH, whilst pH 9 – 10 is commonly used for LOX-1 in SBF [105, 157, 158, 164]. LOX-2 in SBF has also been used at pH 6 [105] to oxygenate free fatty acids for the formation of mono-hydroxy PUFA products as precursors of volatile compounds. The optimum pH found for the sfLOX-catalysed DHA reaction in this work may represent a compromise between the optimum pH of the three isozymes. This optimum pH is supported by other studies. For example, the optimum pH for LOX-1 in converting mono-hydroxy AA into di-hydroxy AA products (second dioxygenation) was found to be pH 7.5 – 8, and occurred at a significantly lower rate compared to the first dioxygenation at pH 9 [55, 124]. Moreover, a

previous study showed that a crude LOX extract from defatted SBF had maximum activity on hydrolysed safflower oil (composed mainly of linoleic acid) at pH 8 although higher pH showed higher product specificity for the formation of 13-hydroperoxy linoleic acid [59].

In this work, the reaction was specific in forming mono-hydroxy DHA products with 17-HDHA as the dominant product at pH 7.5, whereas there was no specific mono-hydroxy product formed at lower pHs (≤ 7). Moreover, 17-HDHA formation increased significantly with increasing pH (7.5 – 11) and reached a maximum at pH 11. These findings are supported by previous studies which showed that LOX had high specificity with single dioxygenation at high pHs [43, 59, 91]. It is likely that at high pH values (≥ 7.5) fatty acid is fully deprotonated for better solubility in water, and preferably inserted methyl end first (not the charged carboxyl end) into the catalytic pocket in the active site of the enzyme [49]. Furthermore, the second dioxygenation reaction is not favoured at high pHs leading to a higher concentration of mono-hydroxy products remaining.

Both di-hydroxy DHA products increased significantly with increasing DHA concentration and reached a maximum at 0.2 mM DHA. At higher DHA concentrations (> 0.2 mM), formation of both di-hydroxy DHA isomers decreased significantly. This could be due to a number of reasons including substrate inhibition, product inhibition and low oxygen concentration. A small amount of hydroperoxides is required to initiate the LOX-catalysed reaction by converting the inactive form of LOX-Fe^{2+} into the active form, LOX-Fe^{3+} [95]. However, hydroperoxide binding can be prevented at high initial substrate concentrations [194, 195]. Moreover, the enzyme prefers to bind to product than substrate [196, 197], therefore high product

concentration can interfere with substrate binding which prevents the reaction from proceeding.

Air-saturated water at 20°C contains only 0.29 mM oxygen whereas 0.2 mM DHA requires 0.4 mM oxygen to fully convert DHA into di-hydroxy DHA products. Oxygen will be limiting in the reaction and anaerobic conditions may develop during the reaction, although extra oxygen can enter the reaction medium slowly from the atmosphere over the course of the reaction. At high substrate concentrations (> 0.2 mM), di-hydroxy DHA formation was suppressed and there was an increase of 17-HDHA, and unknown III and IV formation. This could be because there was not enough oxygen to generate di-hydroxy DHA products from 17-HDHA, and unknown III and IV may form from the anaerobic conditions. Under anaerobic conditions the LOX pathway has been shown to form a range of by-products instead of hydroxylated compounds [88]. For example, anaerobic products of LOX-catalysed linoleic acid reaction were fatty acid radicals which combined to form dimers with a conjugated diene on each fatty acid molecule, 13-oxo-9,11-octadecadienoic acid, 13-oxotridecadienoic acid compounds and racemic hydroperoxides [91]. An oxo compound also formed from 17-HDHA was identified and a dimer formed from DHA radicals was proposed in a previous study by Dobson et al. [43]. The aerobic cycle competes constantly with the anaerobic cycle, especially at high substrate and enzyme concentrations, where the oxygen diffusion is not good enough to take over the reaction rate [96]. Therefore, maintaining oxygen at a sufficient level is necessary for the sfLOX-catalysed DHA reaction to produce a good yield of di-hydroxy DHA compounds.

The formation of di-hydroxy DHA products increased with increasing SBF concentration. Previous work showed that sufficient levels of enzyme are required

for a good reaction yield, to transport oxygen effectively to the substrate [91]. It may also be because of enzyme inactivation by product inhibition [43, 125], therefore enough enzyme is required to balance these effects. However, at a certain point, adding higher volumes of SBF extract decreased product formation. This could result from self-inactivation/product inhibition [53], the development of anaerobic conditions and interference or inhibition of the reaction from other components in SBF.

Surfactant showed a significant contribution to enhancing sfLOX activity on DHA. A few studies showed that LOX-1 activity can be enhanced at low surfactant concentrations but inhibited at high surfactant concentrations [131, 198]. No inhibition was observed with sfLOX in this work. Surfactants have functions in solubilising fatty acids [116], increasing thermal stability of LOX [117], stabilising protein structure [198] and reducing induction period [206]. A study also found that in the presence of Triton X-100, sLOX-1 was inhibited at low enzyme concentration, whereas the activity was enhanced at high enzyme concentration [131]. At 0.01 – 0.05% v/v surfactant concentration, di-hydroxy DHA product yield was maximised, in the presence of a high volume of SBF; however, at higher concentrations of surfactant (> 0.05% w/v), the enhancement of enzyme activity appeared to decrease. This may have been caused by the formation of an emulsion during solvent extraction, which could interfere with product extraction. Also, it has been proposed that the enzyme inhibition and enhancement observed from the addition of surfactants result from changes in non-micellar substrate concentration due to partitioning of the substrate into surfactant micelles [116].

After reaching the optimum reaction time, the two di-hydroxy DHA products decreased slowly, whereas this was not observed with 15-sLOX-1 in the previous

work [43]. This suggests that other enzymes in SBF and soybean meal such as hydroperoxide lyases and hydroperoxide isomerases [137, 138] may be active in SBF extract and may be converting di-hydroxy DHA reaction products into other compounds (although these were not detected). As the sfLOX-catalysed DHA reaction used double the substrate concentration compared to 15-sLOX-1 catalysed DHA reaction, anaerobic conditions may also develop and cause degradation of hydroperoxides [207].

Oxygen concentration also had a significant effect on di-hydroxy DHA formation, whilst previous work showed no effect on 15-sLOX-1 catalysed DHA (0.1 mM) reaction [43]. It is likely that the dissolved oxygen concentration at room temperature (~ 0.26 mM) is enough for converting 0.1 mM DHA (in the reaction catalysed by 15-sLOX-1) into di-hydroxy DHA products, but is not enough for converting 0.2 mM DHA (in the reaction catalysed by sfLOX) into di-hydroxy DHA products, which would require 0.4 mM oxygen. Saturation of oxygen (~ 1.1 mM) into the buffer solution satisfied the oxygen demand for the sfLOX-catalysed DHA reaction resulting in increased product formation. Additional oxygen was not required, and so an oxygen-filled balloon covering the reaction to preserve the oxygen-rich environment was not needed and did not affect the results. Under oxygen-depleted conditions, the normal reaction products were not formed and probable anaerobic products (unknown III and IV) were formed instead.

Temperature was also found to have a significant effect on the reaction. At both low (0 – 10ºC) and high (30 – 40ºC) temperatures, enzyme activity was decreased compared to at ambient temperature (~ 22ºC). With increasing temperature, substrate solubility and mass transfer increases whereas oxygen concentration decreases and vice versa. Enzyme deactivation or product degradation could happen at higher

temperatures. As previously reported, LOX-1 in soybean was identified to be inactive at 54ºC at pH 9 and have a midpoint inactivation temperature at 44 ± 1ºC [117]. The room temperature optimum may be a compromise between substrate and oxygen solubility, mass transfer, enzyme activity and stability. An increase in different product isomers or non-enzymatic products at higher temperatures was not observed in this work, which was consistent with previous work using 15-sLOX-1 and DHA [43].

Two compounds (unknown I and II) were formed under certain conditions: 0ºC, 1 min reaction time and high volume of SBF extract ($\geq 100 \mu L$). Unknown I appeared to contain two conjugated dienes and unknown II appeared to contain a conjugated triene based on the absorbance spectra, so they may potentially be di-hydroxy DHA products. These two compounds have not been identified.

Less than 5% DHA remained at the end of the reaction using 25 μL SBF extract at different pH and buffer types. Approximately 15 – 20% DHA was detected at 40ºC. No DHA was detected under any other conditions, even where di-hydroxy DHA formation was much less compared to the final optimised conditions. However, there was no new compounds observed in the HPLC chromatograms to account for the loss of DHA. The reason for the missing DHA and/or products during the reaction is unknown. However, some side reactions on DHA or DHA hydroperoxides may have metabolised these compounds to volatile or shorter chain polar compounds, which were lost or not extracted in the solvent extraction step prior to detection by NP-HPLC. These side reactions may be inhibited at 40ºC, therefore more DHA was observed.

Comparison between sfLOX and 15-sLOX-1

The sfLOX has achieved similar efficacy to 15-sLOX-1 in the reaction with DHA for di-hydroxy DHA formation. The reaction at optimised conditions can produce double the amount of di-hydroxy DHA products. Based on the cost difference between soy flour and the commercial enzyme, it is approximately 28,000 times cheaper when SBF is used to replace 15-sLOX-1 in the production of di-hydroxylated compounds per mg DHA. These data demonstrated that SBF is a feasible alternative to commercial 15-sLOX-1 to reduce the synthesis cost of resolvin and protectin analogues. sfLOX was able to catalyse oxidation of other biologically important PUFAs as previously shown with 15-sLOX-1 [43]. The ratio of the two di-hydroxy isomer formation was comparable for DHA, EPA and AA as substrates, whereas one isomer was formed in significantly larger amount than the other with DPAn-3 and DPAn-6 as substrates. The reason for the differences in the ratios of the two major di-hydroxylated PUFA products between substrates catalysed by sfLOX is currently unknown. Similar results were also observed with 15-sLOX-1 and these PUFA substrates [43].

However, there are some potential issues when using SBF directly as a crude enzyme source in comparison with 15-sLOX-1. The optimised conditions required a large volume of SBF extract (800 µL ~ 40 mg SBF) because the enzyme concentration is much less concentrated than commercial LOX. The more complex mixture can interfere with product extraction by SPE. When using 300 µL or more of SBF extract, the SPE method cannot be used to extract products from the reaction mixture as high amounts of particulate and insoluble material from the flour blocks the SPE tubes. Therefore, solvent extraction is required as an alternative method to isolate products. The presence of more LOX-2 in SBF than in 15-sLOX-1 identified by the colorimetric test, may cause the sfLOX-catalysed DHA reaction to be less specific in

product biosynthesis than commercial 15-sLOX-1, although this was not observed in the current experiments.

For sfLOX catalysis on DHA, substrate was not detected under almost all reaction conditions while product formation did not account for the loss of substrate. This was also observed in some cases with the commercial enzyme [43]. There are some reasons that could account for this. LOX-2 in SBF may be responsible for making *n*-hexanal as volatile green flavour compounds, as previously reported in a study between LA and sLOX-2 [208]. Other enzymes such as hydroperoxide lyases and hydroperoxide isomerases in the flour could take part in some side reactions in which the reaction products may be metabolised into undesirable compounds such as aldehydes and oxo-acid compounds [209]. However, in this work these compounds were not detected.

5. Conclusion

For the first time, SBF has been used directly, without purification, as an alternative to commercial purified LOX for synthesizing both mono- and di-hydroxy PUFA resolvin and protectin analogues from a range of biologically important n-3 and n-6 PUFAs. The reaction conditions are mild and the conversion is rapid. The synthesised products using sfLOX and 15-sLOX-1 are identical in terms of regio- and stereo-specificity. This method could potentially be applied to a low-cost industrial scale synthesis of these anti-inflammatory SPM compounds for pharmaceuticals, nutraceuticals and functional food applications. However, when using SBF, other compounds and enzymes present are also present and may interact with reaction product and/or substrate in side reactions or may interfere with the LOX catalysed reaction, affecting overall product yield.

Below is a flow diagram (Figure 3.19) summarising the process of resolvin analogue synthesis from DHA under the optimised conditions.
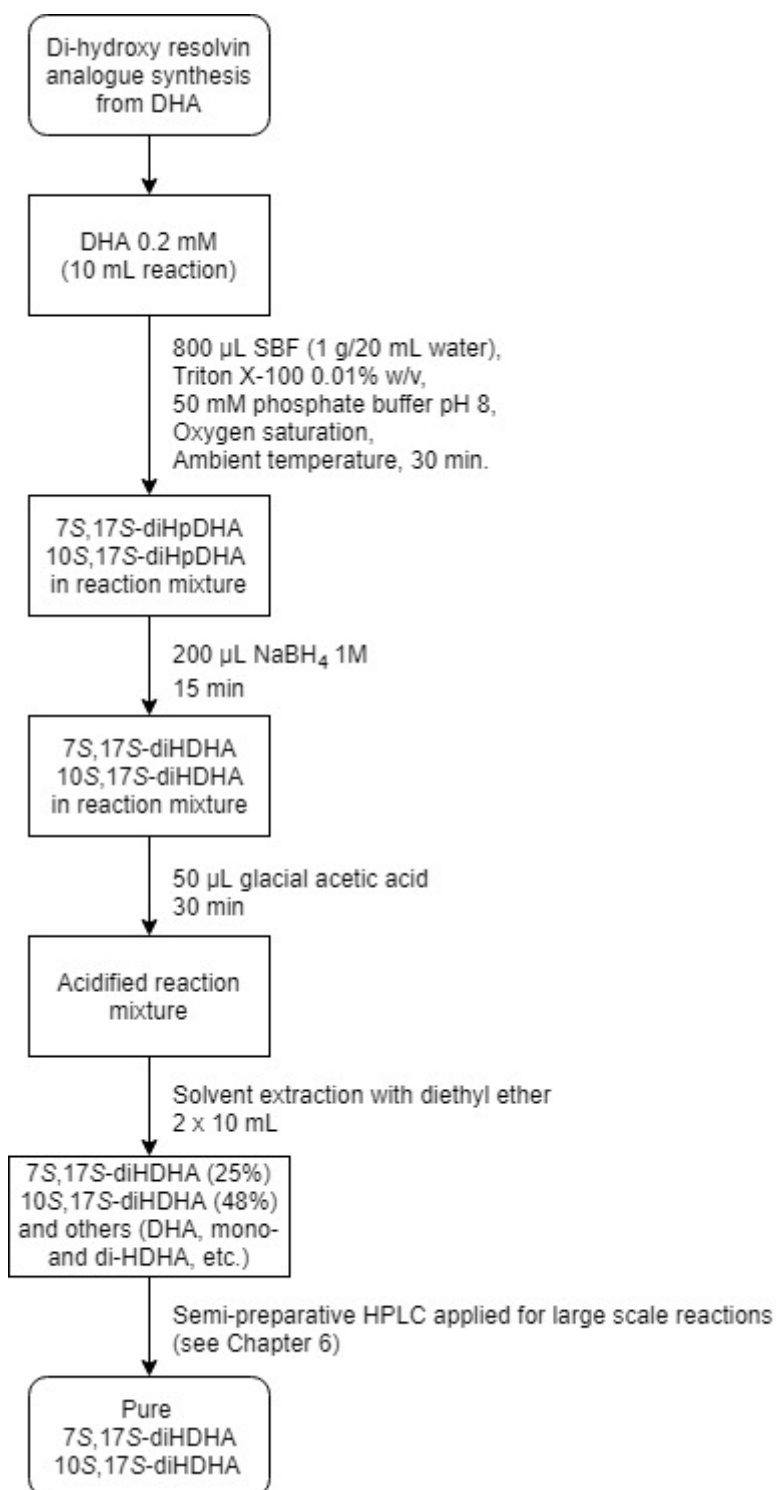
Figure 3.19: Summarised process of resolvin analogue synthesis from DHA into pure 7*S*,17*S*-diHDHA and 10*S*,17*S*-diHDHA under the optimised conditions with product yield included.

CHAPTER IV

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SOYBEAN FLOUR LIPOXYGENASE CATALYSIS OF TRILINOLEIN AS A TRIACYLGLYCEROL MODEL

1. Introduction

A triacylglycerol (TAG) molecule consists of three fatty acids attached to a glycerol backbone. TAGs are the major components (approximately 95%) of natural oils such as fish and vegetable oils [210]. Dioxygenation of PUFAs as free fatty acids by sfLOX has been demonstrated in Chapter 3 for the synthesis of potential bioactive compounds such as resolvin and protectin analogues. Free fatty acid substrates are commonly obtained from natural oils after hydrolysis with or without purification. It would be of great interest for PUFA containing TAGs to be oxygenated directly for bioactive compound synthesis without the extra hydrolysis step.

LOX is a versatile enzyme for catalysing the dioxygenation of PUFAs containing 1,4-pentadiene units. Soybean is a rich source of LOX and soybean LOX is well studied compared to other plant LOXs [55, 90]. In soybean seeds, the two types of LOXs, type-1 LOX (LOX-1) and type-2 LOX (LOX-2 and LOX-3), have distinct characteristics. Type-1 LOX is known to catalyse the oxygenation of free fatty acids effectively at optimum pH 9, whilst esterified fatty acids are not a preferred substrate. In contrast, type-2 LOX can oxygenate not only free fatty acids but also esterified fatty acids (EFAs) directly without prior to hydrolysis at optimum neutral pH [88, 90]. Only LOX-1 is commercially available; type-2 LOXs are not, most likely in part because they are unstable after purification [56]. LOXs can be used to synthesise various bioactive compounds such as eicosanoids and docosanoids [86]. These compounds, especially the di- and tri-hydroxy conjugated PUFAs, can act as specialised pro-resolving mediators with anti-inflammatory and pro-resolving activity at very low doses [6].

Less studies have reported the oxygenation of EFAs catalysed by soybean LOX compared to free fatty acids. Type-2 LOX has been shown to have much higher activity than LOX-1 in the catalysis of EFA substrates such as trilinolein and methyl linoleate [122, 131]. However, a number of studies have still investigated LOX-1 catalysis on various EFAs [129, 130, 168, 169, 211-213]. Importantly, LOX-1 was found to catalyse reactions with EFAs such as trilinolein and 1,3-dilinolein under specific conditions in the presence of a bile salt and a multicomponent buffer to increase the reaction rate, however the rate is still generally low compared to when LA is the substrate [129, 169]. Although there have been some studies on type-2 LOX-catalysed reactions with EFAs [122, 131, 175, 214], mainly investigating reaction pH and comparisons between LOX isozymes, a full optimisation of reaction conditions for maximum product formation has not been performed. Further investigation of type-2 LOX and EFA is needed and would be useful for a better understanding of LOX catalysis on EFAs. Soybean flour (SBF) is a useful LOX source, as it contains both LOX types in a stable, natural form. SBF is especially valuable as a source of type-2 LOX, which can be used directly to react with EFAs effectively and without prior hydrolysis.

In this chapter, optimisation of the sfLOX-catalysed reaction on trilinolein for the maximum tri-hydroperoxylated product formation is described. The approximate yield of the reaction was also calculated. Trilinolein was used as a model TAG substrate, containing three linoleate moieties. The TAG reaction products can contain up to three hydroxy groups (one on each fatty acid), and these compounds were characterised with a range of analytical techniques including HPLC-DAD, GC-MS and NMR. The optimised conditions were compared between the reaction of trilinolein (TL) and a commercial enzyme (15-sLOX-1) or SBF to confirm the versality of SBF in reacting with EFAs. The reaction products are highly stereo- and

regio-specific hydroxy linoleate moieties in mono-, di- and tri-hydroxy TL. This model system may be applicable to more complex natural oils containing a variety of TAGs such as fish oils with high percentage of n-3 LC-PUFAs (DHA and EPA), where the n-3 LC-PUFAs will be selectively oxygenated to produce potential anti-inflammatory compounds with high bioavailability.

2. Materials and methods

2.1. Materials

Soybean flour (Kialla Pure Foods, certified organic) was purchased from Organic Wholefoods (Brunswick East, VIC, Australia). Trilinolein (Nu-Chek Prep) was obtained from Adelab Scientific (Thebarton, SA, Australia). Acetyl chloride (Fluka), butylated hydroxytoluene (BHT), citric acid, ferric chloride, gum arabic, L-cysteine, methyl tricosanoate, N,O-bis(trimethylsilyl)trifluoroacetamide (with 1% trimethylchlorosilane), potassium phosphate monobasic, potassium phosphate dibasic, potassium iodide (KI), platinum(IV) oxide, pyridine (>99%), sodium borohydride, sodium thiosulfate ($Na_2S_2O_3$), starch, sodium citrate dihydrate, Trizma® base, Tween® 20 and (trimethylsilyl)diazomethane (2 M in diethyl ether) were purchased from Sigma Aldrich (Castle Hill, NSW, Australia). Agilent Bond Elut 500 mg, 3 mL SPE tubes and Vac Elut SPE vacuum apparatus were from Agilent Technologies (Mulgrave, VIC, Australia). Heptane (99%, 0.2 micron filtered) and isopropanol were obtained from Fisher Scientific (Scoresby, VIC, Australia). Hydrochloric acid (32%) and sodium tetraborate and were purchased from APS Chemicals Ltd (Seven Hills, NSW, Australia). Ethanol, diethyl ether, methanol, potassium bicarbonate and sodium chloride were purchased from Chem-Supply (Gilman, SA, Australia). Acetonitrile (Scharlau, Spain), acetic acid (BDH Lab Supplies, UK) and sodium hydroxide (Fisher Chemicals, UK) were purchased

from Science Supply (Mitcham, VIC, Australia). Triton X-100^{TM}, toluene and chloroform were purchased from Ajax Finechem (Taren Point, NSW, Australia). Genistein (Toronto Research Chemicals, Canada) was purchased from Sapphire Bioscience (Redfern, NSW, Australia). Hydrogen, nitrogen and oxygen gases were obtained from Coregas Pty Ltd (North Geelong, VIC, Australia).

2.2. Lipoxygenase-catalysed reaction optimisation for maximum product formation

1 mL stock TL emulsion solution (10 mg/mL) was freshly prepared in 50 mM tris-HCl buffer pH 7.5 with 15% w/v gum arabic and sonicated by an ultrasonic processor (500 W, 20 kHz, amplification 20%) with 10 pulses [92]. The SBF suspension [56] was prepared by suspending 1 g SBF in 20 mL water, followed by shaking for 1 min. The reaction mixture was prepared with stirring and contained 0.1 mg/mL TL emulsion in 10 mL buffer (50 mM) with a range of pH and buffer types (Table 4.1). Other additives if required were added and then 25 µL SBF suspension was added to initiate the reaction. The reaction was conducted at room temperature (23°C) with stirring for 15 min. 50 µL glacial acetic acid was added at the end of the reaction and the reaction mixture was stirred for approximately 30 min. For short reactions (less than 15 min), the reactions were stopped by adding 20 mL diethyl ether to the reaction mixture after adding the 50 µL acetic acid.

Table 4.1: Three types of buffer used and the pH ranges studied.

Buffer type	pH
Phosphate	5 − 8
Borate	8 − 11
Citrate	3 − 6

The reaction products were extracted by solid phase extraction (SPE) under vacuum as described in Chapter 3, Section 2.2. For the reactions using high volumes of SBF suspension ($\geq$ 150 µL), solvent extraction was used as an alternative to SPE, because the reaction mixtures blocked the SPE columns. The reaction mixtures were adjusted to pH 3 by HCl (1 M) and were extracted with diethyl ether (2 × 20 mL) and the combined solvent after extraction was evaporated under vacuum. The sample was diluted in 1 mL methanol with 0.1% v/v acetic acid or methanol:isopropanol (80:20) with 0.1% v/v acetic acid for RP-HPLC analysis. Blank reactions either without substrate or SBF were performed. They showed minimal autoxidation products (less than 5% of total products).

The reaction was optimised to determine the ideal conditions for the synthesis of the maximum amount of tri-hydroperoxy products and also total products. Three variables of pH, buffer type (see Table 4.1) and SBF suspension volume (25, 100, 200, 300 and 400 µL), were investigated at the same time. The best conditions were chosen as initial conditions for further optimisation, with the remaining variables tested one at a time (Table 4.2). The conditions with the best results were kept for further optimisation and for the final optimised conditions.

Table 4.2: Reaction variables optimised in the sfLOX-catalysed reaction with TL.

Variable	Range studied
TL amount (mg)	0 – 10
Reaction time (minutes)	1 – 120
Oxygenation	Oxygen, Nitrogen, Air, None
Temperature (°C)	0 – 40
Ferric chloride concentration (µM)	0 – 10
Genistein concentration (mM)	0 – 1
Butylated hydroxytoluene - BHT (mg/mL ethanol)	0 – 0.2

For optimisation of individual reaction variables, experiments were repeated between 1 and 3 times to ensure a clear trend and optimum was observed. Values from a single representative experiment are shown in the results section for each variable. Results at different reaction conditions for each variable were considered to be different when the results varied by greater than ± 5%. The optimised reaction conditions were conducted in triplicate.

2.3. Reduction of trilinolein hydroperoxides

sfLOX-catalysed TL reaction products (TL hydroperoxides) after solvent evaporation under vacuum, were reduced to produce TL hydroxides. Initially, the products were dissolved in 1 mL methanol and the reaction was initiated by adding 200 µL sodium borohydride (NaBH$_4$, 1 M), and the reaction mixture was stirred for 15 min. The reaction was stopped and acidified by adding 50 µL acetic acid, followed by stirring for 15 – 30 min. 10 mL water was added and the reduced products were extracted with diethyl ether (2 × 20 mL). The combined solvent after extracting was evaporated under vacuum before diluting in 1 mL of mobile phase for

RP-HPLC analysis. The following reaction variables were optimised, in duplicate (Table 4.3), to convert TL hydroperoxides into TL hydroxides and minimise the formation of undesirable side products. To obtain the same amount of TL hydroperoxides as starting materials for each reaction, a 100 mL scale reaction (10 × 10 mL reaction conditions) was conducted. The TL hydroperoxide products were dissolved in 13 – 15 mL methanol and 1 mL was used for each reaction. Reactions at each condition were performed in duplicate.

Table 4.3: Reaction variables optimised for the reduction of sfLOX-catalysed TL reaction products.

Variable	Range studied
Extra water volume (µL)	0 – 800
NaBH$_4$ volume (µL)	0 – 200
NaBH$_4$ concentration and volume (M)	1 – 4 M and 200 – 50 µL
Reaction time (minutes)	1 – 45

2.4. Hydrolysis of trilinolein hydroxides

Hydrolysis of EFAs [215, 216] was conducted on TL hydroperoxides with modifications. The reduced sfLOX-catalysed TL reaction products were dissolved in 1 mL ethanol in a 10 mL vial and 1.5 mL aqueous NaOH 50% (w/v) was added. The reaction mixture was sealed and stirred at 65°C for 90 min. Then water was added, up to 10 mL, to dilute the sample to avoid harsh conditions which may cause side reactions before adjusting pH with HCl (4M). The sample was acidified until the solution turned cloudy (approximately pH 2 – 3). Additional acid was avoided because it can cause an increase in undesirable side reaction products. The hydrolysed products were extracted by diethyl ether (2 × 20 mL), followed by evaporating solvent under vacuum. The final sample was diluted in 1 mL methanol

with 0.1% v/v acetic acid for further product characterisation by RP-HPLC, chiral RP-HPLC or GC-MS analysis. Reactions were performed in triplicate.

2.5. Monitoring reaction product formation by RP-HPLC

sfLOX-catalysed TL reaction products including mono-, di- and tri-hydroperoxy TL were separated by RP-HPLC [92]. An Agilent Technologies 1260 series instrument, including a solvent degasser, quaternary pump, autosampler and diode array detector (DAD) was used. A C8 column (Phenomenex, 15 cm × 4.6 mm, 5 μm particle size) was used. The composition of mobile phase was A: 0.1% v/v acetic acid in water and B: methanol with 0.1% v/v acetic acid. Samples were separated under gradient conditions from 90% to 100% B over 20 min, at 1 mL/min, 23ºC. The elution of mono-, di- and tri-hydro(pero)xy products was identified spectrophotometrically through absorption at 210 nm, 234 nm, 250 nm and 270 nm. Injection volume was 15 μL unless otherwise stated and reproducibility was confirmed by repeat injections. Data was processed using Agilent Technologies ChemStation for LC 3D system B.04.03 software.

For reaction yield, all samples were dissolved in 1 mL mobile phase. Standards (TL and TL-3OOH) and reaction product samples were injected with 5 μL, whereas hydroxy LA samples were analysed with 1 μL injection volume.

2.6. Determination of stereochemistry of reaction products by chiral RP-HPLC

Reduced and hydrolysed sfLOX-catalysed TL reaction products at the final optimised conditions were separated by chiral RP-HPLC. An Agilent Technologies 1260 Infinity series instrument, including a solvent degasser, quaternary pump, autosampler and diode array detector (DAD) was used. A Lux 5u Amylose-2 column (Phenomenex, 100 × 4.6 mm, 5 μm particle size) was used to separate products. The mobile phase contained, A: 0.1% v/v acetic acid in water and B: acetonitrile with

0.1% v/v acetic acid. 13-hydroxy LA (13-HLA) obtained from reducing and hydrolysing sfLOX-catalysed TL reaction products was separated for the two enantiomers (13*S*-HLA and 13*R*-HLA) under gradient conditions from 20% to 80% B over 20 min at 0.5 mL/min, 23°C. The elution of two chiral compounds of 13-HLA was identified spectrophotometrically through absorption at 210 nm, 234 nm, 250 nm and 270 nm. Samples were prepared in acetonitrile with 0.1% v/v acetic acid. Injection volume was 15 µL and reproducibility was confirmed by repeat injections. Data was processed using Agilent Technologies ChemStation for LC 3D system B.04.03 software.

2.7. Determination of hydroxy group positions on reaction products by GC-MS

The sfLOX-catalysed TL reaction products were reduced and hydrolysed to obtain hydroxy LA. The positions of hydroxy groups on hydroxy LA were determined by GC-MS [43] after hydrogenation and conversion to trimethylsilylated derivatives (see Chapter 3, Section 2.4.). An Agilent Technologies 6890N Network GC system with a 5975 mass selective detector (electron impact) and a BPX5 column (30.0 m × 0.25 mm, 0.25 µm film thickness; SGE, Ringwood, VIC, Australia) was used. GC-MS analysis was fully described in Chapter 3, Section 2.4.

2.8. Calculation of reaction product yield by GC-FID

The yield of the sfLOX-catalysed TL reaction was estimated through monitoring the consumption of linoleate from the starting material, TL, by GC-FID [217]. An Agilent Technologies 6890N Network GC system with flame ionisation detector and an SGE BPX70 column (30 m × 0.25 mm, 0.25 µm film thickness) were used. Injection volume was 1 µL, helium carrier gas, flow rate 1.5 mL/min (average velocity of 38 cm/second) with an inlet temperature of 250°C and a split ratio of 50:1. Initial oven temperature was 140°C held for 5 min increased to 220°C at a rate

of 4°C/min and held for 5 min. Samples were converted to fatty acid methyl esters (FAMEs) prior to analysis. Approximately 2 mg of sfLOX-catalysed TL reaction products from 10 mL optimised reaction, 2 mg TL or 4 mg TAGs extracted from 25 mg SBF suspension were dissolved in 1 mL toluene. 200 µL methyl tricosanoate (5 mg/mL toluene) as internal standard and 200 µL BHT (1 mg/mL toluene) as antioxidant were added. 2 mL of acidic methanol reagent (10% v/v acetyl chloride was added dropwise into methanol put on ice and stirred for at least 1 hour) was added and kept overnight in a stoppered test tube in an oven at 50°C. After the solution was cooled down, 5 mL of sodium chloride solution (5% w/v in water) was added. Heptane (2 × 5 mL) was used to extract the methyl ester products from the mixture. The combined heptane layers were washed with 5 mL potassium bicarbonate (2% w/v in water) and dried over sodium sulphate before evaporation of the solvent under a stream of nitrogen or under vacuum. All samples were performed in duplicate and the experiment was repeated.

2.9. Product structure identification by NMR spectroscopy

1D ^{1}H-NMR, ^{13}C-NMR and 2D ^{1}H, ^{1}H-COSY-NMR experiments were conducted on an Advance 500 MHz high resolution NMR spectrometer (Bruker) at 10°C unless otherwise stated. The 10 mL sfLOX-catalysed TL reaction under the final optimised conditions (see Section 2.2 above) were scaled up 20 times for the large-scale synthesis of TL hydroperoxides. The reaction products were purified using NP-HPLC as described in Chapter 6 (see Section 2.3). Purified products (~ 10 – 30 mg) were dissolved in 600 µL deuterated chloroform and transferred to an NMR tube. For ^{1}H NMR, 8 – 16 scans were accumulated and the residual chloroform peak was used as a reference (7.26 ppm) for all chemical shifts. The residual chloroform peak was also used as a reference (77.36 ppm) for ^{13}C NMR for all chemical shifts, and ~15,000 scans were accumulated over ~ 20 hours.

2.10. Calculation of reaction product yield by iodometric titration

A modified iodometric titration method for peroxide value [218, 219] was also used to determine the yield of the final optimised sfLOX-catalysed TL reaction. The reaction products (2 mg) after solvent evaporation were dissolved in 6 mL chloroform:acetic acid (2:3) and transferred to 50 mL conical flask. 100 µL of freshly prepared saturated potassium iodide (KI) solution was added with mixing and left for 1 min. 6 mL water was added into the yellow mixture and samples were titrated using sodium thiosulfate ($Na_2S_2O_3$, 0.001 N) with vigorous stirring. 100 µL starch solution 0.5% (w/v) as an endpoint indicator was added when the yellow colour had almost disappeared, turning the mixture dark blue. The titration was continued until the colour fully disappeared. $Na_2S_2O_3$ volume was used to calculate the amount of hydroperoxide in samples. Titrations of sfLOX-catalysed TL reaction product samples were conducted in triplicate. Hydroperoxides in blank SBF and blank TL samples were also identified as controls.

The reaction mechanism is described below

$ROOH + 2H^+ + 2I^- \rightarrow ROH + H_2O + I_2$ (iodine)

$I_2 + 2Na_2S_2O_3 \rightarrow Na_2S_4O_6 + 2NaI$

$I_2 + I^- \Leftrightarrow I^{3-}$ (triiodide and starch give the solution a dark blue colour)
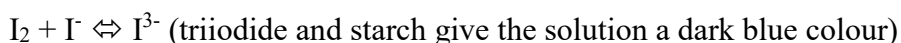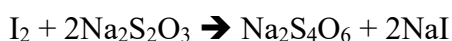
The number of moles of hydroperoxide present is equivalent to half the number of moles of $Na_2S_2O_3$ required for the titration, which was calculated from the titration volume and the known concentration. The yield of hydroperoxide groups added during the sfLOX-catalysed TL reaction was calculated by comparing the moles of hydroperoxide obtained from the reaction with the theoretical maximum number of moles.

3. Results

3.1. Optimisation of sfLOX-catalysed TL reaction conditions for maximum product formation

Luquet et al. used crude SBF suspended in water as a type-2 LOX source to react directly with pure TL, without any purification to produce oxygenated products. The result showed that SBF had significantly higher activity than commercial sLOX-1 in oxygenating pure TL in the absence of solvent, but did not do any product characterisation [56]. Purified germinating barley LOX-2 was demonstrated to oxygenate TL at a substantially higher rate than barley LOX-1. TL was prepared as an emulsion in tris-HCl buffer pH 7.5 containing 15% w/v gum arabic by a sonifier with ultra tip (100 W). Three groups of products were detected by RP-HPLC through absorbance at 234 nm but characterisation of product regio- and stereo-specificity was not obtained [92]. The products contained 1 to 3 hydroperoxy groups, with each hydroperoxy group on a linoleate moiety of TL. They were mono-hydroperoxy TL (TL-OOH), di-hydroperoxy TL (TL-2OOH) and tri-hydroperoxy TL (TL-3OOH, Figure 4.1).
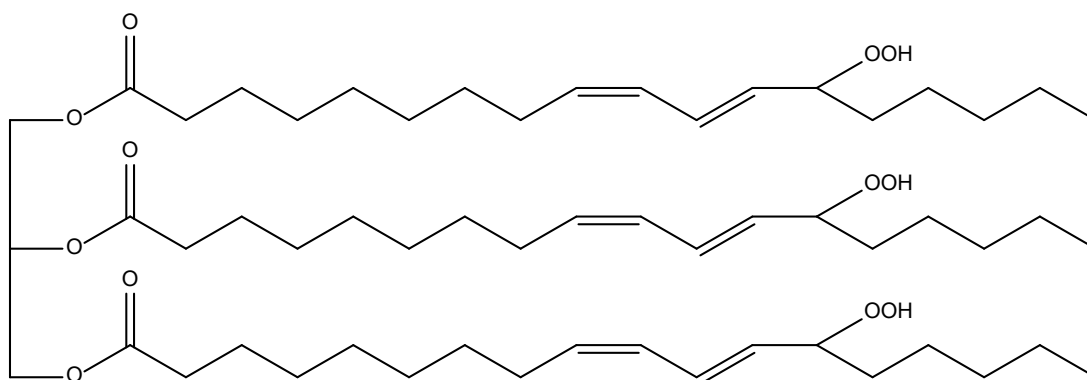
Figure 4.1: Chemical structure of TL-3OOH from sfLOX-catalysed TL reaction. R: 13-hydroperoxy linoleoyl group (as shown in the structure for the first acyl moiety)

Based on these references and the sfLOX and DHA work in Chapter 3, the initial conditions were chosen to be: 100 µL TL emulsion – 10 mg/mL (as a model

substrate for sfLOX-catalysed TAG reaction), 25 µL SBF suspension (1 g SBF in 20 mL water), 10 mL buffer, ambient temperature, no surfactant, 15 min reaction time. Product formation was monitored by RP-HPLC DAD through absorption at 234 nm, corresponding to the formation of conjugated double bonds. The three groups of TL hydroperoxides eluted from highest to lowest polarity on a reverse phase C8 column. Their elution order and maximum absorption were matched to the reaction products from TL emulsion and germinating barley LOX-2 reaction previously analysed under similar RP-HPLC conditions [92]. The compounds were expected to be TL-3OOH (highest polarity), TL-2OOH and TL-OOH (lowest polarity), eluting at approximately 6.5, 11 and 16.5 min respectively (Figure 4.2).
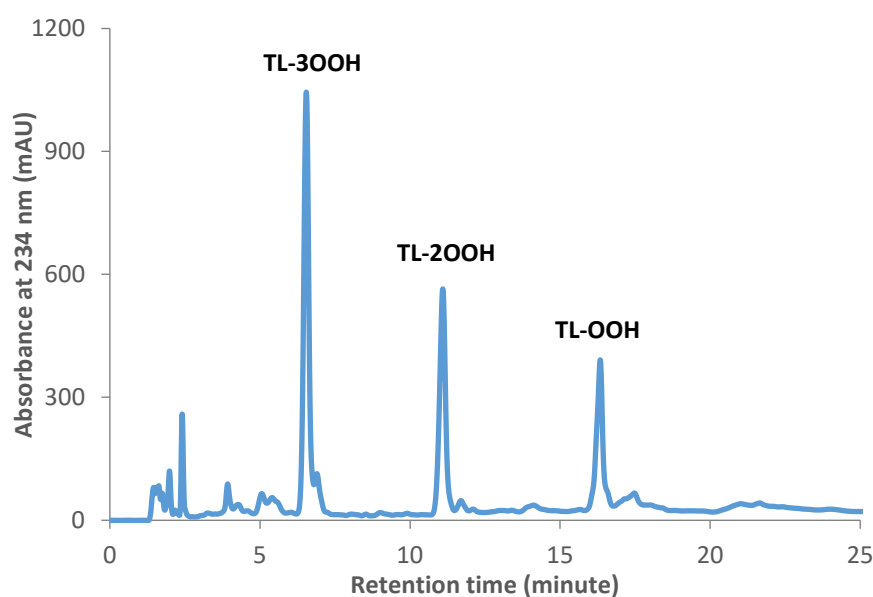
Figure 4.2: RP-HPLC chromatogram of sfLOX-catalysed TL reaction products at these reaction conditions – 10 mL citrate buffer (50 mM, pH 5), 200 µL TL emulsion, 500 µL SBF suspension, ambient temperature, 15 min reaction time.

3.1.1. Effect of buffer type, pH and soybean flour suspension volume on product formation

Different buffer types (citrate, phosphate and borate) were tested in the pH range 3 – 11 with increasing SBF suspension volume (25 – 400 µL) to obtain the optimum pH and SBF suspension volume. The optimum pH was unclear when phosphate and

borate buffers were used. Citrate buffer (studied range: 3 – 6) showed that the optimum pH was at pH 4.5 – 5. TL-3OOH, TL-2OOH and total products increased with increasing pH from 3 – 5, reached a maximum at pH 5 and decreased up to pH 6, whereas TL-OOH formation was maximised at pH 4.5 (Figure 4.3). The optimum pH for the sfLOX-catalysed TL reaction was found to be pH 5 citrate buffer, based on the maximum product formation of TL-3OOH and total products.

For the following HPLC results (15 µL injection), a peak area of 9,500 corresponds to ~ 0.02 mg/mL or 0.021 mM TL-3OOH, and a peak area of 4,500 corresponds to ~ 0.02 mg/mL or 0.023 mM TL in the 10 mL reaction.
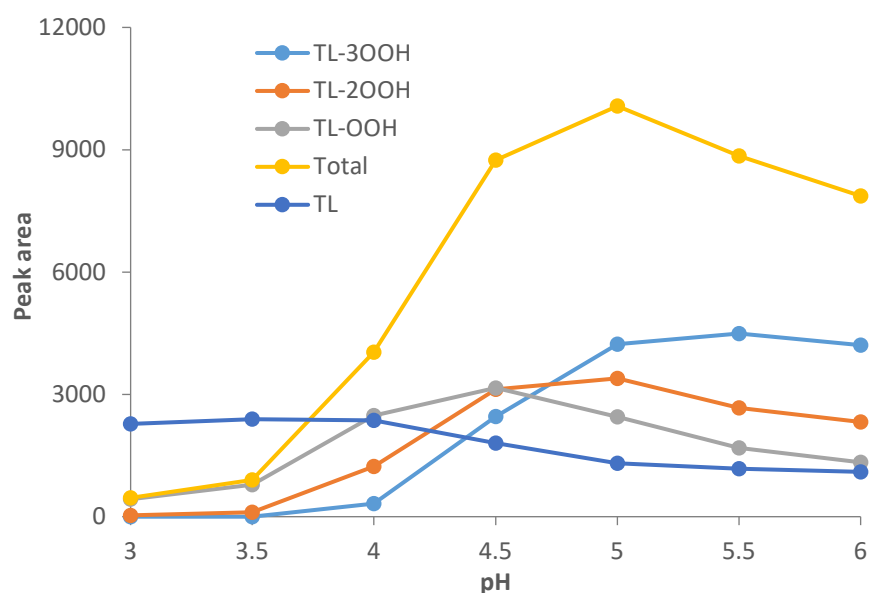
Figure 4.3: Effect of pH (3 – 6) on sfLOX-catalysed TL reaction under the conditions: 100 µL TL emulsion, 200 µL SBF suspension, 10 mL citrate buffer (50 mM), ambient temperature, 15 min reaction time. The results from a single experiment represent pH effect on product formation.

SBF suspension volume was optimised in a larger range (25 – 1500 µL) using the optimum pH 5 citrate buffer. From 25 to 100 µL, product formation changed significantly. TL-3OOH and TL-2OOH increased ~ 3 and 1.5 times respectively, whereas TL-OOH decreased ~ 25%. At higher SBF suspension volume (> 100 µL), product formation did not change substantially (Figure 4.4). 500 µL was used as the

optimum SBF suspension volume to obtain the maximum amount of TL-3OOH, although total products were slightly lower due to the decrease of TL-2OOH and TL-OOH. SBF is an inexpensive LOX source so using a high amount of SBF in the reaction is not an economic issue.
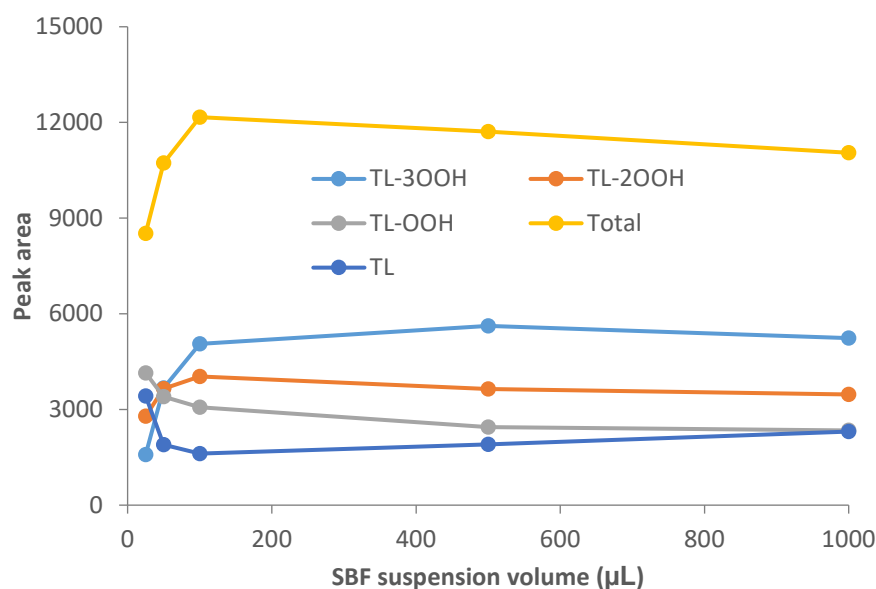
Figure 4.4: Effect of SBF suspension volume (25 – 1000 µL) on sfLOX-catalysed TL reaction under the conditions: 100 µL TL emulsion, 10 mL citrate buffer (50 mM, pH 5), ambient temperature, 15 min reaction time. The results from a single experiment represent SBF suspension volume effect on product formation.

3.1.2. Effect of trilinolein emulsion volume on product formation

TL emulsion volume was optimised in the range 100 – 1000 µL, using 500 µL SBF suspension, 10 mL citrate buffer (50 mM, pH 5), 15 min reaction time. Total product formation increased significantly (~ 2 – 7 fold) with increasing TL emulsion volume in the studied range from 100 to 500 µL, except that TL-3OOH stayed constant from 200 to 500 µL (Figure 4.5A). At higher TL emulsion volume (> 500 µL), no significant change in total products was observed (results not shown). The catalytic efficiency of sfLOX in forming TL hydroperoxides was measured by considering product peak area per mg TL used (Figure 4.5B).
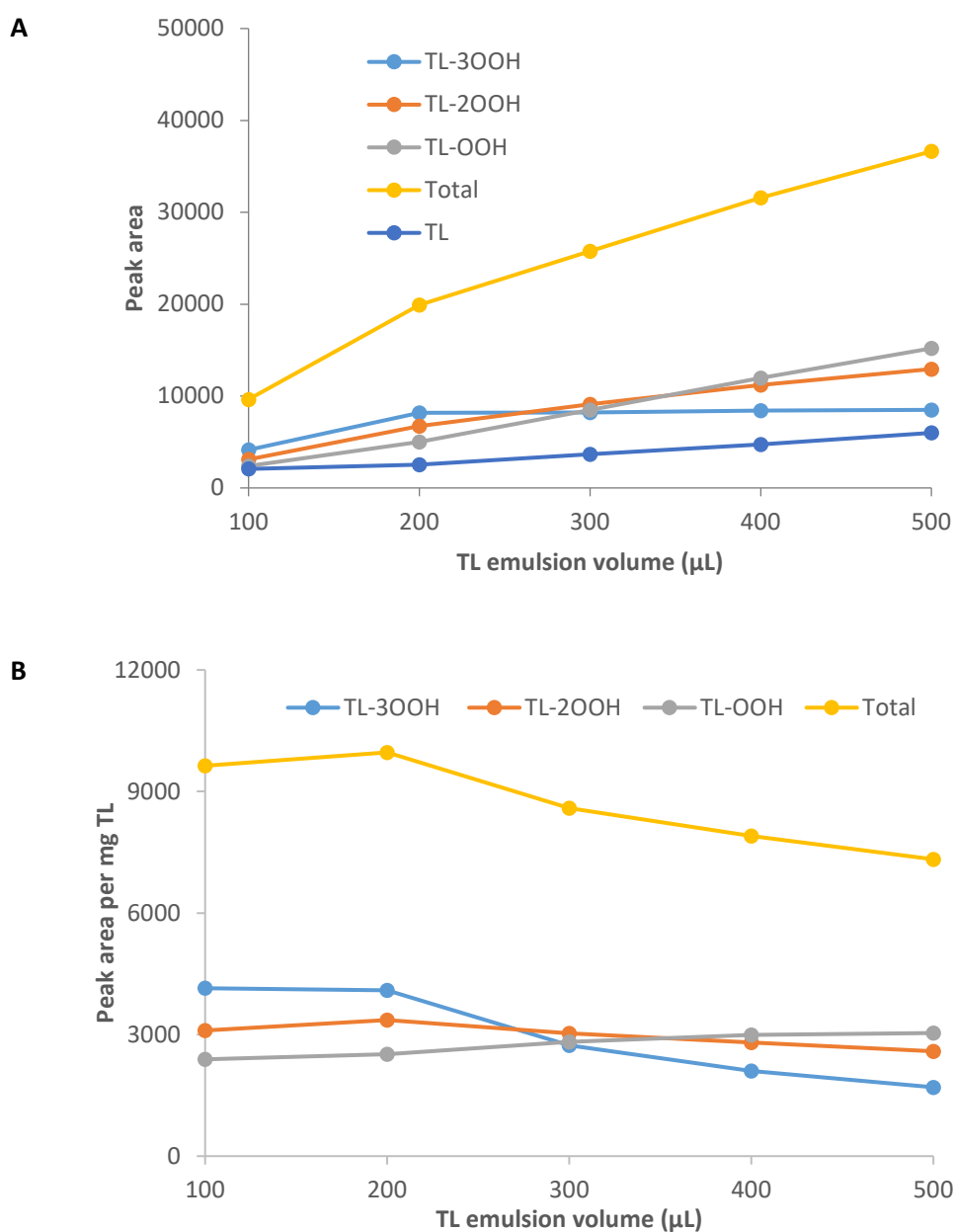
Figure 4.5: Effect of TL emulsion volume (100 – 500 µL) on sfLOX-catalysed TL reaction under the conditions: 500 µL SBF suspension, 10 mL citrate buffer (50 mM, pH 5), ambient temperature, 15 min reaction time. A) Peak area, B) Peak area per mg TL versus TL emulsion volume. The results from a single experiment represent TL emulsion volume effect on product formation.

100 µL TL emulsion (10 mg/mL) was equal to 1 mg TL in the 10 mL reaction. The sfLOX catalytic efficiency for total product formation reached the maximum at 2 mg, and decreased significantly at higher TL amounts (> 2 mg). The optimum TL emulsion volume for the sfLOX-catalysed TL reaction was found to be 200 µL (2 mg TL) in which sfLOX obtained the maximum catalytic efficiency. At the new

optimised TL emulsion volume, SBF suspension volume was re-optimised in the range of 400 to 1000 µL. SBF suspension volume did not affect product formation significantly. Therefore, 500 µL was kept for further optimisation.

3.1.3. Effect of reaction time on product formation

In order to monitor the time course of the reaction, a 100 mL reaction (10 times the normal 10 mL reaction volume) was conducted and aliquots (10 mL) of reaction mixture were taken at specific time points (1 – 75 min) after the reaction was initiated. All product types formed quickly in the first minute of the reaction. TL-OOH concentration reached a maximum at 1 to 5 min, which was much faster than maximums for TL-2OOH and TL-3OOH were reached. It took 15 min to reach maximum TL-2OOH levels, while TL-3OOH concentration was maximised between 30 and 60 min. All product formation tended to decrease after 60 min (Figure 4.6). The substrate TL decreased significantly from 1 to 30 min and then decreased very little up to 75 min, which matched with the increase of TL-3OOH and the conversion of TL-OOH into TL-2OOH and TL-2OOH into TL-3OOH. The optimum reaction time was found to be 45 min for the maximum production of TL-3OOH.
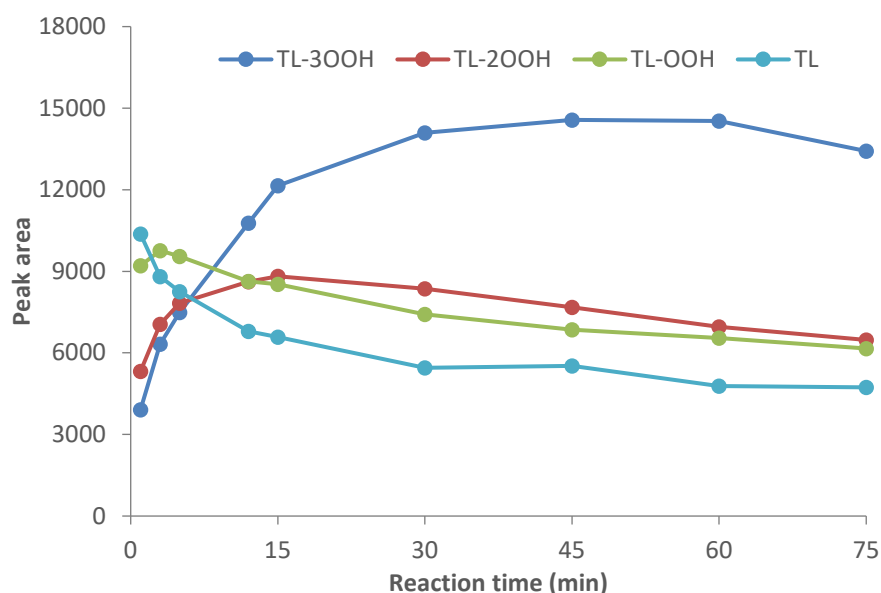
Figure 4.6: Product formation over time (1 – 75 min) in the sfLOX-catalysed TL reaction under the conditions: 200 µL TL emulsion, 500 µL SBF suspension, 10 mL citrate buffer (50 mM, pH 5), ambient temperature. The results from a single experiment represent reaction time effect on product formation.

3.1.4. Effect of oxygenation on product formation

The following sfLOX-catalysed TL reaction conditions were used to optimise oxygen concentration in buffer: 200 µL TL emulsion, 500 µL SBF suspension, 10 mL citrate buffer (50 mM, pH 5) and 45 min reaction time. Oxygen concentration in the 10 mL buffer was varied from a maximum of 35 mg/L to a minimum of close to 0 mg/L by sparging the buffer with oxygen, air or nitrogen until saturation (approximately 5 min) or leaving untreated before the reaction was initiated. During the reaction time, the nitrogen-saturated reaction was sealed in order to maintain low oxygen concentration. Oxygen is a critical component of the LOX reaction to form FA hydroperoxide products. Unexpectedly, the sfLOX-catalysed TL reaction was not affected significantly by oxygen concentration. Under low oxygen conditions (buffer saturated with nitrogen), product formation did not decrease substantially (Figure 4.7). Further experiments were conducted to make sure no oxygen was remaining in buffer over the reaction time. After nitrogen-saturation of the buffer, the reaction was

continuously sparged with nitrogen gas during the reaction time to maintain an oxygen concentration at 0 mg/L (confirmed by dissolved oxygen meter). There was almost no product formation in the total absence of oxygen (results not shown). In the literature, products such as dimers and oxo compounds have been reported under anaerobic conditions [220]. However, there were no new products formed at nitrogen saturated conditions (low oxygen concentration and 0 mg/L oxygen). The reason for the high amount of product formation at low oxygen concentration is unclear. As there was no significant difference in product yield, no gas sparging was used in further experiments.
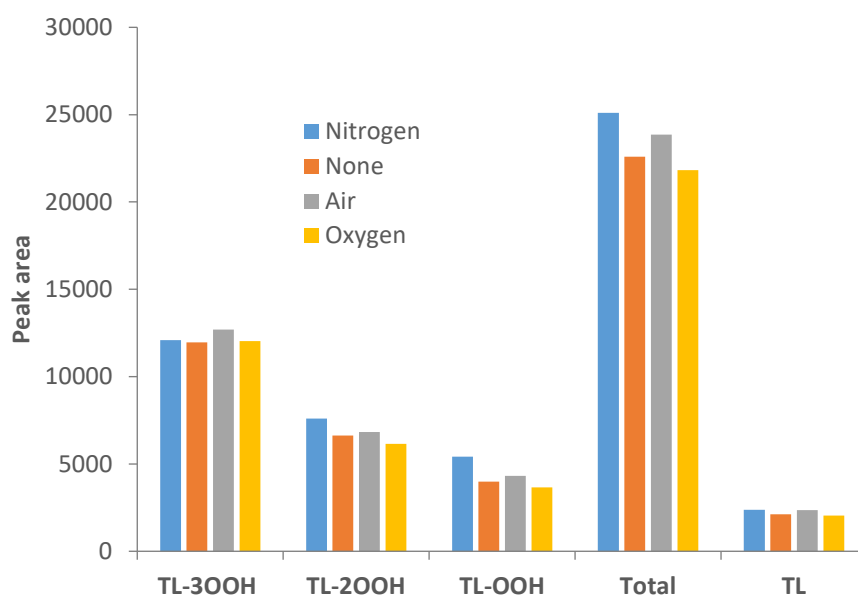
Figure 4.7: Effect of buffer oxygenation on the sfLOX-catalysed TL reaction under the conditions: 200 µL TL emulsion, 500 µL SBF suspension, 10 mL citrate buffer (50 mM, pH 5), ambient temperature. The results from a single experiment represent oxygenation effect on product formation.

Under the above reaction conditions with buffers either at low oxygen concentration (nitrogen sparged condition) or sufficient oxygen (untreated condition, control), the reaction was investigated using various substrate preparations to search for the source of oxygen in the nitrogen sparged reaction conditions. TL in solvent with or without sonication, and sonicated TL emulsion with or without 15% w/v gum arabic

were tested. Oxygen is an essential substrate for oxygenation of PUFAs catalysed by LOXs, therefore product formation depends on oxygen concentration. When a decrease in product formation was observed with a decrease in oxygen concentration, this observation was referred to as 'having an oxygen effect'. In contrast, when product formation did not change when oxygen concentration was decreased, 'no oxygen effect' was observed. For both gum arabic concentrations (0 and 15% w/v), emulsions were formed by sonication, but the emulsion in the absence of the emulsifier was significantly less stable than the one with 15% gum arabic. There was no oxygen effect with TL emulsions containing 0 or 15% w/v gum arabic. However, oxygen effect was observed when TL was prepared in solvent (acetone:ethanol = 1:1), with or without sonication, instead of as a TL emulsion. TL in solvent with sonication cannot form an emulsion. As a result, sonication of aqueous solution to make the TL emulsions may contribute to the elimination of the oxygen effect.

3.1.5. Effect of temperature on product formation

The sfLOX-catalysed TL reaction was optimised in the temperature range 0 – 40°C using the following conditions: 200 μL TL emulsion, 500 μL SBF suspension, 10 mL citrate buffer (50 mM, pH 5), 45 min reaction time, and buffer without gas sparging. The 0°C reaction was conducted on ice, the 10°C reaction was kept in an ice water bath with temperature monitored by a thermometer, and the 30°C and 40°C reactions were heated in a water bath on a heating plate. In the temperature range studied, TL-3OOH formation was found to be affected dramatically, whereas TL-2OOH and TL-OOH were less affected by temperature. TL-3OOH increased rapidly from 0°C to a maximum at 20 – 23°C (ambient temperature), an increase of approximately 2.5 times compared to 0°C, and then decreased significantly up to 40°C (Figure 4.8). The optimum temperature for TL-3OOH and total product formation was found to be at room temperature (20 – 23°C).
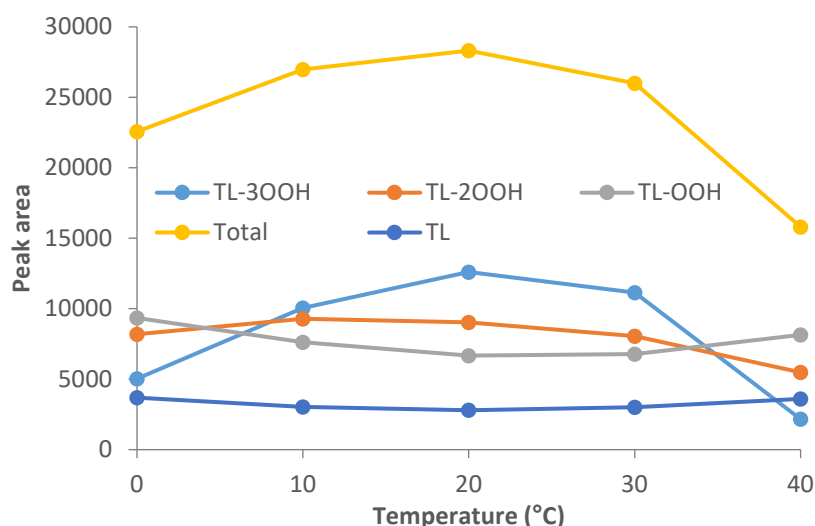
Figure 4.8: Effect of temperature (0 – 40°C) on the sfLOX-catalysed TL reaction under the conditions: 200 µL TL emulsion, 500 µL SBF suspension, 10 mL citrate buffer (50 mM, pH 5). The results from a single experiment represent temperature effect on product formation.

3.1.6. Effect of antioxidant, BHT on product formation

The sfLOX-catalysed TL reaction at the above conditions (200 µL TL emulsion, 500 µL SBF suspension, 10 mL citrate buffer (50 mM, pH 5), 45 min reaction time, without oxygenation, and ambient temperature) was optimised in the presence of antioxidant, BHT (0 – 0.2 mg/mL ethanol). BHT (and some other antioxidants) are funtional as antioxidants at very low concentrations ($\leq 0.01\%$ w/w), but they may exhibit pro-oxidant behaviours such as oxidation initiation at higher concentrations [221, 222]. Pro-oxidation could promote non-specific oxygenation of substrates to form a range of undesirable products. However, BHT at optimised concentrations was reported to stimulate LOX reactions [223]. It is therefore of interest to investigate the effect of BHT effect on product formation in the sfLOX-catalysed TL reaction.

In this work, TL-3OOH and TL-2OOH formation increased significantly with increasing BHT concentrations and reached maxima at 0.05 mg/mL ($\times$ 4.3) and 0.025 mg/mL ($\times$ 2.0), respectively. TL-OOH formation was not affected by BHT concentration in the range studied. TL-3OOH decreased rapidly at higher BHT

concentrations (> 0.05 mg/mL), whereas TL-2OOH stayed relatively constant after reaching the optimum at 0.025 mg/mL (Figure 4.9). BHT in the studied range did not show an inhibition effect on the reaction. 0.05 mg/mL was found to be the optimum BHT concentration for the maximum yield of sfLOX-catalysed TL reaction.

For the following HPLC results (5 μL injection), a peak area of 16,000 corresponds to ~ 0.1 mg/mL or 0.1 mM TL-3OOH, and a peak area of 7,000 corresponds to ~ 0.1 mg/mL or 0.11 mM TL in the 10 mL reaction.
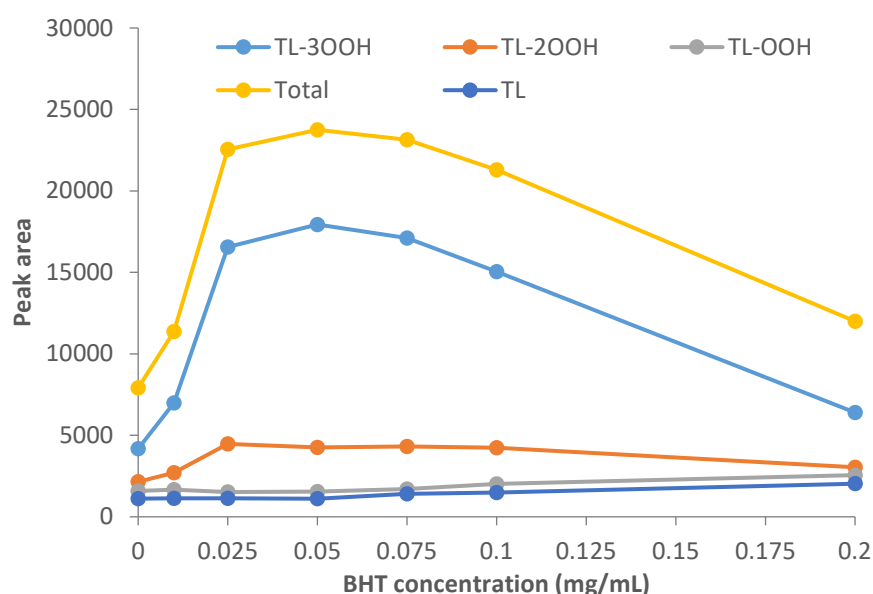
Figure 4.9: Effect of BHT concentration (0 – 0.2 mg/mL) on the sfLOX-catalysed TL reaction under the conditions: 200 μL TL emulsion, 500 μL SBF suspension, 10 mL citrate buffer (50 mM, pH 5). Due to the high concentrations of products, 5 μL injection was used for HPLC analysis instead of 15 μL as normal. The results from a single experiment represent BHT effect on product formation.

3.1.7. Final optimised conditions for maximum product formation

The final optimised conditions were found to be: 500 μL SBF suspension, 200 μL TL emulsion, citrate buffer (50 mM, pH 5), BHT (0.05 mg/mL), ambient temperature, 45 min reaction time. Under the final optimised conditions, TL-3OOH and TL-2OOH yields were increased substantially (approximately 50 and 10 times respectively), whilst increases in TL-OOH yield were insignificant compared to the

yields achieved under the initial conditions. The reproducibility of the optimised sfLOX-catalysed TL reaction was tested by repeating the reaction five times on the same day. The results were reproducible with relative standard deviation lower than 5%.

As it was previously observed, that the removal of oxygen from the reaction buffer did not affect product formation (Section 3.1.4 above), the final optimised reaction was also tested under nitrogen sparged buffer conditions. As was seen previously, there was no change in product formation regardless of changing oxygen concentration. Again, when TL substrate was prepared in solvent (instead of an emulsion), product formation was decreased under nitrogen sparged conditions (by approximately 50%) compared to atmospheric conditions (no gas sparging). This confirmed that the oxygen effect was diminished when TL in buffer was sonicated to make emulsion, whereas the oxygen effect remained when TL was prepared in solvent.

3.2. Preparation for product characterisation

The products obtained from the optimised sfLOX-catalysed TL reaction were derivatised to enable characterisation. TL hydroperoxides were reduced with sodium borohydride (NaBH$_4$) into TL hydroxides and then hydrolysed to form hydroxy-linoleic acid. This allowed the regio- and stereo-specificity of hydroxy groups on linoleate moieties to be identified. The chemical structures of purified TL-3OOH, TL-2OOH and TL-OOH were determined directly without derivatisation by NMR.

3.2.1. Reducing TL hydroperoxides

TL hydroperoxides formed in the LOX reaction were unable to be reduced directly in 10 mL buffer with NaBH$_4$, as was used for sfLOX-catalysed DHA reaction products (Chapter 3). TL hydroperoxides (after extraction) were reduced into TL hydroxides

by NaBH$_4$ in the presence of methanol. The initial reducing conditions were 200 µL NaBH$_4$ (1 M), extracted TL hydroperoxides, 1 mL methanol and 15 min reaction time. The reduced products (hydroxy TL) were less polar so they eluted after the corresponding TL hydroperoxides. Under the initial conditions, TL hydroperoxides were not completely converted into TL hydroxides. In addition, an unknown peak appeared at approximately 3 min and absorbed at λ_{max} = 232 – 234 nm. This may be a product from an undesirable side reaction. The reaction was optimised with the following variables (extra water volume, NaBH$_4$ preparation and reaction time) to maximise hydroxy TL and minimise the unknown compound. As TL-3OOH was the major product of the sfLOX-catalysed TL reaction, TL-3OOH and TL-3OH were reported in the reduction results below to represent TL hydroperoxides and TL hydroxides.

Effect of water volume

Different water volumes (200 – 800 µL) were added before initiating the reaction by adding 200 µL NaBH$_4$ (1 M in water) into 1 mL methanol containing TL hydroperoxides. The major product formation fell dramatically when 200 µL water was added and continued to decline to zero when more water was used (Figure 4.10). The unknown product also decreased significantly to almost zero with increasing water volume. Adding water suppressed the conversion of TL hydroperoxides into TL hydroxide, although the unknown was eliminated.
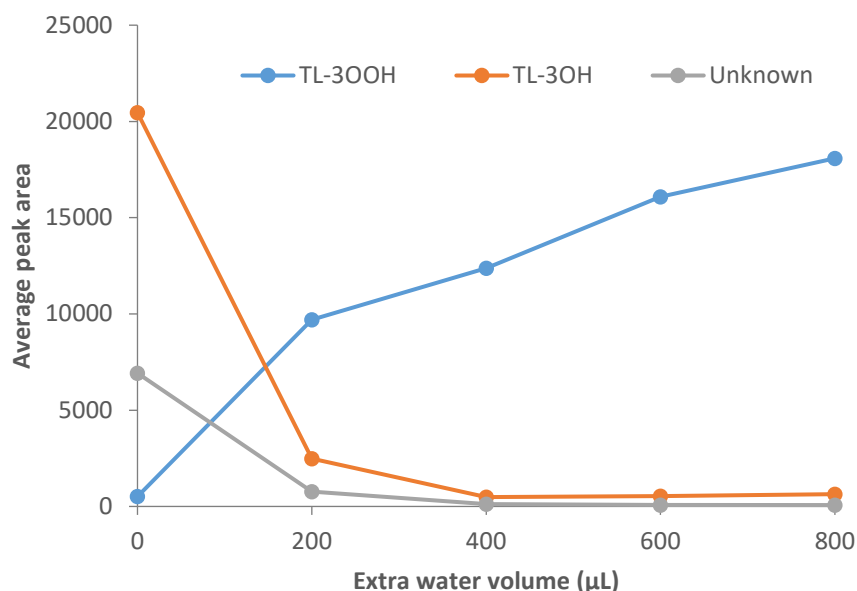
Figure 4.10: Effect of extra water (200 – 800 µL) on reducing sfLOX-catalysed TL reaction products (n-2).

Effect of NaBH$_4$ preparation

NaBH$_4$ volume was optimised in the range below 200 µL, because higher water volumes decreased TL hydroxide formation (see above). A 1 M NaBH$_4$ stock solution was used and 100 µL NaBH$_4$ was the optimum value for TL hydroperoxide conversion into TL hydroxides. TL hydroxides were formed at a high amount; however, the unknown product was also present at a high amount (Figure 4.11).
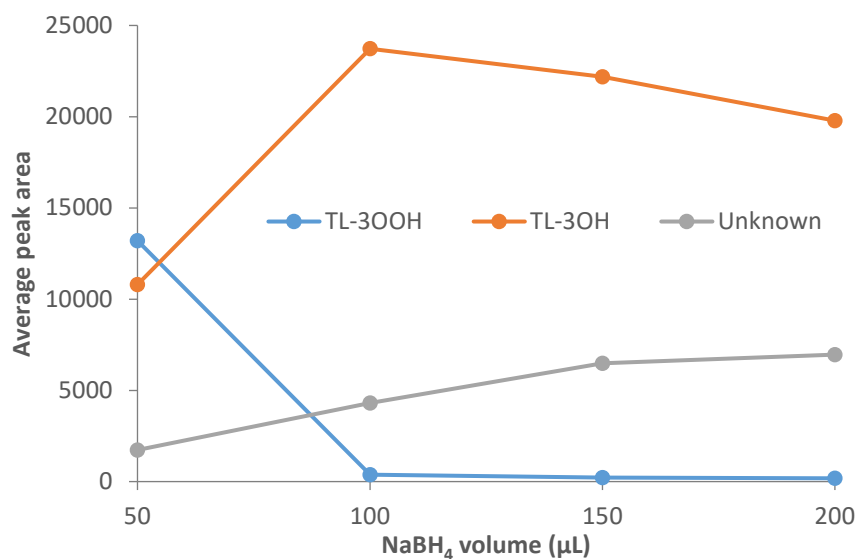
Figure 4.11: Effect of 1 M NaBH$_4$ volume on reducing sfLOX-catalysed TL reaction products (n-2).

The reducing reaction was optimised with different NaBH$_4$ concentrations and volumes corresponding to the same NaBH$_4$ amount in the reaction (~ 8 mg) (Table 4.4). The conversion of TL hydroperoxides into TL hydroxides was maximised with 50 µL NaBH$_4$ 4M. However, the unknown product appeared at all conditions (Figure 4.12).

Table 4.4: NaBH$_4$ concentrations and volumes used to optimise the reduction of sfLOX-catalysed TL reaction products.

NaBH$_4$ concentration (M)	NaBH$_4$ volume (µL)
4	50
2	100
1.5	150
1	200
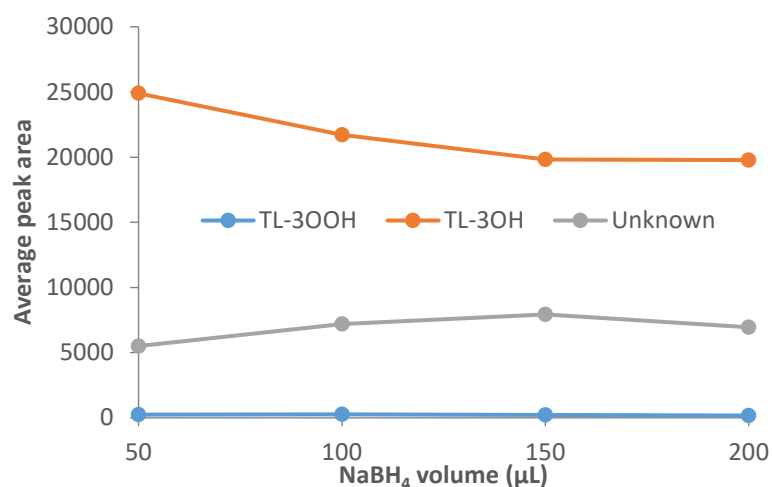
Figure 4.12: Effect of NaBH$_4$ volume and concentration on reducing sfLOX-catalysed TL reaction products (n-2).

Effect of reaction time

The time of the reducing reaction was optimised in the range 1 – 45 min. Surprisingly, the optimum TL hydroxide amount was obtained after 1 min (Figure 4.13), which was opposite to the expected late full conversion. TL hydroxide yield decreased significantly over time. Only a minor trace of unknown product was

detected at 1 min. The unknown product kept increasing over time before being unchanged at 30 min (results not shown). The optimum reaction time for reducing sfLOX-catalysed TL products was identified to be at 1 min.
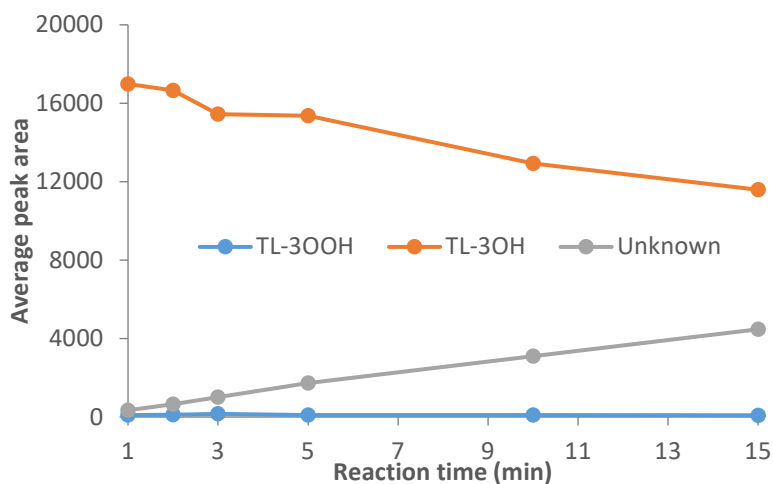
Figure 4.13: Effect of reaction time (1 – 15 min) on the reduction of sfLOX-catalysed TL reaction products (n-2).

Final optimised reduction conditions

The final optimised conditions for the reduction of sfLOX-catalysed TL reaction products were found to be: 1 mL methanol, 200 µL NaBH$_4$ (1 M), 1 min reaction time. Under the final optimised conditions, TL hydroperoxides were fully converted into TL hydroxides with only a minor trace of the previously mentioned side product (Figure 4.14). The reaction under optimised conditions was repeated in triplicate and showed reproducible results (%RSD < 5%).
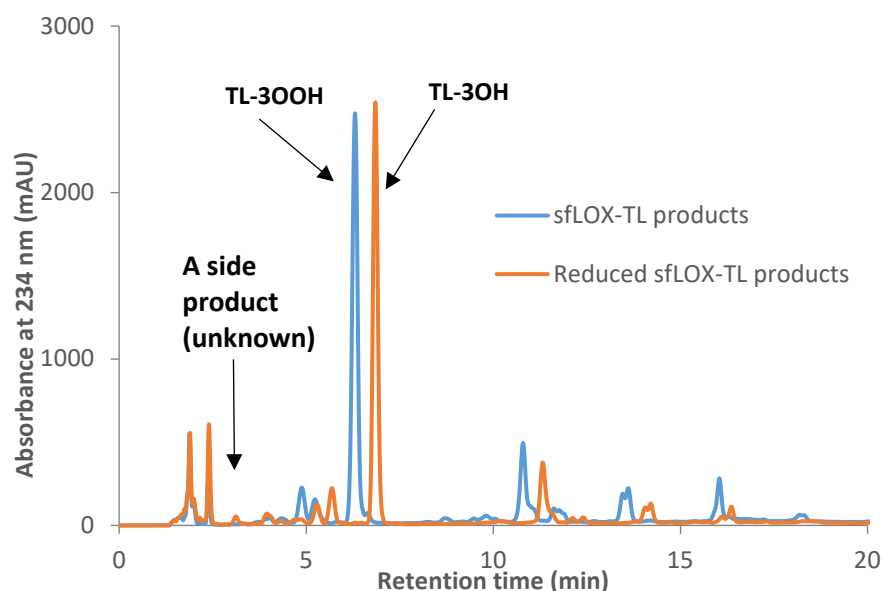
Figure 4.14: RP-HPLC chromatograms of final optimised sfLOX-catalysed TL reaction products: unreduced (TL-3OOH, in blue) and reduced (TL-3OH, in orange).

3.2.2. Hydrolysis of hydroxy TL

Hydrolysis is needed before product characterisation by GC-MS and chiral HPLC as the free fatty acids are required for GC-MS analysis (after further derivatisation) and only free hydroxy fatty acids are available as standards for chiral HPLC. The reduced sfLOX-catalysed TL reaction products at the final optimised conditions were fully hydrolysed into hydroxy linoleic acid (HLA) with reproducible results (three replicates, %RSD < 5%) without detecting any remaining TL or TAGs from SBF. The product was analysed by RP-HPLC and was consistent with HLA produced from the sfLOX-catalysed LA reaction (0.1 mM LA, 50 µL SBF extract, 10 mL borate buffer (50 mM, pH 9), 15 min reaction time). Direct hydrolysis of TL hydroperoxides (without prior reduction into TL hydroxides) was also tested. The peak area of HLA after hydrolysis with or without reducing were quite similar ~ 2% difference (three replicates, %RSD < 5%). Going forward, hydrolysis without prior reduction was chosen to reduce reaction steps required.

3.3. Product characterisation

3.3.1. Determination of hydroxy group positions on reaction products by GC-MS

The final optimised sfLOX-catalysed TL reaction products were completely reduced and hydrolysed into hydroxy linoleic acid (HLA) before fully hydrogenating and trimethylsilylating into derivatives for GC-MS analysis. The hydrogenated and trimethylsilylated (TMS) derivatives are more volatile and thermally stable, and therefore more suitable for GC analysis. The positions of the hydroxy groups on the LA moieties of TL were identified by interpretation of fragmentation patterns of their derivatives. Two fully resolved mono-HLAs (429 *m/z* precursor ion) eluted at approximately 20 min, and the important fragments of these compounds are reported in Table 4.5. Based on the fragmentation patterns, they were identified as fully hydrogenated, TMS derivatives of 13-HLA (Figure 4.15) and 9-HLA. The mono-HLAs were comprised of 90% 13-HLA in percentage of peak area. The results confirmed the presence of 13-HLA as the major product of the reaction.

Table 4.5: Important *m/z* fragments of 9-HLA and 13-HLA from sfLOX-catalysed TL reaction under optimised conditions after reduction, hydrolysis and derivatisation.

Retention time (minutes)	Mono-hydroxy products	Important *m/z* fragments
20.88	9-HLA	73, 229, 317, 413, 429
21.17	13-HLA	73, 173, 373, 413, 429
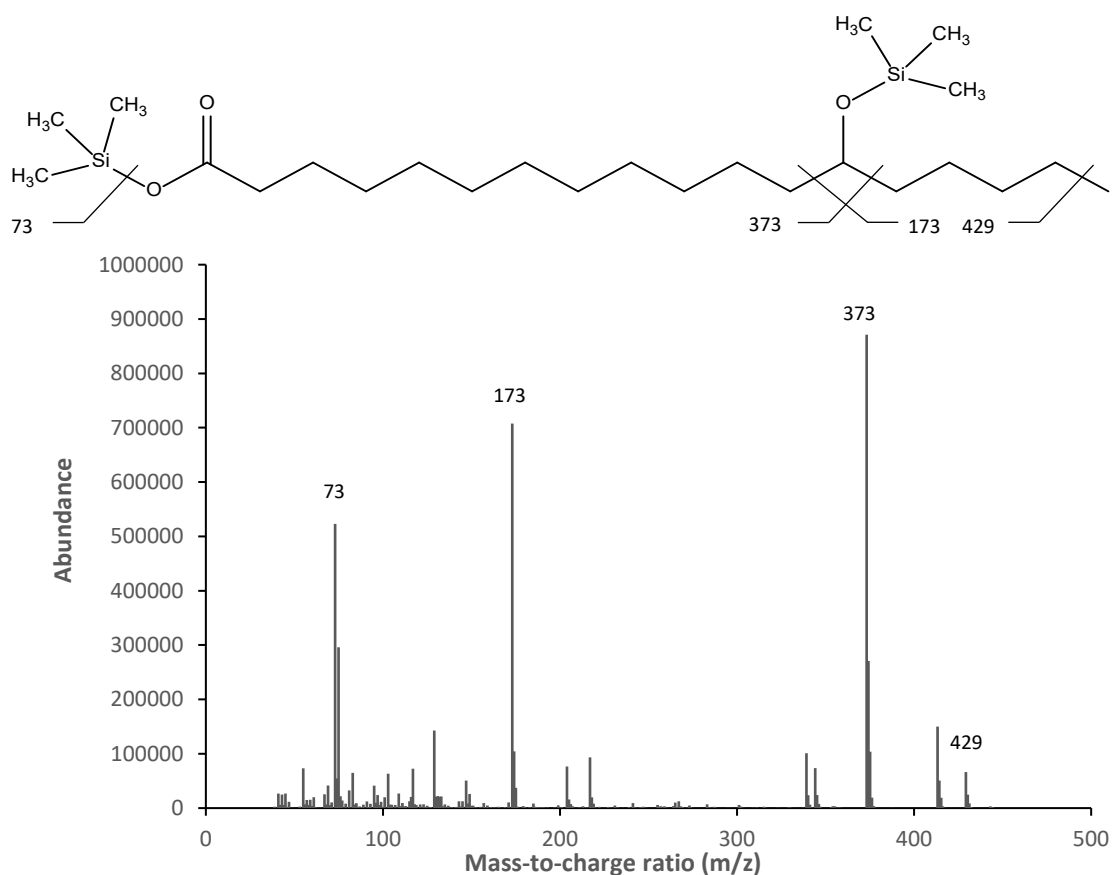
Figure 4.15: Structure showing assignment of important fragments from the mass spectrum of fully hydrogenated di-TMS-13-HLA.

3.3.2. Determination of stereochemistry of reaction products by chiral HPLC

The sfLOX-catalysed TL reaction products at the final optimised conditions were reduced and hydrolysed completely before analysing with chiral HPLC to determine the chirality of the hydroxy group on HLA. The reduced products from the sfLOX-catalysed LA reaction at pH 9 (0.1 mM LA, 50 μL SBF extract), with 13*S*-HLA as the major product, were used as a standard for comparison. HLAs from the sfLOX-catalysed TL reaction were stereo-specifically identified by matching their retention time with HLAs from sfLOX-catalysed LA reaction (Figure 4.16). The results showed that the products were approximately 90% 13-HLA and 10% 9-HLA which matched with the GC-MS results. Approximately 99% of 13-HLA was identified to be 13*S*-HLA and only ~ 1% was 13*R*-HLA (Figure 4.16). The chirality of 9-HLA was not determined.

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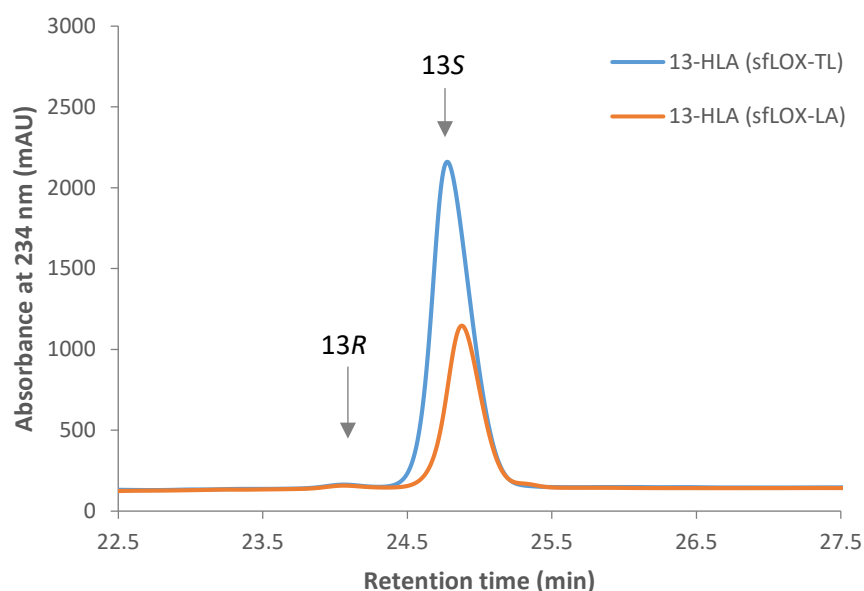
Figure 4.16: Chiral HPLC chromatogram of 13*S*-HLA from sfLOX-catalysed TL reaction (sfLOX-TL) and sfLOX-catalysed LA reaction (sfLOX-LA).

3.3.3. Chemical structure identification of reaction products by NMR spectroscopy

^{1}H NMR

TL-3OOH, purified using preparative HPLC, was characterised and its chemical structure determined using both one and two dimensional NMR spectroscopy experiments (1D ^{1}H-NMR and 2D ^{1}H,^{1}H-COSY NMR). Pure TL was used as a reference for comparison with TL-3OOH. As described below, the major differences were from the formation of hydroperoxy groups and changes in the double bond systems. This resulted in the presence of new peaks and the decrease or disappearance of several peaks. ^{1}H-NMR data for both TL and TL-3OOH are summarised below in Table 4.6 and Table 4.7, respectively.

Table 4.6: ^{1}H-NMR data for TL.

Carbon	[1]H (ppm)	Multiplicity	Integration	J (Hz)
1				
2	2.31	m[a]	6H	7.5/7.7 and 7.4/7.8
3	1.61	m	6H	
4 – 7	1.30	m	24H	
8	2.04	q	6H	6.9
9 - 10	5.28 – 5.42	m	6H	
11	2.76	t	6H	6.6
12 - 13	5.28 – 5.42	m	6H	
14	2.04	q	6H	6.9
15 – 17	1.30	m	18H	
18	0.88	t	9H	6.8/7.1
Glycerol-CH	5.27	m	1H	
Glycerol-CH$_2$	4.15 – 4.29	dd, dd	4H	6.0/11.9 and 4.3/11.9

Table 4.7: ^{1}H-NMR data for TL-3OOH.

Carbon	[1]H (ppm)	Multiplicity	Integration	J (Hz)
1				
2	2.31	m[a]	6H	7.6/7.4 and 7.6
3	1.61	m	6H	
4 – 7	1.30	m	24H[b]	
8	2.18	m	6H	
9	5.46 – 5.51	dt	3H	7.8/10.6
10	5.98 – 6.03	t	3H	11.0
11	6.54 – 6.59	dd	3H	11.2/15.2
12	5.53 – 5.58	dd	3H	8.4/15.2
13	4.35 – 4.39	q	3H	5.4
14	1.61	m	6H	
15	1.41 – 1.50	m	6H	
16 – 17	1.30	m	12H[b]	
18	0.88	t	9H	6.8/7.1
-OOH	8.02	s	3H	
Glycerol-CH	5.27	m	1H	
Glycerol-CH$_2$	4.15 – 4.29	dd, dd	4H	6.0/11.9 and 4.3/11.9

[a] Multiplicity of proton at C2 in both TL and TL-3OOH appeared as offset triplets most likely due to separation of sn-1,3 and sn-2 positional signals. [b] Integration for the signal at 1.30 ppm (protons at C4-7 and C16-17) in TL-3OOH was approximately 49H, which was higher than expected, probably due to difficulty in integrating this large number of hydrogens, although there may also be some overlap with solvent peaks.

The ^{1}H NMR spectrum of TL had signals in the region between 0.5 and 5.5 ppm. In comparison with the spectra of TL, TL-3OOH had a new signal at chemical shift ~ 8.0 ppm due to the hydroperoxy group, -OOH. The olefinic proton signals (C9 –

C12) in TL-3OOH shifted downfield from ~ 5.3 ppm in TL to 5.4 – 7 ppm in TL-3OOH (Figure 4.17). These chemical shift changes are consistent with the presence of both a hydroperoxy group and a new conjugated diene in each linoleate moiety of TL-3OOH. The C13 proton was shifted upfield significantly from ~ 5.3 in TL to 4.4 ppm in TL-3OOH as C13 changed from an olefinic to an allylic proton (Figure 4.17).
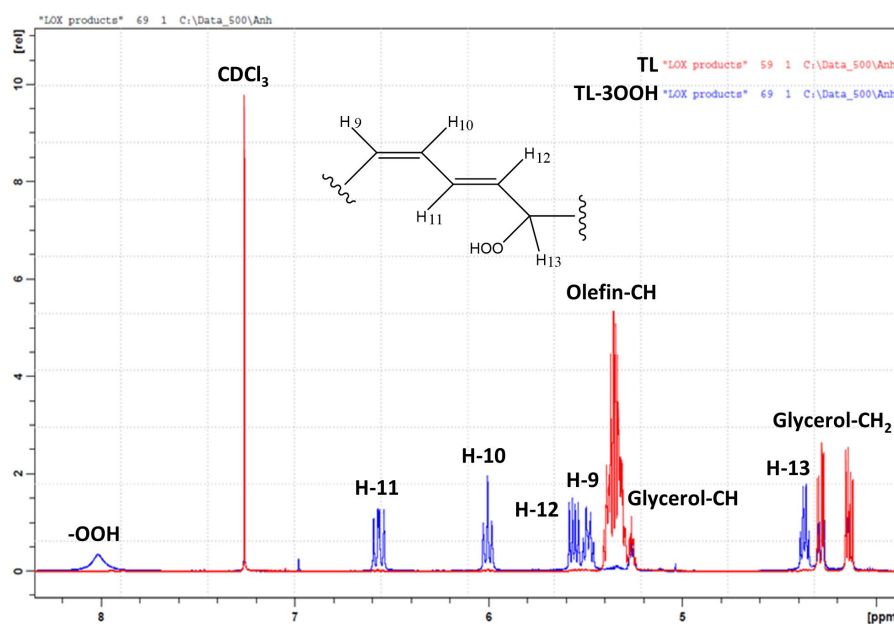
Figure 4.17: Partial 1D ^{1}H-NMR spectra of TL-3OOH (blue trace) and of TL (red trace) overlaid, measured in deuterated chloroform at 500 MHz (10°C) with chemical shift approximately from 4 to 8 ppm. The full spectra are included in the Appendix.

Diallylic methylene protons at C11 (-CH=CH-C**H₂**-CH=CH-) and allylic methylene protons at C8 and 14 (=CH-C**H₂**-) in the ^{1}H NMR spectrum of TL are shifted significantly in the ^{1}H NMR spectrum of TL-3OOH. Diallylic methylene protons at C11 (6H, peak at ~ 2.8 ppm) in TL are present as olefinic protons (3H, -CH=) at ~ 6.6 ppm in the spectrum of TL-3OOH. The three remaining diallylic methylene protons in TL are present as hydroperoxy groups (–OOH) at ~ 8.0 ppm. The chemical shift of allylic methylene protons C8 and C14 (12H, peak at ~ 2 ppm) in TL

was shifted to 2.2 ppm (6H, C8) and 1.6 ppm (6H, C14) in TL-3OOH, respectively. Other changes are also consistent with the new structure (Tables 4.6 and 4.7).

In TL-3OOH, the coupling constant of the C9,10 protons was ~ 11.0 Hz, whereas the C11,12 proton coupling constant was ~ 15.2 Hz. The values of less than 14.0 Hz showed olefinic protons at C9 and C10 were *cis*, whereas C11 and C12 olefinic protons were *trans*. The absence of chemical shift at ~ 6.2 ppm indicated the absence of an *E,E* conjugated diene in TL-3OOH (Table 4.7) [43]. These results indicate that the conjugated double bonds between C9 and C12 have a *Z,E* configuration, which is in agreement with the conjugated double bond system formed by LOX catalysis. The glycerol protons were unchanged in TL-3OOH, as compared to TL (Tables 4.6 and 4.7), indicating that sfLOX-catalysed TL without fatty acid hydrolysis. The structure of TL-3OOH was identified to have a glycerol backbone with three linoleate moieties containing a hydroperoxy group at C13 and conjugated *Z,E* double bonds between C9 and C12 in each moiety. 2D ^{1}H,^{1}H-COSY NMR couplings between neighbouring proton signals were consistent with the assigned structure. These findings confirmed GC-MS results of selective oxygenation at C13. The structure of TL-2OOH and TL-OOH was also elucidated in the same manner with TL-3OOH. Shifting of signals and correlation of neighbouring protons in TL-2OOH and TL-OOH were a combination between TL-3OOH and TL spectra. TL-OOH was confirmed to have a hydroperoxide group and a conjugated diene in one linoleate moiety. Two hydroperoxide groups and two conjugated dienes were determined in two linoleate moieties in TL-2OOH. The NMR spectra of these two compounds are included in the Appendix.

^{13}C-NMR

The ^{13}C-NMR signals for fatty acid carboxylate groups (in the region 170 – 175 ppm) are characteristic for both the type of fatty acid and their positional

distribution at either the sn-1,3 or sn-2 within a TAG compound. The ratio between the signals of carboxylate carbons for a specific fatty acid at the sn-1,3 and sn-2 positions in the ^{13}C-NMR spectrum can be used to quantify the sn-1,3 to sn-2 ratio of the fatty acid. In TL, the sn-2 carboxylate carbon was at ~ 173.0 ppm while the sn-1,3 carboxylate carbons were at ~ 173.5 ppm (Figure 4.18A). As expected, the sn-1,3 to sn-2 ratio was identified to be approximately 2:1 based on integration. In the mixture of sfLOX-catalysed TL reaction products containing TL-OOH, TL-2OOH and TL-3OOH, the results showed that the chemical shifts of carboxylate carbons have moved downfield ~ 0.1 – 0.2 ppm and each carboxylate carbon peak (sn-1,3 and sn-2) is split into 4 peaks, compared to one each in pure TL, illustrating the complexity of the mixture (Figure 4.18B).

Purified TL-3OOH showed a single peak for sn-1,3 and another for sn-2 (sn-1,3 to sn-2 ratio = 2:1), consistent with each of the three fatty acids present having a peroxide present. However, carboxylate groups in TL-OOH and TL-2OOH spectra showed complex patterns with more than two signals, consistent with only some fatty acids being peroxylated, so that fatty acid carbonyl signals appear at different chemical shifts (see Appendix). As a result, for these two compounds the positions of hydroperoxy linoleate moieties on the glycerol backbone were not determined, so the positional preference of the enzyme in forming TL-OOH and TL-2OOH could not be elucidated.
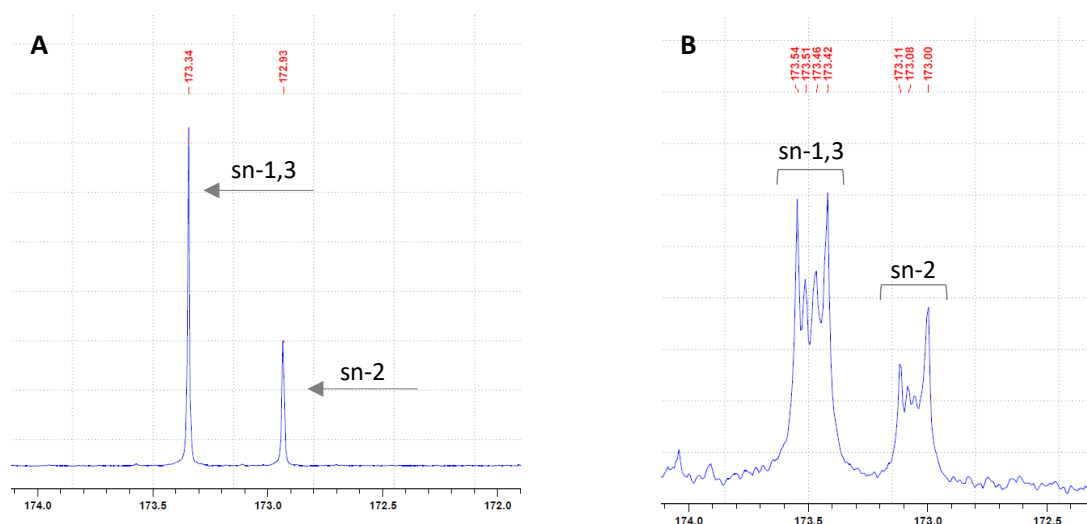
Figure 4.18: Partial ^{13}C-NMR spectra measured in deuterated chloroform at 500 MHz (room temperature) of A) TL and B) mixture of sfLOX-catalysed TL reaction products, between 172 – 174 ppm.

3.4. Calculation of reaction yield using multiple analytical techniques

Concurrent experiments using tuna oil (Chapter 5) demonstrated that the number of sonication pulses used in emulsion formation affected the properties of the emulsion, including stability and droplet size, which in turn affected the lipoxygenase-catalysed reactions. This resulted in emulsions being prepared with 20 sonication pulses (instead of 10 pulses) from this point forward, and in an approximate 30% increase in TL-3OOH and a corresponding 30% decrease in TL-OOH, from the sfLOX-catalysed TL reaction. Further details are described in Chapter 5. Now using the new emulsion preparation, the reaction yield of the final optimised sfLOX-catalysed TL reaction was determined by a range of analytical techniques: RP-HPLC, GC-FID and iodometric titration.

3.4.1. RP-HPLC

TL-3OOH formation

TL-3OOH was purified from the final optimised sfLOX-catalysed TL reaction products to collect approximately 1 mg. Purified TL-3OOH sample (0.98 mg) in

1 mL mobile phase was used to establish a calibration curve using RP-HPLC (Figure 4.21). Due to the limited amount of pure compound, the calibration curve was established by injecting increasing amounts (1 – 10 µL) of the purified TL-3OOH sample (0.98 mg/mL) which was equivalent to TL-3OOH concentrations from ~ 0.2 to 2.0 mg/mL. The amount of TL-3OOH in the optimised reaction was determined to be 1.1 mg/mL from the calibration curve. From this result, at least 47% of the TL was determined to have been converted into TL-3OOH.

$y = 16041x$
$R^2 = 0.9999$
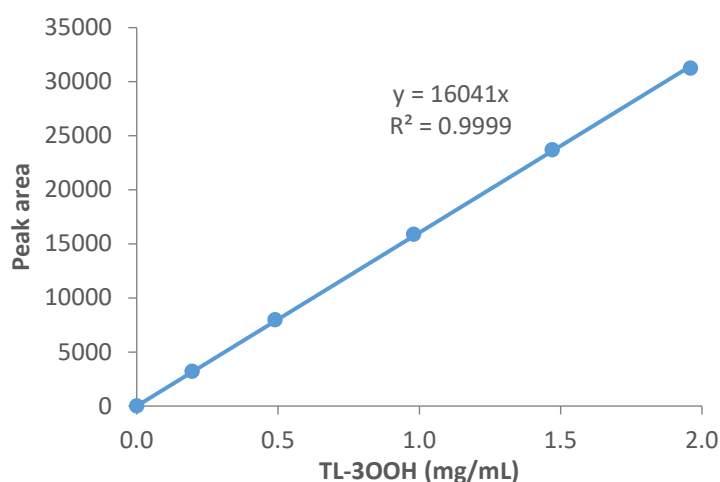
Figure 4.21: Calibration for TL-3OOH using RP-HPLC.

TL residues

A TL calibration curve (Figure 4.22) was established from 0 to 2.0 mg/mL TL. From the calibration curve, the amount of TL remaining at the end of the reaction was determined to be at least 0.3 mg. This is equivalent to approximately 16% of the 2 mg of TL starting material remaining unreacted. TL available in the SBF control was subtracted from samples before calculating remaining TL.
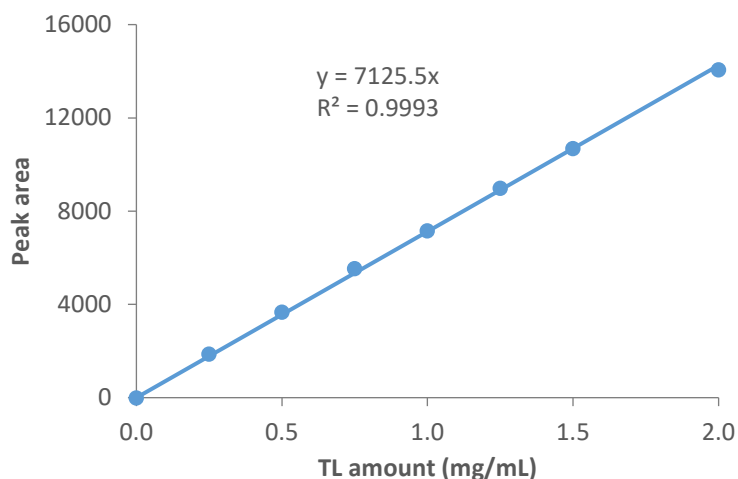
Figure 4.22: Calibration for TL using RP-HPLC.

HLA after hydrolysis

The final optimised sfLOX-catalysed TL reaction products from 200µL TL

(10 mg/mL) for 10 mL reaction (2 mg, 0.23 mM TL as starting material) were

hydrolysed and analysed by RP-HPLC. The HLA peak area was compared to the

peak area of HLA produced from the sfLOX-catalysed LA reaction with

approximately 100% yield under the following conditions: 0.1 mM LA, 50 µL SBF

extract, 10 mL borate buffer pH 9 – 50 mM, 15 min reaction time, giving a final

concentration of 0.1 mM HLA. If TL was converted completely into TL-3OOH,

approximately 0.68 mM HLA would be present after hydrolysis.

The HLA peak area from the sfLOX-catalysed TL reaction products after hydrolysis

was roughly four times higher than the 0.1 mM LA-OH, produced from the sfLOX-

catalysed LA reaction. The formation of approximately 0.40 mM HLA from the

hydrolysed sfLOX-catalysed TL reaction is equivalent to dioxygenation of at least

59% of the available linoleic acid moieties in the TL. HLA formed in the SBF only

reaction (no TL) was not included in the calculation of HLA from the hydrolysed

sfLOX-catalysed TL reaction.

3.4.2. Iodometric titration

An iodometric titration was performed to determine the total number of hydroperoxy groups present after the sfLOX-catalysed TL reaction. Compared to the theoretical maximum yield, where all TL is converted into TL-3OOH, it was determined that at least 85% of the linoleic acid moieties were dioxygenated. This result was higher than the result of HLA identified in the reaction after hydrolysis (~ 60%) but was consistent with unreacted TL in the reaction (~ 15%) determined by RP-HPLC.

The results of 85% linoleic acid moieties in TL being dioxygenated were from using emulsions being prepared with 20 sonicator pulses. Emulsions being prepared with 10 sonicator pulses were tested to compare and only ~ 70% of LA moieties in TL were dioxygenated for these emulsions. This seemed to match with the result of using TL emulsion being prepared with 20 pulses which showed an increase of ~ 30% TL-3OOH and a corresponding decrease of ~ 30% TL-OOH.

3.4.3. GC-FID

The amount of unreacted LA moieties remaining after the reaction were also determined by GC-FID after conversion to methyl esters. Approximately 15% LA ME was identified to remain in the final optimised reaction (after subtracting LA ME in the blank SBF reaction), compared to the TL (2 mg) starting material. Therefore, at least 15% LA moieties in TL remained at the end of the reaction (some TL may have been lost during solvent extraction). This means approximately 85% LA moieties in TL oxygenated by LOX which was matched with iodometric titration results.

4. Discussion

Lipoxygenase-catalysed reaction optimisation

The optimum pH of the sfLOX-catalysed DHA reaction at pH 8 was proposed to be a compromise between the maximum activities of the three sfLOX isozymes in SBF (Chapter 3). This is because all isozymes are able to catalyse free fatty acids. However, the optimised pH 5 in this sfLOX-catalysed TL reaction was significantly lower than the optimum pH of sfLOX-catalysed DHA reaction. This could result from the predominance of type-2 LOX activity as they can oxygenate EFAs more effectively than LOX-1 as shown in previous work from different plant LOXs [56, 92, 184]. In soybean seeds, the optimum pH of type-2 LOX has been found to range from acidic to neutral pH with various EFA substrates [122, 131, 174, 175], suggesting type-2 LOX in SBF is active to EFAs at pH 5. In consistence with the current work, optimum pH between 5 and 7 was reported for the catalysis of SBF or SBM as a LOX source on EFAs such as TL, fatty acid esters and TAGs in a number of previous studies [61, 111, 131, 224, 225]. Moreover for free fatty acids, the optimum pH is high to deprotonate the carboxylic group to obtain the negatively charged substrate required for high LOX-1 activity [49]. sfLOX from SBF was also found to react with hydrolysed natural oils at high pH 8 – 10 [60, 118, 160]. In contrast, TL as an esterified fatty acid cannot be deprotonated, so TL was not oxygenated by sfLOX effectively at high pHs but instead had an optimum pH 5 in this work. Bild et al. showed that type-2 LOX favoured nonpolar or unionised substrates [174].

As observed in the sfLOX-catalysed DHA reaction, product formation from the catalysis of TL by sfLOX initially increased with increasing SBF concentration. At high SBF concentration, there was no decrease in product formation in the reaction with TL as a substrate even though there was a decrease in the sfLOX-catalysed

DHA reaction. TL hydroperoxides may be more stable than DHA hydroperoxides in the reaction mixture with high amounts of SBF. In a study by Frei and co-workers, TL hydroperoxide was found to be more significantly stable than LA hydroperoxide when they were incubated separately in fresh human plasma at 37°C. LA hydroperoxide degraded by almost 90% after 45 min, whereas TL hydroperoxide was still stable up to 120 min [226].

At high TL amounts (> 2 mg), TL-3OOH levels unchanged, whereas TL-2OOH and TL-OOH formation remained increased. This could result from insufficient oxygen in the reaction to convert all TL to TL-3OOH. No anaerobic reaction products were detected at high substrate concentrations or at low oxygen concentrations, as were observed in the sfLOX-catalysed DHA reaction. It was also noted that TL hydroperoxide formation was not affected significantly when oxygen concentration changed. It is likely that oxygen was not limiting in the reaction, so increasing the oxygen concentration (buffer saturated with oxygen) did not increase product formation. However, at low oxygen concentration (buffer saturated with nitrogen), it is unclear why product formation did not decrease. One explanation is that oxygen may be trapped inside the substrate-emulsifier micelles in aqueous solution during sonication and could be released to be used in the reaction to form hydroxylated products. The micelles were possibly only formed in aqueous solution with sonication and could not be formed when TL was prepared in solvent, as the substrate was fully dissolved. However, this potentially trapped oxygen in the micelles was not measured by the dissolved oxygen meter, as the measured oxygen concentration of the emulsion and buffer solutions was not significantly different. No work has investigated the effect of oxygen deprivation on the type-2 LOX-catalysed reaction with emulsified EFAs and no study has been found to support the trapped oxygen proposal.

All sfLOX-catalysed TL reaction products started forming quickly within 1 min, indicating there was a simultaneous conversion of TL into TL-OOH, TL-OOH into TL-2OOH and TL-2OOH into TL-3OOH. The complete conversion of TL into hydroperoxides (45 min) was longer than the DHA conversion into di-hydroxylated DHA (20 – 30 min). This could be because the higher concentration of available linoleate moieties in the TL reaction, requiring ~ 0.7 mM oxygen, was more than the available DHA, requiring 0.4 mM oxygen for maximum conversion, while similar concentrations of enzyme were used. TL-OOH formed quickly and then converted slowly into TL-2OOH and TL-3OOH. The formation of TL-OOH slowed down over time compared to the conversion of TL-OOH into TL-3OOH through TL-2OOH. The rate of TL-2OOH formation was lower over time than its conversion rate into TL-3OOH until TL-3OOH formation reached a maximum. There was also a decrease in products after the reaction reached optimum product formation, consistent with the sfLOX-catalysed DHA reaction.

The dioxygenation of TL by sfLOX was affected by both low and high temperatures and had optimum activity at room temperature. The effect of temperature on the catalysis of sfLOX on TL to produce TL-3OOH showed the same pattern as the sfLOX-catalysed DHA reaction in making 10,17-diHDHA. However, there was no significant effect of temperature on TL-OOH and TL-2OOH product synthesis. The presence of BHT at low concentrations enhanced product formation significantly. It is interesting to note that BHT is an antioxidant, but did not inhibit the dioxygenation catalysed by LOX. Different effects of antioxidants have been reported on LOX-catalysed reactions using various substrates. In one study, tocopherol inhibited peanut LOX activity on TL and LA, whereas BHT showed no effect on these substrates [206]. Mammalian 15-LOX was found to be stimulated by BHT in catalysing the dioxygenation of biomembranes [223]. Especially in soybean seeds,

BHT was previously found to increase LOX-2 + LOX-3 activity, whereas no effect on LOX-1 activity was observed with LA as a substrate [227]. These findings partially support that BHT enhanced type-2 LOX activity on the catalysis of TL in the current work. The enhancement may result from the increase of substrate accessibility to the enzyme, and the suppression of unspecific oxygenation products formed through free radicals [223]. BHT, a lipophilic antioxidant, could largely distribute into lipid phase of oil droplets and disturb surfactant interfacial membrane structure, so the enzyme may gain better access toward the substrate therefore increasing enzyme activity and/or product formation. Moreover, BHT can scavenge free radicals, potentially forming over time, which may otherwise degrade reaction products. However, the mechanism of BHT enhancement of LOX reactions is unknown with futher investigation included in Chapter 5.

Product characterisation

The LA moieties in TL were found to be primarily dioxygenated at the 13C position after the sfLOX-catalysed reaction. 13-HLA is the major product of the LOX-1 catalysed LA reaction. Formation of this product is consistent with the mechanism of substrate insertion into the catalytic pocket, where the methyl end of the EFA enters the active site of the enzyme. This is expected as the large glycerol backbone of acylglycerol substrates is unable to enter the active site [49, 108]. Reaction chirality was highly stereospecific (~ 99%) as expected, due to enzymatic catalysis and had *S* chirality which is well-known for major compounds produced by soybean LOX catalysis. Consistent with the major 13*S* derivative found in this work, two LOX isozymes in soybean leaves were previously found to catalyse the oxidation of TL to form TL-3OOH mainly at C13 with *S* chirality in the linoleate moieties [176].

Reaction yield

The sfLOX-catalysed TL reaction was estimated to have a high yield. From the RP-HPLC results, approximately 85% TL (~ 16% TL remained identified by RP-HPLC using standard plot) was consumed to potentially produce TL-OOH, TL-2OOH and TL-3OOH and approximately 45% TL was converted into TL-3OOH. Iodometric titration indicated that approximately 85% of LA moieties in TL were oxidised, which was consistent with GC-FID results showing approximately 15% of LA moieties remained unreacted. However, only ~ 60% LA-OH was identified after hydrolysis of TL hydroperoxides. Some product yield may have been lost during the extra steps of hydrolysis and solvent extraction compared to other methods. Overall, the reaction yield was identified to be approximately 60 – 85%.

5. Conclusion

This work has demonstrated that SBF is able to catalyse the dioxygenation of TL (as a model EFA substrate). The reaction shows the advantage of proceeding directly without prior hydrolysis of EFA substrates. The enzyme activity on EFAs has been attributed to the presence of stable type-2 LOX in the flour matrix, due to its preference for acting on EFAs but being unstable when purified. The preferred reaction products are TL hydroperoxides fully dioxygenated at all three linoleate moieties while small amounts of mono- and di-hydroperoxy TL are obtained. The reaction yield is high (60 – 85%) in the presence of antioxidant BHT which showed a significant effect on product formation. It is important that the reaction products show highly regio- and stereo-specificity at C13*S* as it can be useful for potential application to the synthesis of nutraceuticals and pharmaceuticals. There is potential to apply this method to other EFA substrates and natural oils and then isolate the modified PUFA-containing TAGs from complex lipid mixtures.

CHAPTER V

SOYBEAN FLOUR LIPOXYGENASE CATALYSIS OF TUNA OIL AS A COMPLEX LIPID MIXTURE

1. Introduction

The fatty acids (FAs) in fish oils, such as those sourced from tuna and anchovy, exist primarily as triacylglycerols (TAGs). The TAGs found in these oils contain a range of FA types, including saturated FAs (SFAs) and unsaturated FAs (both monounsaturated FAs, MUFAs, and polyunsaturated FAs, PUFAs) of varying chain lengths. Approximately 30 – 40 % of all FAs in tuna oil are PUFAs (primarily EPA and DHA), most of which are located at the sn-2 position in TAGs [31, 228]. This distribution of DHA is consistent with other fish oils, whereas the distribution of EPA is species dependent [228]. For example, in a study with natural anchovy/sardine fish oil, 61.7% of DHA was found predominantly at the sn-2 position, while 80.9% of EPA was bound at the sn-1,3 positions [229]. The position of LC-PUFAs in TAGs at either sn-2 or sn-1,3 may affect dioxygenation catalysed by LOX but it is difficult to investigate this due to a lack of available pure substrates.

PUFA distribution in tuna oil TAGs has been reported previously [230-233]. For palmitic acid (P; the major SFA), DHA and EPA (D and E, respectively) – DDP and EEP were found to be the predominant PUFA-containing TAG regioisomers, compared to DPD and EPE. PDP and PEP were slightly more abundant than, or equal to PPD and PPE [232, 233]. The composition of TAG molecular species in a mixture of tuna and sardine oil was estimated to consist of ~ 1% TAGs with three omega-3 long chain PUFAs (n-3 LC-PUFAs; primarily DHA and/or EPA), ~ 20% with two n-3 LC-PUFAs and ~ 35% containing one n-3 LC-PUFA [230]. Similar results in another study reported ~ 4% DDD, ~ 20% di-PUFA TAGs and ~ 30% mono-PUFA TAGs in tuna oil, with DHA and EPA being the major PUFAs [231].

As a result, not all TAG molecules in tuna oil are able to be dioxygenated by LOX as only PUFA-containing TAG molecules are acceptable as substrates in LOX reactions.

n-3 LC-PUFAs are beneficial for human health [27, 234] so it is useful to be able to separate them from the other FAs in natural oils, for subsequent use in pharmaceutical and nutritional applications. The remaining fraction, composed primarily of SFAs and MUFAs, can be used for other applications, such as biodiesel production [235, 236]. However, this separation process can be difficult. Current methods for making n-3 LC-PUFA concentrated fish oils are lengthy and complex. Fish oils rich in n-3 LC-PUFA-containing TAGs are first hydrolysed or ethanolysed (either enzymatically or chemically) to obtain free FAs or FA ethyl esters, respectively [237, 238]. Then the free n-3 LC-PUFAs (or n-3 LC-PUFA ethyl esters) are separated from SFAs and MUFAs by various methods, such as supercritical fluid extraction, freezing crystallisation and urea complexation [234, 238]. Finally, free n-3 LC-PUFAs (or n-3 LC-PUFA ethyl esters) are esterified or transesterified back onto a glycerol backbone to enrich the oil with n-3 LC PUFA, some of which can contain up to 90% DHA and EPA [31, 234, 239]. Alternatively, fish oils can be selectively hydrolysed by lipases with particular FA and/or positional selectivity to obtain n-3 LC-PUFA concentrates [216, 240]. n-3 LC-PUFAs can be concentrated in free FA (FFA), ethyl ester or TAG formats. However, it has been reported that n-3 LC-PUFAs are absorbed most readily in the body as TAGs [241], therefore this form is preferred. Selective separation of n-3 LC-PUFAs containing TAGs from natural oils, by first adding functional hydro(pero)xy groups using LOX could be very useful and much simpler.

Type-2 LOX is known to react with esterified FAs more effectively than LOX-1 [88, 90]. Due to the presence of a stable type-2 LOX in soybean flour, soybean flour LOX (sfLOX) can catalyse the dioxygenation of TAGs. The work in Chapter 4 demonstrated that sfLOX could be used directly to obtain a relatively high reaction yield (approximately 60 – 85%) with pure trilinolein (TL) as a simple model TAG substrate. Therefore, the use of sfLOX provides a promising approach for catalysing the dioxygenation of complex lipid mixtures, such as fish oils, for the synthesis of resolvin analogues directly from EPA and DHA-rich TAGs in these oils. However, this could be challenging compared to TL as a substrate for a number of reasons, such as, varying FA composition, FA chain length and saturation. Therefore, in this Chapter, the sfLOX-catalysed reaction with tuna oil is not only optimised for maximum product formation as was done in Chapter 4, but also the enzyme activity is investigated through monitoring the initial reaction rate under various reaction conditions.

By monitoring the reaction rate it is possible to observe the direct effect of different reaction variables on the catalytic ability of the enzyme, not just overall changes in reaction yield which may be influenced by a number of opposing factors. Using conditions where the catalytic activity of the enzyme is maximised should result in high product yield. One specific variable that is of particular importance in LOX catalysed reactions with esterified substrates is the preparation of the substrate. TAG substrates are commonly prepared as emulsions in the presence of a surfactant or emulsifier for LOX reactions [92, 182]. Correct preparation of the substrate ensures that it is available to the enzyme. Understanding the emulsion properties (the uniformity and droplet size) is of particular importance when employing complex lipid mixture such as tuna oil, as it can have a significant effect on the catalytic activity of the enzyme, reproducibility of the reaction, and overall reaction yield.

Emulsion droplet size has been investigated in a few studies on lipid oxidation and LOX reactions. Non-enzymatic oxidation was found to increase with decreasing droplet size due to the increase of surface area [242], and with decreasing surfactant concentration when having similar droplet size [243]. Activity of a commercial soybean LOX-1 was shown to generally increase with increasing surface area of emulsion droplets for both linoleic acid and methyl linoleate emulsion systems. In this study, droplet surface area was manipulated by changing surfactant concentration and through different emulsification processes such as hand mixing or rotor-stator blending [244].

Only a few studies have used SBF LOX to catalyse reactions with natural oils such as soybean oil, sunflower oil and butterolein but no product characterisation or reaction rate investigations have been reported [56, 61, 111]. Fish oils such as tuna oil are a rich source of EPA and DHA-containing TAGs, and so are good potential LOX substrates for the synthesis of resolvin analogues in TAG form. In this chapter, sfLOX was used to catalyse the reaction with tuna oil to assess the synthesis of potential resolvin analogues in a complex TAG substrate. The initial reaction rate was investigated under various conditions by monitoring the formation of conjugated dienes using a UV-Vis spectrophotometer. Product formation of di- and mono-hydroperoxy TAGs was then measured using RP-HPLC to optimise the reaction yield. The reaction products after hydrolysis were characterised with RP-HPLC and GC-MS to identify regio-specificity of the reaction on n-3 and n-6 LC-PUFA moieties in tuna oil TAGs. This is the first time soybean flour has been used as a crude LOX source for the direct oxidation of TAGs in tuna oil without prior hydrolysis. The expected reaction products would be mainly mono- and di-hydroperoxy TAGs, because ~ 50% of TAGs in tuna oil contain one or two n-3 LC-PUFAs [230, 231]. Tri-hydroperoxy TAGs are likely to be insignificant because only

~ 1 – 4% of TAGs containing three n-3 LC-PUFAs are present in tuna oil [230, 231]. This work has potential applications in the processing of PUFA-rich oils as, after dioxygenation, hydroxylated n-3 LC-PUFA-containing TAGs can be easily separated from the oil. It is also possible that the hydroperoxy-TAGs generated in this reaction may provide a more bioavailable and/or potent form of resolvin analogues compared to those in the FFA form.

2. Materials and methods

2.1. Materials

Soybean flour (Kialla Pure Foods, certified organic) was purchased from Organic Wholefoods (Brunswick East, VIC, Australia). Tuna oil and anchovy oil were obtained from NuMega Ingredients Ltd (Altona North, VIC, Australia). Trilinolein and DHA TAG (Nu-Chek Prep) were obtained from Adelab Scientific (Thebarton, SA, Australia). Acetyl chloride (Fluka), butylated hydroxytoluene (BHT), citric acid, gum arabic, methyl tricosanoate, *N,O*-bis(trimethylsilyl)trifluoroacetamide (with 1% trimethylchlorosilane), potassium phosphate monobasic, potassium phosphate dibasic, platinum (IV) oxide, pyridine (>99%), sodium citrate dihydrate, Trizma® base and (trimethylsilyl)diazomethane (2 M in diethyl ether) were purchased from Sigma Aldrich (Castle Hill, NSW, Australia). Hydrochloric acid (32%) and sodium tetraborate were purchased from APS Chemicals Ltd (Seven Hills, NSW, Australia). Acetone, diethyl ether, ethanol, methanol, potassium bicarbonate and sodium chloride were purchased from Chem-Supply (Gilman, SA, Australia). Acetonitrile (Scharlau, Spain), acetic acid (BDH Lab Supplies, UK) and sodium hydroxide (Fisher Chemicals, UK) were purchased from Science Supply (Mitcham, VIC, Australia). Isopropanol was obtained from Fisher Scientific (Scoresby, VIC, Australia*).* Calcium chloride from Merck (Darmstadt, Germany) was purchased from

Merck Millipore (Bayswater, VIC, Australia). Triton X-100™ and toluene were purchased from Ajax Finechem (Taren Point, NSW, Australia). Hydrogen, nitrogen and oxygen gases were obtained from Coregas Pty Ltd (North Geelong, NSW, Australia).

2.2. Fatty acid profiling by GC-FID

FAs from tuna oil were transesterified into fatty acid methyl esters (FAMEs) and analysed by GC-FID [217]. Methyl tricosanoate internal standard (1 mg) was used for quantification. An Agilent Technologies 6890N Network GC system with flame ionisation detector and an SGE BPX70 column (30 m × 0.25 mm, 0.25 μm film thickness) was used. Samples containing approximately 5 mg of pure tuna oil were used for FAMEs analysis. Details on FAME conversion and GC-FID analysis are described in Chapter 4 (Section 2.5.4).

2.3. UV-visible spectrophotometry for initial rate investigation

To characterise the sfLOX-catalysed tuna oil reaction, UV-Vis spectrophotometry was used to measure the initial rate of the reaction by monitoring the formation of conjugated dienes through absorbance at 234 nm. The substrate (tuna oil) was prepared from a stock tuna oil solution (100 mg/mL), prepared in ethanol:acetone (1:1) and stored at 4ºC until required. To prepare a tuna oil emulsion (10 mg/mL), 100 μL of the tuna oil stock solution was taken and the solvent evaporated under nitrogen flow. The tuna oil was added to 1 mL of filtered 50 mM Tris-HCl buffer, pH 7.5 containing 15% w/v gum arabic, in a 4.5 mL vial. The solution was sonicated using an ultrasonic processor Sonics Vibra cell (John Morris Scientific) (500 W, 20 kHz, amplification 20%) to form the emulsion. Samples were sonicated with five pulses four times and the vial shaken after each repeat to minimise temperature increase. The emulsion was prepared fresh before use. Soybean flour (SBF) extract

was prepared by addition of 1 g SBF in 20 mL of water and stirred for 3 min. This was subsequently centrifuged for 5 min at 4,200 rpm (Allegra X-30 Centrifuge, Beckman Coulter) and the clarified supernatant retained. The supernatant was filtered through a 0.45 µm nylon syringe filter and then diluted in water to obtain the appropriate concentrations required for use in assays.

An appropriate volume of buffer to bring the total volume of the assay reaction to 3 mL was added to a quartz cuvette, with other components (20 µL, as required) added sequentially. The solution was then mixed by inversion of the cuvette. Next, 20 µL of the tuna oil emulsion (10 mg/mL) was added without mixing. Finally, 150 µL of SBF extract was added, the reaction mixture immediately mixed by inversion and the cuvette placed in the spectrophotometer to start the measurement immediately. The effect of different variables on the initial rate of the reaction were investigated, including pH (50 mM citrate buffer pH 4.5 – 6.0, 50 mM phosphate buffer pH 6.0 – 8.0, 50 mM borate buffer pH 8.0 – 9.0), butylated hydroxytoluene (BHT) concentration (1.67 – 13.33 µg/mL), and Triton X-100 concentration (0.0007 – 0.0033% w/v). BHT (0.25 – 2 mg/mL) and Triton X-100 (0.1 – 0.5% w/v) stock solutions were prepared in ethanol and water, respectively.

For tuna oil emulsions prepared with Triton X-100, a tuna oil working emulsion was prepared by mixing 400 µL of the tuna oil emulsion (10 mg/mL) and 56.6 mL of 50 mM citrate buffer pH 5.0 (maintaining the ratio of tuna oil emulsion to buffer used in the 3 mL reactions). The working emulsion was continuously stirred to maintain its stability. An aliquot of SBF extract (150 µL) was transferred into the cuvette inside the UV-Vis spectrophotometer. The tuna oil working emulsion (2.85 mL) was then added and the change in absorbance was recorded immediately. The initial reaction rate was investigated with Triton X-100 prepared in the tuna oil

emulsion to obtain final concentrations of 0.0007 – 0.0033% w/v. The effects of other variables were also tested: oxygenation (nitrogen, none, air and oxygen sparging), temperature (0 – 40ºC) and calcium chloride ($CaCl_2$) concentration (0.017 – 0.067 mM).

The initial reaction rate was measured at 234 nm for 1 min with a Cary 300 Bio UV-visible spectrophotometer (Agilent Technologies) using a 1.0 cm path length quartz cuvette. The first 30 seconds were used to calculate the reaction rate. All sample analyses were conducted in triplicate unless otherwise stated. The values obtained for samples were presented as an average initial rate with error bars indicating one standard deviation. Tuna oil and SBF-only controls were subtracted from the initial rate readings.

2.4. Zetasizer analysis for emulsion properties

The droplet size and size distribution in tuna oil emulsions were measured using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). The refractive indices were set at 1.45 and 1.33 for tuna oil and water, respectively. Samples were appropriately diluted prior to analysis to ensure accurate unobscured measurements were obtained. Data is represented as intensity Z-average, with all samples prepared in duplicate and measured in triplicate. These analyses were performed by Dr. Tim Nalder.

2.5. TAG hydroperoxide enzymatic synthesis and optimisation for maximum product formation

SBF extract was prepared as described in Section 2.3, except that 50 mM citrate buffer pH 5.0 was used in place of water and the SBF in buffer was stirred for 30 min instead of 3 min. Reactions (10 mL) used the optimal conditions determined by the UV-Vis spectroscopy method. The final conditions used were; 50 mM citrate buffer pH 5.0, saturated with oxygen, 0.05 mg/mL SBF extract concentration, 0.067 mg/mL tuna oil (emulsified) and 0.002% w/v Triton X-100. The tuna oil working emulsion

(95 mL) was prepared in a 250 mL Schott bottle by addition of 667 µL tuna oil emulsion (containing 0.3% w/v Triton X-100) and 94.33 mL 50 mM citrate buffer pH 5.0 (maintaining the ratio of tuna oil emulsion and buffer used in the 3 mL reactions), and the solution was stirred for at least 10 min. The solution was then saturated with oxygen by bubbling pure oxygen into the solution for 10 min. An aliquot (9.5 mL) of tuna oil working emulsion was added into a 20 mL vial and stirred, with 0.5 mL of SBF extract added to initiate the reaction. The reaction mixture was stirred for 15 min. Acetic acid (50 µL) was added at the end of the reaction. Reactions shorter than 15 min were stopped by adding the reaction mixture into 20 mL diethyl ether. Reaction products were extracted with 3 × 20 mL diethyl ether and the solvent removed under vacuum. The lipid fraction was then dissolved in 1 mL methanol:isopropanol (1:1) with 0.1% v/v acetic acid for RP-HPLC analysis.

Reaction variables were evaluated for the formation of mono- and di-hydroperoxy TAGs. The variables were reaction time (0 – 90 min), SBF extract concentration (0.0125 – 12.5 mg/mL), BHT concentration (0, 0.0033 or 0.05 mg/mL) and tuna oil emulsion concentration (0.067 – 0.4 mg/mL). Different SBF stocks were prepared or diluted from the original stock (1 g SBF in 20 mL buffer) to obtain appropriate final SBF extract concentrations in the reaction, while maintaining the same SBF extract volume (0.5 mL). Each variable was investigated individually and then the optimal conditions were combined. Blank controls for the tuna oil emulsion and SBF extract were performed with the autoxidation products from the blank controls subtracted from the peak areas determined from the different reactions.

For optimisation of individual reaction variables, experiments were repeated between 1 and 3 times to ensure a clear trend and optimum was observed. Values from a single representative experiment are shown in the results section for each variable.

Results at different reaction conditions for each variable were considered to be different when the results varied by greater than ± 5%. The optimised reaction conditions were conducted in triplicate.

2.6. Hydrolysis of TAG hydroperoxides

The sfLOX-catalysed tuna oil reaction products (TAG hydroperoxides) needed to be hydrolysed prior to analysis by RP-HPLC and GC-MS. The method was performed as described in Chapter 4 (Section 2.4) with some modifications. In a 10 mL vial, samples were dissolved in 1 mL of ethanol and 1.5 mL 25% (w/v) NaOH in water was added. The reaction mixture was stirred at 65°C for 15 or 60 min and water was subsequently added to a final volume of 10 mL (to dilute the sample before acidification, which may cause side reactions). The sample was acidified by the addition of 4 M HCl until the solution turned cloudy and the pH was checked (a pH of approximately 2.0 – 3.0 is required). The hydrolysis products were extracted by diethyl ether (2 × 20 mL), with the solvent subsequently removed by evaporation under vacuum. The final sample was diluted in 1 mL of methanol containing 0.1% (v/v) acetic acid for RP-HPLC or GC-MS analysis. Reactions were performed in triplicate.

2.7. Monitoring reaction product formation by RP-HPLC

The sfLOX-catalysed tuna oil reaction products, including the major mono- and di-hydroperoxy TAGs, were separated using the same HPLC system, column and procedure as described in Chapter 4 (Section 2.5), except that the injection volume was 30 µL. Reproducibility was confirmed by repeat injections. The composition of mobile phase was A: 0.1% (v/v) acetic acid in water and B: methanol with 0.1% (v/v) acetic acid. Hydrolysed reaction products were separated using the following gradient conditions; 50% (v/v) to 80% (v/v) B over 40 min, then to 100% (v/v) B

over 10 min and held at 100% B for another 10 min. The separation used a flow rate of 1 mL/min at 23ºC. The retention times were compared with resolvin analogue standards to identify product regio-specificity.

2.8. Determination of hydroxy group positions on reaction products by GC-MS

The hydrolysed sfLOX-catalysed tuna oil reaction products were fully hydrogenated and trimethylsilylated as described in Chapter 3, Section 2.3.2. Then derivatives were analysed with GC-MS as previously covered in the same section of Chapter 3.

3. Results

3.1. Tuna oil fatty acid profile

The FA profile of tuna oil was determined by GC-FID analysis. This was done to determine the concentration of FAs containing 1,4-pentadiene units (PUFAs), which lipoxygenase is capable of reacting with. Only FAs that contributed > 1% of the total peak area were considered for the calculation of FA composition. The results were calculated from peak area relative to the concentration of internal standard (tricosanoate methyl ester, 1 mg) using theoretical relative FID response correction factors to correct the peak areas of the FAs. Approximately 30% of FAs in the tuna oil were found to be PUFAs, with DHA, EPA, AA, and LA being the four major species (Table 5.1). DHA and EPA accounted for approximately 70 and 20% of PUFAs, respectively, with AA and LA combined forming 10%.

Table 5.1: PUFA content identified in tuna oil by GC-FID (n=3).

PUFA	FA content (%) (Mean ± SD)
LA	1.17 ± 0.02
AA	1.53 ± 0.02
DHA	21.90 ± 0.34
EPA	5.82 ± 0.09

3.2. Effect of emulsion droplet size on LOX product formation

The final optimised sfLOX-catalysed reaction conditions with TL (Chapter 4) were applied to the sfLOX-catalysed tuna oil reaction with some modifications. Preliminary results from RP-HPLC analysis of reaction products showed that tuna oil emulsions (1 mL) prepared with 5 sonication pulses gave a good product yield, however the amount of products formed from these emulsions was inconsistent. To explore this, TL emulsions were prepared to investigate the droplet size and size distribution using a Zetasizer. Using this set up, it was possible to investigate how the number of sonication pulses (5 – 20 pulses in a 1 mL TL stock emulsion) affected both the size of the emulsion droplets and the reproducibility of their preparation. Further to this, the emulsion properties were compared with product formation in sfLOX-catalysed reactions, as determined by RP-HPLC, to investigate what effect emulsion size has on the reaction.

The reproducibility of size distribution increased as the number of sonication pulses increased. All emulsions (prepared with 5, 10, 15 or 20 pulses) had monomodal droplet size distributions, however emulsions with 5 sonicator pulses showed poor reproducibility (Figure 5.1A). Emulsions generated with 5 or 10 pulses had poor polydispersity index (PdI) values (> 0.400), suggesting the emulsions had very broad size ranges, poor homogeneity and may not be suitable for analysis by Zetasizer. PdI is a dimensionless value generally ≤ 1 and is an indicator of the polydispersity of droplet size and the broadness of size distribution. In contrast, the size distribution of emulsions prepared with 20 pulses were well defined (Figure 5.1B), with PdI values < 0.250, suggesting a good level of emulsion homogeneity. The reproducibility of droplet size range also increased as the number of pulses increased from 5 to 20 pulses. The average droplet size also tended to decrease when pulse number increased. The Z-average of the 6 analyses (duplicate samples analysed in triplicate)

was plotted against the number of pulses (Figure 5.1C), clearly showing the improvement in sample preparation reproducibility with respect to the average droplet size.
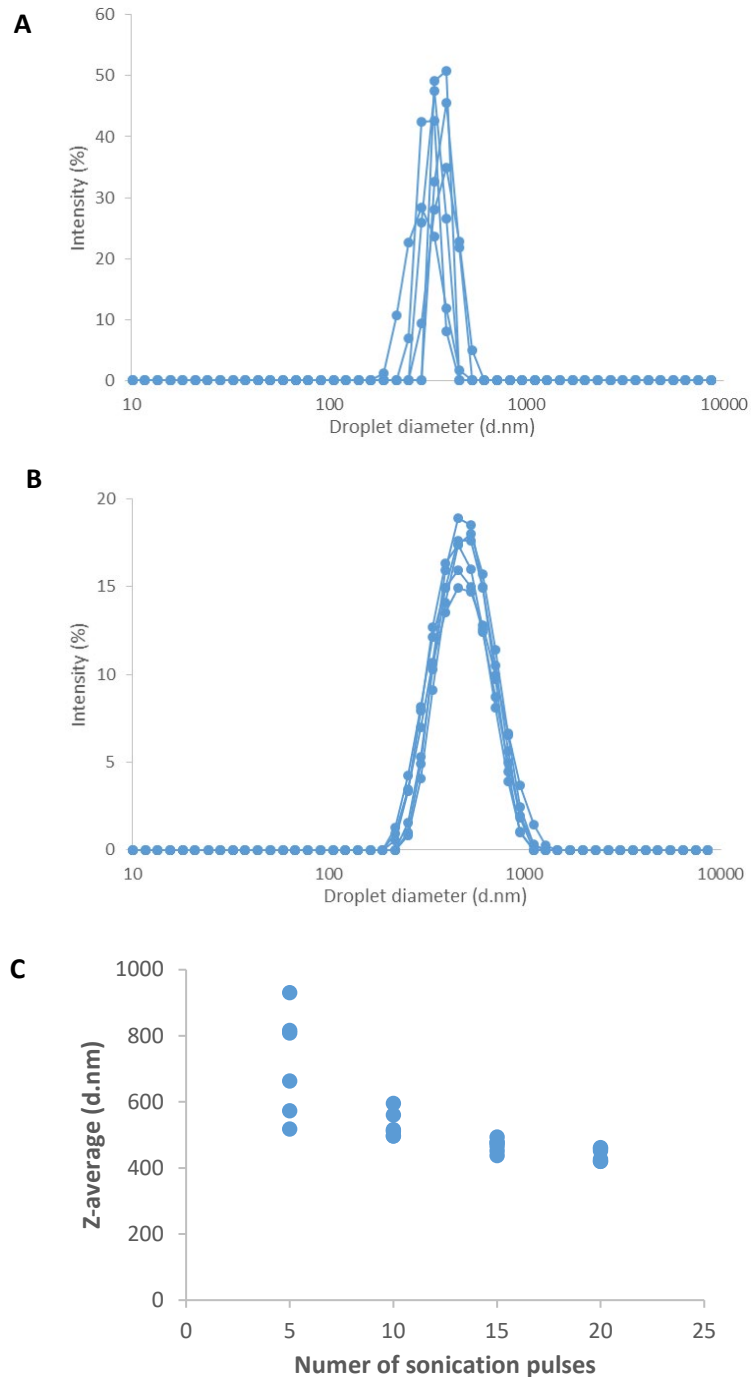
Figure 5.1: Effect of number of sonication pulses on the reproducibility of TL emulsion formation analysed by Zetasizer. A) Size distribution of TL emulsions with 5 sonication pulses, and B) size distribution of TL emulsions with 20 sonication pulses. C) Plots of TL emulsion Z-average values versus number of sonication pulses (5 – 20 pulses). Stock TL emulsions contain 10 mg/mL TL and 15% w/v gum arabic in Tris-HCl buffer pH 7.5. Each experimental condition (pulse number) was analysed in triplicate and repeated (n=6).

The same emulsions, prepared using 5 to 20 pulses, were used as substrates in reactions with sfLOX under the final optimised conditions (TL 0.2 mg/mL, 0.3% w/v gum arabic, SBF extract 2.5 mg/mL, 10 mL 50 mM citrate buffer pH 5.0, 45 min reaction time) and product formation was monitored using RP-HPLC. For TL-3OOH and total product, reproducibility of product formation increased significantly with increasing number of sonication pulses used to form the emulsions. Reproducibility of TL-2OOH and TL-OOH formation was comparable between all conditions, except for TL-OOH where 20 pulses showed better reproducibility. Consistent with the Zetasizer results, emulsions formed with 5 pulses were not uniform, therefore the reproducibility of product formation from these emulsions was poorer. However, emulsions formed with 10 pulses gave reasonable reproducibly in the formation of reaction products from sfLOX-catalysed TL reactions, suggesting the results in Chapter 4 using a TL emulsion prepared with 10 sonication pulses were fit for purpose.

TL-3OOH and total product formation increased, whereas TL-OOH decreased with increasing number of sonication pulses. TL-2OOH formation was constant regardless of the number of pulses (Figure 5.2). TL-3OOH formation from the sfLOX-catalysed reaction using the TL emulsion formed with 20 pulses was approximately 40% higher than when the TL emulsion was formed with 5 pulses. Emulsions formed with 20 pulses produced the greatest amount of total products in all sfLOX-catalysed TL reactions. Therefore, 20 sonication pulses were applied to make tuna oil emulsions, of which the production was found to be reproducible (%RSD < 5%, in triplicate).
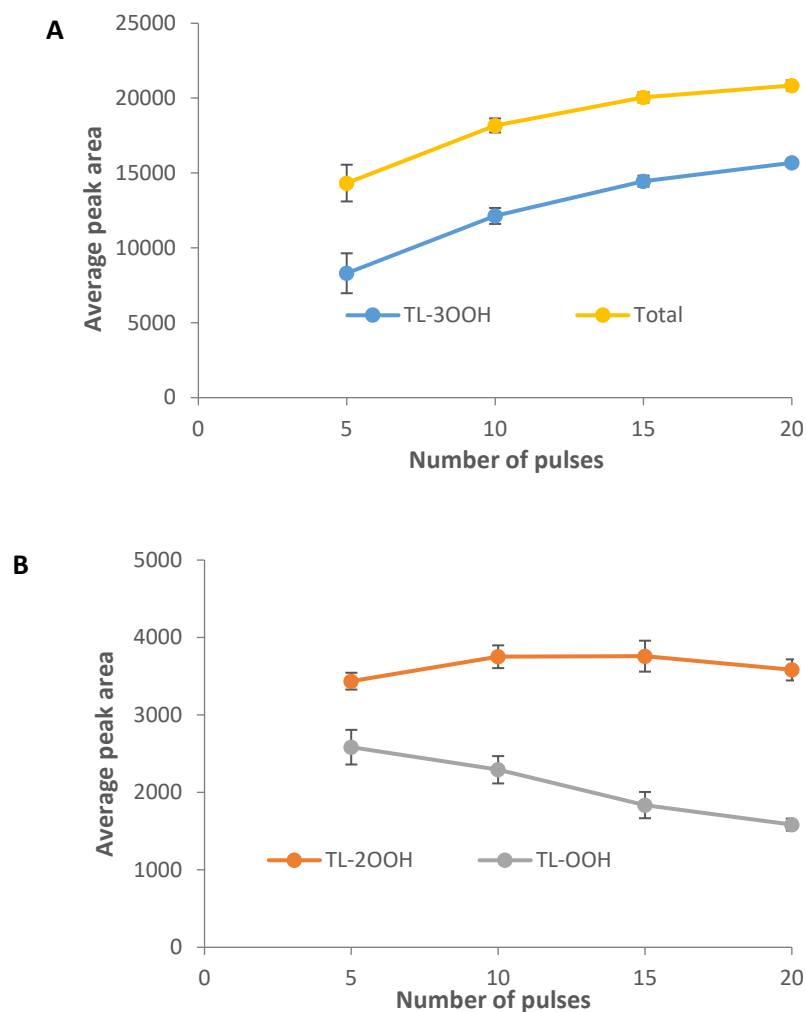
Figure 5.2: Effect of number of sonication pulses (5 – 20) on the formation of products from sfLOX-catalysed TL reactions. Measurement of reaction product absorbance at 234 nm A) TL-3OOH and total product, B) TL-2OOH and TL-OOH. Reaction of TL 0.2 mg/mL, 0.3% w/v gum arabic, SBF extract 2.5 mg/mL, 10 mL citrate buffer (50 mM, pH 5), 45 min reaction time. Error bar stands for ± 1 standard deviation (n=4).

3.3. Investigation of the initial rate of sfLOX-catalysed tuna oil reactions

LOX activity is commonly measured through the formation of LA hydroperoxides containing a single conjugated diene, which can be monitored spectrophotometrically at 234 nm [56, 59, 145]. Preliminary results from RP-HPLC analysis showed that sfLOX-catalysed tuna oil products containing one or more single conjugated double bonds absorbed optimally at 234 nm after subtracting blank controls. The initial reaction rate was investigated under different reaction conditions as described in

Section 2.3, using a UV-Vis spectrophotometer at 234 nm to monitor the formation of all products.

Before proceeding with the investigation, a standard curve was generated using a range of SBF extract concentrations (0.05 – 0.25 mg/mL) to determine the dynamic range of the assay, i.e. to ensure the reaction rate was not limited. The initial rate corresponded to the extract concentration in a linear fashion (Figure 5.3), ensuring the activity measured was relative to the enzyme concentration used in the assays. A concentration of 0.125 mg/mL SBF extract was chosen for use in further assays, as it fell approximately in the middle of the linear range. This assay was used to investigate the effects (positive or negative) of different variables (pH, BHT concentration, Triton X-100 concentration, oxygen, temperature, $CaCl_2$ concentration) on the reaction.

$$y = 1.081x - 0.0305$$
$$R^2 = 0.9989$$
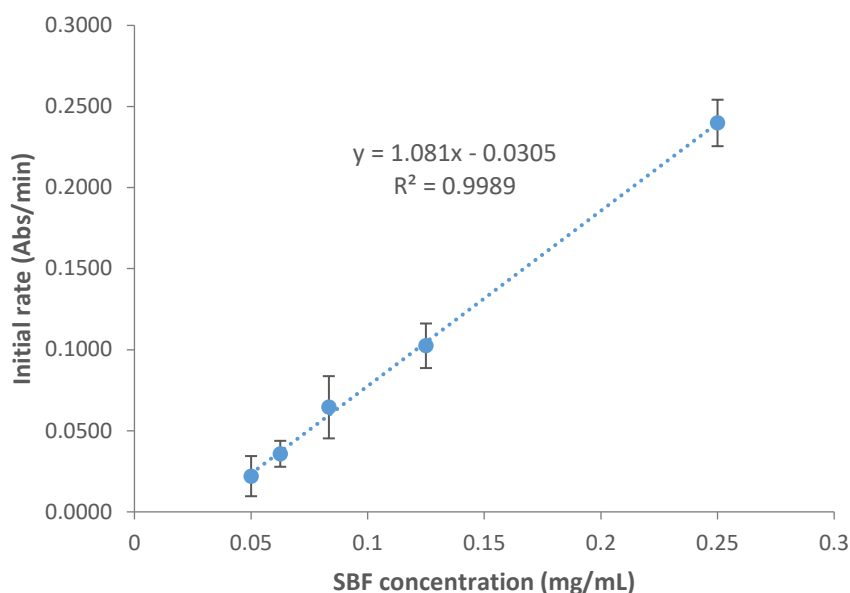
Figure 5.3: Analysis of reaction rate as a function of SBF extract concentration (n=3). Reaction of SBF extract (0.05 – 0.25 mg/mL) with 0.067 mg/mL tuna oil emulsion in 3 mL citrate buffer (50 mM, pH 5.0).

It was also necessary to check the stability of the sfLOX under these conditions over time. This was done by measuring the activity of the SBF extracts every 10 min over

a period of 80 min. The sfLOX (1 in 20 dilution) was found to be relatively stable over 80 min (Figure 5.4) and all analyses measuring the initial reaction rate used the SBF solution within 80 min.
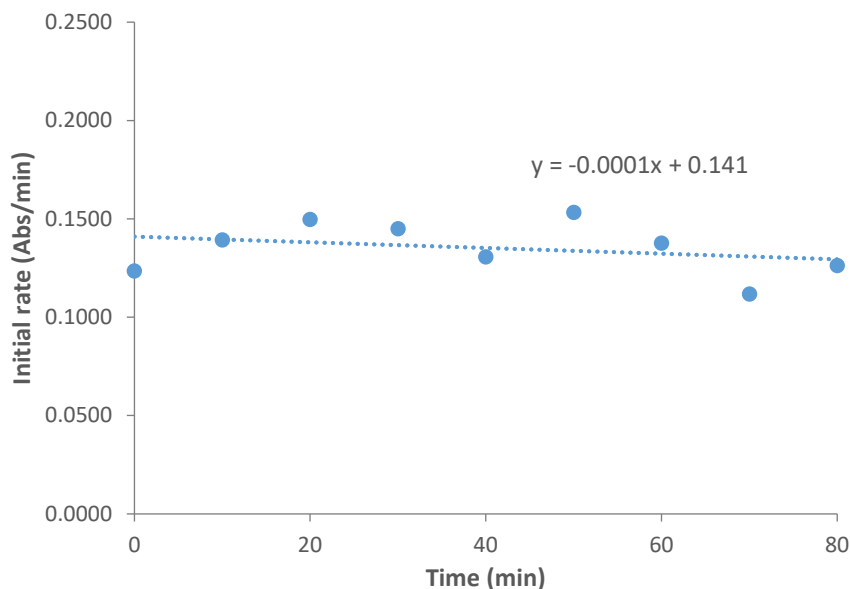
Figure 5.4: Stability of sfLOX activity over time (0 – 80 min). Reaction of SBF extract 0.125 mg/mL with 0.067 mg/mL tuna oil emulsion in 3 mL citrate buffer (50 mM, pH 5.0).

3.3.1. Effect of pH on the initial reaction rate

The pH optimum of the sfLOX catalysis of tuna oil was determined using a number of different buffer systems (see Section 2.3) to cover the pH range 4.5 to 9.0. The assays used 0.067 mg/mL tuna oil emulsion and 0.125 mg/mL SBF extract. The sfLOX was found to be optimally active at pH 5.5, with the reaction slowing significantly at low (pH 4.5) and high pH (pH 9.0) (Figure 5.5). At pH 4.5 and 9.0, the activity was only 40% of that at pH 5.5. For consistency with the previous reaction of a pure TAG (TL), pH 5.0 was chosen for the sfLOX-catalysed tuna oil reaction, as this pH showed strong activity greater than 85% of that at pH 5.5.

Figure 5.5: Effect of pH on the sfLOX-catalysed tuna oil reaction (n=3). Reaction of SBF extract 0.125 mg/mL with 0.067 mg/mL tuna oil emulsion in 3 mL of buffer. pH 4.5 – 6.0: 50 mM citrate buffer (blue); pH 6.0 – 8.0: 50 mM phosphate buffer (red); pH 8.0 – 9.0: 50 mM borate buffer (green).

3.3.2. Effect of BHT concentration on the initial reaction rate

The reaction products generated from the sfLOX-catalysed TL reaction increased significantly in the presence of BHT, as shown in Chapter 4 (Section 3.1.6). Therefore, the effect of BHT was investigated in the sfLOX-catalysed tuna oil reaction to observe if the effect was consistent. The effect was investigated over the range of 1.67 – 13.33 µg/mL; BHT in ethanol (20 µL) was used in the reaction. Control experiments showed that the addition of ethanol (up to 100 µL) did not affect the initial rate (data not shown). The initial rate increased approximately 25% in the presence of 3.33 – 6.67 µg/mL BHT compared to assays without BHT (Figure 5.6). At higher BHT concentrations (> 6.67 µg/mL) the rate decreased significantly.

Figure 5.6: Effect of BHT concentration on the sfLOX-catalysed tuna oil reaction (n=3). Reaction of SBF extract 0.125 mg/mL with 0.067 mg/mL tuna oil emulsion in 3 mL citrate buffer (50 mM, pH 5.0) with different concentrations of BHT (0 – 13.33 µg/mL).

3.3.3. Effect of Triton X-100 concentration on the initial reaction rate

Non-ionic surfactants such as Triton X-100 have previously been used to enhance LOX activity and thermal stability [117, 198]. In the concentration range (0.0007 – 0.0033% w/v of Triton X-100) studied, the initial reaction rate increased 2.3 fold in the presence of 0.002% w/v Triton X-100, compared to reactions preformed without the surfactant (data not shown). Triton X-100 was then used as an emulsifier in the preparation of the tuna oil emulsion. The addition of Triton X-100 decreases the emulsion droplet size and could affect the enzyme activity. In the same concentration range as above, the initial reaction rate was shown to increase significantly (greater than 6-fold) with increasing Triton X-100 concentration. A similar effect on the initial reaction rate was observed with Triton X-100 prepared in tuna oil emulsion as was observed when Triton X-100 was used as an additive in the reaction, although a wider optimum range was obtained (0.0013 – 0.0027% w/v; Figure 5.7). A concentration of 0.002% w/v Triton X-100 (prepared in the tuna oil emulsion) was chosen for further investigations.
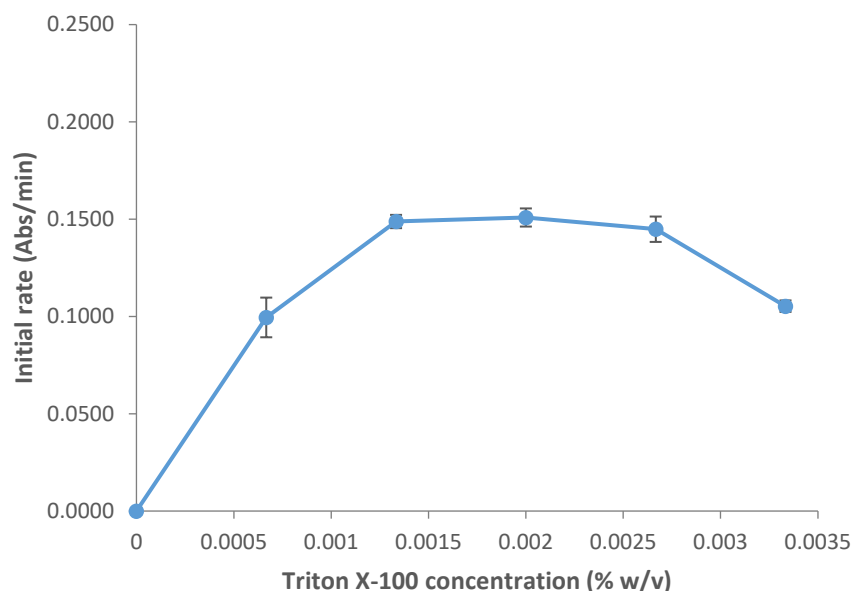
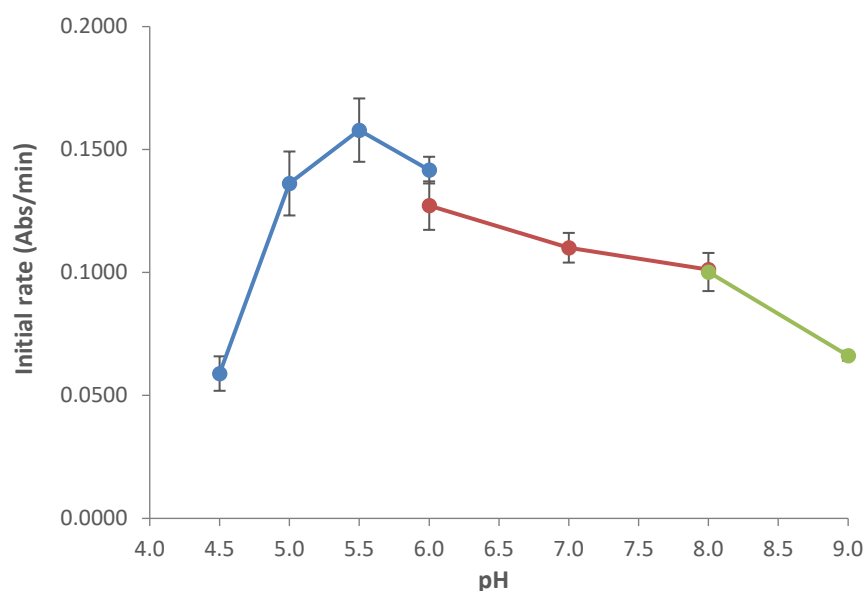**Figure 5.7:** Effect of Triton X-100 concentration on the sfLOX-catalysed tuna oil reaction (n=3). Reaction of SBF extract 0.05 mg/mL with 0.067 mg/mL tuna oil emulsion and Triton X-100 (0.0007 – 0.0033% w/v) in 3 mL citrate buffer (50 mM, pH 5).

3.3.4. Effect of oxygen on the initial reaction rate

Oxygen is essential for a LOX-catalysed reaction to proceed. To investigate the effect of oxygen concentration on the sfLOX-catalysed tuna oil reaction, the reaction was performed with different concentrations of oxygen present in the solution. Tuna oil working emulsions were sparged with pure oxygen, air or nitrogen for 10 min, or left untreated before use, to alter the oxygen concentration from close to zero to a maximum of approximately 35 mg/L. The reaction rate increased significantly with increasing oxygen concentration, with the reaction rate increasing by approximately 35% under oxygen-sparged conditions compared to the untreated reaction. Under nitrogen-sparged conditions, the reaction rate was observed to be approximately 80% of the rate at the untreated condition (Figure 5.8).
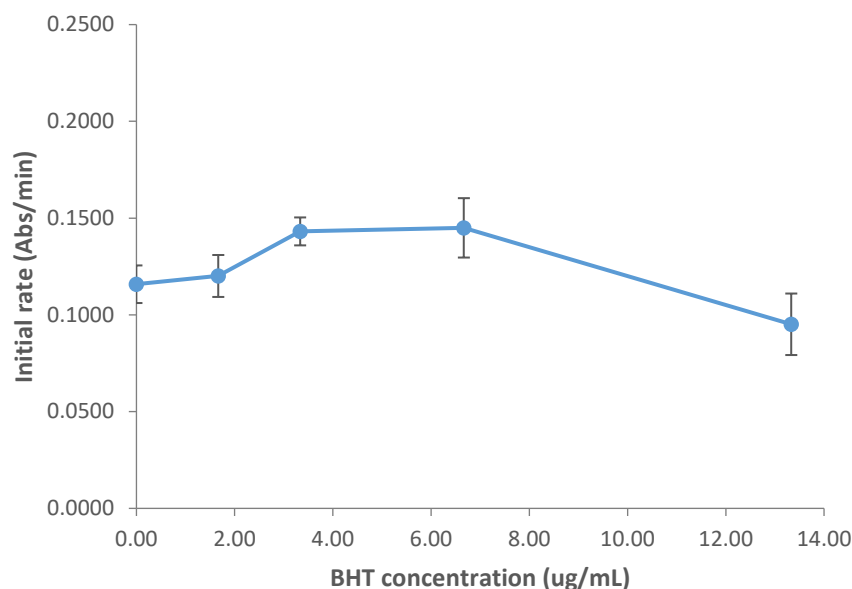
182
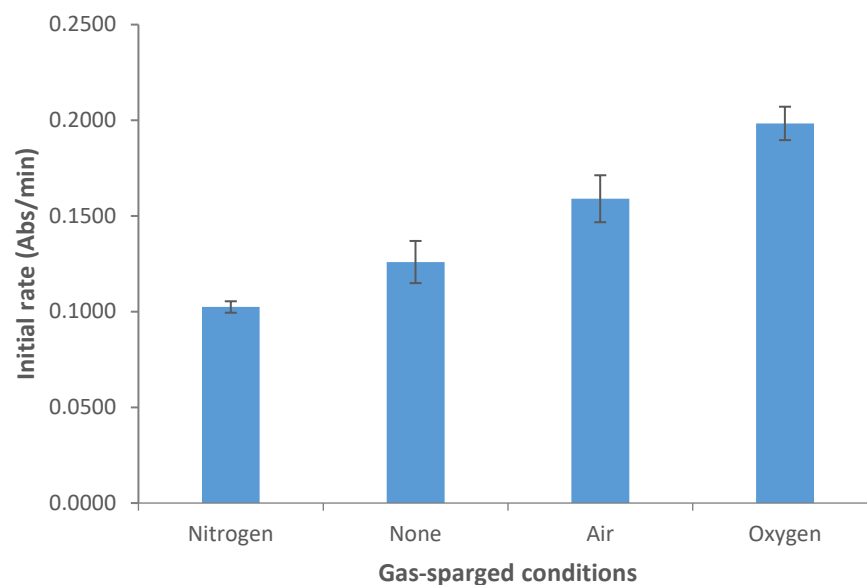
Figure 5.8: Effect of oxygen concentration on the sfLOX-catalysed tuna oil reaction (n=3). Reaction of SBF extract 0.125 mg/mL with 0.067 mg/mL tuna oil emulsion in the presence of 0.002% w/v Triton X-100 in 3 mL citrate buffer (50 mM, pH 5).

3.3.5. Effect of temperature on the initial reaction rate

Temperature is an important variable and can affect the initial rate significantly as it can alter the enzyme stability and oxygen concentration. The reaction was investigated with respect to temperature in the range 0 – 40°C. Tuna oil–buffer mixtures were incubated at the different temperatures and the initial rate of the sfLOX reaction determined: on ice (0ºC), ice water bath (10ºC), room temperature (~ 20ºC) or on a heating block at 30 and 40°C. The initial reaction rate was fastest between 10 and 30ºC, with an increase of approximately 20 – 25% compared to 0ºC and 40ºC (Figure 5.9). For convenience room temperature was chosen to perform further experiments.
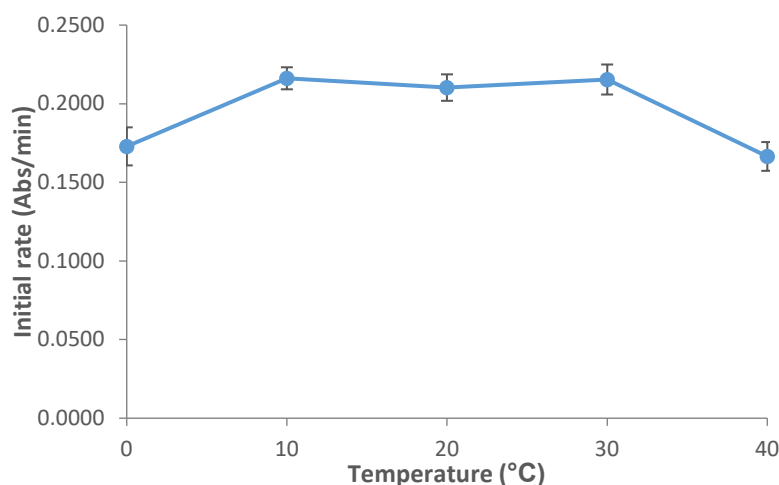
Figure 5.9: Effect of temperature (0 – 40°C) on sfLOX-catalysed tuna oil reaction (n=3). Reaction of SBF extract 0.125 mg/mL with 0.067 mg/mL tuna oil emulsion in the presence of 0.002% w/v Triton X-100 in 3 mL citrate buffer (50 mM, pH 5).

3.3.6. Effect of calcium chloride on the initial reaction rate

Calcium ions have previously been found to remove substrate inhibition and activate enzyme activity in some plant LOX isozymes [186, 245]. The presence of calcium chloride (0.017 – 0.067 mM) in the reaction was investigated. Calcium chloride did not have a significant effect on the initial reaction rate (Figure 5.10), and was not studied further.
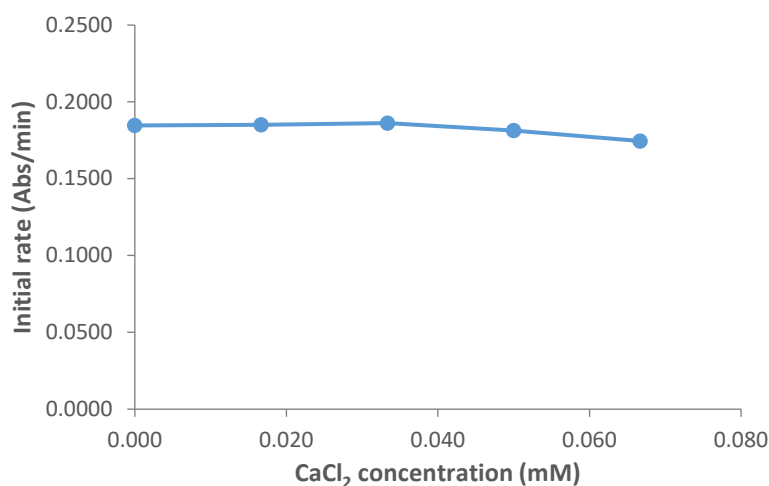
Figure 5.10: Effect of calcium chloride on the sfLOX-catalysed tuna oil reaction. Conditions: 0.067 mg/mL tuna oil emulsion, 0.05 mg/mL SBF extract, 0.002% w/v Triton X-100, 3 mL citrate buffer (pH 5, 50 mM). An aliquot (20 µL) of $CaCl_2$ (0.017 – 0.067 mM) was added before initiating the reaction by adding to the tuna oil–buffer mixture. Experiments were conducted in duplicate.

3.3.7. Effect of variable combination on the initial reaction rate

The combination of variables (Triton X-100, oxygen saturation and BHT) were tested to investigate any combined effects on the reaction. The following working tuna oil emulsions were used as substrates for comparison: 1) without Triton X-100, 2) with Triton X-100, 3) with Triton X-100 and BHT, 4) with Triton X-100 and oxygen and 5) with Triton X-100 and BHT and oxygen. The initial rate for condition 1 was close to zero, the addition of Triton X-100 (condition 2) as an emulsifier in the tuna oil emulsion substantially increased the initial rate to 0.126 Abs/min. Oxygen saturation in combination with Triton X-100 (condition 4) was found to increase the initial rate by ~ 40% compared to the reactions without oxygen sparging (conditions 2 and 3, Figure 5.11). Under the reaction conditions with saturation of oxygen, the results showed that BHT decreased the initial rate ~ 10% compared to the control without BHT (conditions 4 and 5, Figure 5.11). Oxygen saturation did not increase the initial rate in the presence of both BHT and Triton X-100 (conditions 3 and 5, Figure 5.11). The reaction conditions in the presence of Triton X-100 and oxygen saturation showed the fastest initial reaction rate and were applied to scaled-up 10 mL reactions for further optimisation using RP-HPLC to maximise reaction products (hydroperoxy TAGs).
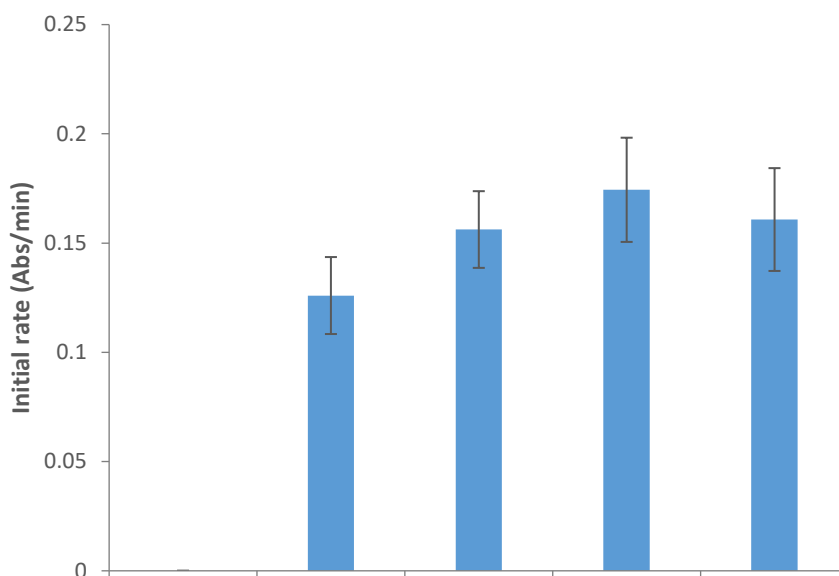
Condition	1	2	3	4	5
Triton X-100 (0.002% w/v)	✗	✓	✓	✓	✓
BHT (0.0033 mg/mL)	✗	✗	✓	✗	✓
O_2 (~ 35 mg/L)	✗	✗	✗	✓	✓

Figure 5.11: Effect of combined variables on the sfLOX-catalysed tuna oil reaction (n=3). The reactions were conducted under the conditions: 0.067 mg/mL tuna oil emulsion, with or without 0.002% w/v Triton X-100, 0.05 mg/mL SBF extract, without or with BHT (0.0033 mg/mL), with or without oxygen saturation, 3 mL citrate buffer (50 mM, pH 5).

3.4. Effect of Triton X-100 concentration on emulsion properties

The stock tuna oil emulsions (15% w/v gum arabic and 10 mg/mL tuna oil) containing different concentrations of Triton X-100 (0, 0.1, 0.3 and 0.5% w/v) were analysed by Zetasizer to investigate the effect of Triton X-100 concentration on the emulsion droplet size and size distribution. At all concentrations of Triton X-100 tested, the emulsions were found to be mono-modal in their size distribution. Polydispersity index (PdI) values obtained were < 0.4 (Table 5.2) suggesting that the data were of reasonable quality and that the homogeneity of the emulsions was suitable for analysis by Zetasizer.

Table 5.2: Values determined by Zetasizer analysis of tuna oil emulsions prepared with different concentrations of Triton X-100, at 30°C (n=6).

Triton X-100 % (w/v) in TO emulsion	Z-average (d.nm) (Mean ± SD)	PdI (Mean ± SD)
0.0	388.4 ± 20.0	0.187 ± 0.021
0.1	276.9 ± 5.3	0.207 ± 0.012
0.3	205.1 ± 4.2	0.176 ± 0.008
0.5	174.4 ± 2.6	0.180 ± 0.019

Figure 5.12A shows examples of the size distribution curves determined for emulsions with different Triton X-100 concentrations, while Figure 5.12B shows the Z-average values obtained for emulsions prepared with the different concentrations of Triton X-100. As shown in Table 5.2 and Figure 5.12B, the Z-average of droplet diameter decreased significantly from 388.4 ± 20.0 nm to 174.4 ± 2.6 nm as the Triton X-100 concentration increased from 0 to 0.5% w/v. Tuna oil emulsions with higher concentrations of Triton X-100 (> 0.5% w/v) were not suitable for analysis by Zetasizer as they presented as bi-modal size distributions and the PdI values were > 0.4 (data not shown). The appearance of a second micelle population is likely to have occurred due to Triton X-100 forming micelles predominantly composed of the surfactant itself. For this reason, emulsions containing high concentrations of Triton X-100 were not used for testing in LOX reactions.
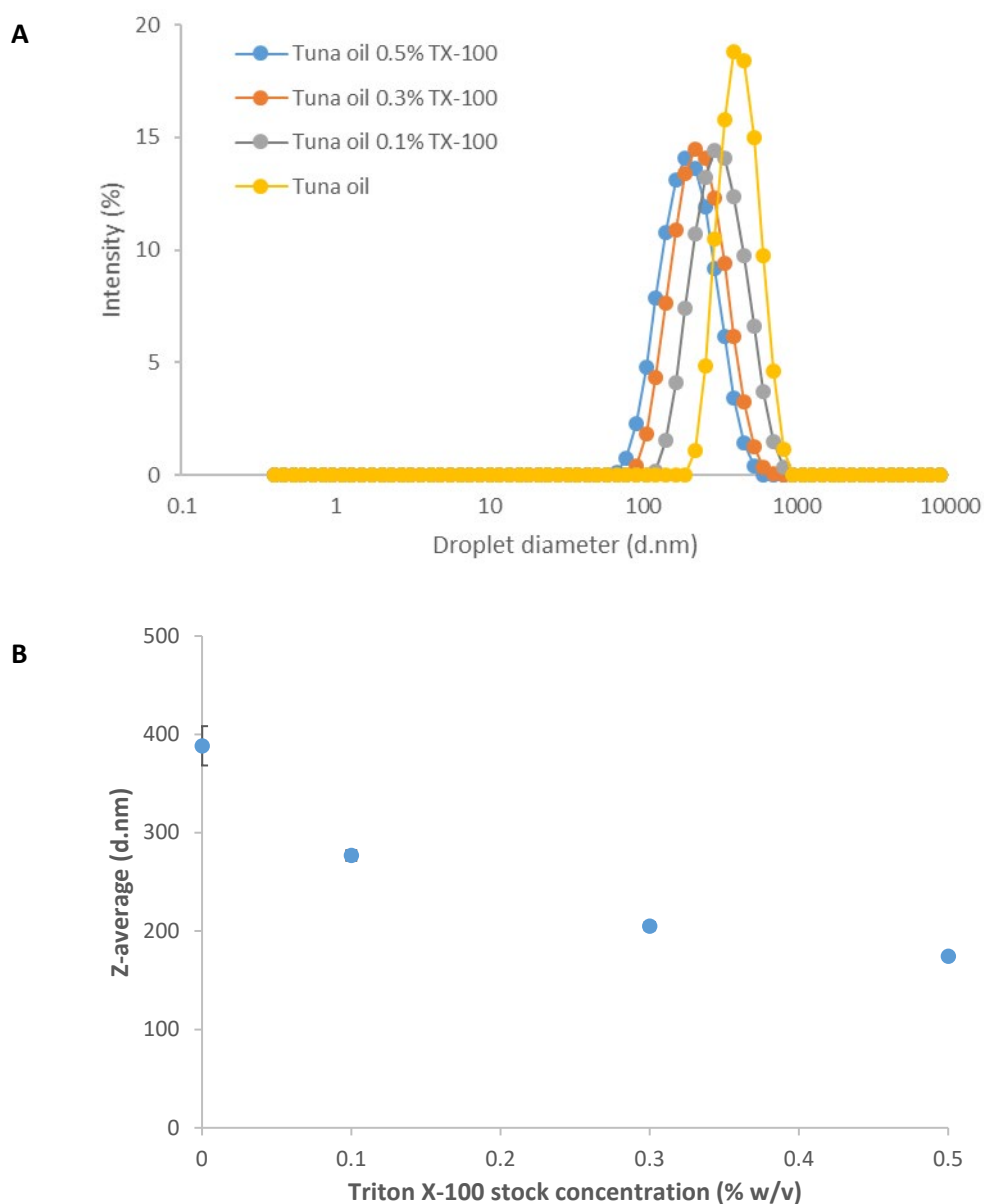
Figure 5.12: Effect of Triton X-100 concentration on tuna oil emulsion droplet size and size distribution. A) Size distribution of tuna oil emulsions prepared with 0.0 – 0.5% w/v Triton X-100 in the stock solution. B) Z-average values obtained for tuna oil emulsions prepared with 0.0 – 0.5% w/v Triton X-100 in the stock solution (n = 6).

3.5. Optimisation of sfLOX-catalysed tuna oil reaction conditions by RP-HPLC DAD

The conditions identified by UV-Vis spectroscopy (0.067 mg/mL tuna oil emulsion, 0.05 mg/mL SBF extract, 0.002% w/v Triton X-100, oxygen saturated buffer) to improve the reaction rate of product formation were applied to scaled-up 10 mL reactions and the products analysed by RP-HPLC. Reaction products were detected

by absorption at 234 nm and the substrate monitored at 210 nm. Because of the very low amount of tri-hydroperoxy TAGs produced, the formation of di- and mono-hydroperoxy TAGs were the focus of this investigation. Di- and mono-hydroperoxy TAGs were separated by RP-HPLC due to differences in their polarity. Di-hydroperoxy TAGs (TAG-2OOH) eluted earlier than mono-hydroperoxy TAGs (TAG-OOH) as they are more polar with two hydroperoxy groups (Figure 5.13).
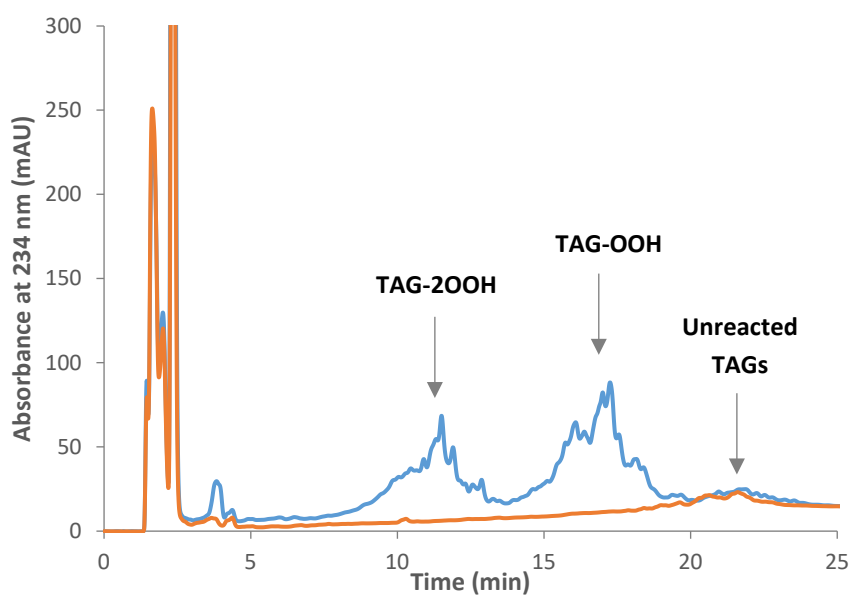
Figure 5.13: RP-HPLC chromatograms of sfLOX-catalysed tuna oil reaction products compared to blank tuna oil control. Reaction of 0.067 mg/mL tuna oil emulsion, 0.002% w/v Triton X-100 and 0.25 mg/mL SBF extract, in 10 mL citrate buffer (50 mM, pH 5.0) saturated with oxygen, for 15 min (blue trace). Blank tuna oil control under the same above conditions without SBF (orange trace).

3.5.1. Effect of reaction time on product formation

To allow for the formation of the maximum amount of reaction products, reaction time was investigated from 1 to 90 min. A 100 mL reaction was initiated, then at different time points 10 mL aliquots were taken and added directly into 20 mL diethyl ether with 50 μL acetic acid to stop the reaction. The lipid fraction was extracted and the products analysed by RP-HPLC. TAG-OOH increased significantly over the first 15 min, then remained unchanged up to 90 min (Figure 5.14). TAG-

2OOH concentration was minimal at 1 min and did not increase over time (results not shown). Unreacted fish oil TAGs appeared to decrease with a small change over time (Figure 5.14). Under these conditions, the reaction had reached completion after 15 min, although a large amount of substrate still remained. A 15 min reaction time was chosen for further experiments.
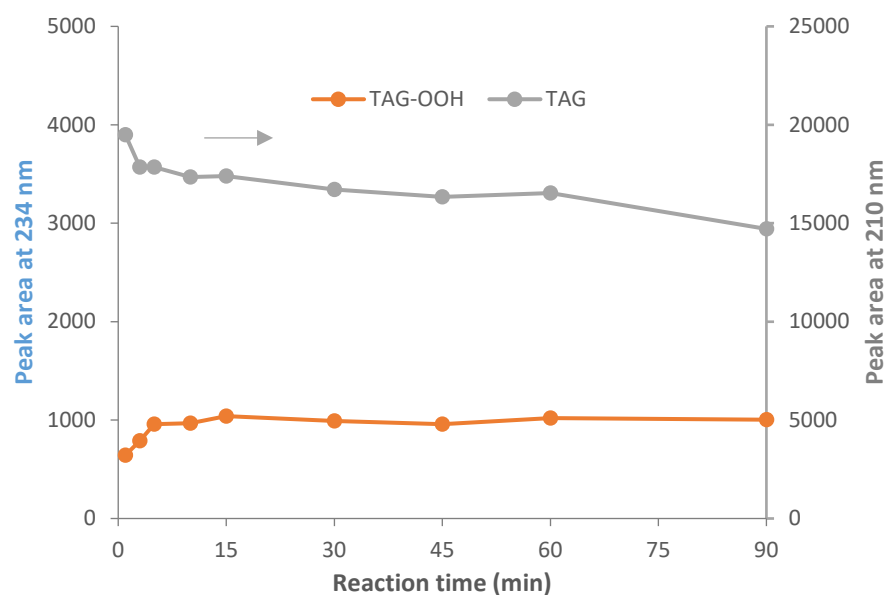
Figure 5.14: Effect of reaction time on the formation of sfLOX-catalysed tuna oil reaction products and unreacted TAGs over time. Reactions contained 0.067 mg/mL tuna oil emulsion, 0.002% w/v Triton X-100, 0.05 mg/mL SBF extract, in 10 mL citrate buffer (50 mM, pH 5.0), saturated with oxygen. TAG-OOH measured at 234 nm (left axis), TAG measured at 210 nm (right axis). The results from a single experiment represent reaction time effect on product formation.

3.5.2. Effect of SBF concentration on product formation

The optimal enzyme concentration for the reaction needed to be determined, as at low concentrations the conversion is incomplete, and at high concentrations product formation may be inhibited. SBF extract concentration was optimised in the range 0.0125 – 2.5 mg/mL, with the production of TAG-OOH and TAG-2OOH monitored. The formation of TAG-OOH increased substantially with increasing SBF extract from 0.0125 to 0.125 mg/mL, reaching a maximum between 0.05 and 0.125 mg/mL, before decreasing significantly from 0.125 to 0.5 mg/mL. Beyond 0.5 mg/mL the

concentration of TAG-OOH remained relatively constant (Figure 5.15). At SBF

concentrations < 0.25 mg/mL, TAG-2OOH formation was minimal. From 0.25 to

1.25 mg/mL SBF extract, TAG-2OOH increased significantly, then stayed constant

up to 2.5 mg/mL. The concentration of TAG decreased dramatically up to

1.25 mg/mL SBF extract, then decreased slightly until 2.5 mg/mL (Figure 5.15). The

amount of TAG remaining at the end of the reaction using 2.5 mg/mL SBF extract

was approximately 5% of that remaining when 0.0125 mg/mL SBF extract was used.

The reaction has gone to completion as the substrate was limiting from 1.25 mg/mL

SBF extract onward. The peak area of substrate consumed did not account for the

peak area of products formed in the reaction. However, there were no other products

formed to compensate the significant decrease of TAG and small amount of TAG-

OOH and TAG-2OOH formation.
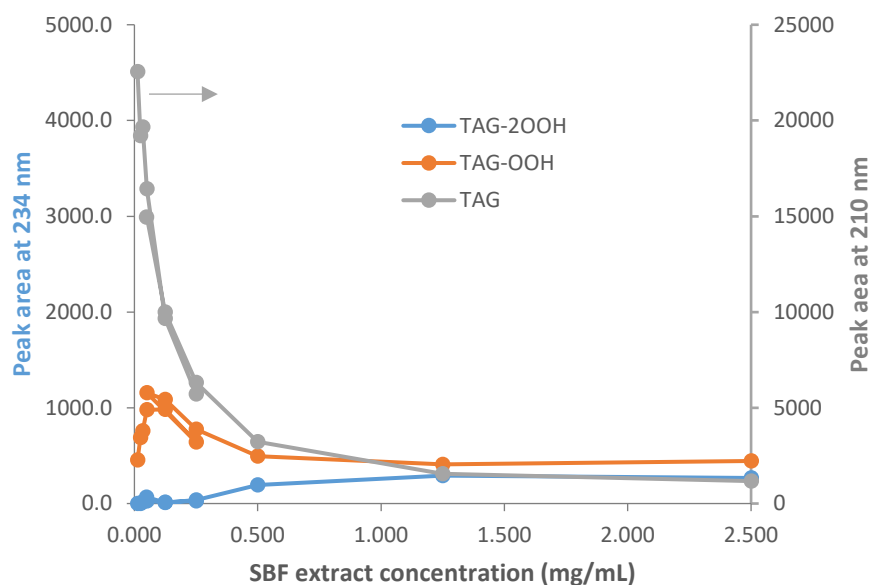
Figure 5.15: Effect of SBF extract concentration on sfLOX-catalysed tuna oil reaction. Reaction of 0.067 mg/mL tuna oil emulsion, 0.002% w/v Triton X-100, SBF extract (0.0125 – 2.5 mg/mL), in 10 mL citrate buffer (50 mM, pH 5.0), saturated with oxygen, 15 min reaction time. Lines in graph are not joined due variation in results conducted on different days. TAG-2OOH and TAG-OOH measured at 234 nm (left axis), TAG measured at 210 nm (right axis). The results from a single experiment represent the effect on product formation.

3.5.3. Effect of SBF concentration in the presence of BHT on product formation

BHT is an antioxidant or radical scavenger that is known to protect lipids from autoxidation. The SBF extract concentration was re-optimised in the presence of BHT (0.0033 mg/mL) to potentially recover the lost products at SBF concentrations above 0.05 mg/mL. The addition of BHT at 0.0033 mg/mL showed a 25% increase in the initial reaction rate (as determined by the UV-vis method), therefore this concentration was tested. TAG-2OOH formation was higher (approximately 2-fold) in the presence of BHT compared to without BHT (compare Figures 5.15 and 5.16). TAG-OOH formation was drastically increased at 0.05 mg/mL SBF extract concentration, roughly 2.5-fold higher than at any other SBF extract concentrations (Figure 5.16). TAG consumption in the reaction was observed to be similar in the presence and absence of BHT (Figures 5.15 and 5.16). Again, the reaction reached completion with minimal TAG remaining at 1.25 mg/mL SBF extract.
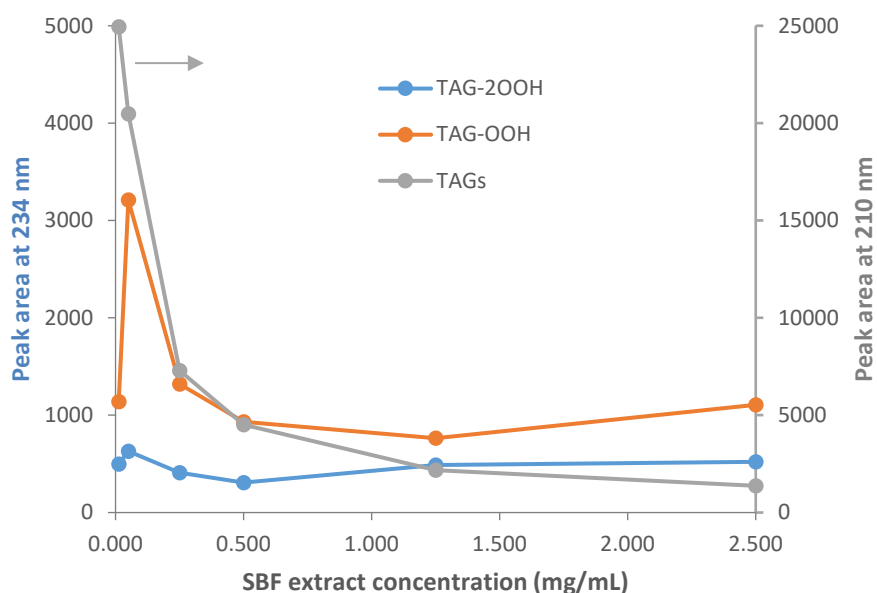
Figure 5.16: Effect of SBF extract concentration on the sfLOX-catalysed tuna oil reaction in the presence of 0.0033 mg/mL BHT. Reaction of 0.067 mg/mL tuna oil emulsion, 0.002% w/v Triton X-100, SBF extract (0.0125 – 2.5 mg/mL), in 10 mL citrate buffer (50 mM, pH 5.0), containing 0.0033 mg/mL BHT, saturated with oxygen, 15 min reaction time. TAG-2OOH and TAG-OOH measured at 234 nm (left axis), TAG measured at 210 nm (right axis). The results from a single experiment represent the effect on product formation.

In the sfLOX-catalysed TL reaction (Chapter 4, Section 3.1.6), product formation was found to increase significantly at a higher BHT concentration of 0.05 mg/mL. Therefore, this BHT concentration was tested, and the SBF extract concentration (0.0125 – 2.5 mg/mL) was re-optimised. As the SBF extract concentration increased the amount of TAG-OOH and TAG-2OOH detected increased substantially, while TAG decreased significantly (Figure 5.17). The SBF extract concentration for maximum yield of both TAG-OOH and TAG-2OOH was found to be 1.25 mg/mL. At higher SBF extract concentrations, there was no further conversion of TAG-OOH into TAG-2OOH. This is likely because no more unreacted PUFA were available in the TAG-OOH compounds to further convert into TAG-2OOH.
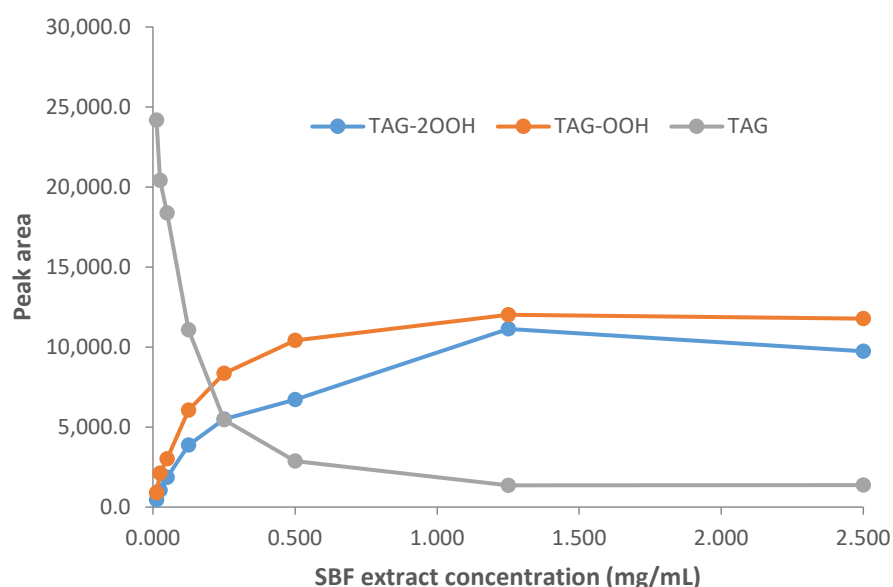
Figure 5.17: Effect of SBF extract concentration on the sfLOX-catalysed tuna oil reaction in the presence of 0.05 mg/mL BHT. Reaction of 0.067 mg/mL tuna oil emulsion, 0.002% w/v Triton X-100, SBF extract (0.0125 – 2.5 mg/mL), in 10 mL citrate buffer (50 mM, pH 5.0), containing 0.05 mg/mL BHT, saturated with oxygen, 15 min reaction time. Reaction products (TAG-OOH and TAG-2OOH) measured at 234 nm (red and blue traces), TAG measured at 210 nm. The results from a single experiment represent the effect on product formation.

3.5.4. Effect of tuna oil amount on product formation

Using the conditions determined above (1.25 mg/mL SBF extract and 0.05 mg/mL BHT) that gave the greatest amount of product formation, the effect of tuna oil

concentration was investigated. This was done because the concentration of TAG substrate used in the initial reactions was potentially limiting the reaction. Tuna oil concentrations ranging from 0.067 to 0.4 mg/mL were investigated by increasing the stock tuna oil emulsion volume from 66.7 to 400 μL, instead of increasing stock tuna oil concentrations and using the same volume in the reaction. This is because stock tuna oil emulsions cannot be formed in water at high concentrations of tuna oil. In this way, the concentrations of emulsifiers (gum arabic and Triton X-100) in the reaction were also increased. In the studied range, TAG-OOH increased steadily with concentrations from 0.067 to 0.2 mg/mL, before decreasing with concentrations greater than 0.2 mg/mL. TAG-2OOH formation was greatest with 0.133 mg/mL tuna oil, and decreased at higher concentrations (Figure 5.18). A tuna oil concentration of 0.2 mg/mL was chosen for further optimisation because at this concentration TAG-OOH was highest and a high amount of tuna oil still remained after the reaction ceased, showing that substrate was not limiting the reaction.
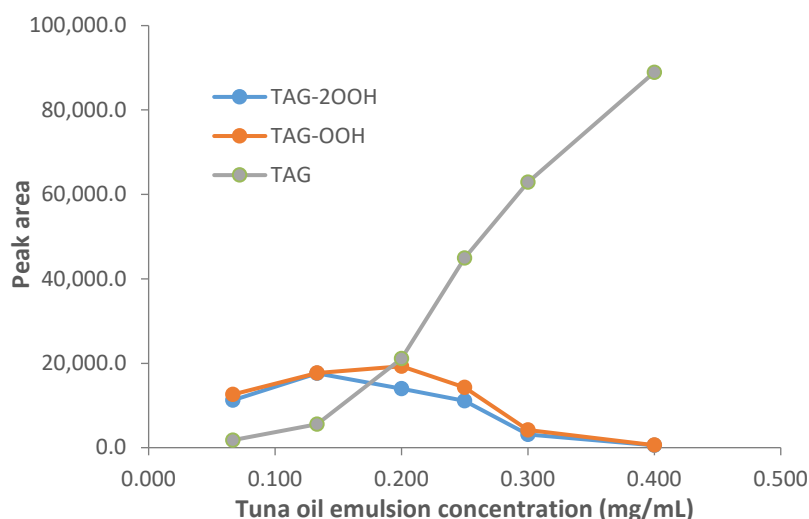
Figure 5.18: Effect of tuna oil emulsion concentration on the sfLOX-catalysed tuna oil reaction. Reaction of tuna oil emulsion (0.067 – 0.4 mg/mL), SBF extract 1.25 mg/mL, BHT 0.05 mg/mL, 10 mL citrate buffer (50 mM, pH 5) saturated with oxygen, 15 min reaction time. Reaction products (TAG-OOH and TAG-2OOH) were measured at 234 nm (orange and blue traces), and TAG was measured at 210 nm (grey trace). The results from a single experiment represent tuna oil emulsion concentration effect on product formation.

3.5.5. Reoptimisation of SBF concentration on product formation

The addition of more SBF extract was tested to see whether more reaction products could be formed with the higher substrate concentration. The reaction with 0.2 mg/mL tuna oil was re-assessed with higher SBF concentrations (1.25 – 7.5 mg/mL). More TAG-OOH and TAG-2OOH were detected as SBF extract concentration increased up to 5 mg/mL. From 5 mg/mL of SBF extract concentration, the maximum amount of TAG-OOH and TAG-2OOH was obtained with minimal amount of TAGs remaining (Figure 5.19).
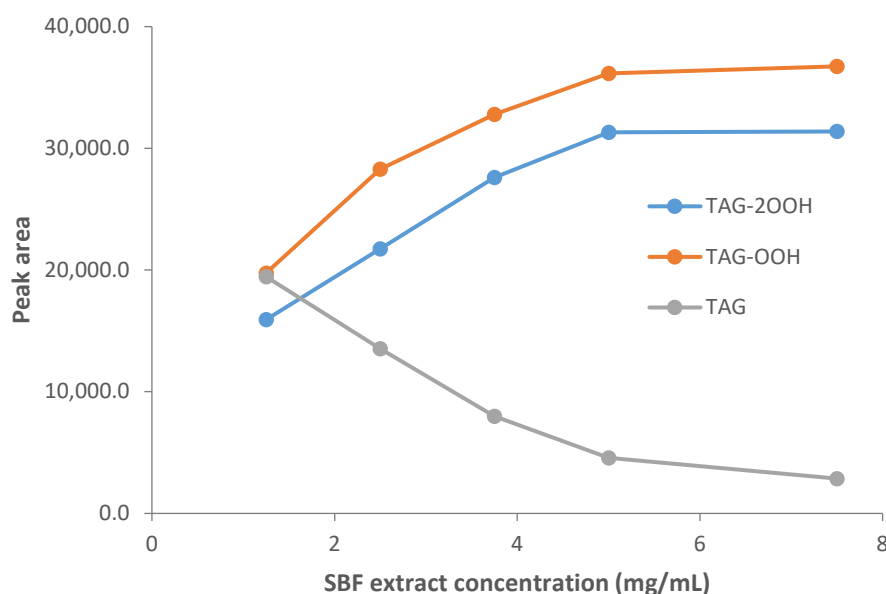
Figure 5.19: Effect of SBF concentration on the sfLOX-catalysed reaction with 0.2 mg/mL tuna oil. Reaction of 0.006% w/v Triton X-100, SBF extract (1.25 – 7.5 mg/mL), 10 mL citrate buffer (50 mM, pH 5) saturated with oxygen, 15 min reaction time. Reaction products (TAG-OOH and TAG-2OOH) measured at 234 nm, TAG measured at 210 nm. The results from a single experiment represent the effect on product formation.

As higher amounts of SBF could convert most of the remaining substrate from 0.2 mg/mL tuna oil into TAG-OOH and TAG-2OOH, the reaction with 0.25 mg/mL tuna oil was also tested with higher SBF extract concentrations (1.25 – 12.5 mg/mL). As expected, both TAG-OOH and TAG-2OOH increased significantly from 1.25 to 7.5 mg/mL SBF extract with unreacted TAGs decreasing. However, at higher SBF

concentrations TAG-2OOH slowly decreased whereas TAG-OOH and TAG

remained stable (Figure 5.20).
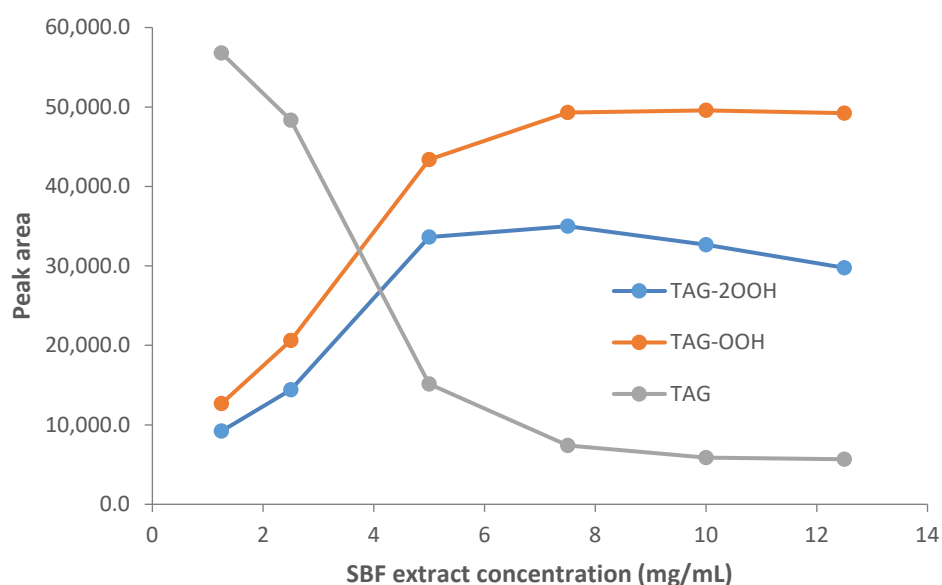
Figure 5.20: Effect of SBF concentration on the sfLOX-catalysed reaction with 0.25 mg/mL tuna oil. Reaction of 0.0075% w/v Triton X-100, SBF extract (1.25 – 12.5 mg/mL), 10 mL citrate buffer (50 mM, pH 5) saturated with oxygen, 15 min reaction time. Reaction products (TAG-OOH and TAG-2OOH) measured at 234 nm, TAG measured at 210 nm. The results from a single experiment represent the effect on product formation.

The PUFA-containing TAGs in tuna oil at increasing concentrations (0.067, 0.2 and 0.25 mg/mL) could be converted into TAG-OOH and TAG-2OOH effectively by using appropriate SBF concentrations (1.25, 5 and 7.5 mg/mL, respectively). These reactions were reproducible with relative standard deviation < 5%. The conversion efficiency relative to the amount of tuna oil used was calculated by dividing the peak areas by the amount of tuna oil in each 10 mL reaction. As shown in Table 5.3 below, the total product formation efficiency of both TAG-OOH and TAG-2OOH was comparable between reactions using different tuna oil amounts (0.667 – 2.5 mg). There was no significant difference in the efficiency of TAG-OOH and TAG-2OOH conversion. However, TAG-OOH conversion tended to decrease whereas TAG-2OOH conversion appeared to increase with increasing tuna oil concentration.

Table 5.3: Relative product formation efficiency for sfLOX-catalysed tuna oil reactions using different amounts of tuna oil (0.667 – 2.5 mg).

Tuna oil amount (mg)	TAG-OOH formation efficiency	TAG-2OOH formation efficiency	Total product formation efficiency
0.667	16.7	18.0	34.7
2	15.7	18.1	33.7
2.5	14.0	19.7	33.7

Although product formation efficiency was not much different between tuna oil amounts in the reaction, the maximum amount of reaction products was obtained with 2.5 mg tuna oil per 10 mL reaction. As a result, the final optimised reaction conditions were 0.25 mg/mL tuna oil emulsion, 0.0075% w/v Triton X-100, 7.5 mg/mL SBF extract, 0.05 mg/mL BHT, 10 mL citrate buffer (50 mM, pH 5) saturated with oxygen, 15 min reaction time.

3.6. Product characterisation

Reaction products formed in the catalysis of tuna oil by sfLOX using the final optimised conditions were hydrolysed to enable characterisation of the different PUFA products. The hydrolysed reaction products from reactions using 0.067 and 0.25 mg/mL tuna oil were compared. The RP-HPLC results for the conditions using the 0.25 mg/mL tuna oil emulsion showed that the yield of hydrolysed products was much lower than expected. Additionally unknown compounds absorbing at 270 nm were also formed in significant amounts. The hydrolysis conditions using 12.5 M NaOH for 90 min (Chapter 4, Section 2.4) may be too harsh for the n-3 LC-PUFAs found in tuna oil. To address this the reaction was trialled with different sodium hydroxide concentrations (2.1 – 12.5 M) and reaction times (1 – 90 min) to minimise the loss of the products. The optimum sodium hydroxide concentration was found to be 6.25 M. As for reaction time, the optimum conditions were inconclusive (either 15

or 60 min). At 15 min, higher amounts (20 – 30% more) of the products were released by hydrolysis and the reaction was more reproducible than at 60 min. However, an extra peak eluting at ~ 30 min was observed in the 15 min reaction that was absent in the 60 min hydrolysis. As neither hydrolysis time was ideal, both were used.

3.6.1. Determination of hydroxy fatty acid composition by RP-HPLC

The hydrolysed sfLOX-catalysed tuna oil products were compared with standards of mono- and di-hydroxy analogues of DHA and EPA under the same RP-HPLC running conditions. The standards were previously synthesised and purified in-house using sfLOX. Retention times and absorbance spectra of sample peaks were matched to standards to confirm the presence of particular compounds in the samples. However, slight variation in retention times between runs made retention time matching difficult, so each standard and sample were co-injected to observe if co-elution occurred. An example, showing the co-elution of the 17-HDHA standard with a peak in the 15 min hydrolysis sample is shown in Figure 5.21. 17-HDHA and 15-HEPA were confirmed to be present in both hydrolysis samples. A peak eluting at 23 min matched the retention time of 7,17-diHDHA, but their spectra were different. It has a maximum absorbance at 234 nm instead of two absorption maxima at 226 and 246 nm. This compound and the extra peak in the 15 min hydrolysis sample were unidentified.
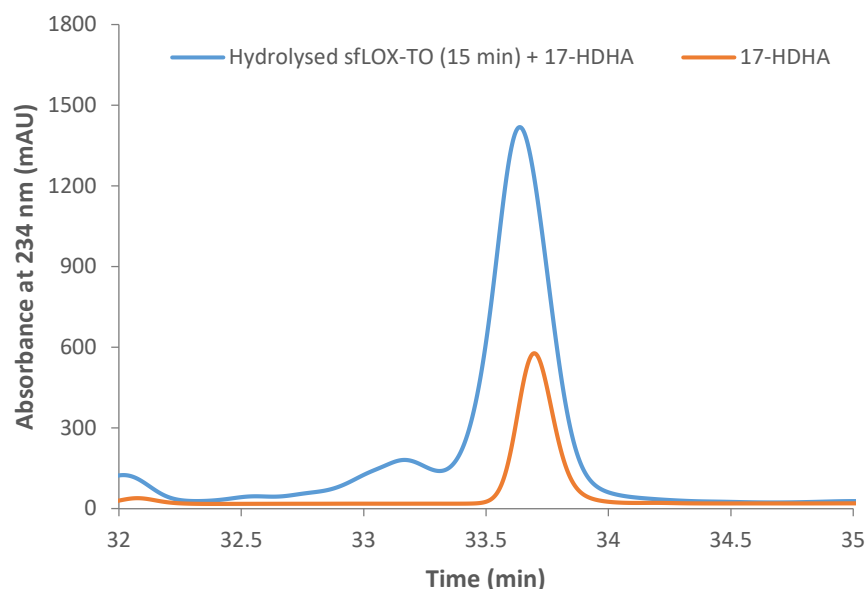
Figure 5.21: Co-elution of the 17-HDHA standard and hydrolysed sfLOX-catalysed tuna oil products after 15 min hydrolysis with 6.25 M NaOH.

3.6.2. Determination of hydroxy group positions on reaction products by GC-MS

After hydrolysis the reaction products were derivatised to fully hydrogenated and trimethylsilylated (TMS) compounds. This was done as the derivatives are more volatile and stable at high temperature, allowing for GC-MS analysis. The fragmentation patterns obtained for the derivatives were used to identify the positions of hydroxy groups on the different FA products. Two major mono-hydroxy products and a range of FAs with different chain lengths were detected. No significant difference was observed between the samples hydrolysed for 15 or 60 min.

Two mono-hydroxy products that eluted between 24 and 27 min (Table 5.4) were identified based on fragmentation patterns (Figure 5.22). They were identified as fully hydrogenated TMS derivatives of 17-HDHA and 15-HEPA, confirming the presence of these two compounds that were also identified by RP-HPLC. Only trace amounts of di-hydroxy products were detected between 28 and 30 min, meaning the identification of the di-hydroxy products was not possible. Various FAs from C14 to C22 that were present as hydrogenated and TMS derivatives were also identified.

These saturated, TMS derivatives most likely originated from palmitic and steric acids (SFAs), and oleic acid (a MUFA) present in the tuna oil, which sfLOX is unable to catalyse, and from unreacted DHA, EPA, LA and AA (PUFAs) remaining after the reaction.

Table 5.4: Important *m/z* fragments of the hydrogenated, trimethylsilyl derivatives of mono-hydroxy products from sfLOX and tuna oil reaction under optimised conditions.

Retention time (minutes)	Mono-hydroxy products	Important *m/z* fragments
24.14	15-HEPA	73, 117, 173, 401, 457
26.93	17-HDHA	73, 173, 373, 429, 485
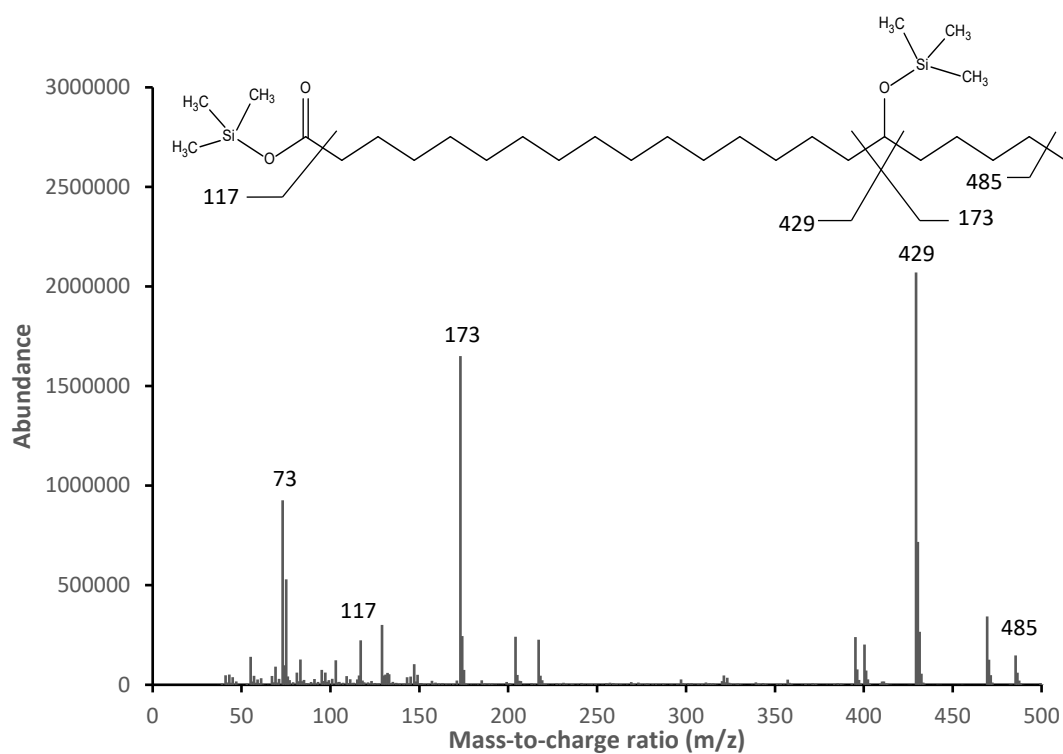
Figure 5.22: Mass spectrum of fully hydrogenated di-TMS-17-HDHA and structure showing assignment of important fragments.

3.7. Applications of optimised sfLOX-TAG reaction conditions

3.7.1. Using other complex lipids as substrates

The conditions optimised with tuna oil were applied to tridocosahexaenoin (DHA TAG) and anchovy oils. Both were found to yield high concentrations of products absorbing at 234 nm when being catalysed by sfLOX (Figure 5.23). A small amount of substrate remained after the reactions, < 10% for both substrates. However, the remaining substrate concentration is not indicative of reaction yield, as both substrate and products can also be metabolised into other compounds that cannot be detected by the HPLC protocols in the current work. From the DHA TAG reaction (Figure 5.23A) the two major peaks were expected to be tri- and di-hydroperoxy DHA TAGs, while the smaller peaks between 15 and 20 min were expected to be mono-hydroperoxy DHA TAGs. These are consistent with the retention times of the sfLOX-catalysed TL reaction products. As for anchovy oil, two groups of potential di- and mono-hydroperoxy PUFA TAGs with the same retention time as those from the sfLOX-catalysed tuna oil reaction were obtained at high concentration (Figure 5.23B). The results confirmed that sfLOX was able to catalyse anchovy oil and DHA TAGs effectively using the conditions optimised with tuna oil. However, sfLOX did not show any significant activity with DHA TAGs using the optimised conditions from the TL reactions.
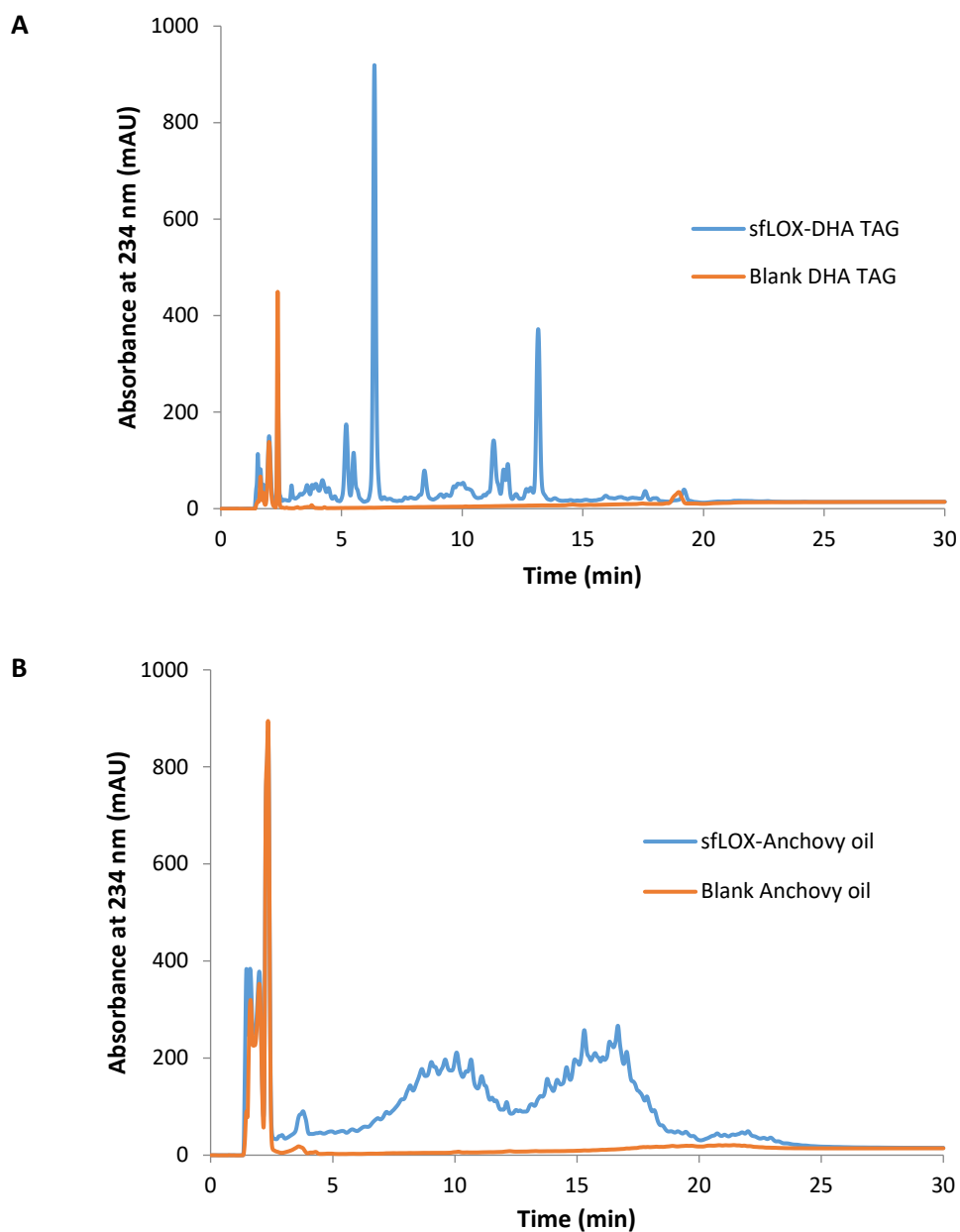
Figure 5.23: Chromatograms of A) sfLOX-catalysed DHA TAG reaction products, B) sfLOX-catalysed anchovy oil reaction products produced using the conditions optimised with tuna oil.

3.7.2. Using commercial 15-sLOX-1 as a LOX source

The sfLOX preparation was shown to react effectively with different complex lipids, including tuna oil, anchovy oil and DHA TAGs. To determine if a commercial enzyme could react with esterified FA substrates, the optimised reaction conditions from the reactions with tuna oil and TL were tested with the commercial 15-sLOX-1. 50% substrate amount was used in the reactions with the commercial enzyme, consistent with the optimised conditions found for the LOX-catalysed reactions with

DHA (Chapter 3). In comparison with sfLOX, 15-sLOX-1 was only capable of oxygenating esterified FAs poorly, with minimal product formation observed. Taking into account the differences in substrate concentration, the formation of mono- and di-HPTAGs from the 15-sLOX-1-catalysed tuna oil reaction was approximately 4 and 1.5%, respectively, of that from the sfLOX-catalysed reaction (Figure 5.24A). Approximately 85 and 10% unreacted tuna oil remained for 15-sLOX-1 and sfLOX-catalysed reactions, respectively. The 15-sLOX-1 catalysis of TL as a substrate gave tri-, di- and mono-HPTL at approximately 0.5, 1.5 and 15%, respectively, of the amounts formed with sfLOX (Figure 5.24B) using the optimised conditions found for the sfLOX-catalysed reaction with TL. Approximately 75 and 30% of unreacted TL remained in the reactions with 15-sLOX-1 and sfLOX, respectively. These results show that 15-sLOX-1 has a lower affinity for the catalysis of esterified FAs than sfLOX.
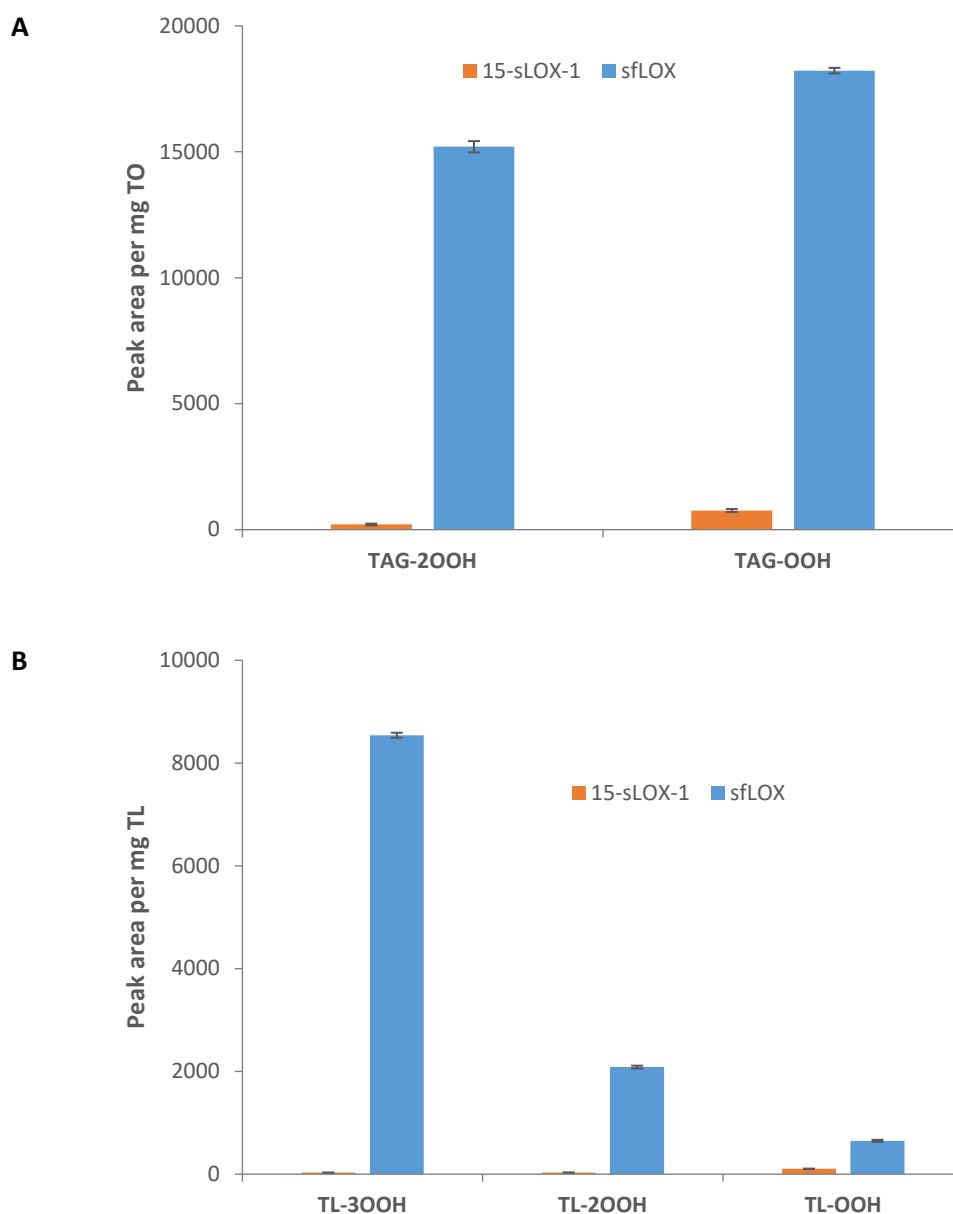
Figure 5.24: Reactions catalysed by 15-sLOX-1 and sfLOX with different esterified FA substrates. A) Tuna oil and B) TL, under the optimum conditions found for sfLOX. Amount produced per milligram substrate, error bars indicate (±) one standard deviation (n=3).

4. Discussion

Increasing the number of sonication pulses increased the homogeneity of TL emulsions, and therefore led to more reproducible product formation from sfLOX-catalysed reactions. Micelle droplet size decreased significantly with the increasing number of pulses. Reaction product profiles showed an overall decrease in TL-OOH and an increase in TL-3OOH, while TL-2OOH remained constant, indicating an

overall increase in dioxygenation. Smaller droplets have previously been found to be more sensitive to lipid oxygenation than larger droplets due to the increase in total surface area [242]. Similarly, the interaction between substrate and enzyme at the buffer-droplet interface is likely to be enhanced by the increase in total surface area, resulting in increased enzyme activity.

Some studies have shown that both LOX-1 and type-2 LOX (LOX-2 and LOX-3) were able to metabolise esterified FA substrates, but type-2 LOX was found to have significantly higher activity than LOX-1 from soybean [122, 131] and barley [92, 179]. The current study showed that the sfLOX-catalysed reaction significantly increased product formation with esterified FAs (TL and tuna oil) compared to the commercial type-1 LOX reactions. In agreement with this observation, sfLOX has previously been found to possess much higher activity with TL compared to a commercial LOX-1 [56, 61]. In this thesis, multiple LOX isozymes (LOX-1, LOX-2 and LOX-3) were found to be active in both sfLOX and the commercial LOX, although the commercial LOX may contain a lower concentration of LOX-2. Or it may be less active compared to that found in sfLOX, as indicated by the colorimetric tests (Chapter 3). LOX-1 and LOX-3 may contribute to esterified FA dioxygenation, but at a considerably slower rate compared to LOX-2.

The optimum pH found for the sfLOX-catalysed reaction with tuna oil of pH 5.5 is likely to be due to the activity of LOX-2 or LOX-2 and LOX-3 combined. In support of this proposal, purified type-2 LOX was also found to have higher activity with TL at pH 5.5 (compared to pH 6.5 and 8.3) in a previous study [131]. Using LA as a substrate, Christopher and co-workers showed that purified LOX-3 had a broad optimum pH range from 5.5 to 8.0 and the activity was significantly reduced at pH 4.5 and 9 [214].

Through investigating the initial reaction rate using UV-vis spectrophotometry the sfLOX-catalysed tuna oil reaction was found to be pH dependent and have a low optimum pH of 5.5, similar to that determined with the TL reaction (see Section 3.1.1, Chapter 4). The initial reaction rate was significantly less at low pH (4.5) and high pH (9). In agreement with the observations from this work, several previous studies also used acidic and neutral pH for the sfLOX-catalysed dioxygenation of various esterified FAs and natural oils such as TL, dilinolein, methyl linoleate, soybean oil and butterolein at pH 6 – 7 [61, 111, 224, 225]. Cai and co-workers also found a similar pH optimum for sfLOX using soybean oil as a substrate in the pH range of pH 5 – 9, with a slight decrease in the reaction yield from pH 5 to 8 and a significant decrease in the reaction yield at pH 9 [111]. However, in a study by Kohlen and co-workers, sfLOX was shown to have a sharp optimum activity with TL at pH 7 and significant decreases towards both low and high pH in the studied range of pH 5 to 10 [61]. Moreover, Koch and colleagues found that a crude soybean meal extract catalysed reaction with TL had two optima, at pH 5.5 and pH 8.3, but the activity at pH 8.3 was less than at pH 5.5 [131], consistent with the observations in the sfLOX-catalysed tuna oil reaction.

The effect of surfactants on soybean LOX-catalysed esterified FA reactions has been found to be inconsistent between studies [169, 172, 206]. Dillard and co-workers showed that soybean LOX activity was inhibited by the presence of Triton X-100 and Tween 40 in the reaction with TL [206]. Piazza and colleagues found that Tween 20 did not affect the oxidation rate of 1,3-dilinolein by soybean LOX-1, but a significant increase in the oxidation rate with 1-monolinolein in the presence of Tween 20 was observed [169]. Fukuzawa et al. reported that soybean LOX-1 activity increased substantially in oxygenated egg yolk phosphatidyl choline in the presence Triton X-100, due to the increase of substrate availability by disturbing the

membrane structure of vesicles [172]. In agreement with some of these findings, the initial rate of the sfLOX-catalysed tuna oil reaction observed here was found to increase greatly in the presence of Triton X-100. The optimum Triton X-100 concentration was very low, at approximately 0.002% w/v (0.031 mM), compared to its critical micelle concentration at 0.24 mM [246]. When Triton X-100 was used as an emulsifier combined with gum arabic in the tuna oil emulsion (instead of being added directly to the reaction buffer), the decrease in the size of tuna oil emulsion droplets with increasing Triton X-100 concentration was expected, as surfactant has been used to decrease the oil in water droplet size in several studies previously [247, 248]. Under this condition, the initial reaction rate increased to a higher extent than the same Triton X-100 concentration directly added into the reaction mixture. This suggested that the effect of decreasing emulsion droplet size was much stronger than that of Triton X-100 on the enzyme activity. Further investigations using different reagents to reduce droplet size were not performed.

In this work, the smallest tuna oil droplet size prepared in the 50 mM Tris-HCl buffer pH 7.5 (15% w/v gum arabic and 10 mg/mL (1% w/v) tuna oil) with 0.5% w/v Triton X-100 was 174.4 ± 2.6 nm. Similarly, previous work by Kentish et al. using 15% v/v flaxseed oil in water with Tween 40, demonstrated that droplet size decreased with increasing concentrations of surfactant before reaching the lowest droplet size of 135 ± 5 nm at 5.6% v/v [247]. The initial rate increased significantly with decreasing droplet size by increasing Triton X-100 from 0 to 0.3% w/v in the stock tuna oil emulsion. As mentioned previously, smaller droplets are more susceptible to lipid oxidation as the total surface area is higher than that of larger droplets [242]. Increasing the surface area may also result in increased interaction between the enzyme (sfLOX) and the substrate (tuna oil) at the oil-water interface. This likely improves the substrate accessibility to the enzyme's active site and increases the

initial rate significantly. In agreement with this, the activity of a commercial soybean LOX-1 was found to increase with increasing surface area of methyl linoleate emulsion droplets, which was induced by the increase of surfactant concentration [244]. However, the rate decreased when the emulsion contained greater than 0.3% w/v Triton X-100, although the droplet size still tended to decrease. It is possible that at higher surfactant concentrations, multilayers of surfactant surround the oil droplets at the oil-water interface and protect oil from oxidation [249]. This was demonstrated in a study by Fomuso et al. where a 10% fish oil-caprylic acid structured emulsion containing 0.25% Tween 20 had a higher level of oxidation over a 48-day period than an emulsion prepared with 1% Tween 20 [243]. Although gum arabic was not added to make the emulsion in the study by Fomuso et al., they used a much higher fish oil concentration (10%) than was used in this work (1%). As a result, it is postulated that Triton X-100 at concentrations higher than 0.3% w/v may be acting to shield the tuna oil interface, which interferes with the access of sfLOX to the substrate.

Oxygen is necessary for LOX-catalysed reactions with PUFAs. As expected for a typical LOX reaction, the reaction rate increased when the oxygen concentration was increased. Under oxygen saturated conditions, the initial rate increased $\sim 35\%$ compared to conditions without oxygen sparging. This was similar to the $\sim 35\%$ increase in product formation with oxygen saturation compared to without oxygen sparging found in the sfLOX-catalysed DHA reaction (see Section 3.1.5, Chapter 3). At low oxygen concentrations (nitrogen sparging), the reaction rate only decreased $\sim 20\%$ compared to the untreated condition. This was in agreement with no significant change in product formation from sfLOX-catalysed TL reaction between nitrogen-sparged and un-sparged conditions. For reaction temperature, the initial reaction rate optimum was found between 10 and 30°C, which was a broad

temperature range compared to the sharp optimum at room temperature for product formation in sfLOX-catalysed reactions of DHA and TL (see HPLC results, Chapters 3 and 4 respectively). This suggests that the sfLOX-catalysed reaction with natural oils, such as tuna oil, may be less affected by temperature than the reactions found with pure substrates (DHA and TL). The initial reaction rate was lower at 0°C and 40°C, which is typically for LOX activity. This is the result of a compromise between enzyme denaturation, oxygen concentration and substrate solubility in the reaction medium, as discussed in Chapter 3.

$CaCl_2$ did not show any effect on the sfLOX-catalysed tuna oil reaction in the presence of Triton X-100. LOX-2 and LOX-3, or LOX-2 only are thought to be the key LOX isozyme(s) catalysing reactions with esterified FAs, such as tuna oil. However, Zimmerman and Snyder found that $CaCl_2$ inhibited purified LOX-2 in the presence of Tween 20 using LA as a substrate [250]. Another study's findings were similar to the current results, where it was reported that LOX-2 was stimulated, but LOX-3 was inhibited by $CaCl_2$ using LA as a substrate [214]. Previous investigations have also demonstrated that purified LOX-2 [250] and crude SB extract [251] are not activated by $CaCl_2$ when methyl linoleate and TL were used as substrates, respectively.

The optimisation of the tuna oil reaction using RP-HPLC showed that the reaction time for optimal product formation was 15 min, which was shorter than that for the sfLOX-catalysed DHA (30 min, Chapter 3) and sfLOX-catalysed TL reactions (45 min, Chapter 4). This could be because the formation of di-hydroxy PUFA products (two hydroxy groups in one fatty acid molecule) is significantly slower than that of mono-hydroxy PUFA products [43, 125], as observed with the sfLOX-catalysed reactions of DHA and tuna oil as a substrate respectively. TL-3OOH (three

hydroperoxy groups with one on each fatty acid moiety) may also require longer reaction times compared to up to two hydroperoxy addition into PUFA-containing TAGs in tuna oil. Moreover, the ratio of enzyme to substrate in the tuna oil reaction is much higher than that in DHA and TL reaction, which likely contributes to the shorter reaction time for tuna oil.

Longer reaction times did not affect sfLOX-catalysed tuna oil reaction product formation. This differed to the work in Chapter 3 and 4, where product formation from sfLOX-catalysed reactions with DHA and TL tended to decrease after reaching the optimum reaction time. In previous studies, hydroperoxide yields have also been reported to degrade after the optimum reaction time was reached [162, 212]. Different enzyme sources and substrates were used in these studies. Haefliger and Sulzer used SBF as a LOX source to catalyse the dioxygenation of LNA [162]. Therond et al. used various phosphatidylcholines as substrates in reactions catalysed by soybean LOX-1, and demonstrated a more dramatic degradation of product formation with C18/AA-PC compared to the other substrates [212].

The optimisation of SBF extract concentration using 0.067 mg/mL tuna oil, increasing SBF extract concentration from 0.0125 to 0.05 mg/mL led to increased product formation. However, for SBF extract concentrations of 0.05 – 0.5 mg/mL, the substrate was consumed significantly but product formation decreased. At SBF extract concentrations > 0.05 mg/mL, products and substrate may be metabolised or degraded as a result of other enzymes in SBF and/or free radicals.

In the presence of BHT (0.0033 – 0.0067 mg/mL), the initial rate was found to increase approximately 25% compared to conditions without BHT. The optimum BHT concentration (0.0033 mg/mL) and the resulting change in LOX activity was much lower compared to findings from the sfLOX-catalysed TL reaction (0.05

mg/mL BHT and 2 – 4.3 fold increase of product formation, see Section 3.1.6, Chapter 4). The optimum SBF extract concentration was the same at 0.05 mg/mL with or without BHT but overall product yield was significantly higher with BHT. However, at higher SBF extract concentrations (> 0.05 mg/mL) both mono-HPTAGs and TAGs still decreased substantially as observed in the conditions without BHT.

At a higher BHT concentration of 0.05 mg/mL, product formation increased significantly with increasing SBF extract concentrations compared to conditions without BHT, as seen with the sfLOX-catalysed TL reaction (see Section 3.1.6, Chapter 4). The increase continued at SBF extract concentrations greater than 0.05 mg/mL which was not observed in the presence of BHT at 0.0033 mg/mL. The decrease in TAG concentration was the same with both 0.0033 and 0.05 mg/mL BHT. This suggested that BHT at 0.05 mg/mL could be enough to protect TAG hydroperoxides and substrate from degradation, possibly from the attack of free radicals, therefore product formation increased with the increasing SBF extract concentration. This is because for 0.067 mg/mL tuna oil, the molar concentration of BHT at 0.05 mg/mL is roughly three times higher than that of tuna oil, whereas 0.0033 mg/mL BHT only contains approximately 0.2 molar equivalents.

Decomposition of lipid hydroperoxides can occur by either enzymatic or non-enzymatic reactions. The most common non-enzymatic decomposition pathway is through free-radical mechanisms and involves the homolytic cleavage of hydroperoxides into peroxyl and alkoxyl radicals. The process is catalysed by transition metal ions and metalloproteins [252]. These radicals are further degraded to form various epoxy and oxo compounds, shorter chain compounds (such as alcohols and alkanes) and volatile compounds (aldehydes) through β-scission (breaking the molecules at covalent bonds adjacent to carbon-oxygen bond),

epoxidation and oxidation mechanisms [253, 254]. Antioxidants such as BHT can be used as free radical scavengers that act by donating a hydrogen. The presence of BHT in the reactions is likely protecting the TAG hydroperoxides, formed by sfLOX catalysis, from free-radicals in solution, resulting in the significant increase in the TAG hydroperoxide yield. This is not due to the increase of enzyme activity as the initial rate (UV-vis results) increased as a much smaller ratio compared to product formation (RP-HPLC results). Reducing the rate of breakdown could account for this increase of product formation. However, using HPLC-DAD and GC-MS no aldehydes or other oxidative compounds were detected in either sfLOX-catalysed reactions with TL or tuna oil in the absence of BHT.

LOX-2 and LOX-3 have been found to form radical intermediates more readily in comparison to LOX-1. LOX-2 and LOX-3 were reported to bleach β-carotene in the presence of LA at pH 6.5 more efficiently than LOX-1 at pH 9, due to their ability to form active peroxyl radicals that facilitate the co-oxidation of β-carotene [156]. The formation of radical intermediates by LOX-3 is independent of oxygen concentration, whereas LOX-1 only produces radical intermediates at low oxygen concentrations [255]. This further supports the sfLOX-catalysis of esterified FAs, which involves mainly type-2 LOX, requirement for BHT to protect TAG hydroperoxides from free-radical degradation. Similarly, BHT was previously found to increase LOX-2 and LOX-3 activity more significantly than LOX-1 activity [227].

In the presence of BHT, more sfLOX-catalysed tuna oil reaction products can be generated by adding extra tuna oil and SBF without a drop in reaction efficiency. The 10 mL sfLOX reaction with 0.667 mg tuna oil was effectively catalysed with minimum amount of tuna oil remaining (< 10%). Tuna oil amounts of 2.0 and 2.5 mg can be efficiently converted into products by adding more enzyme. However, at high

tuna oil amounts (≥ 3 mg) product formation decreased significantly, which may be due to inhibition by oxygen depletion, the decrease of mass transfer and/or the increase of emulsifier concentration (Triton X-100 and gum arabic). No further work testing the effects of the concentration emulsifier components on the reaction was conducted.

Using the conditions optimised with the sfLOX-catalysed tuna oil reaction, DHA TAG, tuna oil and anchovy oil were effectively converted into TAG hydroperoxides with less than 10% of unreacted TAG remaining. It is possible that approximately 90% DHA TAG was consumed in the sfLOX-catalysed reaction. However, tuna oil and anchovy oil reactions have less than 50% TAGs containing one, two or three PUFAs, but only < 10% TAGs (by total HPLC peak area at 210 nm) in these oils were available after the reactions. This could be due to the differences in molar extinction coefficients of various TAGs in oils. TAGs containing PUFAs were shown to have more significant peak area than TAGs without PUFAs at the same concentration [256]. The minimal peak area detected could be mainly from TAGs lacking PUFAs.

Mono- and di-hydroperoxy TAGs were formed in the sfLOX-catalysed tuna oil reaction and only mono-hydroperoxy PUFA moieties in TAGs were observed. The major mono-hydroxy products were identified by RP-HPLC and GC-MS as 17-HDHA and 15-HEPA moieties in TAG form, which corresponds to the major mono-hydroxy products from DHA and EPA reactions catalysed by sfLOX (Chapter 3) and soybean LOX-1 [43]. The formation of only mono-hydroxy PUFA products is in agreement with previous studies where esterified FAs have been shown to react with LOX by inserting only at the methyl end in the active site [211, 257]. Whereas the carboxyl groups required for the formation of di-hydroxy PUFA products (containing

double conjugated dienes and a conjugated triene) are unavailable in esterified FA. The optimised sfLOX-catalysed tuna oil reaction conditions were confirmed to be effective conditions for other complex lipid substrates such as DHA TAGs and anchovy oil. This study has shown that 15-sLOX-1 was unable to react effectively with esterified FAs (tuna oil and TL) under the optimum conditions of sfLOX.

5. Conclusion

LOX from soybean flour has been demonstrated to catalyse the dioxygenation of tuna oil (a complex lipid mixture), whereas 15-sLOX-1 was unable to catalyse esterified FAs (tuna oil and TL) effectively. Prior hydrolysis is not required to oxygenate PUFA-containing TAGs using this method. Soybean flour contains both LOX-2 and LOX-3, which is advantageous compared to many purified LOX preparations available commercially. LOX-2 or its combination with LOX-3 in soybean flour are thought to be responsible for the activity of sfLOX in catalysing esterified FA substrates. In contrast to the results in Chapter 4, surfactant Triton X-100 as a second emulsifier in the tuna oil-gum arabic emulsion was found to increase the reaction rate significantly with an optimum concentration of 0.3% w/v in the emulsion system partly due to the decrease of emulsion droplet size. The optimised sfLOX-catalysed tuna oil reaction conditions can be applied to other TAG substrates, such as DHA TAG and anchovy oil. The antioxidant BHT appears to protect against the degradation of TAG hydroperoxides without interference in sfLOX catalysis, and in doing so increases the product formation significantly.

CHAPTER VI

BIOSSAYS OF POTENTIAL ANTI-INFLAMMATORY LIPID MEDIATORS FROM n-3 AND n-6 LC-PUFA ENZYMATICALLY SYNTHESISED BY SOYBEAN FLOUR LIPOXYGENASE

1. Introduction

PUFAs are long known to have many health benefits and the dietary omega-6/omega-3 ratio is important in maintaining good health and reducing risk of a wide range of diseases. In particular, DHA is found predominantly in the brain as a major component of neural membranes, and in cortical grey matter as the most abundant FA. Therefore, DHA has crucial structural and functional roles in brain health [189]. However, the mechanism for PUFA benefits is not fully understood. PUFAs are endogenously metabolised into specialised pro-resolving mediators (SPMs) by different types of enzymes including lipoxygenases and cyclooxygenases. SPMs are a group of structurally related, bioactive lipid compounds, which have been demonstrated to actively induce the resolution of inflammation [17, 19, 66]. They are hydroxylated compounds derived from omega-3 and omega-6 PUFAs and divided into four groups: lipoxins, resolvins, protectins and maresins. These compounds with highly stereo-specific hydroxy groups and characteristic conjugated double bond systems have shown high anti-inflammatory and pro-resolving bioactivity at low concentrations both *in vitro* and *in vivo* [6]. SPMs and potential analogues are promising in the treatment and prevention of inflammatory diseases as new anti-inflammatory drugs, and may in part explain the health benefits of PUFAs in the body.

Inflammation is a protective response of the host which attempts to remove harmful stimuli and heal damaged tissue. Inflammation is induced by the attack of invading pathogens, from tissue injury and infection, leading to the release of pro-

inflammatory lipid mediators such as leukotrienes and prostaglandins and other chemical mediators such as cytokines and chemokines to initiate and coordinate the inflammatory response. This is followed by the recruitment of white blood cells (leukocytes or PMN), especially neutrophils, to the injured area to target and destroy invading pathogens by secreting reactive oxygen species [8, 10, 11]. Platelets are also involved in inflammation. They are blood cells and have an important role in blood clotting. They gather at the damaged site and release adhesive molecules to recruit and aggregate with white blood cells. The platelet-granulocyte aggregates amplify inflammation by further secretion of immune modulators to recruit neutrophils, monocytes and lymphocytes [258]. Self-limited inflammation is essential for survival of the host from foreign invaders. However, uncontrolled inflammation is associated with a range of diseases such as cardiovascular diseases, diabetes and Alzheimer's disease. Anti-inflammatory drugs targeting the treatment and prevention of these diseases are in demand. Anti-inflammatory activity is expressed through the ability to inhibit the inflammatory pathways such as pro-inflammatory mediator generation and leukocyte recruitment and others, or by stimulating the anti-inflammatory pathway such as generation of anti-inflammatory cytokines.

The anti-inflammatory activity of SPMs and related analogues has been measured in a number of studies using many different approaches, including: 1) a decrease in the concentration of pro-inflammatory lipid mediators such as LTB4 and PGE2 [259, 260] or a decrease in the enzyme activity (such as 5-LOX) involved in their synthesis [261, 262]; 2) a reduction in the concentration of pro-inflammatory cytokine and chemokine secretion such as IL-1β, TNF-α, IL-6 and MCP-1 [75, 260, 263-269] and/or an increase in anti-inflammatory cytokines such as IL-10 and TGF-β [260]; 3) an inhibition of mRNA expression of pro-inflammatory factors such as IL-1β, IL-6,

inducible nitric oxide synthase (iNOS) and COX-2 [22, 270, 271]; 4) a decrease in platelet aggregation [70]; 5) PMN inhibition or a decrease in PMN migration [272], leukocyte cell number [22, 67, 260, 273], neutrophil count [266], or pathology score based on inflammation levels (white blood cell count from none (0) to severe (3)) [274]; 6) lower production of reactive oxygen species by neutrophil enzymes such as myeloperoxidase and iNOS [259] or a decrease in their enzyme activity [259, 275]; and 7) activation of receptors inducing anti-inflammatory activity [188, 275].

Cytokine secretion is a common way to detect and quantify inflammation. Interleukin-1β (IL-1β) and tumor necrosis factor-alpha (TNF-α) are major pro-inflammatory cytokines involved in the process of inflammation and are produced mainly by activated macrophages. They are involved in the inflammatory response to various stimuli including infectious agents and pathological pain [276]. IL-1β, TNF-α and other cytokines have been measured as markers of inflammation in various diseases such as cardiovascular diseases and chronic obstructive pulmonary disease [277, 278]. Secretion of IL-1β and TNF-α from cultured cells such as macrophages are also commonly used to evaluate the anti-inflammatory activities of specific compounds. IL-1β and TNF-α are pro-inflammatory cytokines, whereas some of other cytokines such as IL-6 and interferon-γ (IFN-γ) can be either pro-inflammatory or anti-inflammatory agents in various cases [276, 279], which may interfere with the interpretation of results in such assays.

The mouse microglial cell line (C8-B4) [280] and the human monocytic cell line (THP-1) are inflammatory cells, macrophages and monocytes, involved in the inflammatory process. They can respond to inflammatory stimuli and secrete cytokines such as IL-1β and TNF-α in response, therefore allowing the assessment of anti-inflammatory activity of a compound of interest. Moreover, the C8-B4 cell line

is derived from mouse brain cells and DHA is rich in brain so the bioactivity of its metabolites may elucidate the neuroprotective function of DHA through anti-inflammatory activity. Lipopolysaccharide (LPS) is a common stimulus used in inflammatory assays of C8-B4 and THP-1 cell lines as LPS can polarise monocytes into macrophages and also activate macrophages for cytokine secretion.

17S-monohydroxy DHA (17-HDHA), 7S,17S-dihydroxy DHA (17,17-diHDHA or resolvin D5) and 10S,17S-dihydroxy DHA (10,17-diHDHA or protectin DX) were enzymatically synthesised from DHA using soybean flour as an inexpensive LOX source alternative to commercial LOX (Chapter 3). Other biologically important LC-PUFAs (EPA, AA, DPAn-3 and DPAn-6) having different chain lengths and number of double bonds were also used to form analogues of the compounds synthesised from DHA. These compounds are of interest to assess anti-inflammatory activity as they are analogues of highly bioactive resolvins and protectins belonging to the SPM superfamily. As shown in previous work, resolvin D5 from DHA and DPAn-3 (RvD5$_{DHA}$ and RvD5$_{DPAn-3}$) and protectin DX from DHA and DPAn-6 (PDX$_{DHA}$ and PDX$_{DPAn-6}$) are known members of the SPM superfamily, and their anti-inflammatory bioactivities have been examined and confirmed in a number of studies [67, 70, 75, 259, 263-268, 270, 272, 274]. Mono-hydroxy PUFAs (17-HDHA, 17-HDPAn-6, 15-HETE and 15-HEPA) have also shown anti-inflammatory activity in several studies [67, 261, 262, 270]. However, other di-hydroxylated compounds of resolvin D5 and protectin DX analogues from other biologically important PUFAs (EPA, ETE and DPAn-6) have not been investigated for anti-inflammatory activity. The anti-inflammatory activity of all these resolvin D5 and protectin DX analogues has not been tested with IL-1β and TNF-α secretion using THP-1 and C8-B4 cell lines.

In this chapter, the anti-inflammatory activity of a range of resolvin and protectin analogues was tested in inflammatory bioassays. The mono- and di-hydroxy PUFAs were synthesised in milligram scale from a range of n-3 and n-6 LC-PUFAs (DHA, EPA, ETE, DPAn-3 and DPAn-6) using soybean flour lipoxygenase as a biocatalyst. For each PUFA, one mono- and two di-hydroxy compounds were synthesised and tested. The 15 purified mono- and di-hydroxy PUFA compounds with their PUFA precursors (PUFAs and resolvin analogues) were all tested for the first time on LPS-induced inflammation models using human monocytic cells (THP-1) and mouse brain cells (C8-B4). IL-1β and TNF-α were measured to assess their anti-inflammatory properties *in vitro*. For the first time, the bioactivities of resolvin D5 and protectin DX-structured compounds and their precursor mono-hydroxy PUFAs in each PUFA group were systematically compared as a function of structure, and structure-activity relationships are discussed.

2. Materials and methods

2.1. Materials

Soybean flour (Kialla Pure Foods, certified organic) was purchased from Organic Wholefoods (Brunswick East, VIC, Australia). The fatty acids DHA, EPA, ARA, DPAn-3 and DPAn-6 (Nu-Chek Prep) were obtained from Adelab Scientific (Thebarton, SA, Australia). 2,2-dimethoxypropane (Fluka), potassium phosphate monobasic, potassium phosphate dibasic, sodium borohydride and tris(2-carboxyethyl)phosphine hydrochloride (TCEP) were purchased from Sigma Aldrich (Castle Hill, NSW, Australia). Heptane (99 %, 0.2 micron filtered), chloroform and isopropanol were obtained from Fisher Scientific (Scoresby, VIC, Australia). Acetic acid (BDH Lab Supplies, UK) was purchased from Science Supply (Mitcham, VIC, Australia). Triton X-100 was obtained from Ajax Chemicals (Sydney, NSW,

Australia). Oxygen gas was obtained from Coregas Pty Ltd (Yennora, NSW, Australia).

2.2. Resolvin analogue synthesis

The optimised 10 mL soybean flour LOX (sfLOX) catalysed DHA reaction conditions (Chapter 3) was scaled up 20 times and applied to the large-scale synthesis of di-hydroxy resolvin analogues from LC-PUFAs: 200 mL phosphate buffer (pH 8, 50 mM), oxygen saturation, 0.2 mM LC-PUFAs (from 20 mM stock in ethanol), 1 mL Triton X-100 solution in water 1% w/v, 16 mL SBF extract (5 g/100 mL water), ambient temperature, 30 min reaction time. For the synthesis of di-hydroxy DPAn-6 products used in the C8-B4 bioassays, TCEP (0.6 mM) was added in the sfLOX- catalysed DPAn-6 reaction before adding SBF to increase the 7,17-diHDPAn-6 yield (details shown in Chapter 3, Section 3.3). The reactions were stopped after 30 min by adding 4 mL sodium borohydride (1 M) in water with stirring for 15 min. The reaction mixture was acidified with 1 mL acetic acid and stirred for 30 min. When TCEP was included as an additive, the reaction mixture was acidified directly without prior reduction with sodium borohydride. Then the reaction mixture was adjusted to pH 3 with hydrochloric acid solution (4 M) and reaction products were extracted by solvent extraction in a separating funnel with 3 × 200 mL chloroform. Emulsion formation was possible after shaking in the separating funnel, and required sitting without disturbance, or centrifugation to separate the two phases. For ease of handling, the total extract volume (600 mL) was divided into 2 parts (300 mL each) and each washed with 100 mL water, then dried over sodium sulphate. The recombined extract (~ 600 mL) was concentrated under vacuum until approximately 20 – 50 mL remained, which was filtered through a 0.45 μm nylon filter. The remaining solvent was evaporated under vacuum until only a few milliliters remained, which was transferred into a 2 mL HPLC vial before evaporation of the

remaining solvent under nitrogen. After evaporation, dark brown impurities were observed in the sample, which were likely from SBF. The impurities were removed by adding 3 × 1 mL mobile phase (90% heptane and 10% isopropanol with both containing 0.1% v/v acetic acid) as the impurities were not soluble in this solution. The collected solvent was evaporated under nitrogen gas, and redissolved in 1 mL mobile phase. This sample was then purified by semi-preparative HPLC. To achieve a high recovery of products during purification steps, chloroform was used for rinsing, after sample drying, filtering and transferring to ensure minimal loss of products.

The optimised 10 mL sfLOX-catalysed DHA reaction (Chapter 3) was modified and applied to the large-scale synthesis of mono-hydroxy DHA compounds and analogues from LC-PUFAs under the following conditions (10 times scale up): 100 mL borate buffer (pH 9, 50 mM), 0.2 mM LC-PUFAs (from 20 mM stock in ethanol), 500 µL SBF extract (1 g/20 mL water), ambient temperature, 15 min reaction time. The reaction was stopped after 15 min by adding 2 mL sodium borohydride 1 M in water with stirring for 15 min. The reaction mixture was acidified by adding 500 µL acetic acid and stirred for 30 min. Solvent extraction (3 × 100 mL chloroform) was used to isolate the products from the reaction mixtures. Further steps from washing to evaporation were performed in the same way as above for the di-hydroxy compound synthesis. As less SBF was used, only insignificant amounts of the impurities were extracted with the mono-hydroxy products. The optimised 10 mL 15-sLOX-1 and DHA reaction in a previous study by Dobson et al. [43] was also used to scale up (15 times) for the large-scale synthesis of 17-HDHA using 15-sLOX-1.

2.3. Resolvin analogue purification by NP-HPLC

Mono- and di-hydroxy resolvin and protectin analogues were purified with a NP-HPLC system, an Agilent Technologies 1200 series HPLC, including a solvent degasser, quaternary pump, autosampler and diode array detector (DAD). A semi-preparative diol-120-NP column (YMC-Pack, 250 × 10 mm, 5 μm particle size) was used. The mobile phase composition was 90% v/v solvent A (heptane with 0.1% v/v acetic acid, 0.1% v/v 2,2-dimethoxypropane) and 10% v/v solvent B (isopropanol). Samples were separated under isocratic conditions within 60 min at 10°C with a flow rate of 2.5 mL/min. Initially, 2 μL of each sample was injected to confirm the presence and retention time of the appropriate hydroxylated compounds. To purify products, samples were injected in increasing volume from 20 – 100 μL if the column pressure did not exceed 200 bar, until 1 mL of each sample was purified. Each hydroxylated product was collected manually from a short tube after the UV-Vis detector when the peak response was detected on the chromatogram. The solvent from the total collected sample for each compound was evaporated under vacuum until approximately two millilitres remained. This volume was transferred to a pre-weighed 2 mL HPLC vial. The remaining solvent was evaporated under a stream of nitrogen gas until sample was completely dry. The vial and sample were weighed to determine the amount of purified compound that had been collected. Generally, less than 10 mg of products was collected.

For bioassays using human monocytic cells THP-1, the purified products were dissolved in 1 mL mobile phase and analysed with NP-HPLC using the same conditions as above to confirm the purity. Then the solvent was evaporated and the samples were re-dissolved in DMSO at a concentration of 60 mg/mL. For the mouse brain cell (C8-B4) assay, purified products were dissolved in DMSO at a concentration of 50 mM. To confirm compound purity, 4 μL of each compound in

DMSO was taken, solvent evaporated and re-dissolved in 200 µL mobile phase, followed by analysis with NP-HPLC. All samples were stored at -80°C when not in use. The fatty acid precursors were prepared at the same concentration as the resolvin analogues in DMSO and purified following the same procedure if high amounts of autooxidation products were detected.

2.4. Bioassays

2.4.1. Human monocytic cells (THP-1)

PUFAs and resolvin analogues (60 mg/mL in DMSO) were sent to Dr Celine Deffrasnes (CSIRO) for bioassay testing with human monocytic cells (THP-1). The bioassays were conducted under the following experimental conditions: THP-1 monocytes were stimulated with 1 µg/mL LPS and co-incubated with precursor PUFAs (DHA, EPA, ETE, DPAn-3 and DPAn-6) or their resolvin analogues at a concentration of 50 µg/mL for 24h. For cell viability tests, alamar blue was added to the cells and the cells were incubated for 4h, then the absorbance (optical density (OD)) was measured at 570 nm and fluorescence intensity was measured with excitation at 530 – 560 nm and emission at 590 nm. Cells were also counted using a hemocytometer. For IL-1β and TNF-α expression bioassays, supernatants were collected and both cytokines were measured by ELISA (Elisakit.com). For 17-HDHA synthesised using 15-sLOX-1 (without purification), PUFAs and di-hydroxy resolvin analogues, assays were performed twice (IL-1β) or three times (TNF-α, high variability). IL-1β and TNF-α levels were measured twice with the mono-hydroxy resolvin analogues. All assays were performed in triplicate and compared to DMSO as the control. Prism from GraphPad was used to calculate p values for statistical analysis with a one-way anova Tukey post-test, and independent samples t-tests. Results were considered to be significantly different from the control if $p < 0.05$.

2.4.2. Mouse brain cells (C8-B4)

PUFAs and resolvin analogues (50 mM in DMSO) were sent to Dr Chiara Bortolasci (Metabolic Research Unit, Deakin University) for bioassay testing on mouse brain cells (C8-B4). The bioassays were performed under the following experimental conditions. PUFAs and resolvin analogues were conjugated with 10% (w/v) fatty acid free bovine serum albumin, filtered into sterile glass vials and stored at -80ºC prior to the treatment of the cells. C8-B4 microglial cells (ATCC®CRL-2540™) were cultured in Dulbecco Modified Eagle Medium – DMEM (Life Technologies) with 10% Fetal Bovine Serum – FBS (Life Technologies) and seeded onto 48-well plates at 3.3×10^4 cells/well for the measurement of TNF-α release. All cells were treated with LPS at a dose of 1 ng/mL, and with PUFAs and resolvin analogues at doses of 50, 100 or 200 µM (n = 3 – 6 replicates per treatment group), and incubated at 37°C for 24 hours. After 24 hours, supernatant was collected and stored at -80°C. Levels of TNF-α in the media were measured using ELISA (Mouse TNF-α Quantikine ELISA Kit, R&D Systems, Cat. No MTA00B) according to the manufacturer's instructions. Data were checked for normality using a Kolmogorov-Smirnov test. Mean differences between treatment groups were assessed using independent samples t-tests. SPSS Statistics 25.0 was used to calculate p value. Data were considered to be significantly different if $p < 0.05$.

3. Results

3.1. Purity of purified compounds

In total, 20 different PUFAs and resolvin analogues were tested in bioassays. The names of the precursors and their resolvin analogues with abbreviations are given in Table 6.1. The purity of each of the prepared compounds was estimated by peak area

of the compound compared to the total peak area including other impurities using

NP-HPLC.

Table 6.1: Full names and abbreviations of PUFA precursors and their resolvin analogues.

Number	Compound Name	Abbreviation
1	**All cis-5,8,11,14-eicosatetraenoic acid**	**ETE (AA)**
2	15-hydroxyeicosatetra-5Z,8Z,11Z,13E-enoic acid	15-HETE
3	5,15-dihydroxyeicosatetra-6E,8Z,11Z,13E-enoic acid	5,15-diHETE
4	8,15-dihydroxyeicosatetra-5Z,9E,11Z,13E-enoic acid	8,15-diHETE
5	**All cis-5,8,11,14,17-eicosapentaenoic acid**	**EPA**
6	15-hydroxyeicosapenta-5Z,8Z,11Z,13E,17Z-enoic acid	15-HEPA
7	5,15-dihydroxyeicosapenta-6E,8Z,11Z,13E,17Z-enoic acid	5,15-diHEPA
8	8,15-dihydroxyeicosapenta-5Z,9E,11Z,13E,17Z-enoic acid	8,15-diHEPA
9	**All cis-4,7,10,13,16-docosapentaenoic acid**	**DPAn-6**
10	17-hydroxydocosapenta-4Z,7Z,10Z,13Z,15E-enoic acid	17-HDPAn-6
11	7,17-dihydroxydocosapenta-4Z,8E,10Z,13Z,15E-enoic acid	7,17-diHDPAn-6
12	10,17-dihydroxydocosapenta-4Z,7Z,11E,13Z,15E-enoic acid	10,17-diHDPAn-6
13	**All cis-7,10,13,16,19-docosapentaenoic acid**	**DPAn-3**
14	17-hydroxydocosapenta-7Z,10Z,13Z,15E,19Z-enoic acid	17-HDPAn-3
15	7,17-dihydroxydocosapenta-8E,10Z,13Z,15E,19Z-enoic acid	7,17-diHDPAn-3
16	10,17-dihydroxydocosapenta-7Z,11E,13Z,15E,19Z-enoic acid	10,17-diHDPAn-3
17	**All cis-4,7,10,13,16,19-docosahexaenoic acid**	**DHA**
18	17-hydroxydocosahexa-4Z,7Z,10Z,13Z,15E,19Z-enoic acid	17-HDHA
19	7,17-dihydroxydocosahexa-4Z,8E,10Z,13Z,15E,19Z-enoic acid	7,17-diHDHA
20	10,17-dihydroxydocosahexa-4Z,7Z,11E,13Z,15E,19Z-enoic acid	10,17-diHDHA

3.1.1. Human monocytic cells (THP-1)

After purification, most compounds had high purity (> 95%) except for 5,15-diHETE, 7,17-diHDHA and 7,17-diHDPAn-6 (batch 1) with purity of approximately 85% as shown in Table 6.2.

Table 6.2: Purity of PUFAs and synthesised resolvin analogues for bioassays on THP-1 cells. Where multiple batches of a single compound were prepared, the batch number is given after the abbreviated compound name (eg. 5,15-diHEPA_**1**).

Abbreviation	Mass (mg)	Volume DMSO (μL)	Concentration (mg/mL)	Purity (%)
ETE (AA)	5.5	90	61.1	99
15-HETE	4.91	82	59.9	99
5,15-diHETE	2.2	35	62.9	87
8,15-diHETE	3.1	50	62.0	>99
EPA	6.6	110	60.0	99
15-HEPA	5.77	96	60.1	>99
5,15-diHEPA_1	1.5	25	60.0	99
5,15-diHEPA_2	3.51	60	58.5	97
8,15-diHEPA_1	0.6	10	60.0	>99
8,15-diHEPA_2	1.52	25	60.8	>99
DPAn-6	7.7	130	59.2	99
17-HDPAn-6	6.18	103	60.0	>99
7,17-diHDPAn-6_1	0.8	15	53.3	84
7,17-diHDPAn-6_2	1.55	25	62.0	99
10,17-diHDPAn-6_1	1.3	20	65.0	>99
10,17-diHDPAn-6_2	5.23	85	61.5	>99
DPAn-3	6.1	100	61.0	99
17-HDPAn-3	5.25	87.5	60.0	99
7,17-diHDPAn-3	3.5	60	58.3	>99
10,17-diHDPAn-3	2.0	35	57.1	>99
DHA	11	185	59.5	99
17-HDHA_1[a]	8.2	130	63.1	>99
17-HDHA_2[a]	5.79	96.5	60.0	>99
7,17-diHDHA	2.3	40	58.3	86
10,17-diHDHA	1.1	20	57.1	99

[a] 17-HDHA_1 was prepared using commercial 15-sLOX-1, with no HPLC purification after synthesis and solvent extraction. 17-HDHA_2 was prepared approximately 10 months after 17-HDHA_1, using sfLOX and was purified by HPLC after synthesis.

3.1.2. Mouse brain cells (C8-B4)

All of the synthesised compounds had a purity of at least 95%, except 10,17-diHDPAn-3 (86%) as shown in Table 6.3. Some compounds (5,15-diHETE and 10,17-diHDPAn-3) were obtained in smaller amounts (< 3 mg).

Table 6.3: Purity of PUFAs and synthesised resolvin analogues for bioassays on C8-B4 cells.

Abbreviation	Mass (mg)	Volume DMSO (µL)	Concentration (mM)	Purity (%)
ETE (AA)	9.38	616	50.0	>99
15-HETE	5.34	334	49.9	>99
5,15-diHETE	2.87	170.6	50.0	96
8,15-diHETE	5.37	320	49.9	>99
EPA	9.23	610	50.0	>99
15-HEPA	6.47	406	50.0	98
5,15-diHEPA	4.38	262	50.0	97
8,15-diHEPA	3.57	214	49.9	>99
DPAn-6	8.92	540	50.0	97
17-HDPAn-6	7.87	454	50.0	>99
7,17-diHDPAn-6	4.42	244	50.0	95
10,17-diHDPAn-6	6.44	356	49.9	>99
DPAn-3	9.31	564	49.9	98
17-HDPAn-3	7.26	420	49.9	96
7,17-diHDPAn-3	8.02	442	50.1	>99
10,17-diHDPAn-3	1.20	66.2	50.0	86
DHA	9.45	576	49.9	98
17-HDHA	6.75	392	50.0	>99
7,17-diHDHA	3.41	190	49.8	>99
10,17-diHDHA	6.93	384	50.1	97

3.2. Bioassays

3.2.1. Human monocytic cells (THP-1)

Cell viability

When determining the bioactivity of compounds, it is important to first measure the impact of the chemical compounds on cell function including metabolic activity, cell proliferation, and cell death. The alamar blue assay uses a redox indicator dye (resazurin) that changes colour in response to cellular metabolic activity. The

absorbance and fluorescence intensity of the reagent are directly proportional to the number of living cells present. Cells were treated with the PUFAs and mono- and di-hydroxy resolvin analogues under investigation, and the cell viability was measured using the alamar blue assay. Total cell number (cell count) can also give an indication of cell survival following treatment with a compound of interest. This was also performed after treatment of the cells with the compounds of interest.

Di-hydroxy resolvin analogues and precursor PUFAs

Initially, the impact of the precursor fatty acids and the di-hydroxy resolvin analogues on cell viability were tested (as well as 17-HDHA). Most compounds had little or no adverse effect on cell function (Figure 6.1). When metabolic activity was evaluated by the alamar blue assay, only 7,17-diHDHA, 17-HDHA and 7,17-diHDPAn-6 showed a reduction in absorbance compared to DMSO, indicating a decrease in cellular activity (Figure 6.1A). Interestingly, 2 compounds showed an increase in absorbance values suggesting an increase in metabolic activity in cells treated with 8,15-diHEPA and 8,15-diHETE (Figure 6.1A). On the other hand, when cell viability was evaluated by changes in alamar blue fluorescence intensity, only 5,15-diHETE showed a reduction in fluorescence intensity (Figure 6.1B), suggesting that this compound may adversely affect cell function. A second measure of cell viability is cell number. Only 2 compounds resulted in a decrease in cell counts, 17-HDHA and 5,15-diHETE (Figure 6.1C). Overall, although there is some variability between the assays, most of the tested compounds showed no effect on cell viability. The compounds that impacted cell function were 5,15-diHETE (by alamar blue fluorescence measurement, Figure 6.1B and cell count, Figure 6.1C), 17-HDHA (by alamar blue OD, Figure 6.2A and cell count, Figure 6.2C) and to a lesser extent, 7,17-diHDHA and 7,17-diHDPAn-6 (by alamar blue OD, Figure 6.2A).
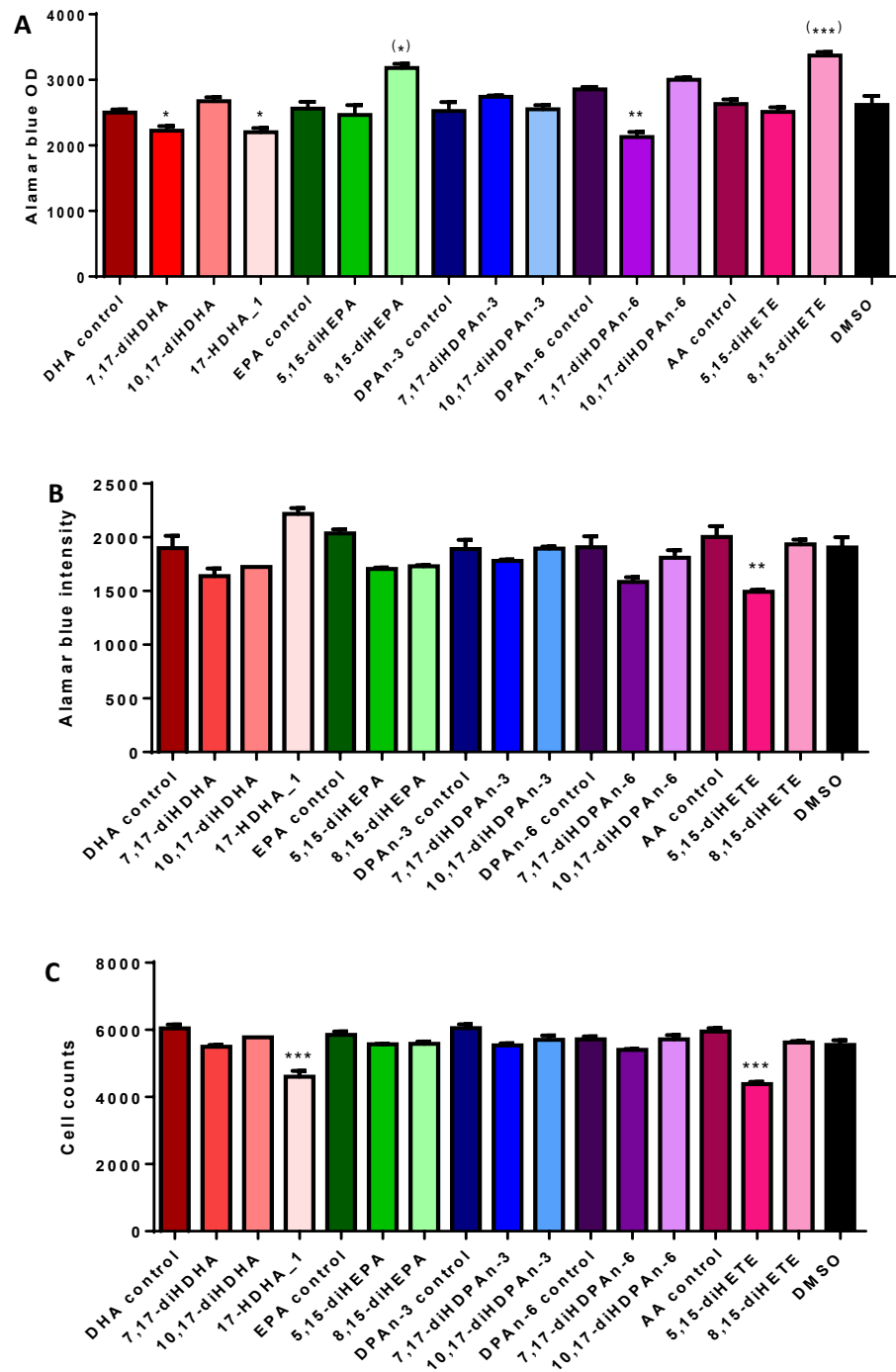
Figure 6.1: Cell viability of LPS-stimulated THP-1 monocytes co-incubated with PUFAs, di-hydroxy resolvins analogues and 17-HDHA_1 for 24 h, as measured by A) alamar blue absorbance (OD), B) alamar blue fluorescence intensity, C) cell counts. ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$, no indicator: not significant. Indicators in brackets: samples are significantly different from the controls but behave differently compared to other samples. Average results presented and error bars indicate + one standard error (n=3).

Mono-hydroxy resolvin analogues

The effects of the mono-hydroxy resolvin analogues on cell viability were tested following the same methodology. None of the mono-hydroxy resolvin analogues adversely impacted cell function as shown by alamar blue OD, fluorescence intensity and cell count measurements (Figure 6.2). There were some differences observed in cell viability between 17-HDHA_1 and 17-HDHA_2 (Figure 6.2), although these two compounds should have been chemically identical. 17-HDHA_1 caused a decrease in alamar blue absorbance and cell count compared to 17-HDHA_2. 17-HDHA_1 was prepared approximately 10 months before preparing 17-HDHA_2. It is possible that long term storage of this compound may have increased its impact on cell viability (potentially through the formation of breakdown products) and may have also affected its bioactivity. The alamar blue OD and cell count data obtained here for 17-HDHA_1 (Figures 6.2A and 6.2C) showed a reduction, but it was not statistically significant compared to the control. This could be because this assay was less reproducible with larger error bar than the previous one, therefore the results here were not significant.
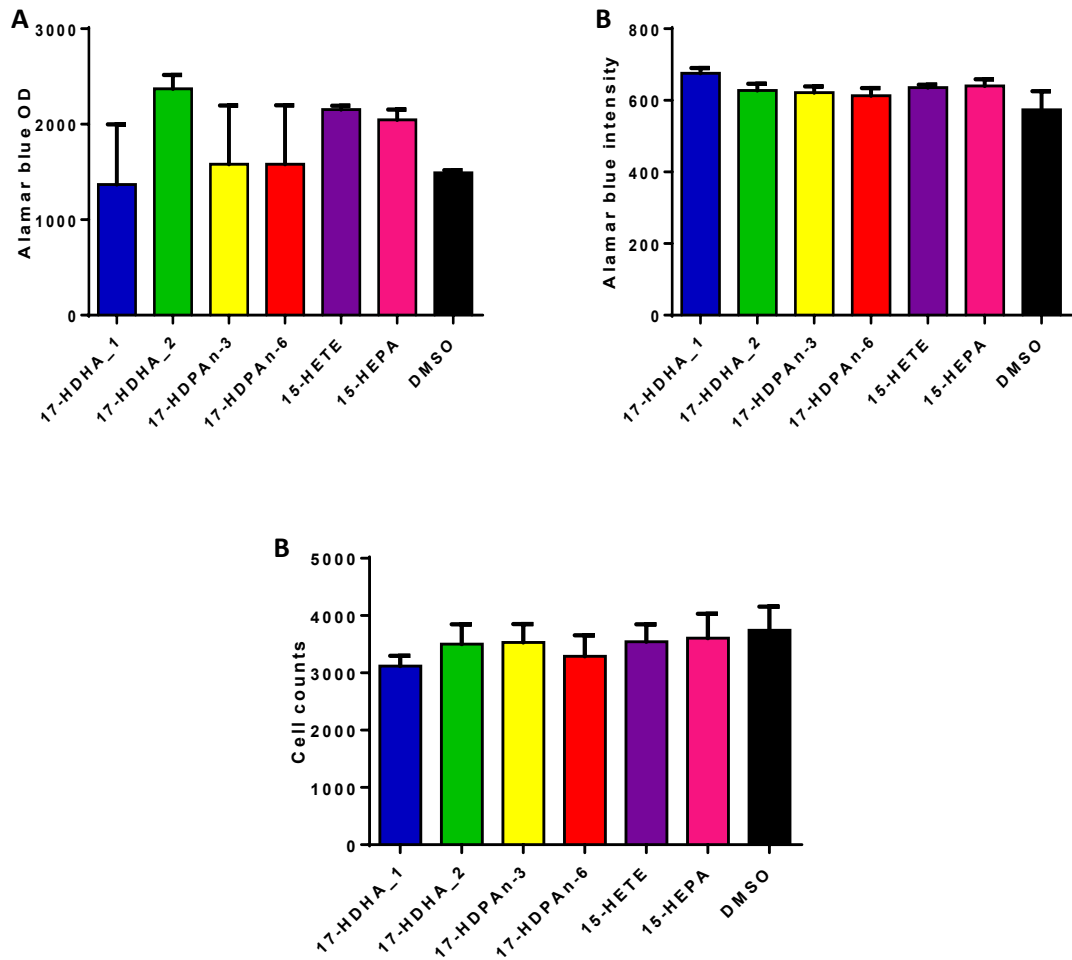
Figure 6.2: Cell viability of LPS-stimulated THP-1 monocytes co-incubated with mono-hydroxy resolvin analogues, as measured by A) alamar blue absorbance, B) alamar blue fluorescence intensity, and C) cell counts. No significant differences between results were observed. Average results presented and error bars indicate + one standard error (n=3).

IL-1β secretion

In the assay, LPS was used to stimulate an inflammatory response in human monocytic cells (THP-1). The inflammatory response can be monitored by measuring the secretion of inflammatory cytokines, such as IL-1β and TNF-α. All of the tested PUFAs and mono- and di-hydroxy resolvin analogues significantly reduced IL-1β secretion compared to the DMSO control in two separate assays, except for 10,17-diHDPAn-3, which did not result in a reduction in IL-1β levels in one of the two assays (Figure 6.3). Furthermore, the degree of IL-1β reduction was different between the compounds and there were some variations between the two assays (Figure 6.3). Overall, the results suggest that all the PUFAs and resolvin analogues (possibly except 10,17-diHDPAn-3) showed potential anti-inflammatory activity in the ability to reduce LPS-induced IL-1β secretion.

Figure 6.3: IL-1β secretion in LPS-stimulated THP-1 monocytes co-incubated with precursor PUFAs, 17-HDHA_1 and di-hydroxy resolvin analogues in A) the first and B) second assay, and mono-hydroxy resolvin analogues in C) the first and D) second assay. ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$, ns: not significant. Average results presented and error bars indicate + one standard error (n=2-4).

TNF-α secretion

The inflammatory cytokine, TNF-α, was also monitored in the LPS-stimulated THP-1 monocytes. There was significant variation in the results from three repeat assays of PUFAs, 17-HDHA and di-hydroxy resolvin analogues. The results of these three assays were combined after normalisation to DMSO, and are shown in Figure 6.4. In this graph, significant decreases in TNF-α secretion after treatment with 17-HDHA, 7,17-diHDPAn-6 and 5,15-diHETE are only indicative not representative, and the conditions for this specific assay need to be optimised further before conclusions can be drawn. However, two compounds, 7,17-diHDPAn-6 and 5,15-diHETE, resulted in reductions in TNF-α secretion consistently and significantly throughout the three assays (although to varying extents), suggesting anti-inflammatory properties.
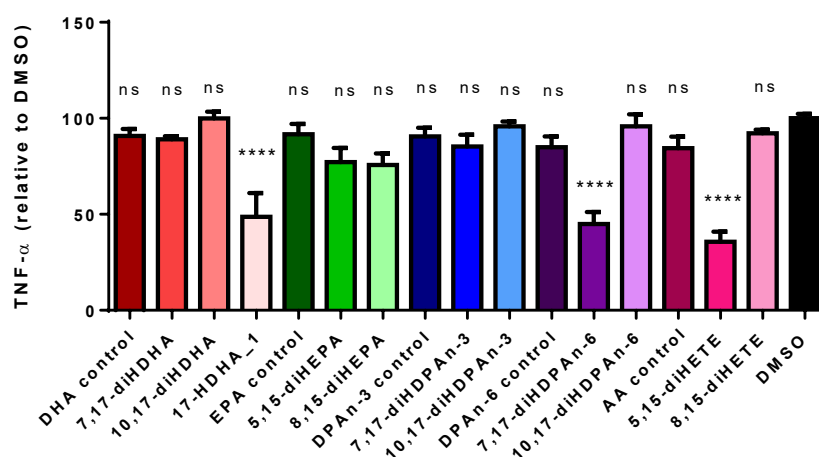
Figure 6.4: TNF-α secretion in LPS-stimulated THP-1 monocytes co-incubated with precursor PUFAs, 17-HDHA_1 and di-hydroxy resolvin analogues. Three separate assays are combined after normalisation to DMSO. ****: $p < 0.0001$, ns: not significant. Average results presented and error bars indicate + one standard error (n=10).

As for the mono-hydroxy resolvin compounds, a strong reduction of TNF-α secretion was observed with 17-HDHA_1 and 17-HDHA_2, 17-HDPAn-3 and 17-HDPAn-6, but the results were inconsistent between the 2 assays for 15-HETE and 15-HEPA (Figure 6.5). This suggests that 17-HDHA, 17-HDPAn-3 and 17-HDPAn-6 may

possess anti-inflammatory properties through their ability to suppress TNF-α secretion. A larger extent of TNF-α reduction was observed with old 17-HDHA compared to the new one.
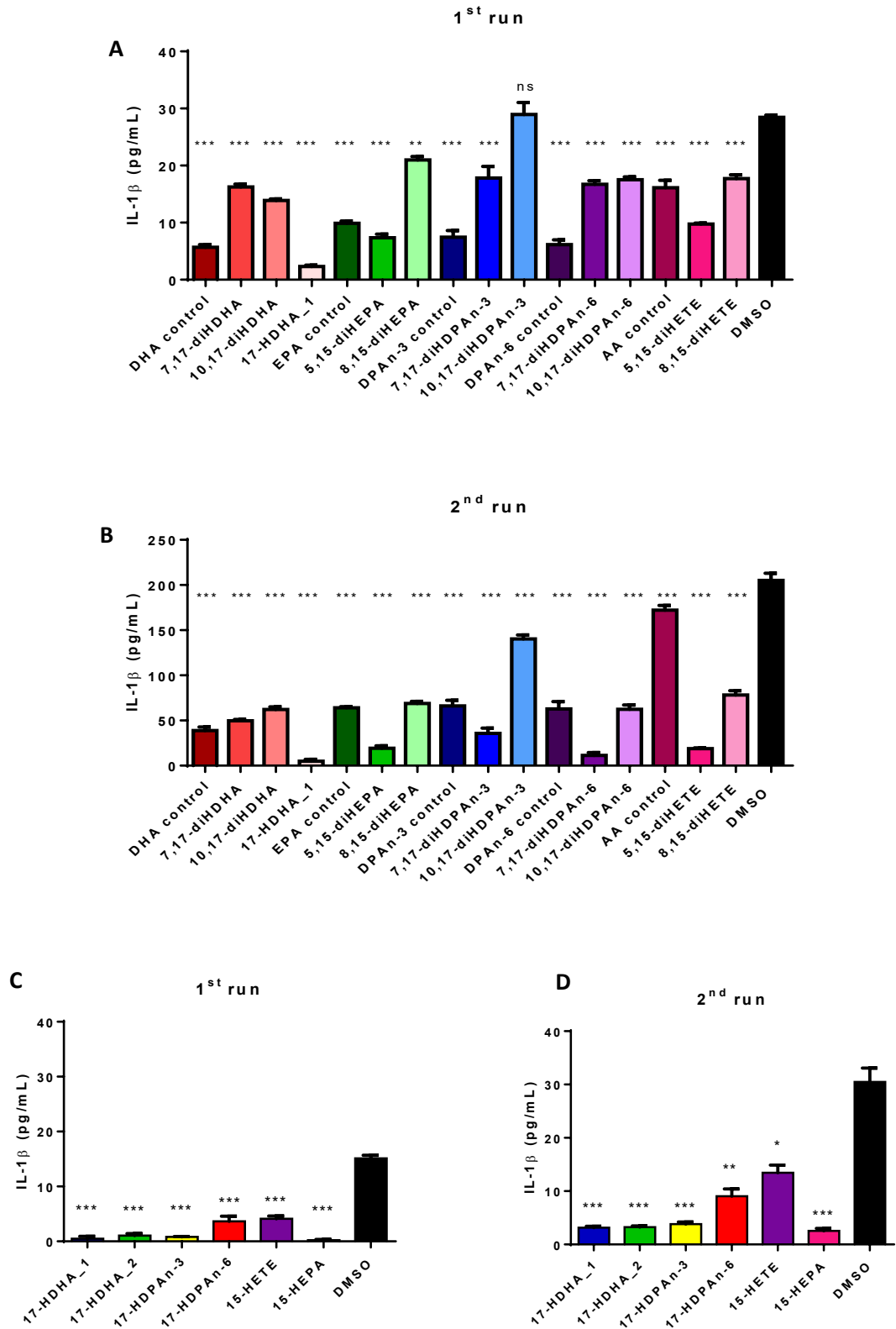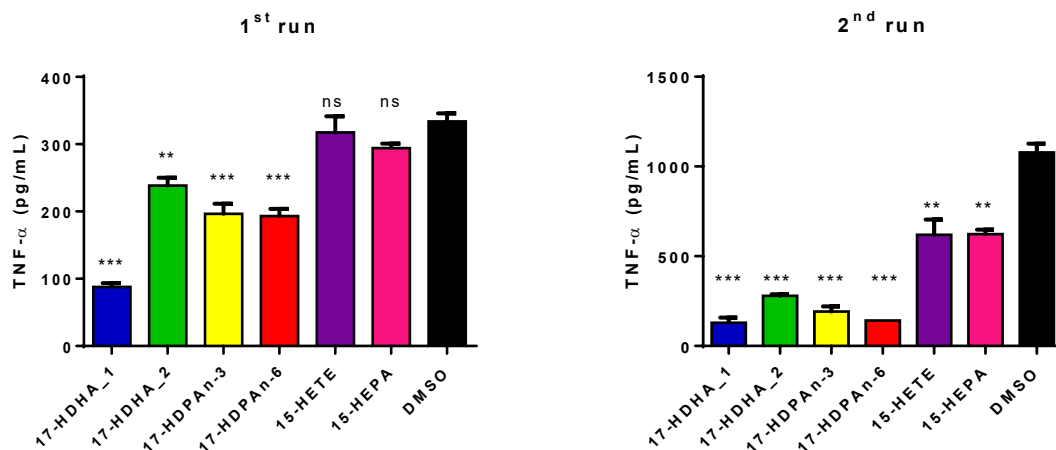

Figure 6.5: TNF-α secretion in LPS-stimulated THP-1 monocytes incubated with mono-hydroxy resolvin analogues. Two separate assays are shown. ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$, ns: not significant. Average results presented and error bars indicate + one standard error (n=1-3).

3.2.2. Mouse brain cells (C8-B4)

PUFAs and their resolvin analogues were also used to treat LPS-stimulated mouse brain cells (C8-B4). The levels of the inflammatory cytokines, IL-1β and TNF-α, were measured in the cells, however, no IL-1β secretion was detected in either treated or untreated cells (for unknown reasons), so only results for TNF-α secretion are reported. All PUFAs and resolvin analogues were tested at three concentrations (50, 100 and 200 μM). Testing with multiple doses can show a dose-dependent response, which is additional evidence of activity. For the data analysis, resolvins were grouped with their precursor PUFA; all compounds were compared with the vehicle, the mono-hydroxy molecule was compared with the precursor PUFA, and the two di-hydroxy molecules were compared with the corresponding mono-hydroxy compound. These comparisons were used to identify possible structure-activity relationships between the compounds and their effects on TNF-α secretion.

DHA group

As compared to vehicle, DHA and 17-HDHA did not impact TNF-α secretion except for a non-significant decrease in the TNF-α level at 100 μM DHA ($p = 0.071$) (Figure 6.6A). The two di-hydroxy DHA compounds decreased TNF-α levels significantly at certain concentrations. 7,17-diHDHA reduced TNF-α secretion at 50 μM ($p = 0.048$) and 200 μM ($p = 0.024$), whilst 100 μM and 200 μM doses of 10,17-diHDHA were both shown to decrease TNF-α levels ($p = 0.024$ for both) (Figure 6.6B). 17-HDHA also did not affect TNF-α secretion at any dose compared to the precursor DHA (Figure 6.6C). Both di-hydroxy DHA compounds (7,17-diHDHA and 10,17-diHDHA) were shown to decrease TNF-α secretion at certain concentrations compared to 17-HDHA. Similarly, 7,17-diHDHA reduced TNF-a levels significantly at low and high doses of 50 and 200 μM ($p = 0.002$ for both), whereas 10,17-diHDHA suppressed TNF-α secretion significantly at the higher doses of 100 and 200 μM ($p = 0.004$ and 0.002, respectively), and also tended to reduce TNF-α secretion at the low dose of 50 μM ($p = 0.093$) (Figure 6.6D). These results suggest that the two di-hydroxy DHA compounds possess anti-inflammatory properties, and the chemical modification of DHA to produce both di-hydroxy DHA compounds (but not the mono-hydroxy DHA) increased their anti-inflammatory properties.

Figure 6.6: TNF-α levels in C8-B4 treated cells with A) DHA and 17-HDHA in comparison with vehicle, B) 7,17-diHDHA and 10,17-diHDHA compared to vehicle, C) 17-HDHA compared to DHA, D) 7,17-diHDHA and 10,17-diHDHA compared to 17-HDHA at different doses of 50, 100 and 200 µM. **: $p < 0.01$, *: $p < 0.05$, no indicator: not significant. Average results presented and error bars indicate + one standard error (n=3-6).

DPAn-3 group

In comparison to the vehicle, all DPAn-3 compounds affected TNF-α secretion at different levels. 17-HDPAn-3 decreased TNF-α secretion significantly at all concentrations ($p = 0.024$ for 50 and 200 µM, $p = 0.036$ for 100 µM), whereas TNF-α concentration tended to decrease with DPAn-3 only at 200 µM ($p = 0.095$) (Figure 6.7A). 10,17-diHDPAn-3 decreased TNF-α secretion significantly at 100 and 200 µM ($p = 0.024$ for both), whereas 7,17-diHDPAn-3 slightly decreased TNF-α levels only at 200 µM (p = 0.095) but the result was not significant (Figure 6.7B). As a result, 17-HDPAn-3 possesses anti-inflammatory property at all doses, whereas the other compounds showed some anti-inflammatory behaviour but the results were inconclusive.
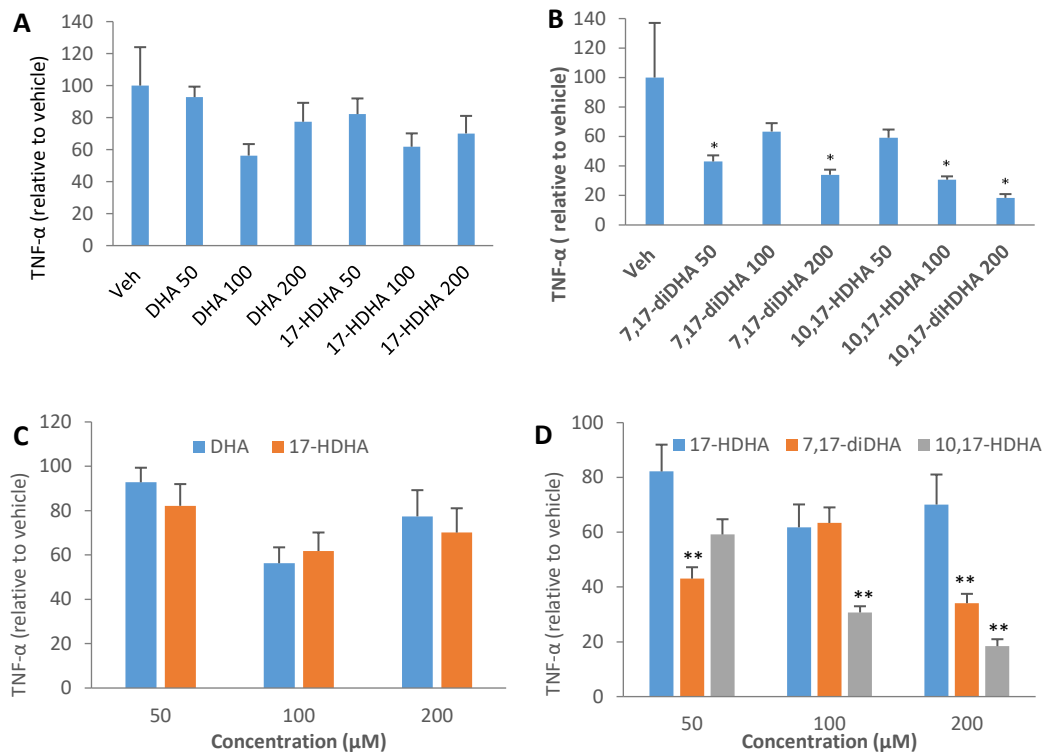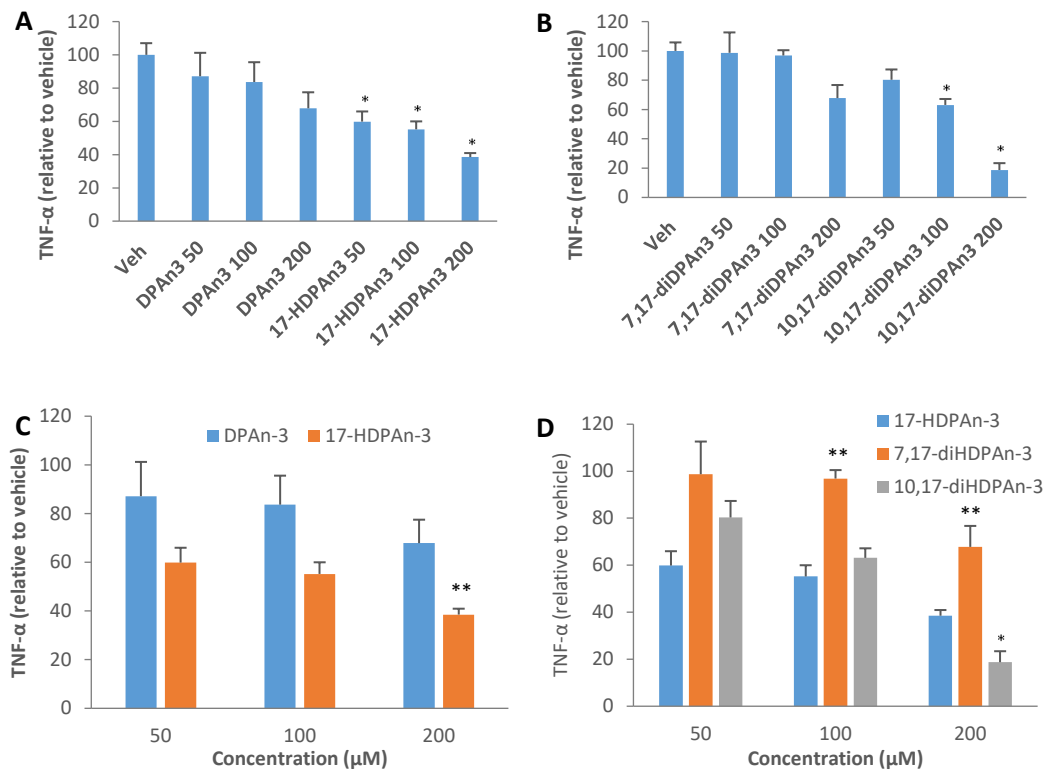
Figure 6.7: TNF-α levels in C8-B4 treated cells with A) DPAn-3 and 17-HDPAn-3 in comparison with vehicle, B) 7,17-diHDPAn-3 and 10,17-diHDPAn-3 compared to vehicle, C) 17-HDPAn-3 compared to DPAn-3, D) 7,17-diHDPAn-3 and 10,17-diHDPAn-3 compared to 17-HDPAn-3 at different doses of 50, 100 and 200 µM. **: $0.001 < p < 0.01$, *: $0.01 < p < 0.05$, no indicator: not significant. Average results presented and error bars indicate + one standard error (n=3-6).

17-HDPAn-3 at the concentration of 200 µM ($p = 0.002$) was found to decrease TNF-α levels and at the concentration of 100 µM ($p = 0.056$) tended to decrease TNF-α levels compared to the precursor DPAn-3, which suggests that 17-HDPAn-3 has higher anti-inflammatory activity than DPAn-3 (Figure 6.7C). In comparison with 17-HDPAn-3, 7,17-diHDPAn-3 showed less anti-inflammatory activity at all doses of 50, 100 and 200 µM ($p = 0.065$, 0.004 and 0.002 respectively) by increasing TNF-α levels, although the result at 50 µM was not statistically significant, whereas 10,17-diHDPAn-3 showed more anti-inflammatory activity by decreasing TNF-α secretion at the highest dose of 200 µM ($p = 0.026$), but showed a trend towards less anti-inflammatory activity by increasing TNF-α levels at the lowest concentration of 50 µM ($p = 0.052$) (Figure 6.7D). Both di-hydroxy compounds of DPAn-3 had

different anti-inflammatory levels compared to 17-HDPAn-3, in which 7,17-diHDPAn-3 was less anti-inflammatory than 17-HDPAn-3, whereas 10,17-diHDPAn-3 generally seemed to be more anti-inflammatory than 17-HDPAn-3. The results suggest that adding a hydroxy group at position C17 improves the anti-inflammatory properties of DPAn-3 at all doses. However, adding a second hydroxy group to form di-hydroxy DPAn-3 tends to decrease the anti-inflammatory properties.

ETE group

When compared with vehicle, none of the compounds in the ETE group changed TNF-α levels significantly, at any dose, indicating they may not have anti-inflammatory activity (Figures 6.8A and B). 15-HETE did not change TNF-α levels at any dose when compared with ETE (Figure 6.8C). Neither of the di-hydroxy compounds, 5,15-diHETE or 8,15-diHETE, affected TNF-α secretion significantly compared to 15-HETE, although 5,15-diHETE seemed to decrease TNF-α levels slightly at all doses (Figure 6.8D). Overall these results do not show significant anti-inflammatory activity from the ETE derived compounds. Furthermore, some of the responses from these compounds show higher levels of TNF-α secretion compared to the vehicle, although the results are not significant. Adding one hydroxy group at C15 appeared to slightly increase anti-inflammatory activity, and adding a second hydroxy group at C5 may further increase the anti-inflammatory activities, but not significantly.
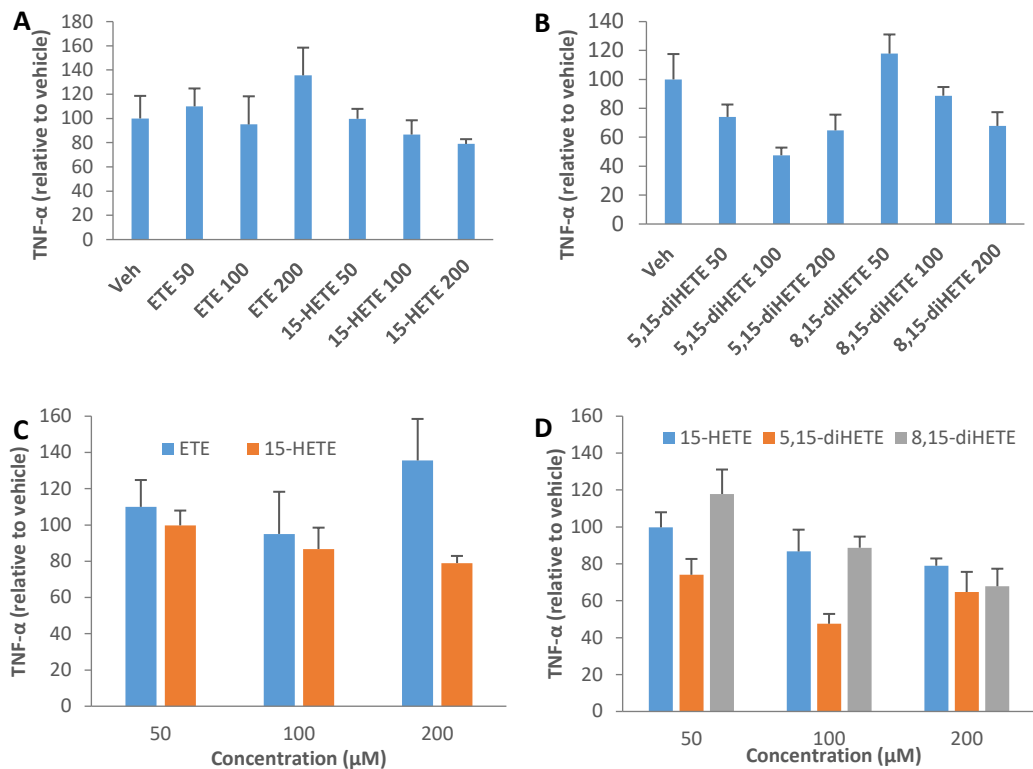
Figure 6.8: TNF-α levels in C8-B4 treated cells with A) ETE and 15-HETE in comparison with vehicle, B) 5,15-diHETE and 8,15-diHETE compared to vehicle, C) 15-HETE compared to ETE, D) 5,15-diHETE and 8,15-diHETE compared to 15-HETE at different doses of 50, 100 and 200 µM. No significant differences between results were observed. Average results presented and error bars indicate + one standard error (n=3-6).

EPA group

Compared with the vehicle, EPA increased TNF-α secretion at 100 and 200 µM ($p = 0.024$ for both) and 15-HEPA also increased TNF-α levels at 50 µM ($p = 0.036$) and 100 µM ($p = 0.024$) (Figure 6.9A). However, 5,15-diHEPA did not impact the secretion of TNF-α at any dose, and 8,15-diHEPA only reduced TNF-α secretion significantly at the highest dose of 200 µM ($p = 0.048$) (Figure 6.9B). These results suggest that both EPA and 15-HEPA have pro-inflammatory properties whilst 8,15-diHEPA has some anti-inflammatory activity.

Figure 6.9: TNF-α levels in C8-B4 treated cells with A) EPA and 15-HEPA in comparison with vehicle, B) 5,15-diHEPA and 8,15-diHEPA compared to vehicle, C) 15-HEPA in comparison with EPA, D) 5,15-diHEPA and 8,15-diHEPA compared to 15-HEPA at different doses of 50, 100 and 200 μM. **: $p < 0.01$, *: $p < 0.05$, no indicator: not significant. Average results presented and error bars indicate + one standard error (n=3-6).

Within the EPA group, 15-HEPA seems to have higher pro-inflammatory activity than EPA by increasing TNF-α secretion at concentrations of 50 and 100 μM ($p = 0.016$ and $p = 0.009$ respectively) when comparing these two compounds (Figure 6.9C). Both 5,15-diHEPA and 8,15-diHEPA were found to significantly decrease TNF-α levels at all doses compared to 15-HEPA ($0.001 < p < 0.01$), indicating the two di-hydroxy compounds are more anti-inflammatory than the pro-inflammatory mono-hydroxy EPA (Figure 6.9D). The results suggest that adding one hydroxy group makes EPA more pro-inflammatory, whilst adding another hydroxy group counteracts the pro-inflammatory properties of 15-HEPA.

DPAn-6 group

In comparison with the vehicle, only 7,17-diDPAn-6 reduced TNF-α level significantly at 50 and 200 μM doses ($p = 0.024$ for both), indicating that it is anti-inflammatory (Figures 6.10A and B). When compared to DPAn-6, 17-HDPAn-6 was found to decrease TNF-α secretion at a dose of 200 μM ($p = 0.009$) (Figure 6.10C). When compared to 17-HDPAn-6, 7,17-diHDPAn-6 reduced TNF-α levels at a concentration of 200 μM ($p = 0.026$), whereas 10,17-diHDPAn-6 tended not to decrease TNF-α secretion as much as 17-HDPA did (Figure 6.10D). The results indicate that adding hydroxy groups on DPAn-6 to form 17-HDPAn-6 and 7,17-diHDPAn-6 increased the anti-inflammatory properties. However, adding a second hydroxy group to 17-HDPAn-6 to form 10,17-diHDPAn-6 may reduce these anti-inflammatory properties.
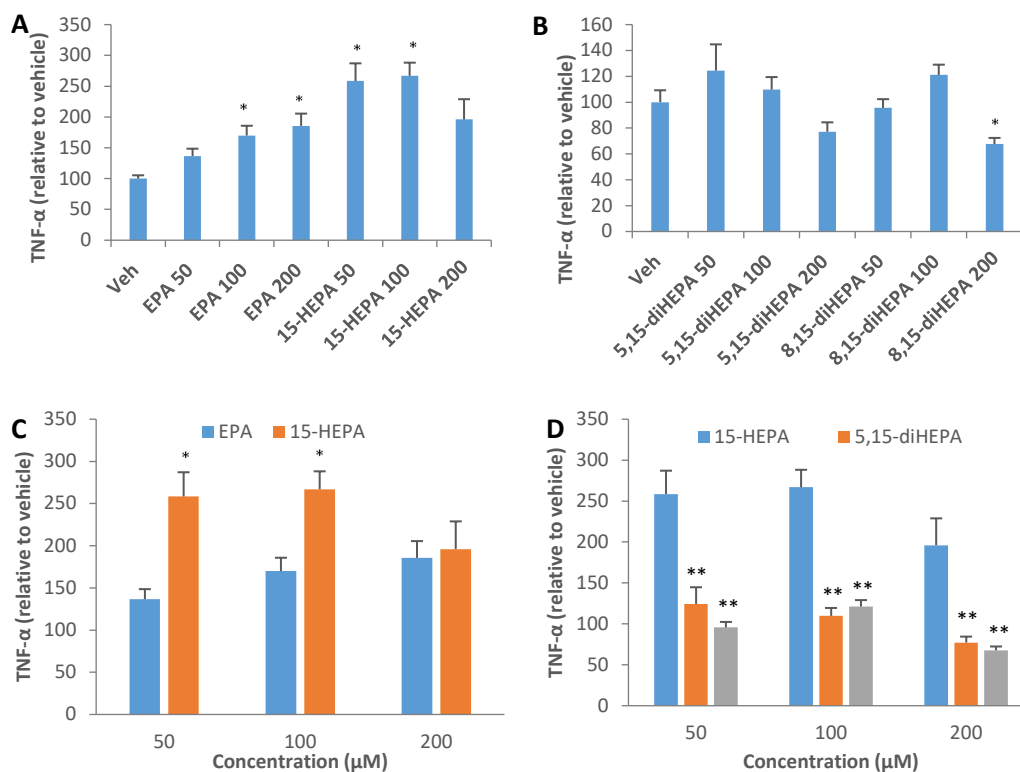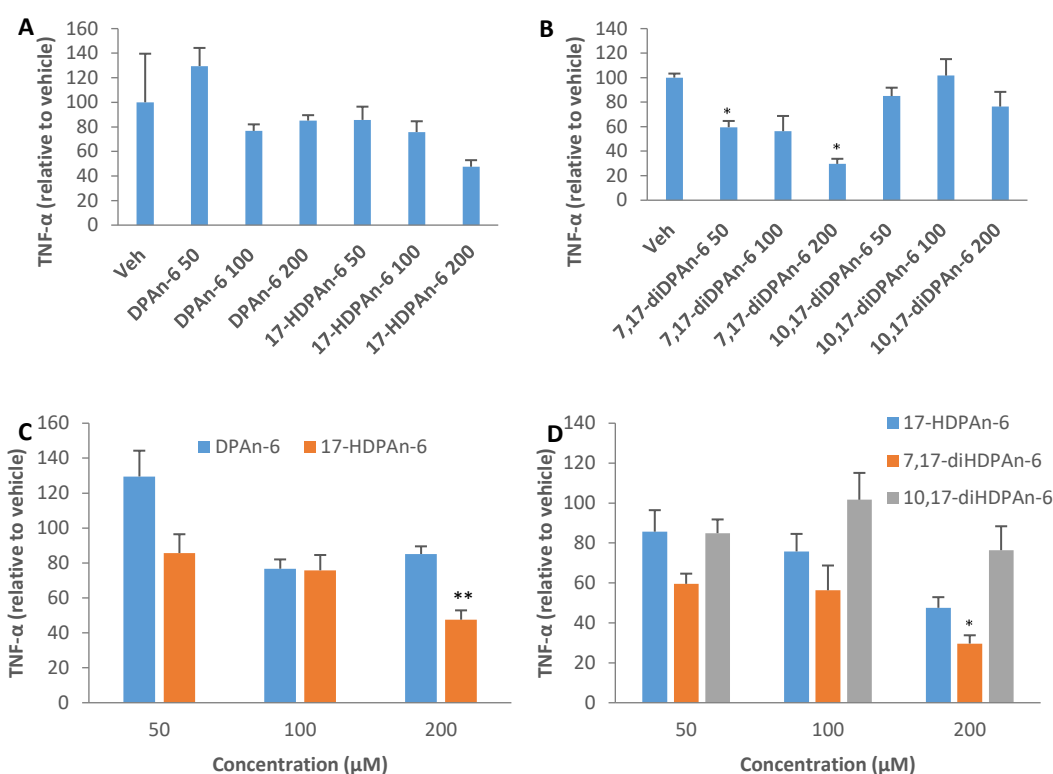

Figure 6.10: TNF-α levels in C8-B4 treated cells with A) DPAn-6 and 17-HDPAn-6 in comparison with vehicle, B) 7,17-diHDPAn-6 and 10,17-diHDPAn-6 compared to vehicle, C) 17-HDPAn-6 compared to DPAn-6, D) 7,17-diHDPAn-6 and 10,17-diHDPAn-6 compared to 17-HDPAn-6 at different doses of 50, 100 and 200 μM. **: $p < 0.01$, *: $p < 0.05$, no indicator: not significant. Average results presented and error bars indicate + one standard error (n=3-6).

During the assays, cells were observed for any changes after treatment with the PUFAs and analogues. At 200 µM EPA altered cell shapes were observed and dead cells were found after treatment with 200 µM DPAn-6, so these two PUFAs appeared to be toxic at this dose. For both THP-1 and C8-B4 cell line assays, the anti-inflammatory activity of the PUFAs and resolvin analogues in each group are summarised based on structure-activity relationship in Table 6.4.

Table 6.4: Anti-inflammatory activity of each PUFA group (IL-1β level in THP-1 treated cells, TNF-α in THP-1 and C8-B4 treated cells). PUFA: PUFA versus vehicle, Mono-hydroxy compound: mono-hydroxy PUFA versus PUFA, Di-hydroxy isomer 1: di-hydroxy PUFA with 10 carbons between two hydroxy groups versus mono-hydroxy PUFA and Di-hydroxy isomer 2: di-hydroxy PUFA with 7 carbons between two hydroxy groups versus mono-hydroxy PUFA. ↑: increase of anti-inflammatory activity, ↓: decrease of anti-inflammatory activity and '-': no change in anti-inflammatory activity. ( ): tending to increase or decrease anti-inflammatory activity (not significant).

	PUFA			Mono-hydroxy compound			Di-hydroxy isomer 1			Di-hydroxy isomer 2		
	THP-1 IL-1β	THP-1 TNF-α	C8-B4 TNF-α	THP-1 IL-1β	THP-1 TNF-α	C8-B4 TNF-α	THP-1 IL-1β	THP-1 TNF-α	C8-B4 TNF-α	THP-1 IL-1β	THP-1 TNF-α	C8-B4 TNF-α
DHA	↑	-	(↑)	↑	↑	-	↓	↓	↑	↓	↓	↑
DPAn-3	↑	-	(↑)	↑	↑	↑	↓	↓	↓	↓	↓	↑
EPA	↑	-	↓	↑	↑	↓	↓	-	↑	↓	-	↑
DPAn-6	↑	-	-	-	↑	↑	-	-	↑	(↓)	↓	(↓)
ETE	↑	-	-	↑	-	(↑)	(↑)	↑	(↑)	(↓)	-	-

PUFAs were compared to the vehicle. Mono-hydroxy PUFAs were compared to the corresponding PUFA. Both di-hydroxy products were compared with the corresponding mono-hydroxy PUFA. Changes in anti-inflammatory activity between the chemical structures of PUFAs, mono-hydroxy and di-hydroxy PUFA compounds are discussed in detail in the discussion section below. The potential structure-

activity relationships were identified through the comparison across different groups, cell lines and anti-inflammatory assays.

4. Discussion

Cell viability of human monocytic cells (THP-1)

The cell viability assays showed that all of the precursor PUFAs, and most of the mono- and di-hydroxy resolvin analogues had no adverse effect on cell function. However, one mono-hydroxy compound (17-HDHA) and three di-hydroxy compounds (7,17-diHDHA, 7,17-diHDPAn-6 and 5,15-diHETE) showed some impacts on cell viability, but the results were inconclusive as the results between the three sets of cell viability assays were inconsistent. 17-HDHA and 5,15-diHETE showed an adverse effect in two of the cell viability assays, whereas 7,17-diHDHA and 7,17-diHDPAn-6 showed an adverse effect on only one cell viability assay. Previous studies have assessed the effects of several PUFAs, SPMs and analogues on cell viability using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay, which is similar to the alamar blue assay but uses a different reagent [281-283]. Hamid and co-workers reported that the alamar blue assay (the method used in this study), was found to be more sensitive in detecting cytotoxic effects of most tested drug compounds (117 drugs) than the MTT assay [284].

PUFAs are a part of the normal human diet, so it is expected that they would not show any effect on cell viability at the tested concentration of 50 µg/mL (approximately 150 µM). Some previous studies with THP-1 cell lines also showed that DHA and EPA did not affect cell viability in the concentration range of 6.25 – 100 µM [282] or at concentrations of 10, 50 and 75 µM [283]. However, the possible impact on cell viability of the four resolvin analogues listed above was unexpected, as SPMs are endogenously synthesised from PUFAs in the body and typically show

anti-inflammatory and pro-resolving activity. Various SPMs and analogues such as Resolvin E1, Resolvin D1 and Maresin R1 have not shown cytotoxicity or impacted viability of various cell lines [82, 281, 285, 286]. Due to the potential bioactivity of these compounds, a number of studies have focused on using PUFAs and SPMs to reduce cytotoxicity of toxic agents on normal cells or to induce cytotoxicity in tumour cells [287-290]. For example, Resolvin D1 showed no cytotoxicity on human gingival fibroblasts (HGFs), and it decreased the cytotoxicity of ethanol on HGFs and inhibited the cytotoxic effects of bacteria such as Porphyromonas gingivalis on HGFs [288]. DHA and 17-hydroperoxy DHA were found to induce higher cytotoxicity than 17-HDHA in human neuroblastoma cells (tumorigenic neural cells) [287].

The possible impact on cell viability from the compounds must be considered when interpreting the results of the anti-inflammatory assays, as a decrease in IL-1β and TNF-α secretion may be due to an increase in the number of dead cells rather than from actual anti-inflammatory activity. This is because a decrease in the number of living cells or an impact on cell function, could cause a decrease in IL-1β and TNF-α levels. The two di-hydroxy compounds (8,15-diHEPA and 8,15-HETE) showed an increase in the number of living cells in the alamar blue OD assay, suggesting these compounds have a positive impact on cell growth. Cell proliferation assays of the two molecules would be necessary in future work. However, there are also other possibilities for this finding; these two compounds may increase enzyme activity in viable cells inducing the redox reaction of the indicator dye (resazurin) or the dye may be contaminated with the reduced compound (resorufin), resulting in an increase of the alamar blue OD value but not directly due to cell growth. Furthermore, 7,17-diHDHA and 7,17-diHDPAn-6 may inhibit the enzyme activity causing the decrease only in alamar blue OD value but not in the cell count assay. As for the alamar blue

assay, measurement with absorbance seemed to be more sensitive to detect adverse effects on cell function than with fluorescence. There was quite high variability in the alamar blue OD results for some of the mono-hydroxy compounds (17-HDHA_1, 17-HDPAn-3 and 17-HDPAn-6). The alamar blue assay is sensitive to a range of experimental conditions such as temperature, pH, light, cell density, incubation time and microbial contamination [291], and therefore this may contribute to high variation in these results.

Anti-inflammatory activity

Human monocytic cells (THP-1)

Almost all PUFAs and resolvin analogues tested, demonstrated potential anti-inflammatory activity through a reduction in IL-1β secretion. Although the assay results showed variation in the extent of the reduction for each compound across duplicate assays, only 10,17-diHDPAn-3 failed to show a statistically significant reduction in one of the two assays. The results for TNF-α were more selective with only seven of the twenty tested compounds showing a reduction in TNF-α secretion. All five mono-hydroxy compounds showed anti-inflammatory activity, as well as two di-hydroxy compounds (7,17-diHDPAn-6 and 5,15-diHETE). None of the PUFAs showed a reduction in TNF-α secretion. However, the results for two of the mono-hydroxy compounds (15-HETE and 15-HEPA) were not replicated across the two assays. Furthermore, the effect observed for 5,15-diHETE in reducing TNF-α (and IL-1β) secretion could also have been caused by the reduction in cell numbers and the impact on cell function seen for this compound and may not be related to anti-inflammatory activity. In this cell line, the anti-inflammatory activity of all five mono-hydroxy compounds was preserved in both IL-1β and TNF-α assays, whereas all five PUFAs and almost di-hydroxy compounds only showed anti-inflammatory activity in the IL-1β assay.

Two preparations of 17-HDHA were used in the THP-1 cell assays (labelled 17-HDHA_1 and 17-HDHA_2). As can be seen from the results, the responses from these two compounds were not the same despite being chemically identical; 17-HDHA_1 caused a much greater reduction in TNF-α secretion than 17-HDHA_2 (although the difference from the control was not statistically significant). The reason for this is unclear, however, 17-HDHA_1 was prepared using commercial soybean LOX-1 approximately 10 months before 17-HDHA_2 was prepared from soybean flour LOX. Unlike the 17-HDHA prepared from soybean flour LOX, the 17-HDHA prepared from commercial soybean LOX-1 did not require purification by HPLC after synthesis and extraction. Whilst the major component in both preparations (17-HDHA, purity >99%) was the same, it is possible that one of the differences (storage time, enzyme used in synthesis, or purification method), has resulted in differences in trace impurities between the two preparations (although this was not observed in the HPLC results), which has affected the assay results. Long term storage of PUFAs and resolvin analogues may affect their impacts on cell viability and/or their bioactivity, so further investigation would be beneficial.

Mouse brain cells (C8-B4) and comparison with human monocytic cells (THP-1)
Unlike in the inflammatory assays employing THP-1 cells, where all PUFAs showed a reduction in IL-1β but not TNF-α secretion, different levels of inflammatory activities (anti-inflammatory, pro-inflammatory and neutral) for PUFAs were observed in TNF-α secretion for C8-B4 cells. DHA tended to have anti-inflammatory activity, showing a reduction in TNF-α levels at all doses but failed to have significantly statistical results. DPAn-3 only tended to have anti-inflammatory activity at the highest dose of 200 μM, whereas EPA unexpectedly showed pro-inflammatory activity at high concentrations. ETE and DPAn-6 did not show any inflammatory activity.

DHA and EPA have received more interest in investigating anti-inflammatory activity compared to other PUFAs in the literature. Many previous studies with THP-1 cells have demonstrated the anti-inflammatory activity of DHA and EPA through the decrease of a range of cytokines including TNF-α, IL-1β, IL-6 and MCP-1 [282, 283, 292]. Moreover, in LPS stimulated human peripheral blood mononuclear cells, DHA, EPA and DPAn-6 were shown to decrease TNF-α and IL-1β production, whereas ETE did not affect the concentration of these two cytokines [293]. However, in this work, DHA, EPA and other PUFAs (ETE, DPAn-3 and DPAn-6) were found to reduce IL-1β levels but not TNF-α levels in LPS treated THP-1 cells. In part agreement with these findings, Mullen and colleagues found that DHA reduced IL-1β levels significantly but did not affect TNF-α secretion in LPS-treated THP-1 cells, however, EPA was found to significantly decrease both IL-1β and TNF-α levels [292]. In a previous study, DPAn-3, EPA and DHA showed anti-inflammatory activity in significantly reducing another cytokine IL-6 secretion in LPS-stimulated RAW264.7 cells [294]. For the LPS treated C8-B4 cells in this study, DHA and DPAn-3 showed some anti-inflammatory activity in agreement with the studies mentioned above, but in contrast to those studies, EPA showed pro-inflammatory activity and DPAn-6 showed no activity. In agreement with a study by Nauroth et al. [293], ETE did not show anti-inflammatory behaviour in LPS treated C8-B4 cells. The anti-inflammatory activity of PUFAs may vary depending on cell lines and cytokines tested.

As for the mono-hydroxy PUFA compounds, almost all showed potential anti-inflammatory activity in decreasing TNF-α levels, which is similar to the results from the treated THP-1 cells. 17-HDPAn-3 and 17-HDPAn-6 showed a trend of decreasing TNF-α levels in the concentration range 50 – 200 µM, demonstrating a dose-dependent response. However, only 17-HDPAn-3 gave statistically significant

results (at all doses). These results matched with the anti-inflammatory activities observed in LPS-stimulated THP-1 monocytes, which showed a reduction of IL-1β and TNF-α levels, giving a strong indication of anti-inflammatory activity. As an exception, 15-HEPA showed potential pro-inflammatory activity, increasing TNF-α levels at low doses, which was not observed in THP-1 monocyte assays.

In support of the current work where all mono-hydroxy compounds tested showed anti-inflammatory activity through a reduction in both IL-1β and TNF-α secretion in the THP-1 cell line, 17-HDHA was previously found to significantly decrease TNF-α levels both *in vitro* and *in vivo* in a number of studies [67, 270, 295]. Other studies have also found that 17-HDHA, 17-HDPAn-6, 15-HEPA and 15-HETE have anti-inflammatory activity *in vitro* and *in vivo*. In a study by Chiu and colleagues, both 17-HDHA and 17-HDPAn-6 were found to decrease mRNA expression of TNF-α and iNOS in cell line RAW264.7 [270]. Dangi et al. showed that 17-HDHA and 17-HDPAn-6 had anti-inflammatory activity through a reduction of the leukocyte count in exudates *in vivo*, however, 17-HDHA did not show a statistically significant reduction [67]. 17-HDHA, 15-HEPA and 15-HETE *in vitro* were shown to have an inhibitory effect on 5-LOX activity, which can decrease the synthesis of the pro-inflammatory lipid mediator, LTB4 [261, 262]. 15-HETE can also activate a receptor to induce anti-inflammatory activity for neuroprotection in ischemic rat brain [275]. These studies also agree with the current results, where 17-HDPAn-6 showed anti-inflammatory activity in treated C8-B4 cells, but not the pro-inflammatory activity displayed by 15-HEPA or the inactivity of 17-HDHA and 15-HETE.

In C8-B4 treated cells, seven of ten di-hydroxy PUFAs had potential anti-inflammatory activity, showing a reduction of TNF-α secretion. Five compounds (7,17-diHDHA, 10,17-diHDHA, 7,17-diHDPAn-6, 10,17-diHDPAn-3 and 5,15-

HETE) showed a reduction of TNF-α secretion at all doses, whereas 7,17-diHDPAn-3 and 8,15-diHEPA only decreased TNF-α levels at the highest dose of 200 μM. Among these seven compounds, only 5,15-diHETE and 7,17-diHDPAn-3 failed to show statistically significant results. The reduction in TNF-α secretion by 10,17-diHDHA and 10,17-diHDPAn-3 were statistically significant at high doses of 100 and 200 μM, whereas the 7,17-diHDHA and 7,17-diHDPAn-6 reduction of TNF-α secretion was statistically significant at random doses of 50 and 200 μM. 7,17-diHDPAn-6 and 5,15-diHETE were also found to show promising anti-inflammatory activity in THP-1 treated cells in both IL-1β and TNF-α assays, providing a strong indication that these two compounds have anti-inflammatory activity. The other five compounds (7,17-diHDHA, 10,17-diHDHA, 7,17-diHDPAn-3, 10,17-diHDPAn-3 and 8,15-diHEPA) also showed activity in the IL-1β assay in treated THP-1 cells, but not in the TNF-α assay, so the results are less conclusive in showing anti-inflammatory activity.

RvD5$_{DHA}$, PDX$_{DHA}$, PDX$_{DPAn-6}$ and RvD5$_{DPAn-3}$, members of SPM superfamily, have anti-inflammatory and pro-resolving activities with confirmed activity [54, 67, 264-268, 270, 274, 296]. A study *in vitro* on tert-butyl hydroperoxide-stimulated human retinal pigment epithelium cells for the prevention and treatment of age-related macular degeneration found that PDX$_{DHA}$ reduced TNF-α secretion significantly [264]. Similarly, several *in vivo* studies showed that PDX$_{DHA}$ inhibited production of inflammatory cytokines such as TNF-α and IL-1β in a range of disease models [265-268]. RvD5$_{DHA}$ was found to have anti-inflammatory activity in reducing neutrophilic inflammation [274] and through increasing bacterial clearance and survival in a murine peritonitis model [296]. Both RvD5$_{DHA}$ and PDX$_{DHA}$ were also demonstrated to possess a significant inhibitory effect on mammalian 5-LOX activity *in vitro* [54]. PDX$_{DPAn-6}$ was found to significantly decrease the leukocyte count in

exudates *in vivo* [67] and decrease mRNA expression of TNF-α and iNOS *in vitro* [270].

These results agree with the current work in the THP-1 cell line where RvD5$_{DHA}$, PDX$_{DHA}$ and PDX$_{DPAn-6}$ showed a reduction in IL-1β secretion, but differ in that they did not reduce TNF-α levels in this study. In contrast, PDX$_{DPAn-6}$ did not show anti-inflammatory activity in the TNF-α assay in treated C8-B4 cells. Gobbetti et al. found that RvD5$_{DPAn-3}$ did not decrease TNF-α and only partly reduced IL-1β production *in vivo* [75]. These results agree with the current findings in treated THP-1 cells, except that in this study, the compound showed a significant reduction in IL-1β secretion. However, RvD5$_{DPAn-3}$ showed anti-inflammatory activity through a reduction of TNF-α secretion at the highest dose (200 µM) in treated C8-B4 cells. No work on inflammatory assays involving cytokine secretion has been done on the other analogues of resolvin D5 and protectin DX except those discussed above.

Previous studies showed that di-hydroxy PUFAs containing conjugated trienes with *E,Z,E* geometry, called poxytrins, such as PDX$_{DHA}$, 8,15-diHETE and 9,16-dihydroxy linolenic acid had anti-inflammatory activity through their anti-aggregatory action, whereas compounds with all *trans* or *E,E,Z* conjugated trienes had no activity [297, 298]. However, the results in this work were mixed with conjugated triene molecules only sometimes showing inhibition of cytokine secretion across the three inflammatory assays. This activity was observed strongly for 10,17-diHDHA, 10,17-diHDPAn-3 and 8,15-diHEPA in two assays, whereas 10,17-diHDPAn-6 and 8,15-diHETE showed activity in only one assay.

Some of the tested compounds (17-HDPAn-3, 10,17-diHDHA, 10,17-diHDPAn-3 and 7,17-diHDPAn-6) were observed to decrease TNF-α secretion at all doses in a dose-dependent manner although several compounds only showed statistically

significant results at some doses. These compounds displayed stronger evidence of anti-inflammatory activity compared to the others, so 17-series resolvin analogues especially from DPAn-3 precursors are possibly more powerful anti-inflammatory than 15-series resolvin analogues. Moreover, some compounds (DHA, DPAn-3, 17-HDPAn-3, 17-HDPAn-6, 7,17-diHDPAn-6 and 5,15-diHETE) showed potential anti-inflammatory activity in multiple assays (at least two), again providing a strong indication of anti-inflammatory activity. Overall, among the 20 compounds tested in this work 17-HDPAn-3 and 7,17-diHDPAn-6 have been strongly shown to be anti-inflammatory molecules.

PDX is an isomer of PD1. Both are derived from DHA and their chemical structures differ only in the stereochemistry of the hydroxy group at C10 and the double bond geometry of the conjugated triene (mentioned in Chapter 2) [74]. PD1 has been reported to be more active than PDX in the resolution of inflammation through inhibition of PMN infiltration *in vivo* [272]. Therefore, this could support why PDX compounds derived from various PUFAs had less bioactivity and did not show consistent anti-inflammatory activity across assays.

Structure-activity relationship

In THP-1 treated cells (Table 6.4), all PUFAs showed higher anti-inflammatory activity than the vehicle in reducing IL-1β secretion but not TNF-α secretion. The high degree of variation between three replicate TNF-α assays for PUFAs may have affected these results, as overall, no change in TNF-α secretion was observed when the results from the three assays were combined. From the THP-1 IL-1β assay, the PUFAs in order of decreasing bioactivity are DHA > EPA, DPAn-3 and DPAn-6 > ETE. For the C8-B4 treated cells, anti-inflammatory activity of PUFAs was observed in the following order from high to low activity compared to vehicle: DHA and DPAn-3 (anti-inflammatory) > ETE and DPAn-6 (no activity) > EPA (pro-

inflammatory). DPAn-3 tended to have higher anti-inflammatory activity than DHA in the C8-B4 cell line, whereas DHA had higher anti-inflammatory activity than DPAn-3 in the THP-1 cell line. DHA was found to have higher anti-inflammatory activity than EPA consistently in both cell lines. Similarly, Nauroth and co-workers also found that DHA had higher anti-inflammatory activity than EPA in inhibiting inflammatory cytokines, IL-1β and TNF-α, in human peripheral blood mononuclear cells stimulated by LPS [293].

Other studies also showed that DHA had higher ability than EPA to suppress IL-1β and IL-6 secretion in LPS-stimulated THP-1 cells [282, 283, 292], and IL-6 secretion in LPS-activated RAW264.7 cells [294]. Moreover, DPAn-6 had higher anti-inflammatory activity than DHA in the same study by Nauroth and co-workers [293]. In contrast, DPAn-6 was found to have lower anti-inflammatory activity than DHA in THP-1 cells and showed no anti-inflammatory activity in the C8-B4 cell line in the current study. This is in agreement with the probable involvement of DPAn-6, an omega-6 PUFA and metabolite of ETE in the omega-6 pathway, in pro-inflammatory lipid mediator pathways [293]. In a similar manner, ETE showed no activity and EPA had pro-inflammatory activity in C8-B4 cell line as they are also known precursors of pro-inflammatory lipid mediators.

Most mono-hydroxy PUFAs showed an increase in anti-inflammatory activity through decreases in both IL-1β and TNF-α secretion, compared to their corresponding PUFAs in both THP-1 and C8-B4 cell lines (Table 6.4). Similarly, Tsunomori et al. reported that 15-hydroperoxy EPA was much more efficient than EPA in inhibiting platelet aggregation by reducing the formation of aggregation markers such as 12-HETE and thrombotic B2 *in vitro* [299]. As for di-hydroxy PUFAs (Table 6.4), only 5,15-diHETE had higher anti-inflammatory than 15-HETE,

measured with both the IL-1β and TNF-α secretion assays in the THP-1 cell line, whereas other di-hydroxy PUFAs decreased or did not affect anti-inflammatory properties compared to their corresponding mono-hydroxy PUFAs. In agreement with this work, 10,17-diHDPAn-6 was also previously found to have less anti-inflammatory activity compared to 17-HDPAn-6 *in vitro* and *in vivo* [67, 270]. In contrast with the results of the THP-1 cell line, most of the di-hydroxy PUFA products tested with the C8-B4 cell line showed an increase in anti-inflammatory activity compared to their corresponding mono-hydroxy PUFAs except for 8,15-diHETE, 7,17-diHDPAn-3 and 10,17-diHDPAn-6. However, there was a consistent increase in anti-inflammatory activity for 5,15-diHETE compared to 15-HETE for both THP-1 and C8-B4 cell lines. This strongly suggests that 5,15-diHETE had higher anti-inflammatory activity than 15-HETE, and di-hydroxy PUFAs with double conjugated dienes may have enhanced activity compared to the corresponding mono-hydroxy PUFAs, and more activity than di-hydroxy PUFAs with a conjugated triene.

The mechanism of the anti-inflammatory and pro-resolving activity of PUFAs and SPMs analogues has been partially elucidated as involving the regulation of cytokines by ligand-receptors and polarisation of different macrophage phenotypes. Several SPMs are known to have anti-inflammatory activity due to their mechanisms of acting as ligands (agonists and antagonists) binding to select surface receptors to induce specific actions of anti-inflammatory and/or pro-resolving activity. For example, RvE1 and RvD1 have functions as antagonists for the LTB4 receptor to inhibit LTB4 production, and RvD1, RvD3 and RvD5 can act as an agonist of the human GPR32 receptor to stimulate macrophage phagocytosis [10, 187, 300].

There are two macrophage phenotypes (M1 and M2), which have different functions in inflammation. The M1 phenotype is known to produce pro-inflammatory cytokines such as TNF-α and IL-1β, reactive oxygen and nitrogen species, when cells are stimulated with inflammatory stimuli such as LPS. In contrast, the M2 phenotype produces anti-inflammatory cytokines and SPMs, and has scavenging and protective activity [10, 301]. Several SPMs and analogues such as RvD1 and MaR1 precursor (13*S*,14*S*-epoxy-DHA) have been shown to shift the macrophage phenotype from M1 to M2 *in vitro* and *in vivo* to express their anti-inflammatory and pro-resolving activities, in a few studies [302, 303]. 17-HDHA, 17-HDPAn-6 and 10,17-diHDPAn-6 in particular, were found to activate the anti-inflammatory and pro-resolving M2 polarised macrophages, with a reduction of TNF-α and iNOS gene expression, and an increase of IL-1 receptor antagonist, scavenger receptor expression and phagocytosis activity *in vitro*. 10,17-diHDPAn-6 appeared to have lower activity than 17-HDPAn-6 [270].

As for the TNF-α assay in the C8-B4 cell line, it is interesting to note that the di-hydroxy PUFA compounds having double bond systems with symmetrical structures (Figure 6.12) were consistently more anti-inflammatory than their corresponding mono-hydroxy PUFAs. These symmetrical structures are found in the compounds 5,15-diHETE (Figure 6.12A), 8,15-diHEPA and 10,17-diHDPAn-3 (Figure 6.12B), 7,17-diHDHA (Figure 6.12C). These structures could potentially be applied to the design of novel anti-inflammatory SPMs with high bioactivities. The other di-hydroxy compounds without symmetrical double bond systems did not always show higher anti-inflammatory activity compared to their corresponding mono-hydroxy PUFAs. They showed either lower anti-inflammatory activity or no change in activity. However, the mechanism through which the symmetrical structures would exert enhanced activity is unknown.
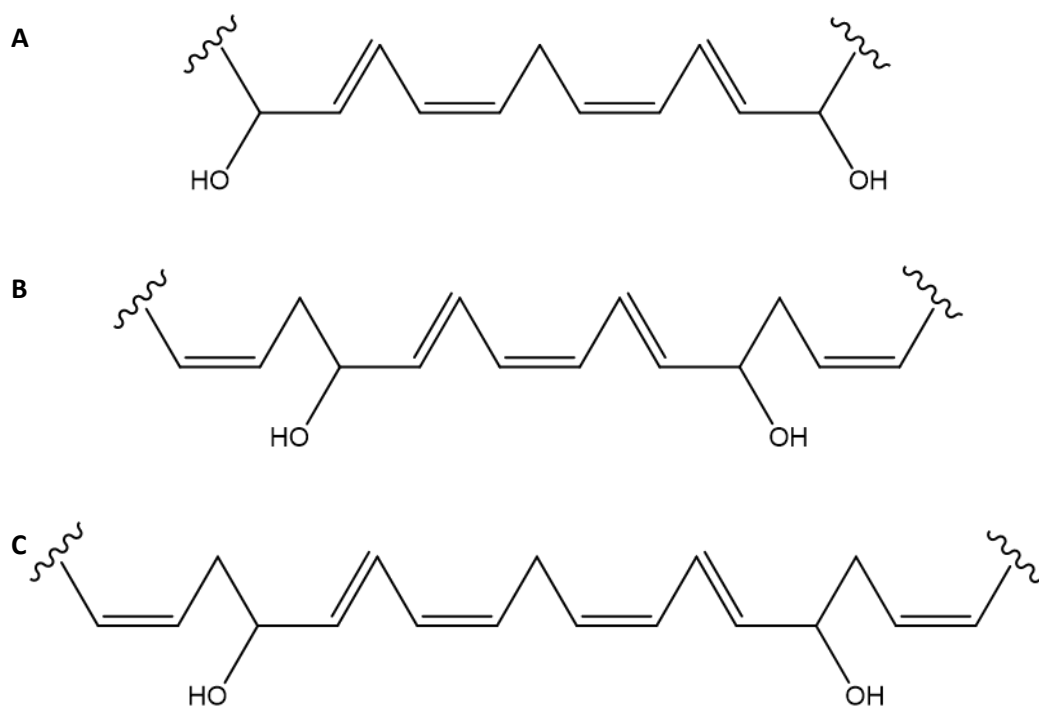
Figure 6.12: Three different symmetrical structures (A, B, C) found in resolvin and protectin analogues.

5. Conclusion

In this chapter, most PUFAs and resolvin analogues have shown potential anti-inflammatory properties *in vitro* by reducing IL-1β and TNF-α secretion in two different cell types, human monocytic cells (THP-1) and mouse brain cells (C8-B4). However, compounds derived from precursors of ETE, DPAn-6 and EPA, which are known to be involved in pro-inflammatory pathways, showed either no activity or pro-inflammatory activity in some cases. The anti-inflammatory properties of PUFAs can be adjusted by chemical modifications by adding hydroxy groups and forming conjugated double bond systems. The formation of mono-hydroxy PUFAs almost universally enhanced the anti-inflammatory activity compared to the parent PUFAs across different cytokine assays and cell lines. The effects of adding a second hydroxy group were more varied, only sometimes improving the anti-inflammatory activity of the mono-hydroxy PUFAs.

This work demonstrated that almost all resolvin and protectin analogues synthesised from PUFAs using a crude enzyme from soybean flour have potential anti-inflammatory activity similar to endogenous SPMs. These novel resolvin D5 and protectin DX analogues (di-hydroxy PUFAs) and their precursors (mono-hydroxy PUFAs), may be useful anti-inflammatory compounds in the treatment and prevention of inflammatory disorders. This work also reinforces the idea that the anti-inflammatory activity of PUFAs at least partially arises from their conversion into more anti-inflammatory resolvins and protectins.

GENERAL CONCLUSIONS AND FUTURE WORK

It has been demonstrated in this project that SBF can be used directly without any complex purification as a natural and inexpensive source of lipoxygenase enzymes to produce a variety of potentially bioactive resolvin and protectin analogues from a range of substrates. SBF is more versatile than 15-sLOX-1 in efficiently oxygenating both FFAs and various esterified FAs (TL, DHA TAG, tuna oil and anchovy oil). LOX sourced from SBF can be used as an effective enzyme preparation compared to the purified and commercially available 15-sLOX-1 in producing bioactive resolvin and protectin analogues. The sfLOX can catalyse twice the amount of substrate and give a greater total product yield. The sfLOX-catalysed reaction yield with DHA or TL is high (approximately 60% or greater), and the products suggest a highly regio- and stereo-specific reaction. Other esterified FAs (DHA TAG, tuna oil and anchovy oil) are also good substrates for sfLOX with small amounts of substrate remaining at the end of the respective reactions.

Importantly, most PUFAs and the resolvin and protectin analogues synthesised from sfLOX have been confirmed to have positive bioactivity in anti-inflammatory assays of IL-1β and/or TNF-α in two different cell lines *in vitro*, as was expected. Structure-activity relationships of these compounds associating the number of hydroxy groups and conjugated double bond systems with their activities have been identified and discussed. The anti-inflammatory properties of PUFAs were improved in many cases by adding a hydroxy group and forming a conjugated diene, whereas adding a second hydroxy group and forming either a conjugated triene or two conjugated dienes showed varied effects on their bioactivities.

For future work, DHA and EPA-enriched TAGs containing mono-hydroxy PUFAs (a hydroperoxy group and two conjugated double bonds), synthesised from reactions

with sfLOX and fish oil, would be promising to test for anti-inflammatory activity. It would be useful to identify the stereo-specificity of the sfLOX-catalysed tuna oil reaction products before assessing their biological activity.

There is potential to use the approach outlined in Chapter 5 for the separation of biologically-important PUFA-containing TAGs from complex natural lipid mixtures in industrial applications. After dioxygenation, hydroperoxylated PUFA-containing TAGs could be easily separated from the lipid mixture based on differences in polarity, and then used in pharmaceuticals and nutraceuticals, while the remaining unreacted TAGs could be used for applications such as biodiesel production.

APPENDIX

¹H-NMR_TL
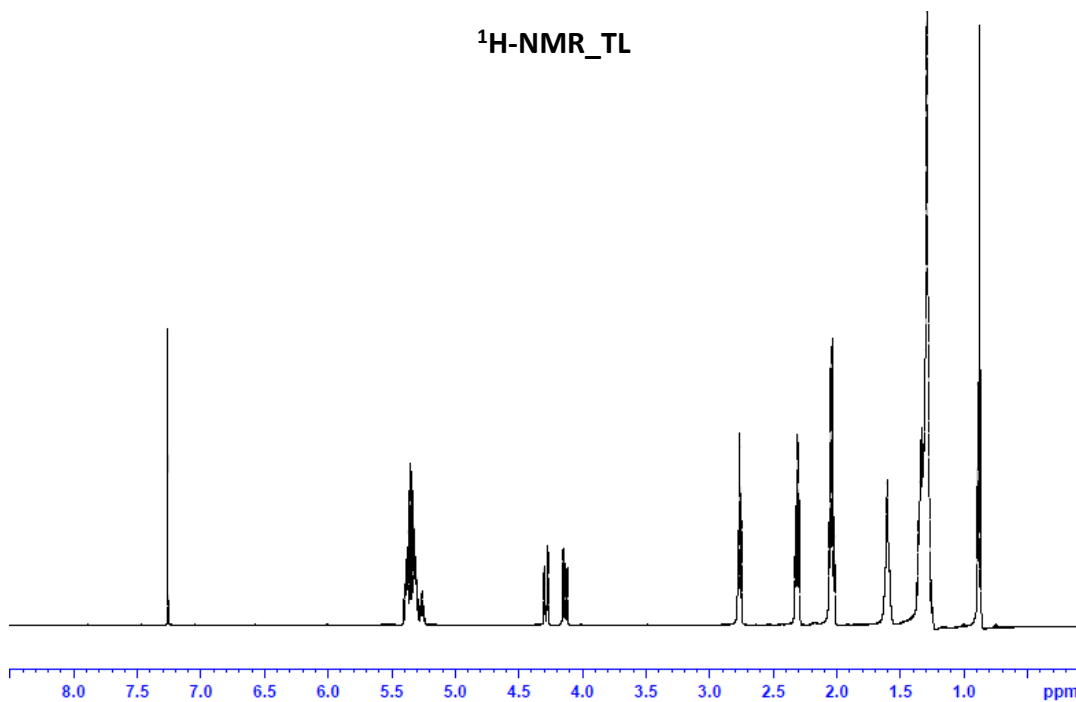
¹H-NMR_TL-OOH
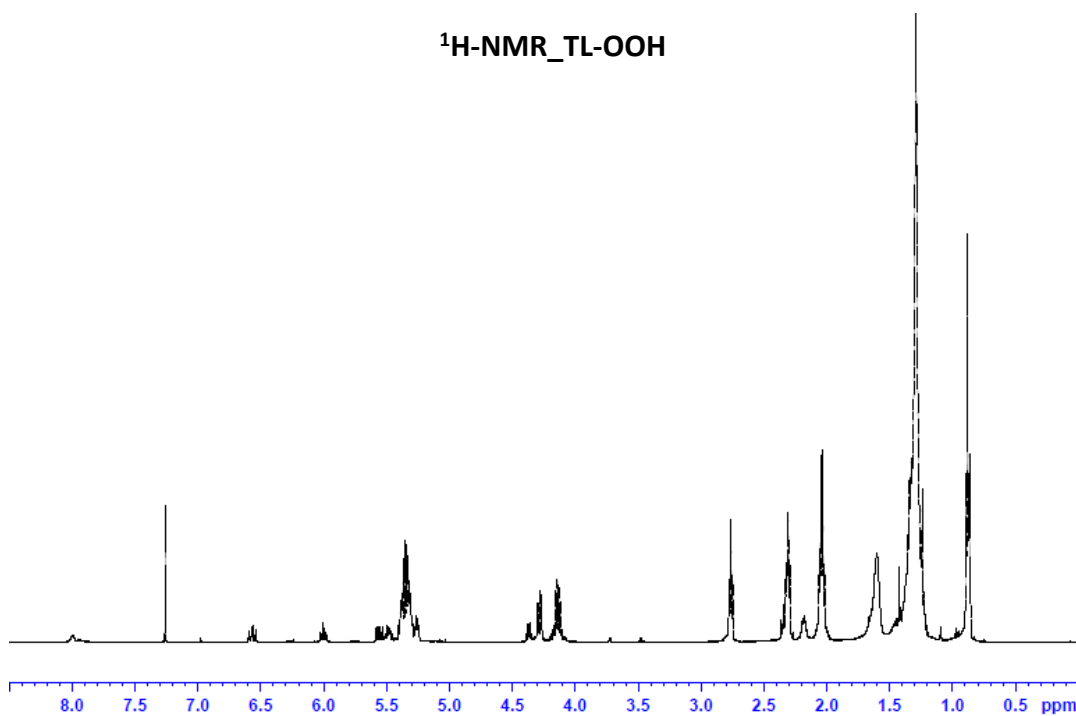
¹H-NMR_TL-2OOH

¹H-NMR_TL-3OOH

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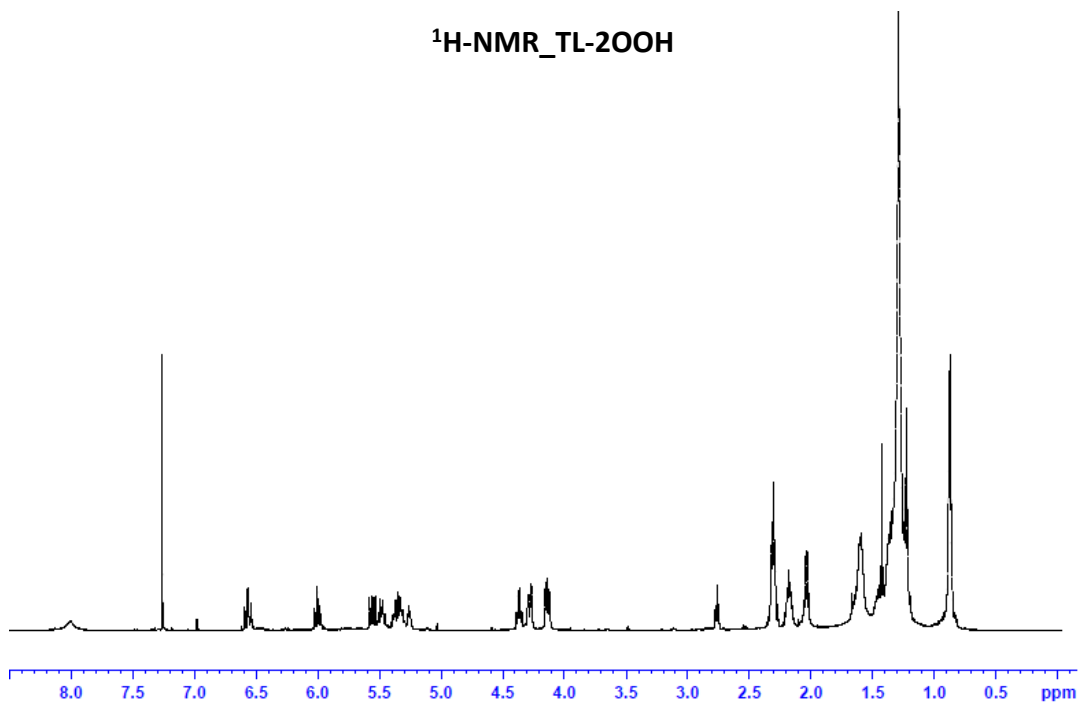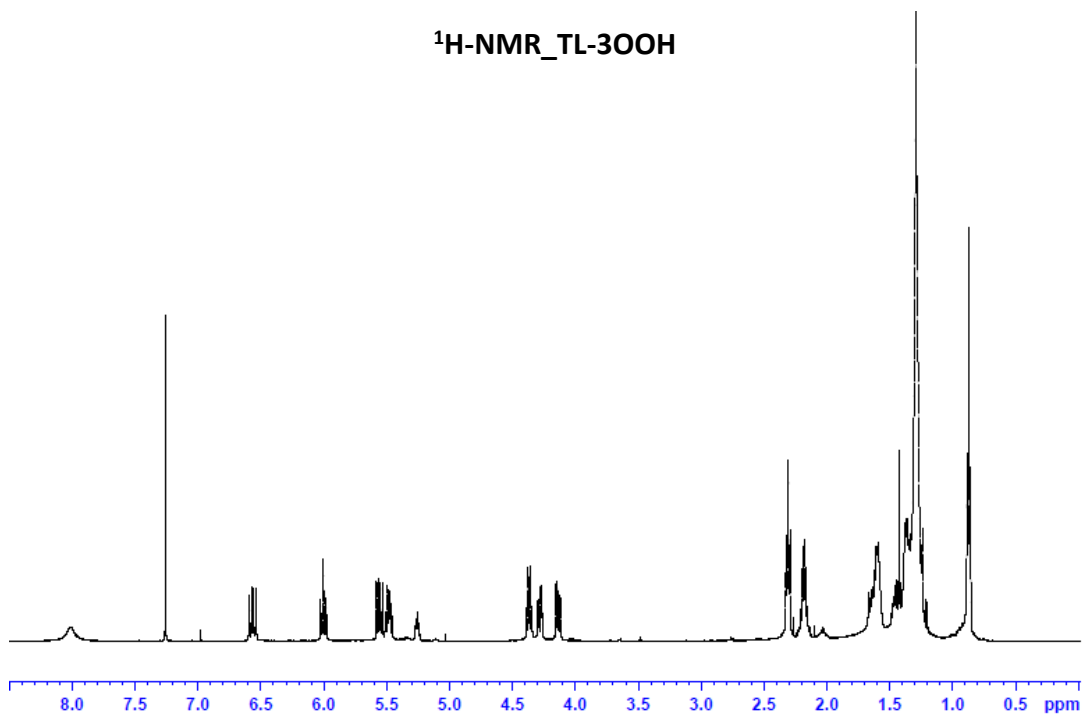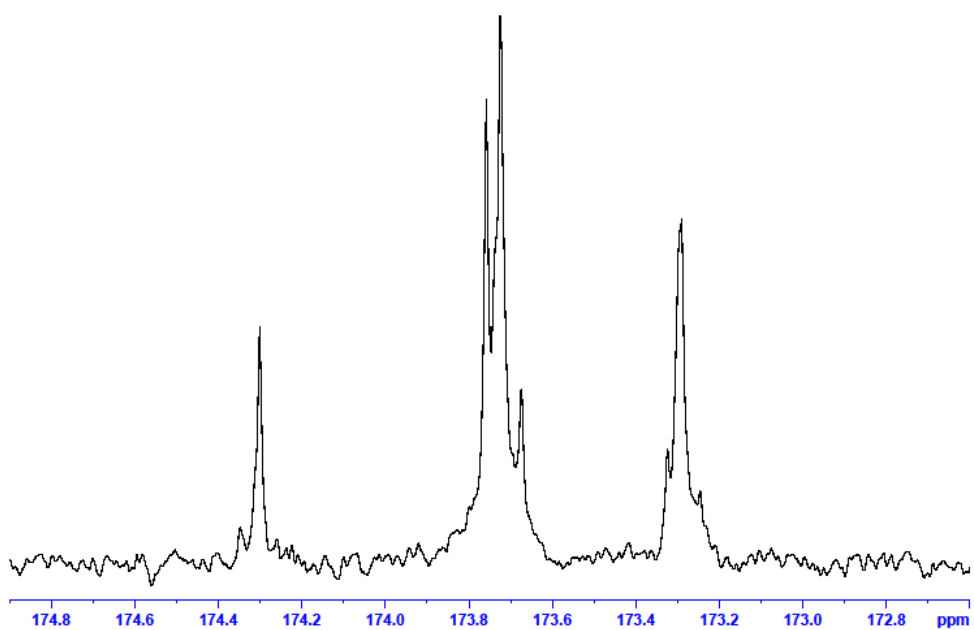
¹³C-NMR_TL-OOH
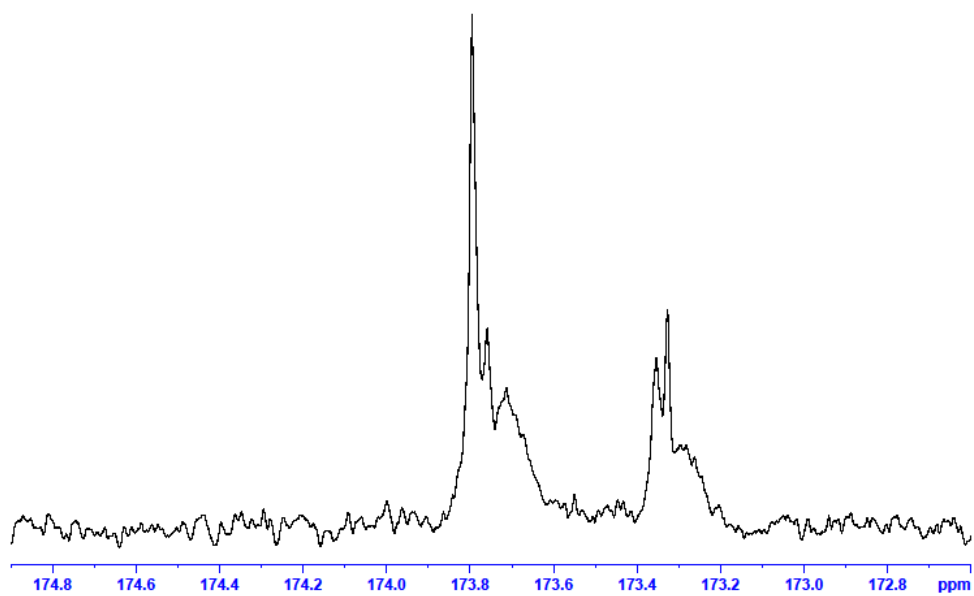
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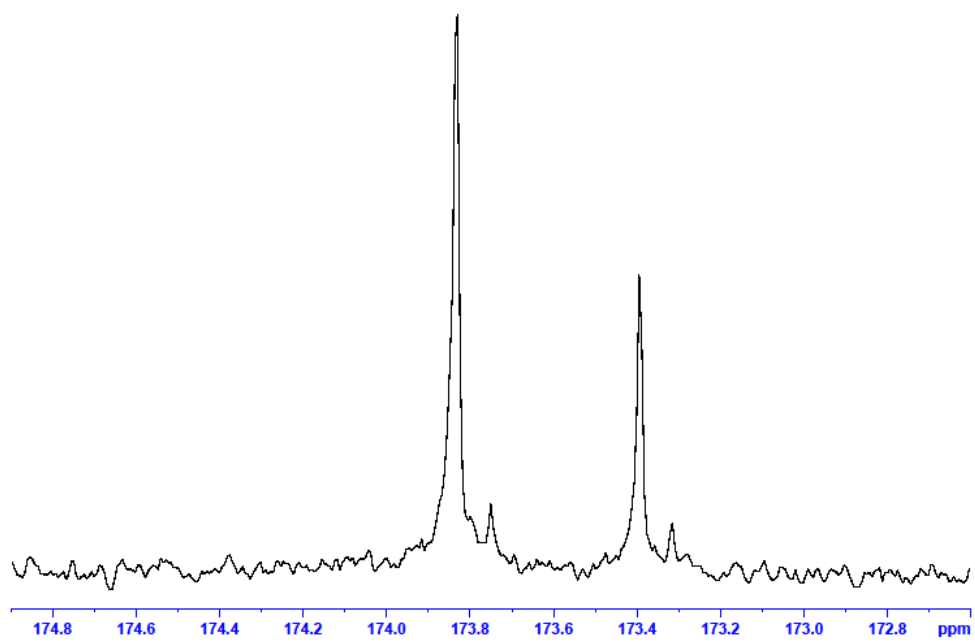
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