Optimisation of Isolation, Characterisation and Exploitation of Oxidative Enzymes for Terpene and Terpenoid Biosynthesis in Industry

by

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Dissertation

Submitted in fulfilment of the requirements for the degree

Master of Science

in

Molecular and Cell Biology

in the Faculty of Science, University of the Witwatersrand, Johannesburg, South Africa

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16 May 2022

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Abstract

Soybean (*Glycine max*) lipoxygenase (LOX) isozymes can convert terpenes into industrially favourable terpenoids through oxidation. In this study, the temperature and pH optima at which LOX-1 and LOX-2 functioned were investigated along with the shelf-life and recyclability of the enzymes. It was confirmed that both isozymes can be recycled as cross-linked enzyme aggregates (CLEAs) which can save time and costs in industrial settings. LOX-1 as compared to LOX-2 has a shelf-life of greater than 75 days whereas after 45 days in aqueous form at 4℃ LOX-2 had lost catalytic ability. LOX-1 was seen to work favourably after 16 weeks in lyophilised form, but LOX-2 had lost all activity. Through the assays used to determine the two enzymes' activity LOX-1 shows optimal activity at pH 10 while LOX-2 worked best at pH 6.8. LOX-1 is stable for 24 hours over a range of pH values, pH 2 to 11 and at temperatures up to and including 55℃. LOX-2 was seen to be less robust with activity only being retained in buffers at a pH of 6 to 11 and in temperatures up to and including 45℃. LOX-1 displayed a Vmax value of 0.1137 µmol/s/mg protein, thus showing higher catalytic ability than LOX-2 with a Vmax value of 0.0183 µmol/s/mg protein. This is mirrored in the Km values whereby LOX-1 shows peak activity with 1.313 µmol sodium linoleate in 0.2 M boric acid (pH 10.0) at 25℃ while LOX-2 shows this activity when 2.335 µmol sodium linoleate in 0.2 M sodium phosphate buffer (pH 6.8) at 25℃ is present.

Acknowledgements

I would like to thank my co-supervisors Professor Dean Brady and Professor Karl Rumbold for all their support, guidance and encouragement along this journey.

This project would not have been possible without the financial aid and invaluable support of Applied Protein Biotechnologies. Stephanus Marais, Daniel Pienaar and Alex Zawaira were amazing mentors and the help of both Deidre Davids and Lindelo Mguni made many techniques in the lab much less daunting. I would also like to thank my colleague Ida Makhubela for her assistance with creating the cross-linked enzyme aggregates and testing them in the valencene to nootkatone conversion reaction.

My biggest thanks go to my colleagues Dylan Moodley, Ateret Ben-David, Alison Brigden and Nikita Nankoo in the Microbiology and Biotechnology department, which would not run quite so smoothly without the assistance of Michael Tobin, for all their help and support throughout the course of this investigation. I would also like to thank Dr Sanchia Moodley for her assistance and support.

Lastly, I would like to thank my mother, Leigh Levinger, for all that she has done to allow me to pursue my passions and complete this degree.

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List of abbreviations

APS: Ammonium persulfate

AS: Ammonium sulfate

CM: Carboxymethyl

CLEA: Cross-linked Enzyme Aggregates

DEAE: Diethylaminoethyl

DS: De-fatted soybean

ddH_2O: Autoclaved distilled water

GC-MS: Gas chromatography–mass spectrometry

HIC: Hydrophobic interaction chromatography

IEX: Ion exchange chromatography

Km: Michaelis constant

LOX: Lipoxygenase

MW: Molecular weight

pH: Potential of Hydrogen

pI: Isoelectric point

PUFA: Polyunsaturated fatty acid

SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SEC: Size exclusion chromatography

TEMED: Tetramethylethylenediamine

Vmax: Maximum Rate of Enzyme Catalysed Reaction

WS: Whole soybean

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1. Introduction

1.1. Biocatalysis

The use of catalysts in chemical reactions is a long-standing practice in the scientific community. Catalysts speed up the rate at which a reaction may occur by lowering the amount of energy required for a substrate to be chemically converted into a product (Illanes, 2008). Once this reaction has occurred the catalyst is freed, unaltered, and once again becomes available to aid in the same reaction, theoretically indefinitely (Illanes, 2008).

Enzymes are the catalysts used by biological organisms to perform highly specific biochemical reactions (Lodish *et al.*, 2003). Loosely speaking, there is an enzyme that has evolved to catalyse almost every specific metabolic reaction a cell may require. As such, enzymes are capable of performing complex and unique reactions that can be extremely difficult to replicate via chemical synthesis (Lodish *et al.*, 2003). Due to the massive array of enzymes that could potentially exist, if one considers that enzymes are proteins made up of 20 different amino acids and at each position in a polypeptide the amino acid is variable as is the length of the protein chain itself, it is probable that there is an enzyme capable of catalysing any chemical reaction of interest (Illanes, 2008). Add to this that enzymes are biologically evolved to work under mild conditions (physiological) *in vivo* the isolation and exploitation of unique enzymes for chemical processing is a tempting opportunity (Fessner & Anthonsen, 2009). Some protein and buffer engineering may be required to convert optimal enzyme performance under biological conditions into optimal enzyme performance under biotechnological conditions.

By catalysing reactions so specifically, enzymes do not produce the many unwanted and possibly toxic by-products that chemical synthesis is known to do (Sheldon & Brady, 2018). This reason, more so than any other, has spurred the growing field of biocatalysis which promotes green chemistry as well as sustainable practices in science and industry (Sheldon & Brady, 2018).

1.2. Enzymes in nature

The planet Earth is home to an as-of-yet unknown number of different organisms but the number of distinct eukaryotic species is estimated to lie between three and 100 million (Mora *et al.*, 2011). Scientists have yet to name, let alone discover, all of the life-forms that call planet Earth home and of those that there is scientific knowledge there is only a fraction known on a genomic, transcriptomic and proteomic level. This means that there is potentially a vast sea of untapped enzymatic resources waiting to be discovered. A single eukaryotic organism can have

numerous proteins associated with each of the genes in its genome due to alternative splicing, post translational modifications and single amino acid polymorphisms (Ponomarenko *et al.*, 2016). These factors indicate that even within a single uncharacterized species the number of untapped enzyme resources is huge. It thus becomes evident that looking to the natural world for enzymes that can help catalyse myriad favourable reactions is a viable avenue that should be explored.

Plants are fantastic stockpiles of such enzymes – be they previously characterized enzymes or those that remain to be discovered. In today's profit-motivated world, it is important to capitalize on the natural resources we have available to us in South Africa, the biggest being our biodiversity (Schnitzler *et al.*, 2011). There may be new variants of already known useful enzymes hidden within the proteome of a seemingly inane plant species that could catalyse a favourable reaction to produce a highly sought-after product. Some examples include new oxidoreductase, transferase or ligase enzymes which can be used in industry for biocatalysis or synthesis of fine chemicals, or perhaps hydrolases, lyases and isomerases which are used in the food, beverage and paper industries for creation of useful products (Khan *et al.*, 2012).

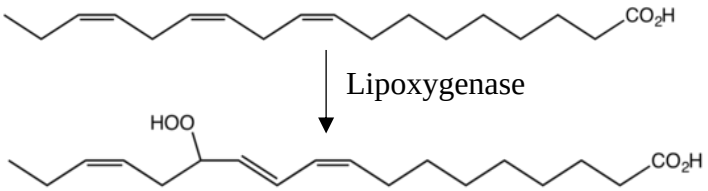
Other enzymes already in use can be found in numerous industries such as the manufacture of beverages, animal feed, textiles, paper, baking, detergents, and fuel (Khan *et al.*, 2012). Certain proteases have been used for numerous purposes – they aid in the removal of stains caused by proteins, act as yeast nutrition-fuel, help in the clotting of milk, form a part of low allergenic infant formula and increase flavour. Proteases have also been used in the baking industry for the production of biscuits and cookies. Another useful class of enzymes are the amylases which have been used to remove stains caused by starches, allow for the increase in bread softness and volume, and help treat juices for consumption. Furthermore, lipases have been utilised in industry for the removal of lipid stains, improve in cheese flavour, improve dough stability and conditioning and control contaminants in the production of paper(Khan *et al.*, 2012). These are but a few examples of the uses of only a few classes of enzymes used in industry which can be aided by the discovery and characterisation of novel enzymes and variants thereof.

One plant source that is of interest for the enzymes it contains is the soybean (*Glycine max*), that has been grown commercially on a large scale in South Africa since the 1990s (Dlamini *et al.*, 2014). The crop was initially introduced to South African agriculture in 1903 and has since boomed in its production and become a vital part of the agricultural economy.

1.3. Soybean lipoxygenases

Lipoxygenase enzymes (linoleate: oxidoreductase, EC 1.13.11.12, LOX) are a group of non-heme metal-containing dioxygenases which catalyse the regio- and stereo-specific dioxygenation of polyunsaturated fatty acids, such as linoleic, linolenic and oleic acid, into conjugated unsaturated fatty acid hydroperoxides (

*Figure **1***) (Schörken & Kempers, 2009). These enzymes are found in numerous plant, animal and microbial organisms but are most prominent in legumes and potatoes. The primary function of plant lipoxygenases is the synthesis of defensive molecules, whereas in animals this function is rather to produce eicosanoids in the endocrine system (Schörken & Kempers, 2009). Within the plant kingdom there are multiple lipoxygenase isoforms, each with their own substrate



specificity, pH optima, stability and product formation (Hayward *et al.*, 2017).

Figure 1: Lipoxygenase catalysed dioxygenation of a fatty acid (linolenic acid) to produce a fatty acid hydroperoxide; taken from Smokefoot (2017)

While present in numerous plants, animals and even microorganisms (Siedow, 1991), there is much less research into any of the lipoxygenases found in any of these organisms than that of the soybean seed, even the leaves of soybean plants have been less studied (Baysal & Demirdöven, 2007). Soybean seeds are known to be the richest source of lipoxygenase to date (Axelrod *et al.*, 1981). There have been four isozymes isolated from soybeans: namely lipoxygenase-1 (LOX-1), lipoxygenase-2 (LOX-2), lipoxygenase-3a (LOX-3a) and lipoxygenase-3b (LOX-3b). Both LOX-3a and LOX-3b are very similar in structure, function and substrate preference and can be considered nearly identical (Axelrod *et al.*, 1981). The three isozymes are very similar in size and amino acid length. The molecular weight is approximately 94 kDa, 97 kDa and 96.5 kDa for LOX-1, LOX-2 and LOX-3 respectively while the length is approximately 840 amino acids (Shibata *et al.*, 1987; Chedea *et al.*, 2008).

Of these isozymes, LOX-1 has been studied the most (Hayward *et al.*, 2017). This is due to how well it performs under laboratory conditions as compared to the other isoforms. The investigations of Axelrod and co-workers (1981) revealed that LOX-1 exhibits ideal kinetic behaviour. It was determined that the reaction rate of this enzyme is directly proportional to

the amount of enzyme added to the reaction in the presence of free polyunsaturated fatty acids (PUFAs). It will form 9- and 13-hydroperoxides (Figure 2) in a ratio of 1:9 at room temperature. Previous investigations have determined the pH optimum of LOX-1 to be pH 9.0 and its isoelectric point (pI – the point at which the overall charge on a protein's surface is zero) sits at 5.68 (Baysal & Demirdöven, 2007; Siedow, 1991). Conversely LOX-2 has a less defined pH optimum based on the literature but lies between pH 6.5 and pH 6.8, the pI is agreed to be 6.25 (Baysal & Demirdöven, 2007). Lipoxygenase-2 forms both 9- and 13-hydroperoxides in a ratio of approximately 1:1 at room temperature but has a much lower catalytic activity than LOX-1 (Baysal & Demirdöven, 2007; Siedow, 1991). Both forms of LOX-3 are also much less kinetically favourable than LOX-1. The pH optimum for the LOX-3 isoforms is pH 7.0 and they have a pI of 6.15 (Siedow, 1991). The hydroperoxide products formed are generally an equal amount of 9- and 13-hydroperoxides (Siedow, 1991).

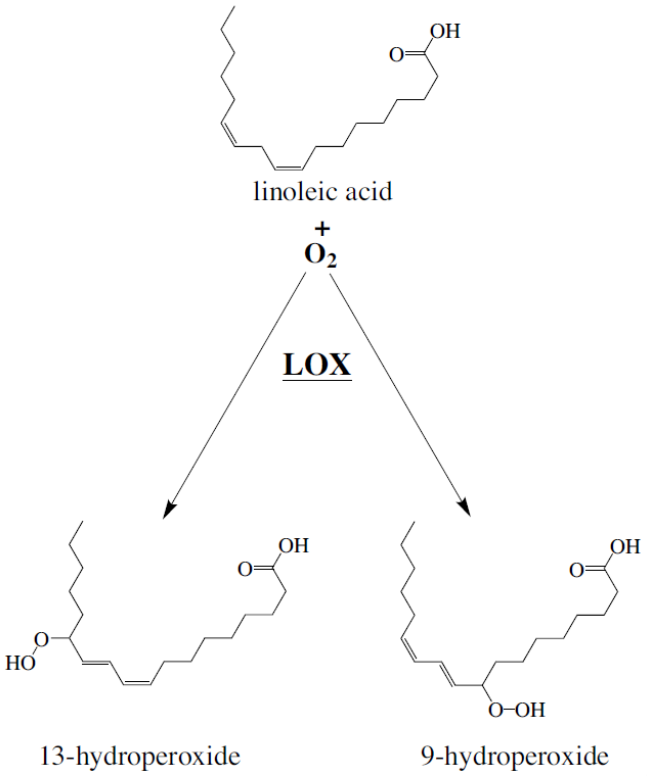


Figure 2: Lipoxygenase-catalysed conversion of linoleic acid into either 9- or 13-hydroperoxides; taken from Gerde & White (2008)

While interacting directly with PUFAs such as linoleic and linolenic acid to produce these hydroperoxides, the LOX isoforms have been observed to catalyse other secondary reactions (Siedow, 1991). Garssen and co-workers (1971) showed that carbonyl compounds formed from linoleic acid hydroperoxides are dependent on both the enzyme and the fatty acid itself. During the formation of hydroperoxides, from the fatty acid, radicals are produced – this can occur

under both aerobic and anaerobic conditions – which are thereafter involved in the process of converting numerous substrates into different products (Figure 3). Therefore, not only do lipoxygenases have numerous direct substrates which they can convert into sought-after products, they are also likely responsible for secondary reactions in the presence of their preferred substrates. These traits make lipoxygenase enzymes of great industrial interest.

The complete list of uses of these soybean seed lipoxygenase isoforms are still under investigation but some of their industrially relevant uses, spurred by their production of various hydroperoxides, include: the production of useful substances such as dyes, plastics, soaps, resin and varnish (Tukel *et al.*, 2005). Lipoxygenase enzymes are also used in the bread making industry for the whitening of flour by oxidising and therefore bleaching carotenoids, the formation of volatile flavour compounds as well as aiding in the increase of loaf volume (Lakshmi *et al.*, 2009). There are further industrial uses currently under investigation by the company Applied Protein Biotechnology (APBio, South Africa) that include the conversion of abundant terpenes and terpenoids into sought after industrially relevant compounds.

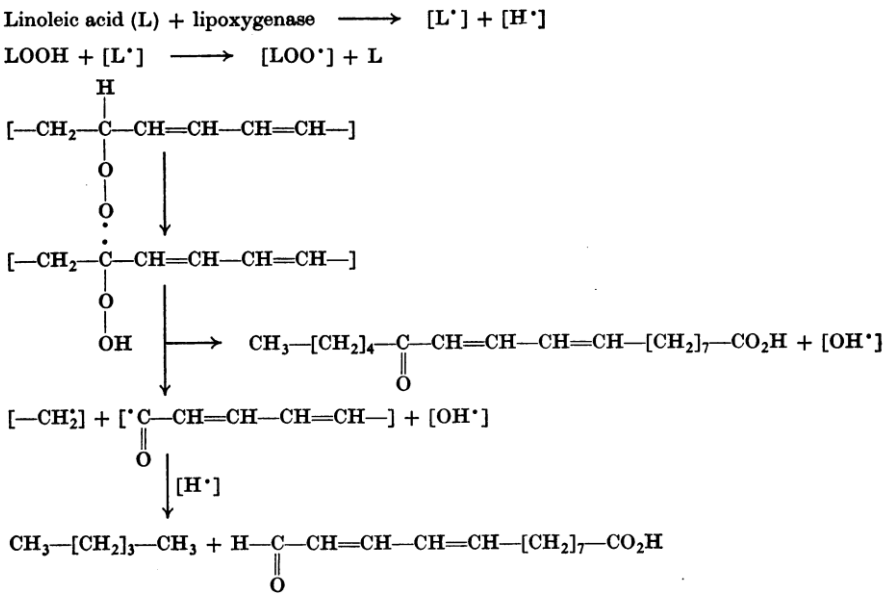


Figure 3: Lipoxygenase catalysed secondary reactions by the production of peroxyl radicals, taken from Garssen and co-workers (1971)

In the quest to work as green as possible APBio found a supplier of waste soybean remains whereby the commercially viable oils had previously been extracted. These de-fatted soybeans (DS) would in most cases be discarded as waste, however APBio would rather turn them into a viable source of lipoxygenase enzymes for use in their many biocatalytic reactions. The LOX-1 and LOX-2 content of the DS would therefore need to be compared to that of whole soybeans (WS), containing all of the natural fats and compounds not affected in the oil removal

processes.

1.4. Terpenes and terpenoids

Numerous commercially viable products can be synthesised using terpenes, an ubiquitous class of natural compounds found in plants (Breitmaier, 2006). Terpenes are predominantly responsible for the smell, taste and pharmacological properties of many plants (Figure 4). There are numerous structures of terpenes composed of differing numbers of carbon such as monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), triterpenes (C_{30}), and tetraterpenes (C_{40}) (Stephane & Jules, 2020). Plants may then alter these terpene precursors to become other useful compounds (Breitmaier, 2006), for example, terpenes can be converted into terpenoids, modified terpenes, usually by the addition of oxygen-containing functional groups (McNaught & Wilkinson, 1997). Terpenoids are also responsible for plant flavours, colours and scents, many of which have a place on the market if they can be isolated, but more so if they can be produced in large quantities (Fahlbusch *et al.*, 2003). For this purpose, the identification of enzymes capable of modifying ubiquitous terpenes into commercially viable terpenoids (which are produced in much smaller quantities by plants) is a priority for green industrial production. Lipoxygenases are a group of such useful enzymes.

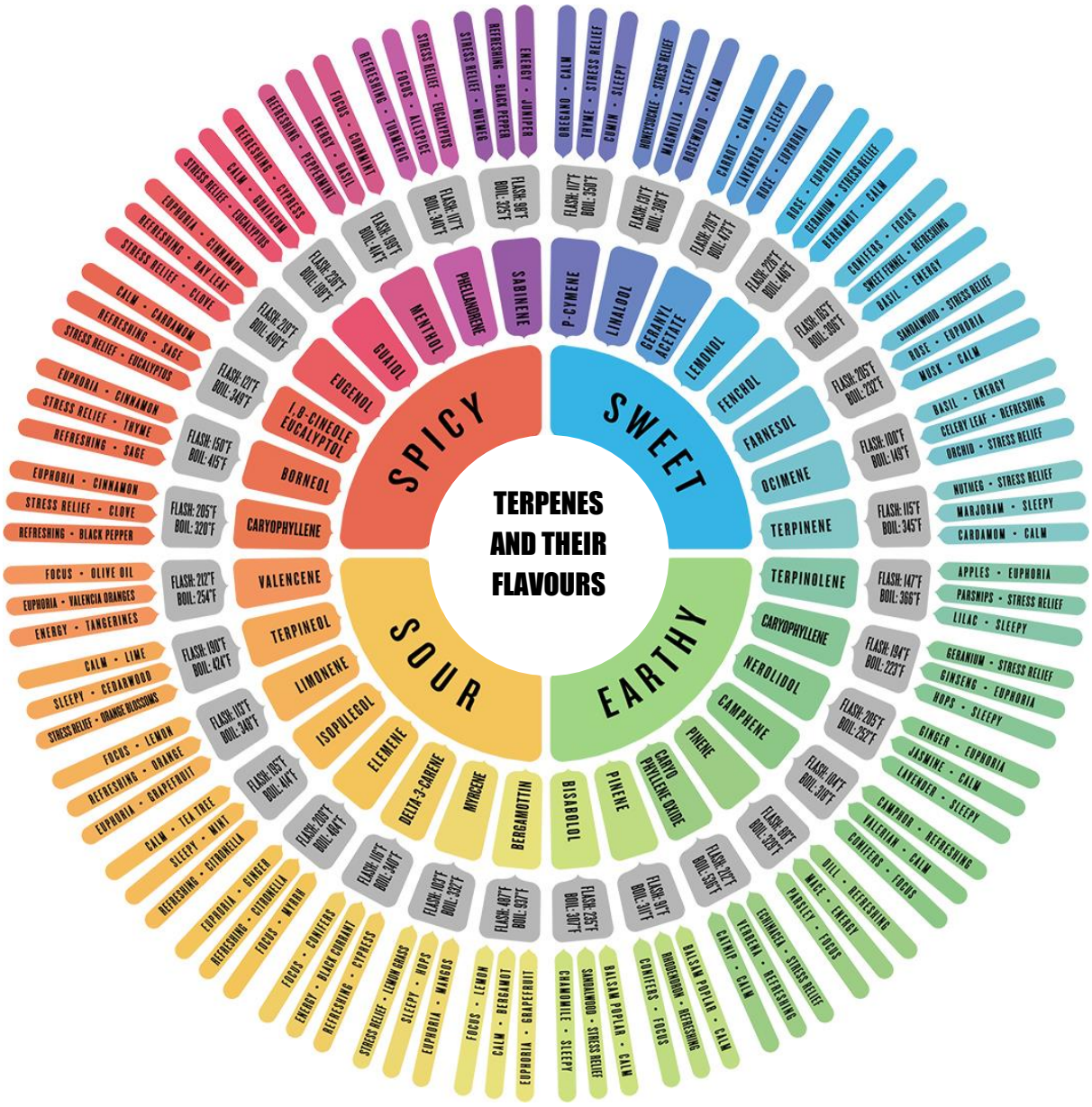


Figure 4: Terpene flavour wheel, adapted from Terp Talks: Terpenes and the Entourage Effect (2019)

One such terpene is limonene, a cyclic terpene (Figure 5a), which is found in numerous essential oils (Fahlbusch *et al.*, 2003). It occurs at a concentration of over 90% in citrus peel oils and in much lower concentrations in the oils of *Mentha spp.* and conifers. It is even a by-product in the production of orange juice. As the name suggests, limonene has a lemon-like odour which in-of-itself is useful in fragrances (Fahlbusch *et al.*, 2003). Due to its abundance, altering it to other valuable compounds with which it shares chemical structural similarity is of interest. Oxidation of limonene can lead to numerous products, an economically favourable

example of which is the terpenoid carvone (Figure 5b) which conveys a spearmint flavour. The use of enzymes to catalyse this reaction, rather than the chemicals that are currently used in industry such as nitrosyl chloride and acetone, yields purer product along with less waste products (Fahlbusch *et al.*, 2003). Such reactions can be helped along by oxidative enzymes such as lipoxygenases which add oxygen atoms to their substrates transforming the substrate molecules into products of industrial importance.

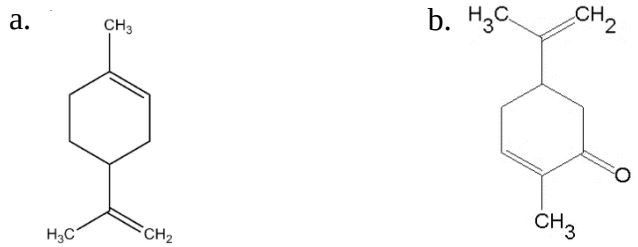


Figure 5: a. Limonene chemical structure; b. Carvone chemical structure

Lipoxygenase enzymes do not directly interact with terpene substrates. Lipoxygenases use polyunsaturated fatty acids, that contain a *cis,cis*-1,4-pentadiene moiety, as their substrates. They cause a dioxygenation reaction which in turn leads to the production of fatty acid hydroperoxides (Matsui *et al.*, 2014). However, if performed in conjunction with other substrates, the reaction can lead to the co-oxidation of other such substrates. For example, carotene, a terpene, is bleached in the presence of lipoxygenase and linoleic acid. Other reactions concerning different terpenes or terpenoids may be catalysed in a similar manner – whereby peroxyl radicals formed by lipoxygenases are able to co-oxidise polyenes (Klein *et al.*, 1985). Therefore, the isolation of such enzymes could be useful in the industrial synthesis of terpenoids from terpene precursors, of which there are numerous, as can be seen in Figure 6, Figure 7 and Figure 8.

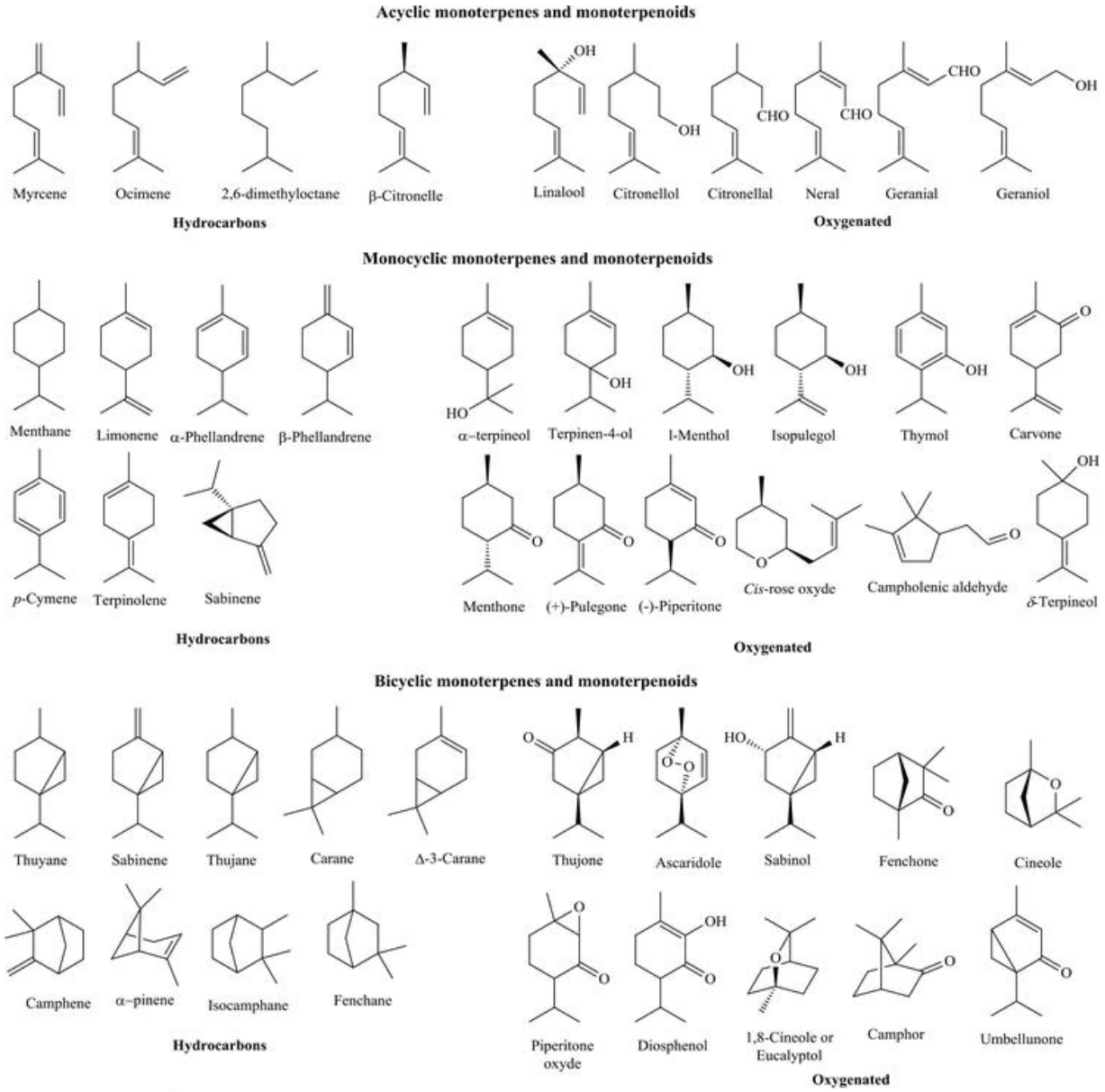


Figure 6: A selection of monoterpene and monoterpenoid structures, taken from Stephane & Jules (2020)

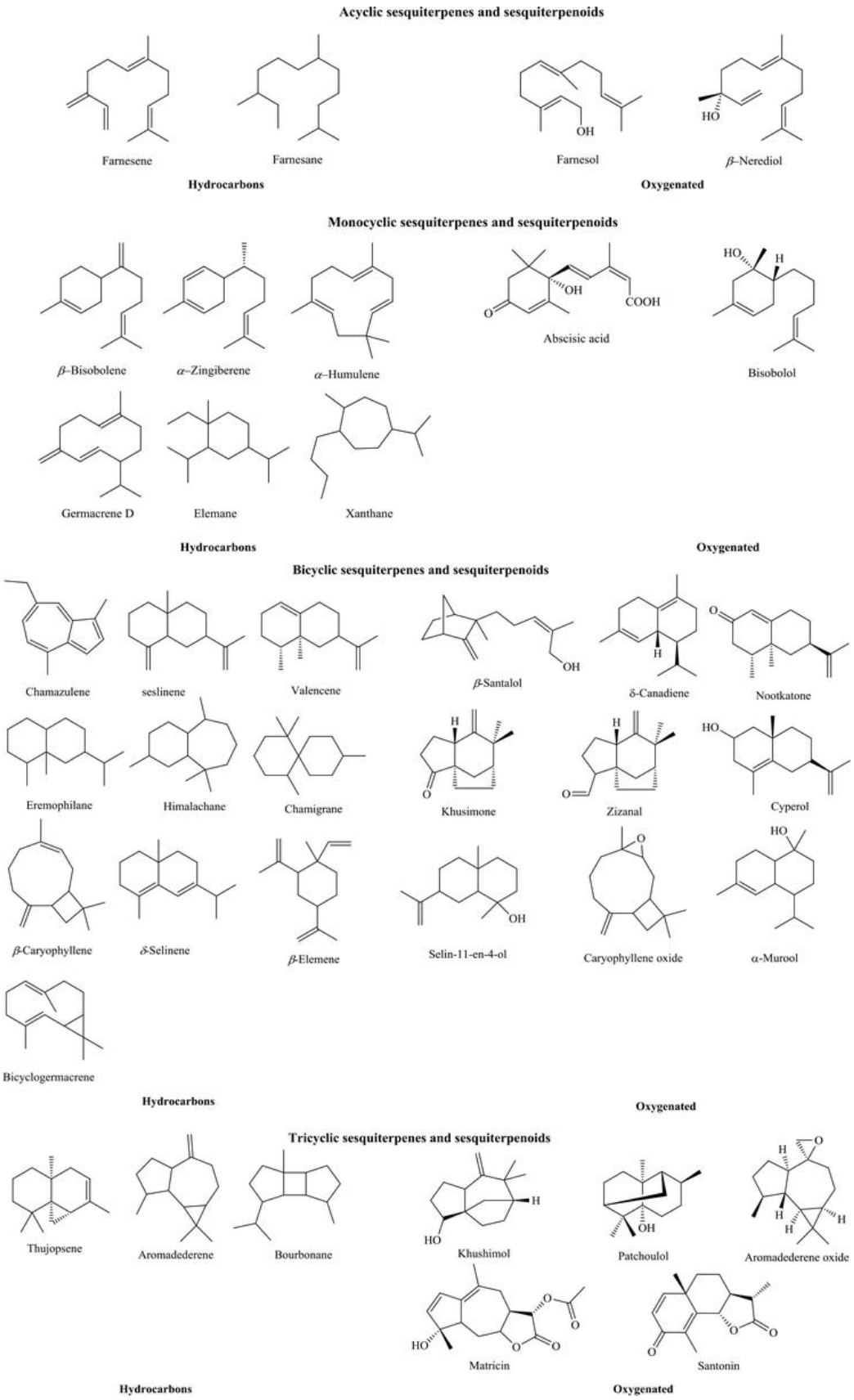


Figure 7: A selection of sesquiterpene and sesquiterpenoid structures; taken from Stephane & Jules (2020)

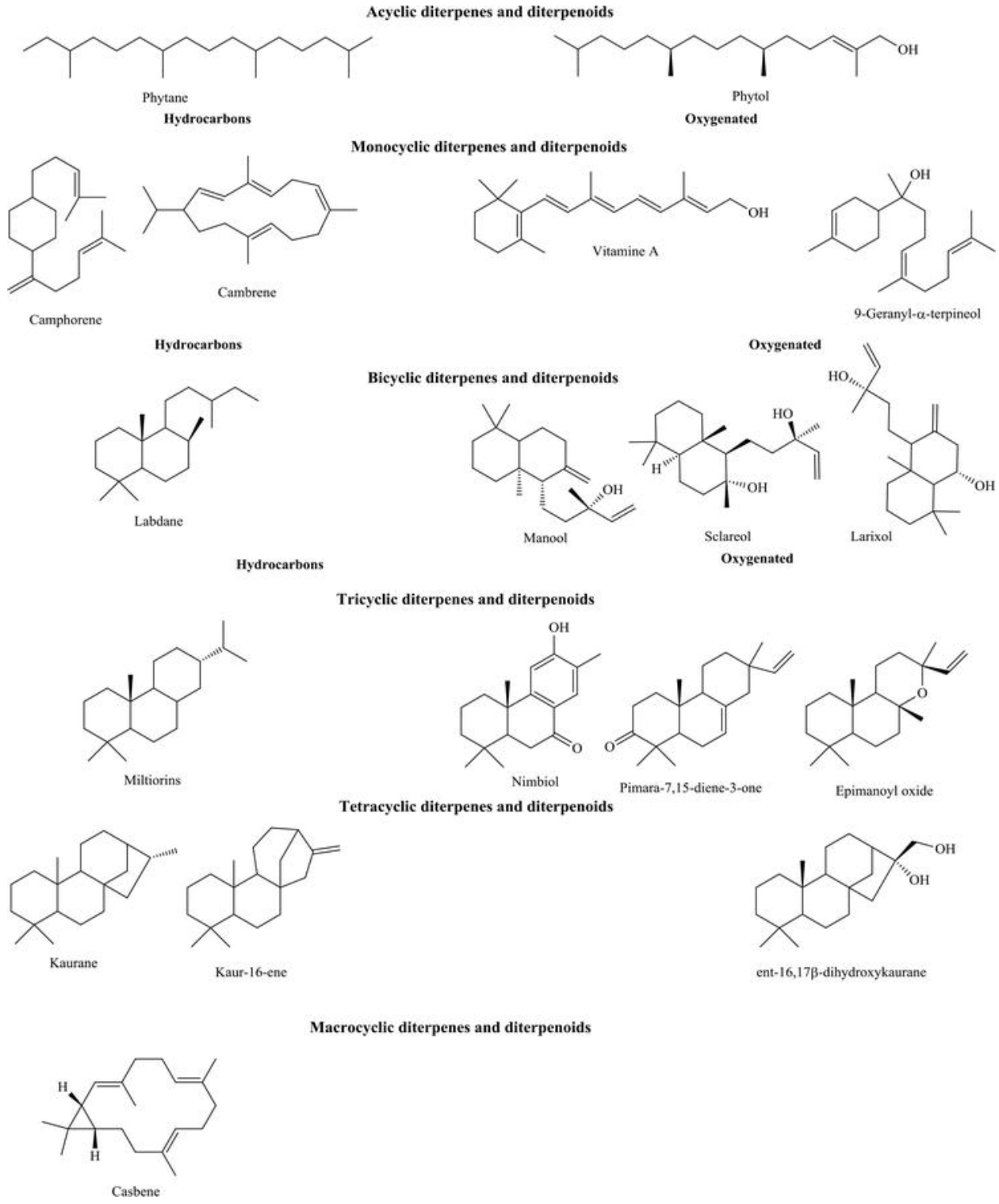


Figure 8: A selection of diterpene and diterpenoid structures; taken from Stephane & Jules (2020)

One particular conversion of interest to APBio, is that of valencene to nootkatone (Figure 9). This conversion makes use of the highly abundant terpene valencene, found in many citrus oils,

as a precursor for the less abundant terpenoid nootkatone, which is responsible for a grapefruit flavour (Hunter & Brogden, 2006). This bioconversion was patented by Muller and co-workers (1998) using lipoxygenase enzymes from soybeans. Previously, nootkatone had been synthesised from valencene using chemical means such as oxidation in the presence of tert-butyl chromate or tert-butyl peracetate, however these are dangerous substances. Tert-butyl chromate has been identified as a carcinogen while tert-butyl peracetate has been determined to act as both fire and health hazards due to its explosive nature (Centers for Disease Control and Prevention, 1995; National Oceanic and Atmosphere Administration, 2010). Therefore, the use of enzymes for this profitable conversion was of interest to the investigators. It was determined that in the presence of hydroperoxides from unsaturated fatty acids and oxygen that the valencene precursor may be converted to nootkatone (Muller *et al.*, 1998). The efficiency of this conversion was increased by the addition of lipoxygenase which allows for the oxidation of the fatty acids which in turn increased the amount of hydroperoxides available to oxidise valencene. Through their investigation it was determined that while soybean flour, with its endogenous lipoxygenase enzymes, may be used to increase the yield of nootkatone, it was rather LOX enzymes in a purer form that resulted in greater yields of nootkatone (Muller *et al.*, 1998).

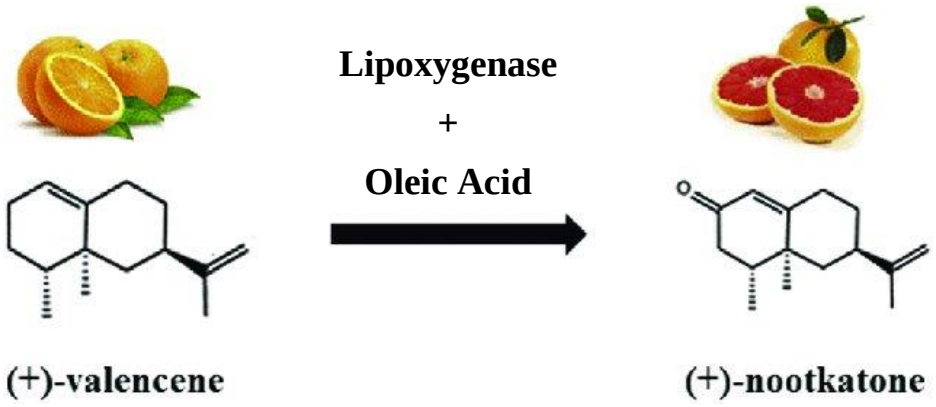


Figure 9: Bioconversion of valencene to nootkatone in the presence of lipoxygenase and oleic acid, adapted from Palmerín-Carreño and co-workers (2015)

1.5. Enzyme isolation from a crude extract

There are numerous plants known to catalyse favourable reactions and as such it becomes prudent to isolate the enzymes from such plants so that they may be characterized and furthermore, used in biocatalytic reactions (Khan *et al.*, 2012). Isolating a single enzyme from a crude extract requires processing, especially because the native conformation of the enzyme needs to be maintained if it is to be used to catalyse a reaction thereafter (Illanes, 2008).

Many protein purification techniques work by initially breaking open the cells before separation of proteins can take place. These methods can include detergents such as sodium dodecyl sulfate (SDS) which works to chemically disrupt the cell membrane, or perhaps sonication which mechanically disrupts cell membranes by bombardment with sound waves, resulting in cavitation (Garcia-Vaquero *et al.*, 2017). If the native conformation of a protein is to be maintained certain techniques must be employed, most often chromatography which takes advantage of either the size, hydrophobicity, charge or affinity for certain ligands that a protein, in its native conformation, might exhibit in order to separate it from other different proteins (Bonner, 2007). These traits can be exploited by changing either the mobile phase, stationary phase, or both of a chromatographic column. Therefore, by exploiting one or multiple of these characteristics, proteins may be partially purified.

Initially however, the solubility of proteins may be taken advantage of. This refers to the phenomenon whereby the solubility of different proteins can be affected by the concentration of ions in the solution in which they are suspended (Duong-Ly & Gabelli, 2014). If the ion-concentration is low (lower than 0.5 M) protein solubility will increase with the increase in ionic strength in a process known as salting-in. The presence of salt helps stabilise the proteins under these conditions because the anions and cations prevent aggregation by neutralising the charged amino acids found on the surface of such proteins (Duong-Ly & Gabelli, 2014). However, as the ionic strength continues to rise the solubility of the proteins starts decreasing in a process known as salting-out. Salting out is caused by the surface of the proteins becoming charged once again by the high concentration of ions causing the protein molecules to begin aggregating (Duong-Ly & Gabelli, 2014). This phenomenon is affected by the ions of the salt dissociating and being recruited by the water found in the protein-containing solution. Thus, instead of the water molecules surrounding the hydrophobic patches exposed on the exterior of the proteins they are removed to solvate the salt ions and the hydrophobic patches become exposed in a solvation shell. If enough salt is added and thus enough water taken from the proteins these hydrophobic patches will begin to interact and result in protein aggregation and eventually lead to precipitation (Doonan & Cutler, 2004).

This phenomenon can be taken advantage of in the purification of proteins. Increasing ionic strength can be achieved by increasing the amount of certain salts, consisting of an anion and a cation, in the protein solution (Duong-Ly & Gabelli, 2014). Of these salts ammonium sulfate ($(NH_4)_2SO_4$) is most often used due to its high solubility as well as its ease of access to acquire and low cost, it has also been found to be less denaturing than the cheaper and more commonly

known sodium chloride (NaCl) salt. Therefore, this can be a useful initial step in the purification of proteins by separating those that more easily aggregate from those that are highly soluble. It also does not require any other specific factors regarding the proteins, such as a purification tag that can select only for the protein of interest. This can, therefore, work well for a collection of proteins naturally produced in soybean seeds rather than engineered and overexpressed in an alternate host. Another advantage of precipitation is its ability to concentrate the protein of interest from a much larger initial volume into one much more manageable with the aid of an appropriate centrifuge (Doonan & Cutler, 2004). Unfortunately this procedure will drag out other contaminates from the crude extract and will thus need to be followed by other purification techniques, such as chromatography (Duong-Ly & Gabelli, 2014).

The types of column chromatography available for protein, or more specifically enzyme, purification are numerous and it is important to consider all the factors that influence the proteins of interest before choosing the best order in which to separate the collection of proteins from one another (Doonan & Cutler, 2004).

Soybean lipoxygenase isoforms have different pI values, substrate specificities, pH optima, etc. (Baysal & Demirdöven, 2007; Siedow, 1991). Many other factors are quite similar, such as size and amino acid composition. Previous investigations have shown that the different pI values are 5.68 for LOX-1, 6.25 for LOX-2 and 6.15 for the LOX-3 isoforms (Baysal & Demirdöven, 2007; Siedow, 1991). These values all lie below pH 7, a neutral pH which will not influence protein structure adversely (Doonan & Cutler, 2004), which makes the collective separation thereof from other soybean seed proteins an easy chromatographic separation step. This is due to the fact that when in a solution at one pH value lower than the pI of the protein it will have an overall positive charge whereas a higher pH will confer a net negative charge to the protein (Bonner, 2007).

Ion exchange chromatography (IEX) will take advantage of this (Bonner, 2007). Anion exchange resins are positively charged and therefore attract negatively charged molecules while cation exchangers sit on the other end of the spectrum, attracting positively charged molecules. Furthermore, ion exchange resins are generally inexpensive and capable of being washed and reused numerous times without losing efficacy (Bonner, 2007). The common two types of ion exchange resins carry either a negatively charged carboxymethyl (CM) group or a positively charged diethylaminoethyl (DEAE) group which will bind to molecules of opposite

charge. Therefore, if the protein of interest is pre-equilibrated in a buffer of the pH value that will cause it to become more positively or negatively charged these resins will be able to bind only those proteins in the crude extract that fall within the correct pI range. The rest will elute from the column holding the resin as it is washed with the binding buffer (the same pH and buffer components to which the crude extract proteins were equilibrated). Only once there is a change in pH or ion concentration will the net charge of the bound proteins change and the proteins will be released from the column and elute separately from those that did not bind, as well as those that detached earlier or later as the pH or ion concentration was changed (Bonner, 2007).

While it seems more straightforward to change the pH to elute the bound proteins, this process is much more complicated in practice. pH does not necessarily change linearly and cannot be mixed in equal proportions to give a gradient the same way adding more of a buffer with a higher salt concentration will result in a linear gradient (GE Healthcare Life Sciences, 2016). The addition of more salt will result in only the ion concentration increasing which can provide reproducible results, a pH gradient on the other hand will not only lead to a change in pH but may also result in the ion concentration changing too which will also affect how the proteins elute (GE Healthcare Life Sciences, 2016). This indicates that eluting using a salt is more reliable than using a pH gradient. As long as the correct salt is used, such as NaCl, which is most often used for its chaotropic nature that allows it to outcompete the proteins' charged ions for the charged resin, there is little chance of the proteins precipitating (Bonner, 2007). Whereas if the pH is altered too far from the range at which the protein is soluble it can denature problematically (GE Healthcare Life Sciences, 2016).

Due to the more general nature of IEX it cannot clear a mixture of proteins completely from the protein of interest and is most often an initial step in the purification procedure (Bonner, 2007). Conversely hydrophobic interaction chromatography (HIC) could be used directly after ammonium sulfate precipitation due the requirement for a high salt concentration in the protein containing solution for binding to the resin (Bonner, 2007). The proteins would thereafter be eluted from the resin by using a low-salt buffer to create a gradient to lower the salt concentration. This will allow the protein to regain its solvation shell and refold back into its native conformation. Much in the same way dialysis works (Bonner, 2007). This chromatographic technique however, results in large volumes that are quite dilute which would then need to be concentrated to more manageable volumes. This can be taken care of by following up with IEX which works well with such starting material (Bonner, 2007).

A further chromatographic technique that does not require any specific tags or factors is that of size exclusion chromatography (SEC, Figure 10) (Bonner, 2007). Unlike the previous two resins, this technique has no direct interaction between the resin and proteins. It will therefore have very little or even no effect on the biological activity of the proteins. Unfortunately, this technique must be used towards the end of the purification procedure as it has a low capacity for how much protein may be loaded onto the column and will dilute the sample immensely thereafter (Bonner, 2007). This technique will separate proteins too large to enter the small pores in the beads of the resin from those small enough to become trapped temporarily. However, these smaller molecules will not only be found within the pores of the resin and thus some will elute along with the larger proteins early on. Not only size matters as the shape of the protein may also affect how quickly the protein elutes (Bonner, 2007). Long and thin proteins will be able to move through the column faster than globular proteins and thus even if they are smaller they will defy the standard elution behaviour for SEC (Bonner, 2007). The purification ability is thus not as intense as that conferred by either IEX or HIC. For the LOX isoforms this technique may not necessarily be suitable to separate the isozymes from one another as they are all of the same size and shape.

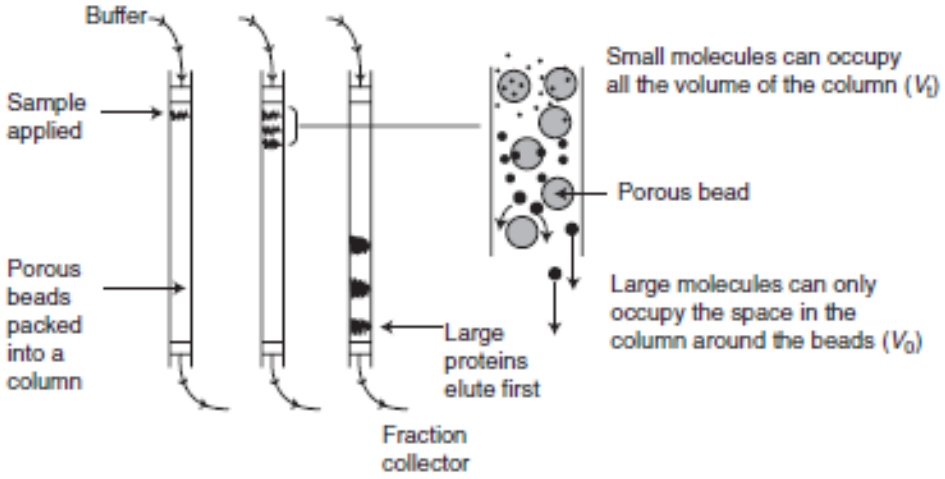


Figure 10: An illustration of how size exclusion chromatography separates proteins by size; taken from Bonner (2007)

Once the correct chromatographic techniques have been identified it is imperative to determine whether the use of a gravity flow column would be appropriate to separate the protein mixture or whether a high-pressure system, such as the ÄKTA Start system, would be more suited to the experiment. Due to the nature of column chromatography the separation of a complex mixture of proteins can become very time consuming, based on the flow rate of the sample through the matrix of the column (Chakravarti *et al.*, 2016).

A gravity flow column does as the name suggests; the sample and liquid mobile phase of the chromatography procedure will flow only based on the force of gravity (Chakravarti *et al.*, 2016). A high-pressure system makes use of pumps to move the buffer and sample through the column at chosen pressure values (these may differ based on the capabilities of the column chosen so as not to damage the resin) which will inevitably speed up the purification process (Chakravarti *et al.*, 2016). A further advantage of the high-pressure system is it most often connected to an ultra violet (UV) cell reader that can give information on the proteins that are washed and eluted from the column at the different stages of chromatography making the collection of important fractions that much easier. The mixing of different buffers to create even gradients is also done automatically by the system rather than manually which eases the workload and eliminates human error (Chakravarti *et al.*, 2016). Therefore, if the system and its compatible columns are available the high-pressure system is a much more convenient method to separate proteins.

1.6. Enzyme identification

Once separated from other proteins it is vital to identify into which fraction the protein (enzyme) of interest has eluted (Bonner, 2007). If the size of the protein is known, the protein may be identified using denaturing techniques such as sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), to confirm that the protein is there based purely on its size (Lodish *et al.*, 2003). However, unless the purification procedure is very thorough and removes all other proteins of a similar length this technique may not be satisfactory in proving that the protein has been selectively purified. Therefore, it is imperative that the activity of the enzyme be confirmed via an enzyme assay to ensure that it is the correct enzyme that has been collected. Thus the identification of a quick, affordable and quantitative assay for the measurement of enzyme activity is an important factor to be considered (Eisenthal & Danson, 2002).

The identification of lipoxygenase enzymes via spectrophotometric assays has most often focussed on the LOX-1 isoform (Anthon & Barrett, 2001). There are numerous methods that have been recorded to effectively determine the activity of LOX-1 from soybean seeds. These methods all focus on the preferred substrate of lipoxygenase – linoleic acid – in different ways. The most straightforward of these assays is described by Axelrod and co-workers (1981) whereby the formation of conjugated double bonds from linoleic acid causes an increase in UV absorbance at 234 nm. This assay and many of its derivatives, however, are not necessarily suitable for crude protein extracts as there are other molecules that can interfere with the UV absorbance (Anthon & Barrett, 2001). The paper by Axelrod and co-workers (1981) also

describes the use of a Clark oxygen electrode in a Gilson Medical Electronics Oxygraph wherein the usage of oxygen is measured during the reaction rather than UV absorbance. However, the expense of such a specific piece of equipment is not necessary when there are alternate spectrophotometric methods to determine lipoxygenase activity.

A different type of assay was described by Anthon & Barrett (2001), it too made use of linoleic acid, however instead of measuring continuous diene formation at 234 nm, the assay measured a final absorbance value at 598 nm to detect a colorimetric change based on the lipid hydroperoxides produced that reacted with bovine haemoglobin in the presence of 3-methyl-2-benzothiazolinone. The haemoglobin-lipoxygenase coupled reaction is rather difficult to control which makes it less suitable for fast and replicable experimentation (del Carmen Pinto *et al.*, 2007). However, there were other assays described in the literature that could be investigated, del Carmen Pinto and co-workers (2007) described an assay that measured lipoxygenase activity using a modified ferrous oxidation-xylenol orange assay. This assay is also based on a colorimetric change caused by the oxidation of Fe^{2+} to Fe^{3+} from the hydroperoxides produced by the lipoxygenase catalysed reaction with linoleic acid. The Fe^{3+} forms a complex with xylenol orange which can be measured at an absorbance of 550 nm.

Lastly, an alternate colorimetric assay has been described in numerous studies wherein methylene blue is bleached by the hydroperoxides produced from linoleic acid in the presence of lipoxygenase enzymes (Toyosaki, 1992; Suda *et al.*, 1995). The investigations undertaken by Suda and co-workers (1995) showed that by performing such an assay under different conditions one might determine the activity of soybean LOX-1 selectively from that of soybean LOX-2 based on their pH optima and activity in different buffer systems. The presence of methylene blue in the assay was determined at an absorbance of 660 nm.

1.7. Enzyme storage and industrial needs

Enzymes are generally quite stable in their native hosts due to the correct requirements being maintained in the cellular environment in which the proteins find themselves (Simpson, 2010). These stabilising factors are removed however when the proteins are extracted from their native host for any reason. The pH of the cellular environment, the temperature, the concentration of buffering ions that maintain the conformation and activity of the enzyme, as well as the protection from damaging agents such as proteases may all need to be recreated synthetically in laboratory settings to ensure the enzyme is not degraded before use (Simpson, 2010).

The lipoxygenase enzymes, as with all proteins, have different requirements in order to remain active and it cannot be said that what works for one enzyme or protein will work for another (Simpson, 2010). This premise may also be true for the different LOX isoforms and thus, by stabilising one isoform another may be left susceptible to degradation under the same storage conditions. It consequently becomes necessary to determine the effect such stabilisation factors have on the different LOX isoforms and whether or not they are even necessary after the enzymes have been purified from the soybean seeds as some proteins are more stable than others (Simpson, 2010).

The most obvious option to delay protein degradation would be to store the enzymes at lower temperatures, the way one would store food in the fridge or freezer to delay spoilage (Erkman & Bozoglu, 2016). However, there is not only the 4℃ fridge and -20℃ freezer options that one might find in a kitchen, but there is also liquid nitrogen for flash freezing the proteins as well as -80℃ freezers (Vaniotis, 2020). The fridge will only keep the proteins stable for as long as it takes oxidation and other contaminants to overcome the low energy barrier and degrade the proteins if no other protective additives are acting in conjunction with the cold. A freezer however, may be able to halt these factors indefinitely. These contaminating factors can include proteases (enzymes which degrade proteins), microbes and molecular oxygen which may oxidise the enzyme and lead to inactivation or even aggregation (Simpson, 2010). The proteases may be removed during the purification of the enzyme if they do not share similar characteristics as the protein of interest, as described previously, or may be halted by the addition of protease inhibitors. Microbial contaminants can be avoided if the enzyme is handled aseptically during all processing and aliquoting procedures (Simpson, 2010). Oxygen on the other hand is more likely to be found in the liquid due to the abundance of oxygen in the atmosphere. It can be removed by purging the sample with other gases (i.e. nitrogen) for 20 to 40 minutes (Butler *et al.*, 1994) but will be reintroduced if the sample is ever exposed to the air. By freezing the proteins however, many of these issues can be avoided by removing the environment necessary for them to proceed in protein degradation.

There is conflict in the literature regarding the effect the rate of freezing and thawing has on the enzymes frozen. The study by Nema & Avis (1993) determined that by freezing enzymes faster they retain more activity than those that were frozen slowly. This is in line with the common knowledge that fast freezing will produce smaller ice crystals within the sample and damage the substances within it less than the larger ice crystals formed during slower freezing. Cao and co-workers (2003) observed the opposite whereby the fast freezing (>10℃/minute)

and slow thawing (<10°C/minute) so often recommended will damage the proteins more so than slow freezing (~1°C/minute) and fast thawing (>10°C/minute). The researchers claim that this is due to the production of the fine ice crystals mentioned previously. These fine ice crystals occur throughout the sample and thus have a larger surface area that adsorbs to the proteins which in turn will cause denaturation of the protein surface, thereby damaging them. This effectively nullifies the benefits of the fast freezing whereby the pH and buffer concentration will not be altered nearly as much as when frozen slowly when large, non-uniform ice crystals force the proteins and solutes into non-frozen areas which results in the solute concentration and pH to shift due to the buffer salt crystallising. These phenomena may result in the dehydration and denaturation of the proteins and thus result in their inactivation.

Cao and co-workers (2003) also observed that slow thawing may be problematic, most notably when performed on fast frozen samples, once again due to the fine ice crystals. Their claim is that when frozen so rapidly the sample does not reach thermodynamic equilibrium and thus when the temperature begins to change (i.e. thawing) there is the chance that the small ice crystals will not just melt but rather begin to form larger ice crystals at the same time to reduce the system energy in a process called recrystallisation. These large ice crystals will consequently damage the proteins in the sample. If faster thawing is employed (>20°C/minute) the melting rate can overcome the recrystallisation rate and result in less denaturation. This problem is less drastic when slow thawing is employed on slow frozen samples as the thermodynamic equilibrium of the sample is more stable than the fast frozen samples and thus the enzymes appeared to be less affected by the thawing rate.

The addition of cryoprotectants and other additives as well as altering the pH, buffer and concentration thereof as well as the addition or removal of NaCl (sodium chloride has been seen to increase the amount of crystallisation in slow freezing with certain buffers such as sodium phosphate) in the sample before it is frozen has also been observed to make a difference in the enzyme activity recovery after freezing due their ability to prevent some of the problems mentioned previously when slow freezing is employed (Cao *et al.*, 2003). However, when the concentration of protein in the sample is high (>1 mg/ml) these effects are less problematic as the proteins tend to be affected less severely by the damaging processes observed during freeze-thawing (Cao *et al.*, 2003).

Another storage option often used for purified enzymes is in a lyophilised form. This particular storage method removes the need for a cool environment which makes the long-term storage

less prone to outside influences (Day & Stacey, 2007) . The loss of power or break-down of the fridge or freezer in which the samples are stored will not factor in if the sample can be stored at room temperature which can be achieved so long as the dried sample is kept moisture-free. If the sample is rehydrated or left in a humid environment where it is not properly sealed it will begin to degrade once again as water will allow for the conditions needed by microorganisms to grow and destroy the proteins. The pH of the sample may also change over time in a water-rich environment as well as oxidation could take place, both factors can lead to the degradation of the proteins in the sample (Day & Stacey, 2007).

Lyophilisation takes advantage of the phenomenon whereby the sample is cooled below its triple point (Parker & Kristol, 1974). Therefore, when the vacuum begins to remove the liquid from the sample sublimation occurs and avoids the chance that melting will ensue as the water is removed from the sample (Parker & Kristol, 1974). In order to cool the sample to this triple-point temperature the first step in freeze-drying is to bring the sample down to a very low temperature to create ice crystals within the sample which will aid in the drying process (allow for sublimation to occur). Freezing also solidifies the sample which prevents foaming when the high pressure exerted by the vacuum is applied. The sample may be affected by the problems described by Cao and co-workers (2003) in the freezing process but will be protected from the thawing effects as the sample will not have any ice crystals left to undergo the recrystallisation process and damage the proteins.

There are other processes, however, in the freeze-drying process that may damage sensitive samples and as such it is important to note the effect freeze drying without added protectants will have on the activity of the enzyme (Adams, 2007). Additives may influence the activity of the sample when it needs to be used after it has been rehydrated and thus may need to be removed before the sample can reach its full activity. Thus if these substances can be avoided so too can such problems (Adams, 2007).

While freeze-drying is a helpful tool for the industrial storage and transport of purified enzymes a further set of techniques that can increase the biocatalytic properties, stability and recyclability of an enzyme is immobilisation. Immobilisation has been described as the confinement of enzymes in a specified space while not only retaining their catalytic ability but improving their recyclability for reuse (Velasco-Lozano *et al.*, 2016). According to Sheldon & van Pelt (2013) there are three main categories into which enzyme immobilisation techniques can be divided: 1. binding the enzyme to a prefabricated support/carrier, 2.

entrapment/encapsulation of the enzyme during the synthesis of a support matrix, and 3. cross-linking of enzyme aggregates or crystals without a carrier/matrix. The first two methods are both dependant on a carrier which will dilute the activity of the enzyme due to the large volume of a non-catalytic substance (which can make up anywhere from 90-99% of the immobilised enzyme's mass) added to the reaction vessel. Adding further enzyme into these immobilisation techniques has not been seen to improve activity as the enzyme molecules can become buried within the matrix during the encapsulation process or under other enzymes if bound to a prefabricated matrix – only those accessible at the surface will be able to interact with the substrate (Sheldon, 2008). Thus, over time, more focus has turned to the cross-linked enzymes that work completely carrier-free and will not dilute a reaction so drastically as the other two immobilisation techniques (Sheldon, 2008). The production of cross-linked enzyme aggregates (CLEA) or cross-linked enzyme crystals (CLEC) has a further advantage over the matrix-based techniques in that the production thereof is much cheaper without the need for the expensive carrier (Sheldon & van Pelt, 2013). For these reasons at an industrial level the use of an immobilised enzyme in CLEA or CLEC form rather than attached to a bulky, expensive matrix may be more profitable if the enzyme is to be immobilised.

The production of CLEAs is cheaper than that of CLECs due to the lack of need to crystallize the proteins before cross-linking takes place(Sheldon & van Pelt, 2013). Instead, the enzymes must be aggregated initially which can be achieved by precipitation which is a generally simple and cheap technique. As described previously, ammonium sulfate ($(NH_4)_2SO_4$) has the ability to bring proteins selectively out of solution based on their individual properties, by knowing how the protein of interest will be affected at the different salt saturations the enzyme may be successfully aggregated before cross-linking (Velasco-Lozano *et al.*, 2016). Cross-linking is thereafter, most often, achieved by the addition of glutaraldehyde, which is a small dialdehyde molecule which, like ammonium sulfate, is relatively inexpensive. The small size of the glutaraldehyde molecule allows it to penetrate the protein interior in some cases, which can become problematic, but in most instances this problem is not notable and rather the glutaraldehyde interacts with the lysine residues found on the protein surface to form the cross-linkages (Sheldon, 2008; Sheldon & van Pelt, 2013; Velasco-Lozano *et al.*, 2016).

CLEAs have been tested on a wide range of enzymes with much success in improving stability wherein the immobilised enzymes show increased resistance to high temperatures, mechanical forces, extreme pH, high substrate concentrations, etc. (Sheldon & van Pelt, 2013). The technique has also shown that the enzymes may be recycled after use so they may be separated

from the reaction components and utilised once again (Velasco-Lozano *et al.*, 2016). In some cases, the enzymes have even shown to have increased enantioselectivity which can improve the production of sought-after enantiomers of products. Another important consideration with regards to the use of CLEAs is their ability to be formed directly from crude enzyme extractions (Sheldon & van Pelt, 2013). If the other proteins found within the sample that precipitate out of solution similarly to the protein of interest will not have an effect on the reaction of interest then the proteins in the crude sample may be immediately subjected to the precipitation technique before it is cross-linked, this will cut down on many other purification techniques that may otherwise be necessary.

1.8. Enzyme kinetics

As previously discussed, enzymes are the catalysts used under biological circumstances to lower the activation energy required for a reaction to proceed (Lodish *et al.*, 2003). Enzymes contain active sites that allow for the binding of the substrate which is required for the reaction. Thereafter the enzyme may perform its function by catalysing the conversion of the substrate into its corresponding product. Each enzyme, however, has evolved to perform a different function and as such has evolved to work slightly differently. These differences will result in which substrate may bind, what product will be formed and at what rate this catalysation may take place (Cornish-Bowden, 1979). Investigations are performed to determine such factors to help qualify and quantify these factors for future use of enzymes.

Once purified and separated from other proteins and contaminants it becomes possible to determine the kinetic parameters to which it conforms (Cornish-Bowden, 1979). These parameters have been determined by the maximum potential of the enzyme and can be tested once the substrate for an enzyme has been discovered. Furthermore, if the enzyme has multiple substrates these parameters will be different for each substrate. The two parameters investigated are Vmax and Km which both depend on the amount of substrate added to a reaction. By using increasing concentrations of substrate it becomes possible to determine at which concentration the enzyme exhibits maximum activity and stops reacting any faster (Cornish-Bowden, 1979).

The rate of a reaction increases with an increase in substrate concentration as it become easier for an enzyme to come into contact with a substrate molecule (Cornish-Bowden, 1979). As this concentration increases so does the likelihood of an interaction and thus production of product, however, over a certain substrate concentration the rate will be limited by the amount of

enzyme present because above this point all enzymes will be saturated with substrate. This rate is termed Vmax and can be used to calculate the other parameter used to describe an enzyme – Km. This value was defined by Leonor Michaelis and Maud Menten through the Michaelis-Menten equation which had been used to characterise an enzymatic reaction (Michaelis *et al.*, 2011). Their equation allowed for Km to be determined at half the value of Vmax and used to extrapolate the concentration of substrate responsible for half the maximum velocity an enzyme may reach. This value is useful because a reaction may show the same Vmax values under different circumstances (such as temperature, substrate and other assay components) but will result in different Km values which can be used to indicate the affinity the enzyme has for the substrate. The higher the affinity an enzyme has for a substrate the better it is at finding the substrate and binding to it to perform its catalytic function and will reach its maximum capacity all the more quickly (Cornish-Bowden, 1979).

1.9. Aims and objectives

To provide a catalyst for the green synthesis of nootkatone we intend to explore the following:

1. Identifications of quantitative assays for lipoxygenase-1 (LOX-1) and lipoxygenase-2 (LOX-2) activity to track the partial purification of such enzymes as well as effects of working conditions.
2. Determine cost- and time-effective extraction procedures of LOX-1 and LOX-2 from defatted and whole soybeans.
3. Determine cost- and time-effective partial purification procedures of LOX-1 and LOX-2 from crude protein extracts through selective protein precipitation and high-pressure chromatography.
4. Investigate effect of creating cross-linked enzyme aggregates on enzyme activity, recyclability and ability to convert valencene to nootkatone.
5. Determine optimal working conditions of enzymes (pH and temperature) for industrial use.
6. Determine how storage conditions will affect enzyme activity as well as the shelf-life of the enzyme.
7. Investigate the enzyme kinetics of the partially purified LOX-1 and LOX-2 enzymes.

2. Methods and materials

2.1. Quantitative assays of soybean lipoxygenase isozymes

2.1.1. Substrate preparation

Sodium linoleate substrate (10 mM) was prepared as described by Suda and co-workers (1995). In 4 ml autoclaved distilled water (ddH_2O), 70 mg of linoleic acid and 70 mg of Tween 20 were homogenized, avoiding air bubbles. To clarify this solution approximately 0.55 ml of 0.5 N sodium hydroxide (NaOH) was added. Thereafter, the solution was made up to 25 ml total volume with ddH_2O. Air bubbles were avoided by using a micropipette to mix the small volume of liquid and one the mixture was brought up to 25 ml it was inverted slowly to ensure no bubbles were created.

2.1.2. Lipoxygenase-1 assay

The assay was performed as described by Suda and co-workers (1995). In a cuvette 0.5 ml of 0.2 M sodium borate buffer (pH 9.0), 0.1 ml of 100 µM methylene blue, and 0.1 ml of 10 mM sodium linoleate substrate were mixed, along with a volume of soybean (Seeds for Africa, South Africa) extract sample containing 100 µg of crude protein – as determined by Qubit® 2.0 Fluorometer (485/590 nm; Invitrogen by Life Technologies, Carlsbad, California, United States). Distilled water (dH_2O) was added to give a total volume of 1.0 ml. A blank consisting of all components (including the enzyme) other than the methylene blue, which was replaced with an equal volume of ddH_2O, was used to zero the spectrophotometer before the assay was performed. The reaction was initiated by the addition of the soybean extract sample, and the absorbance was recorded at 660 nm using the S-20 Spectrophotometer (Boeco, Germany) at 25°C. The reaction was recorded until the optical density (OD) of the sample stopped decreasing (for a maximum of 5 minutes) as the LOX-1 in the sample interacted with the linoleic acid to bleach the methylene blue.

2.1.3. Lipoxygenase-2 assay

The assay was performed as described by Suda and co-workers (1995). In a cuvette 0.4 ml of 0.2 M sodium phosphate buffer (pH 6.0), 0.1 ml of 100 µM methylene blue, 0.1 ml of freshly prepared 200 mM dithiothreitol (in 0.2 M sodium phosphate buffer, pH 6.0), 0.1 ml of acetone and 0.1 ml of 10 mM sodium linoleate substrate were mixed, along with a volume of soybean (Seeds for Africa, South Africa) extract sample containing 1.0 mg of crude protein – as determined by Qubit® 2.0 Fluorometer (485/590 nm; Invitrogen by Life Technologies, Carlsbad, California, United States). Distilled water (dH_2O) was added to give a total volume

of 1.0 ml. A blank consisting of all components (including the enzyme) other than the methylene blue, which was replaced with an equal volume of ddH_2O, was used to zero the spectrophotometer before the assay was performed. The reaction was initiated by the addition of the soybean extract sample, and the absorbance was recorded at 660 nm using the S-20 Spectrophotometer (Boeco, Germany) at 25°C. The reaction was recorded until the OD of the sample stopped decreasing (for a maximum of 10 minutes) as the LOX-2 in the sample interacted with the linoleic acid to bleach the methylene blue.

2.1.4. Investigation of assay false positive reactions

Once purified enzyme had been attained it was prudent to test the assays to ensure that it was indeed the action of LOX-1 and LOX-2 responsible for the bleaching of methylene blue. The assays described by Suda and co-workers (1995) were tested with the enzyme and all reactants other than the substrate (10 mM sodium linoleate) to determine if the methylene blue was bleached by another mechanism than the oxidation of linoleic acid. The assays were also tested without the addition of the enzyme to determine if the reaction components could account for the bleaching of the methylene blue by auto-oxidation of the substrate and methylene blue in an oxygen-rich atmosphere. These were compared to a complete reaction containing the purified enzyme.

2.2. Protein purification

2.2.1. Crude protein extraction from plant material

Based on the methods already in practice by Applied Protein Biotechnologies crude protein extraction was performed and the process was refined. Air dried plant material (both de-fatted soybeans (DS) and whole soybeans (WS)) was milled using a coffee grinder. Crude enzyme extract was prepared thereafter using different buffers. The concentration of boric acid was varied in the analysis for performing an osmotic shock buffer (OSB) soak. The milled beans, in a 20% w/v ratio of plant material to buffer, were initially soaked overnight at 4°C in a buffer of 5 mM sodium metabisulfite ($Na_2S_2O_5$, pH 4.46), the liquid was poured off the beans thereafter and the same volume of buffer now consisting of 0.5 M sucrose, 10 mM $Na_2S_2O_5$ and differing concentrations of boric acid (50 mM, 100 mM, 150 mM or 200 mM) was added and left for a further night at 4°C (~pH 6.0). The other soak to which the extracted protein activity was compared was a single overnight soak at 4°C, in a 20% w/v ratio of plant material to buffer; this was performed with differing concentrations of boric acid (50 mM to 200 mM, ~pH 8.5). The plant material was thereafter strained through four layers of cheese cloth and the filtrate was retained for assaying. After the assays had been performed it was determined that

the most time and cost-effective method, that also resulted in a high enzyme concentration, was a single overnight soak of 125 mM boric acid at 4°C. It was also decided that 10 mM $Na_2S_2O_5$ should be included for its preservative effect on the extracted proteins (Barberá *et al.*, 2000). The samples were run on SDS-PAGE for analysis of the size of the proteins in the samples.

Once an optimal extraction buffer had been determined, altering the pH of the buffer was tested (the pH of the buffer was altered through the addition of sodium hydroxide or hydrochloric acid). To ensure no resultant buffer from the extraction altered the pH of the activity assays and hindered the true results, the overnight extracts were left to dialyse against 125 mM boric acid 10 mM $Na_2S_2O_5$ at pH 7.0 for a further 24 hours. Thereafter, enzyme activity was checked via the assays (section 2.1), and it was determined that pH 5.0 worked best for the extraction of LOX proteins from DS and pH 7.0 for WS. The strained enzyme extract was stored at 4°C until use to slow enzymatic activity. Total protein concentration of the supernatant was quantified using the Qubit® 2.0 Fluorometer (485/590 nm; Invitrogen by Life Technologies, Carlsbad, California, United States). The proteins were run on SDS-PAGE for analysis of the size of the proteins in the samples.

2.2.2. Protein precipitation

The protein extracts from the WS source were subjected to a graded ammonium sulfate precipitation as described by Duong-Ly & Gabelli (2014) as per Table 10 (Appendix 7). Initially the crude protein solution was brought to 20% saturation using solid ammonium sulfate crystals. The sample was left at 4°C for 30 minutes with continual stirring using a magnetic stirrer. The sample was then centrifuged at 20,000 x g for 30 minutes at 4°C. The supernatant was brought to 40% saturation using solid ammonium sulfate crystals and the procedure above was repeated. The resultant supernatant was brought to 60% saturation using solid ammonium sulfate crystals and then 80% saturation using solid ammonium sulfate crystals after each successive round of pelleted protein and cell debris was removed via centrifugation. At each saturation level a 1 ml sample of supernatant and the pellet (resuspended in a quarter of the original volume of the sample worth of their respective extractions buffers) was retained for SDS-PAGE (to determine the size of the proteins in the samples) and assay analysis (section 2.1).

2.2.3. Dialysis

The samples exhibiting enzyme activity with the presence of ammonium sulfate were subjected to dialysis to remove the contaminating salt. The samples (DS and WS), housed within

SnakeSkin™ Dialysis Tubing, 10K MWCO, 35 mm (Thermo Scientific™, Waltham, Massachusetts, United States), were dialysed against their respective extraction buffers altered to pH 7.00 for further processing. The buffer was changed hourly for three hours before being left, above a magnetic stirring plate, overnight at 4°C.

2.2.4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Sample buffer (2x concentration) was prepared as described in the Bio-Rad Formulations Bulletin (Bulletin_6199.pdf, n.d.) consisting of 62.5 mM Tris-HCl (pH 6.8), 2% SDS (v/v), 25% glycerol (v/v) and 0.01% (w/v) bromophenol blue. The buffer was stored at room temperature until needed at which time 5% total volume (v/v) 2-mercaptoethanol was added.

Total protein concentration of the samples was quantified using the Qubit® 2.0 Fluorometer (485/590 nm; Invitrogen by Life Technologies, Carlsbad, California, United States) before and after dialysis. An equal volume of 2x sample buffer was added to the samples and thereafter, based on the quantification, the volume loaded into each well was calculated to contain 20 µg total protein. The samples were boiled at 95°C in a Dri-Block® DB-2D (Techne, Stone, Staffordshire, United Kingdom) heating block for 3 minutes to linearise the proteins and to ensure that no endogenous proteases were left active in the room temperature samples.

A 10% (w/v) separating gel was prepared according to the Bio-Rad Bulletin (Bulletin_6199.pdf, n.d.) using 40% (v/v) acrylamide/bisacrylamide solution, 0.375 M Tris-HCl (pH 8.8) and 0.1% (v/v) SDS, 10% (w/v) ammonium persulfate (APS) and 0.025% (v/v) tetramethylethylenediamine (TEMED) were added last to initiate polymerisation. Once poured into a 1.0 mm gel casting apparatus a layer of 2-propanol was poured on top to ensure that the gel polymerized level. After the gel had been allowed to polymerize the 2-propanol was removed so that the 4% (w/v) stacking gel, made using 40% (v/v) acrylamide/bisacrylamide solution, 0.375 M Tris-HCl (pH 6.8) and 0.1% (v/v) SDS, that would be forced to polymerise by the addition of 10% APS and 0.025% TEMED, could be poured on top.

A 1x running buffer was prepared from a 10x stock solution (pH 8.3) consisting of 250 mM Tris Base, 1.92 M glycine and 1% (v/v) SDS. The buffer was poured into both chambers of the electrophoresis tank and samples (of their respective calculated volumes) were pipetted into the wells of the gel. The first lane of the gels contained PageRuler™ Unstained Protein Ladder (Thermo Fisher Scientific, Waltham, Massachusetts, United States) with a molecular weight range between 10 and 200 kDa. The gels were run at 80 V until the dye front reached the bottom

of the stacking gel, and aligned horizontally, and thereafter the gel was run at 120 V until the dye front had run off the end of the gel.

The polyacrylamide gels were stained with 0.1% (w/v) Coomassie R250 stain (40% (v/v) methanol, 10% (v/v) glacial acetic acid), with shaking (100 rpm), overnight. The gels were then de-stained (20% (v/v) methanol, 10% (v/v) glacial acetic acid), with shaking (100 rpm) until the gel had turned clear, and then stored in dH2O. Pictures were taken of the gels using a light box.

2.2.5. Native polyacrylamide gel electrophoresis

Sample buffer (2x concentration) was prepared as described in the Bio-Rad Formulations Bulletin (Bulletin_6199.pdf, n.d.) consisting of 62.5 mM Tris-HCl (pH 6.8), 40% (v/v) glycerol and 0.01% (w/v) bromophenol blue. The buffer was stored at room temperature until needed.

Total protein concentration of the samples was quantified using the Qubit® 2.0 Fluorometer (485/590 nm; Invitrogen by Life Technologies, Carlsbad, California, United States) before and after dialysis. An equal volume of 2x sample buffer was added to the samples and thereafter based on the quantification the volume loaded into each well was calculated to contain 20 µg total protein.

A 10% (w/v) separating gel was prepared according to the Bio-Rad Bulletin (Bulletin_6199.pdf, n.d.) using 40% (v/v) acrylamide/bisacrylamide solution, 0.375 M Tris-HCl (pH 8.8), 10% (w/v) ammonium persulfate (APS) and 0.025% (v/v) tetramethylethylenediamine (TEMED) were added last to initiate polymerisation. Once poured into a 1.0 mm gel casting apparatus a layer of 2-propanol was poured on top to ensure that the gel polymerized level. After the gel had been allowed to polymerize the 2-propanol was removed so that the 4% (w/v) stacking gel, made using 40% (v/v) acrylamide/bisacrylamide solution, 0.375 M Tris-HCl (pH 6.8), that would be forced to polymerise by the addition of 10% (w/v) APS and 0.025% (w/v) TEMED, could be poured on top.

A 1x running buffer was prepared from a 10x stock solution (pH 8.3) consisting of 250 mM Tris Base, 1.92 M glycine. The buffer was poured into both chambers of the electrophoresis tank and samples (of their respective calculated volumes) were pipetted into the wells of the gel. The first lane of the gels contained Precision Plus Protein™ Dual Xtra Standards Ladder (Bio Rad, Hercules, California, United States) with a molecular weight range between 2 and 250 kDa. The gels were run at 80 V until the dye front reached the bottom of the stacking gel,

and aligned horizontally, and thereafter the gel was run at 120 V until the dye front had run off the end of the gel.

The polyacrylamide gels were stained with 0.1% (w/v) Coomassie R250 stain (40% (v/v) methanol, 10% (v/v) glacial acetic acid), with shaking (100 rpm), overnight. The gels were then de-stained (20% (v/v) methanol, 10% (v/v) glacial acetic acid), with shaking (100 rpm) until the gel had turned clear, and then stored in dH2O. Pictures were taken of the gels using a light box.

2.2.6. Ion exchange chromatography

The ÄKTA™ start chromatography system (Cytiva, Global Life Sciences Solutions USA LLC, Marlborough, Massachusetts, United States) was used for ion exchange chromatography. The dialysed solution was filtered through a 0.45 µm syringe filter (GVS Life Sciences, Sanford, Maine, United States) before it was loaded onto a HiTrap™ DEAE Fast Flow column (1.6 x 2.5 cm; GE Healthcare Bio-Sciences AB, Uppsala, Sweden) preequilibrated with 10 column volumes of binding buffer (125 mM boric acid, 10 mM $Na_2S_2O_5$, pH 7.0) before the column was washed of any unbound protein using binding buffer. Thereafter, the column was subjected to a 100 ml continuous elution gradient using a final buffer concentration of 1 M NaCl (in 125 mM boric acid, 10 mM $Na_2S_2O_5$, pH 7.0) that was collected in 10 ml fractions. The column was then regenerated according to manufacturer's instructions and the resin was stored in 20% (v/v) ethanol.

The samples were kept at 4°C thereafter to halt enzyme activity. Total protein concentration of each of the eluted fractions was quantified using the Qubit® 2.0 Fluorometer (485/590 nm; Invitrogen by Life Technologies, Carlsbad, California, United States) to determine the volume of each sample (20 µg total protein) that needed to be loaded into the wells of a polyacrylamide gel for SDS-PAGE.

Enzyme activity of each fraction containing protein (as indicated by the SDS-PAGE and chromatogram based on UV refraction from the ÄKTA™ start chromatography system UV flow cell) was tested using the assays detailed in section 2.1. Fractions showing the highest activity were pooled and aliquoted into Cryotubes tubes (Lasec SA, Johannesburg, Gauteng, South Africa) and flash frozen using liquid nitrogen before they were stored at -80 °C until use.

2.3. Cross-linked enzyme aggregates

An adapted method based on that described by van Pelt and co-workers (2008) was used to produce LOX cross-linked enzyme aggregates (CLEA). A 20% w/v soak using milled WS was

performed overnight with 150 mM boric acid (pH 8.1). The WS were strained through four layers of cheese cloth and to 60 ml of this liquid solid ammonium sulfate was added to bring the saturation to 40% (Duong-Ly & Gabelli, 2014), the solution was stirred for 30 minutes at 4℃. The sample was thereafter centrifuged for 30 minutes at 20,000 x g at 4℃ and the supernatant was retained while the pellet was discarded. The ammonium sulfate saturation was increased to 70% and left to stir for 30 minutes at 4℃. This liquid was separated into six 10 ml aliquots to which differing volumes of 25% glutaraldehyde was added - 125 µl, 250 µl, 500 µl, 1000 µl, 1500 µl and 2000 µl. The samples were left shaking at 250 rpm at 4℃ for 3 hours. The samples were centrifuged for 30 minutes at 20,000 x g at 4℃ to remove any unlinked proteins and additional glutaraldehyde. The supernatant was removed and assayed for LOX-1 and LOX-2 activity as described in section 2.1. The pellets were resuspended in 10 ml 150 mM boric acid (pH 8.1) and assayed. Of each sample 5 ml was separated to be dialysed against 150 mM boric acid (pH 8.1) overnight to remove any further contaminants and ammonium sulfate as well as wash the CLEAs of unlinked glutaraldehyde before being assayed.

The un-dialysed and dialysed CLEAs were also tested for the conversion of valencene to nootkatone reaction by Ida Makhubela, a colleague from Applied Protein Biotechnologies. A heating mantle was set to 37℃ above which a 50 ml round bottom flask was suspended. To the flask, 1.57 ml oleic acid, 0.0625 g iron salts ($FeSO_4.7H_2O$), 0.0625 g manganese salts ($MnCl_2.4H_2O$) and 28.13 ml resuspended LOX CLEAs (in 150 mM boric acid) were added. The mixture was stirred fast with the constant addition of air blown into the flask for oxygenation. After 30-60 minutes the reaction had turned dark brown at which time 6.25 ml valencene was added. The reaction was left under the same conditions for a further 24 or 48 hours. To work-up the reaction for gas chromatography-mass spectrophotometry (GC-MS), 100 ml ethyl acetate (EtOAc) was added to the reaction. To this mixture 30 ml saturated potassium carbonate (K_2CO_3) and 100 ml brine was used to wash the reaction twice followed by two washes with 100 ml saturated sodium bicarbonate ($NaHCO_3$). Thereafter the organic solvent was dried in magnesium sulfate ($MgSO_4$) which was then removed using a vacuum. What remained was a clear yellow oil which was diluted in methanol (MeOH, 100 ppm) before it was injected into the gas chromatography–mass spectrometer (GC-MS). Readings were performed and interpreted by Ida Makhubela.

These readings were determined using the sample oil dissolved in high-performance liquid chromatography (HPLC) grade methanol to a concentration of 100 to 500 ppm. Standard curves of valencene and nootkatone were created using a range of 10 to 100 ppm each. The

detection limit for valencene was a minimum of 5.8 ppm and a maximum of 17.4 ppm whereas these limits were 11.66 ppm minimum and 33.78 ppm maximum for nootkatone. These limits were determined by Ida Makhubela.

After it was determined that 125 µl glutaraldehyde yielded the highest activity of both LOX-1 and LOX-2 from crude WS extracts using the assays described in section 2.1, the purified enzyme from ion exchange chromatography on the ÄKTA start was used to create the CLEAs. To 10 ml of the purified enzyme 125 µl of 25% glutaraldehyde (v/v) was added and the mixture was left shaking at 250 rpm at 4℃ for 3 hours. The sample was then centrifuged for 30 minutes at 20,000 x g at 4℃ to remove the initial unlinked proteins and additional glutaraldehyde. The sample was tested in the valencene to nootkatone reaction and recycled by centrifugation for 30 minutes at 20,000 x g at 4℃ to separate the CLEAs from the reaction components. The CLEAs were dialysed in 125 mM boric acid, 10 mM sodium metabisulfite overnight. The sample was then tested once again as described above in the valencene conversion reaction.

2.4. Temperature and pH optimum determination

2.4.1. Effect of pH on lipoxygenase

The optimal pH in which each isoform from the WS sample performed was tested using the purified enzyme in the individual assays. The pH of the borate buffer in which LOX-1 was assayed was altered over a pH range from 6.0 to 11.0 as the optimal pH suggested by Suda and co-workers (1995) was pH 9.0. The optimal pH for LOX-1 was determined by comparing the rate of the bleaching of methylene blue. The rates were also compared for LOX-2 in sodium phosphate buffer within a pH range of 4.0 to 10.0 as Suda and co-workers (1995) suggested pH 6.0 as the optima. The assays were performed in triplicate as described above, however at the optimal pH the protein concentration required for the LOX-2 assay was able to be lowered to 100 µg with high activity still detected.

2.4.2. Effect of temperature on lipoxygenase

Once the optimal pH for each isoform was determined the temperature range for each isoform was determined. The reaction mixture was kept as described in section 2.1, with the optimal pH of 10.0 for LOX-1 and 6.8 for LOX-2. The temperature of the reaction was maintained by a circulating water bath attached to a Cary Series UV-Vis Spectrophotometer (Agilent Technologies, Santa Clara, California, United States). The temperature was altered from a range of 10 to 80℃ for LOX-1 and 10 to 70℃ for LOX-2. The assays were performed in triplicate.

2.4.3. pH of enzyme storage buffer

An aliquot of the purified enzyme, which had been eluted into 125 mM boric acid, 10 mM sodium metabisulfite ($Na_2O_5S_2$, pH 7.0) and maintained at the neutral pH frozen at -80°C until use, was incubated with an equal volume of 125 mM boric acid, 10 mM $Na_2S_2O_5$ altered to a range of pH values from 2 to 13 using either hydrochloric acid (HCl) or sodium hydroxide (NaOH) which overran the buffering capacity boric acid, however, to change the buffer in which the enzymes were stored could affect the catalytic capability of the enzymes. The enzyme aliquots were left in the buffer for 1 and 24 hours before the LOX-1 and

2.4.4. Temperature of enzyme storage

An aliquot of the purified enzyme was incubated at varying temperatures (5, 15, 25, 35, 45, 55 and 65°C) for either 1 or 24 hours before the LOX-1 and LOX-2 assays (Suda *et al.*, 1995) were performed in triplicate to determine the effect temperature had on the storage of the enzyme.

2.4.5. Long-term enzyme storage

Aliquots of the purified enzyme (in 125 mM boric acid, 10 mM $Na_2S_2O_5$, pH 7.0) were left at 4°C for 75 days and tested in triplicate every fifteen days using the LOX-1 and LOX-2 assays (Suda *et al.*, 1995) to determine how time affected the enzyme's activity.

A further two 500 µl aliquots were taken from -80°C storage before being lyophilised for 24 hours at -80°C and 222 µB pressure using the VirTis BenchTop Pro with Omnitronics Freeze Dryer (SP Scientific, Gardiner, New York, United States). One sample was immediately tested for both LOX-1 and LOX-2 activity after being resuspended in 500 µl of ddH_2O and the other was left at room temperature in a closed 1.5 ml Eppendorf Microcentrifuge tube (Eppendorf, Hamburg, Germany) for 16 weeks before being resuspended in 500 µl of ddH_2O and tested for both LOX-1 and LOX-2 activity (Suda *et al.*, 1995).

2.5. Enzyme kinetics

2.5.1. Substrate concentration

The Km and Vmax of LOX-1 and LOX-2 was determined. The assays were performed as described above, using 100 µg of purified enzyme, however the concentration of sodium linoleate was varied. A stock of 100 mM sodium linoleate was prepared by suspending 0.7 g of linoleic acid and 0.7 g of Tween 20 in 4 ml ddH_2O and clarified with 5.5 ml 0.5 N NaOH before being brought to a final volume of 25 ml with ddH_2O. This stock was diluted to concentrations of 2 – 20 mM in 2 mM increments and used to determine at which substrate

concentration the enzyme isoforms exhibited highest activity. The rate of reaction was calculated and the Km and Vmax values for LOX-1 and LOX-2 were calculated to determine their maximal rate and substrate saturation which will aid in the reactions performed by APBio.

3. Results and discussion

Lipoxygenase-1 (LOX-1) and lipoxygenase-2 (LOX-2) enzymes are industrially useful biocatalysts that may be used for the conversion of low-cost terpenes into sought-after terpenoids such as valencene to nootkatone. These enzymes are found in large quantities in soybeans and the purification therefrom will aid in the industrial capacity of these biocatalysts.

3.1. Quantitative assays

The most important factor in any enzyme purification protocol is the identification of a repeatable and reliable assay capable of showing whether or not there is enzyme activity at each stage of protein purification. This can be further improved by the identification of a quantitative assay that will show how activity improves with purification of the enzyme of interest from its source material. The removal of contaminants and unnecessary cell components can allow the activity of the enzyme-rich solution to increase drastically (Bonner, 2007).

A method described by Suda and co-workers (1995) made use of methylene blue bleaching to show the production of hydroperoxides from lipoxygenase acting upon linoleic acid. This assay showed results as were expected, even using an extract from the de-fatted soybeans. The DS had been used initially, in accordance with the aims of Applied Protein Biotechnologies (APBio) to work using a waste product to be environmentally friendly. This paper not only showed an assay for LOX-1 (Figure 11) but another geared towards LOX-2 (Figure 12) that also showed consistent results.

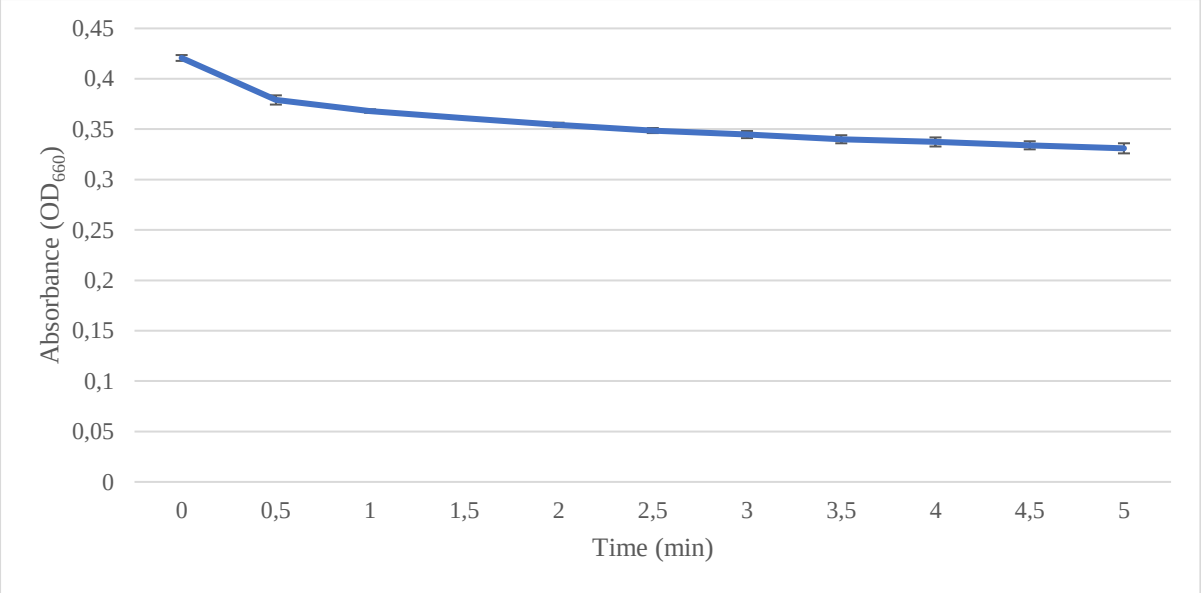


Figure 11: Methylene blue assay to determine lipoxygenase-1 activity

The assay was performed as described by Suda and co-workers (1995). Three biological replicates were performed using de-fatted soybean and the standard deviation values were calculated and shown as error bars for each time point.

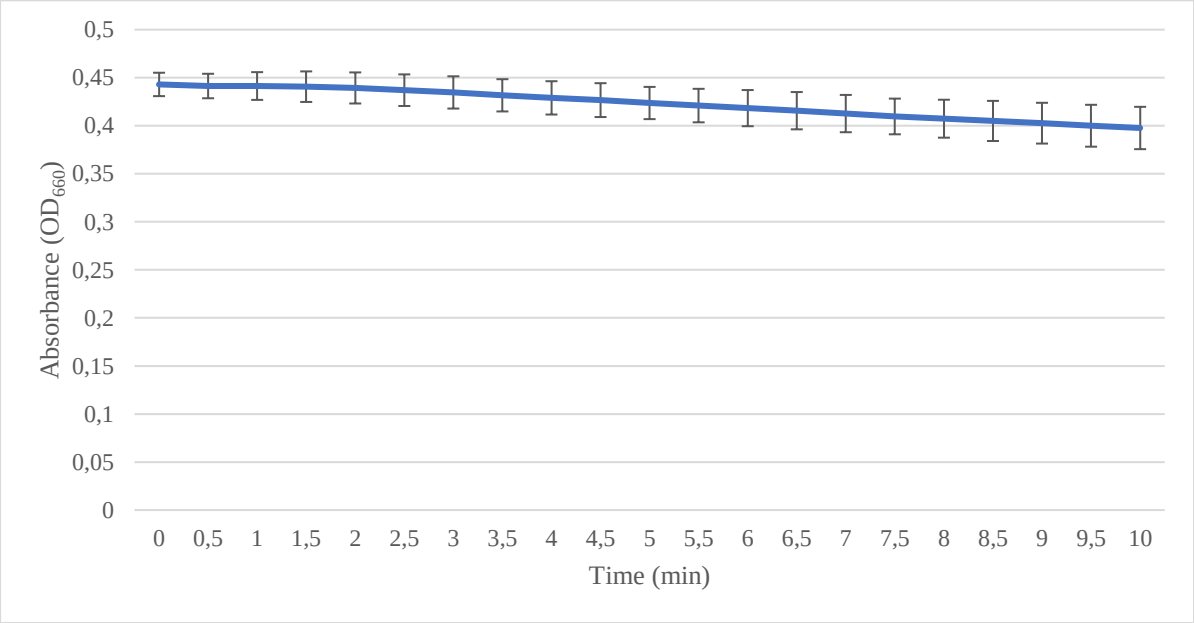


Figure 12: Methylene blue assay to determine lipoxygenase-2 activity

The assay was performed as described by Suda and co-workers (1995). Three biological replicates were performed using de-fatted soybeans and the standard deviation values were calculated and shown as error bars for each time point.

It was determined that as the two assays showed consistent results between technical replicates for both LOX-1 and LOX-2 they could be used going forward to determine enzyme activity during the purification and testing of the enzyme during experimentation. The paper by Suda

and co-workers (1995) did not describe a unit for enzyme activity and thus the rate of bleaching will be judged both by how fast the lag period ends and how quickly the bleaching occurs (ΔOD_{660}/time). The straight-line portion of the curves generated for each sample, after the lag period and before the curve plateaus, was used to determine the change in optical density over the specific amount of time taken between these two time periods.

The assays described by Suda and co-workers (1995) were determined experimentally whereby they used the known pH and buffer optima for LOX-1 as compared to LOX-2. Their investigations determined that a change in pH alone (from pH 9.0 for LOX-1 to pH 6.0-7.0 for LOX-2) could not yield optimal methylene blue bleaching from LOX-2. Further investigation showed that therefore based on buffering capacity, instead of a borate buffer, a phosphate buffer better showed the action of LOX-2. Their reaction system was composed with another additive – dithiothreitol (DTT) – which has the ability to lower the redox potential of the reaction. The investigators had determined that when the pH of the reaction had been lowered to the more neutral range in which LOX-2 better performs (pH 6.0-7.0) the redox balance erred more towards the oxidised state. By the addition of the DTT they were able to lower this potential and induce the bleaching of methylene blue by the action of LOX-2 upon linoleic acid. They also included acetone which was able to help clarify the solution for spectrophotometric readings by dissolving the substrate at the lowered pH of the LOX-2 assay reaction mixture.

During the course of the investigation of quantitative assays for LOX enzyme activity certain assays were discarded due to lack of consistent results. The first such assay is that described by Allen (1968) which measures the production of conjugated double bonds from linoleic acid as an increase in UV absorbance at 234 nm. However, instead of absorbance only increasing, as it should, the readings fluctuated inconsistently. These results may have been attributed to the use of LOX from DS as well as oleic acid. This was used as it is the substrate used by Applied Protein Biotechnologies for its ease of access and relative cheapness in comparison to the favoured substrate linoleic acid (Axelrod *et al.*, 1981). Oleic acid has been seen to have approximately 10% of the affinity for the lipoxygenase enzyme as compared to the preferred substrate linoleic acid (Allen *et al.*, 1977). A further assay that was investigated was a colorimetric assay as described by del Carmen Pinto *et al.* (2007). The assay also focusses on the production of hydroperoxides from unsaturated fatty acids in the presence of lipoxygenase enzymes in the presence of xylenol orange. Once again, the readings were inconsistent. The reasons for this may be tentatively attributed to the use of oleic acid instead of linoleic acid

(used for the same reasons stated above). However, in this instance both whole soybeans and de-fatted soybeans were investigated for their activity and showed inconsistent readings. Once linoleic acid had been acquired and was used in the assays described by Suda and co-workers (1995) it was decided that it was unnecessary to repeat the previously discarded assays. This was due to the usefulness conferred by having two assays that could distinguish between LOX-1 and LOX-2.

3.2. Crude protein extraction from plant material

3.2.1. Buffer components and concentration

In order for the greatest concentration of enzyme to be extracted from any source it is necessary to investigate the conditions under which this will occur (Doonan & Cutler, 2004). APBio had previously investigated extraction methods upon both the DS and WS. During their investigation, they discovered that the literature indicated that dried, milled/ground plant material yielded greater enzyme due to the mechanical destruction of the tough plant cell walls compared to when the plant material was not macerated (Muller *et al.*, 1998). The enzyme extraction from the different samples thereafter had been investigated through the composition of the buffers used to soak the beans and extract proteins. These buffers were also those that were previously in use by APBio but the concentrations were altered and compared.

The different biological replicates for each extraction method did not necessarily extract the same amount of total protein each time it was employed upon the milled plant material. This may be due to the minute differences between the portion of soybeans in each sample. Some samples may have had more hulls and others more endosperm which could have accounted for the difference in total protein concentration. This could account for the standard deviations between the protein concentration of the replicates being quite high (shown in Appendix 1), the difference between samples of soybeans may be higher between smaller samples than larger samples as the homogeneity in a larger sample is more likely to be similar between replicates. These experiments were performed on a very small scale – 2 g of milled soybeans in 10 ml of each buffer for the different soaks – which may account for the large variance of protein concentration between the replicates. On a larger scale the amount of plant material used may keep the protein concentration similar as a more even dispersion between parts of the bean used will be in the replicates.

The SDS-PAGE for both DS (Figure 13) and WS (Figure 14) do not give dense bands in the region, indicated by the red box between 85 and 100 kDa, expected for lipoxygenase isoforms to migrate. However, as this band is within a collection of total proteins from soybeans, assays were used to determine enzyme activity more definitively.

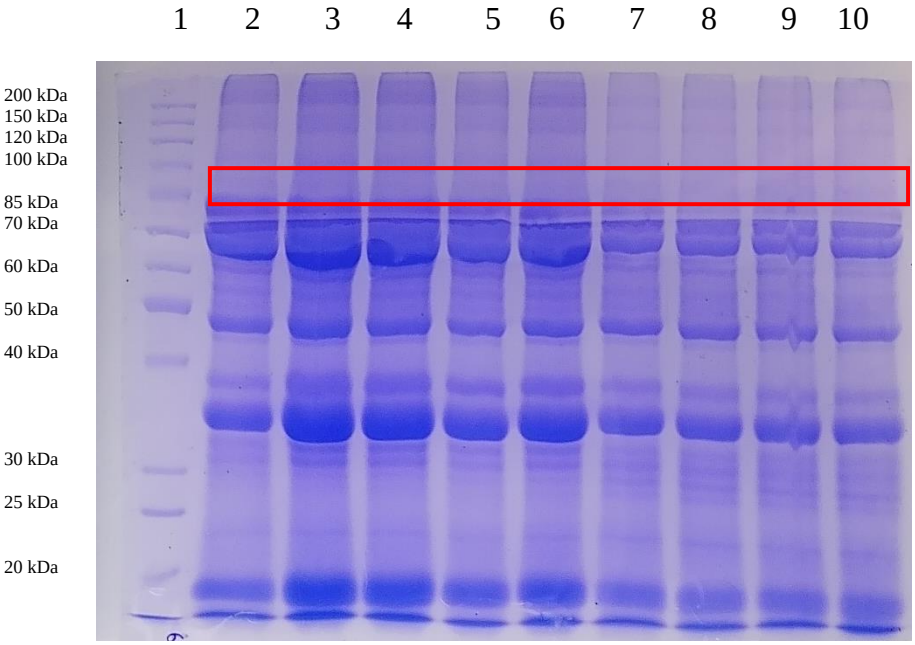


Figure 13: Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) of de-fatted soybean (DS) crude enzyme extraction methods based on boric acid (BA) concentration

Proteins were extracted from DS using either: the osmotic shock buffer (OSB) method. Initial overnight soak with 5 mM sodium metabisulfite ($Na_2S_2O_5$) – hypotonic soak (HS) followed by a further overnight soak in the presence of 0.5 M sucrose, 10 mM $Na_2S_2O_5$ and varying concentrations of boric acid; or a single over overnight soak of with varying concentrations of boric acid. The red box indicates the region to which lipoxygenase (LOX) proteins should have migrated. Lane 1: Thermo Scientific™ PageRuler™ Unstained Protein Ladder; L2: HS DS; L3: OSB 50 mM BA; L4: OSB 100 mM BA; L5: OSB 150 mM BA; L6: OSB 200 mM BA; L7: 50 mM BA; L8: 100 mM BA; L9: 150 mM BA; L10: 200 mM BA

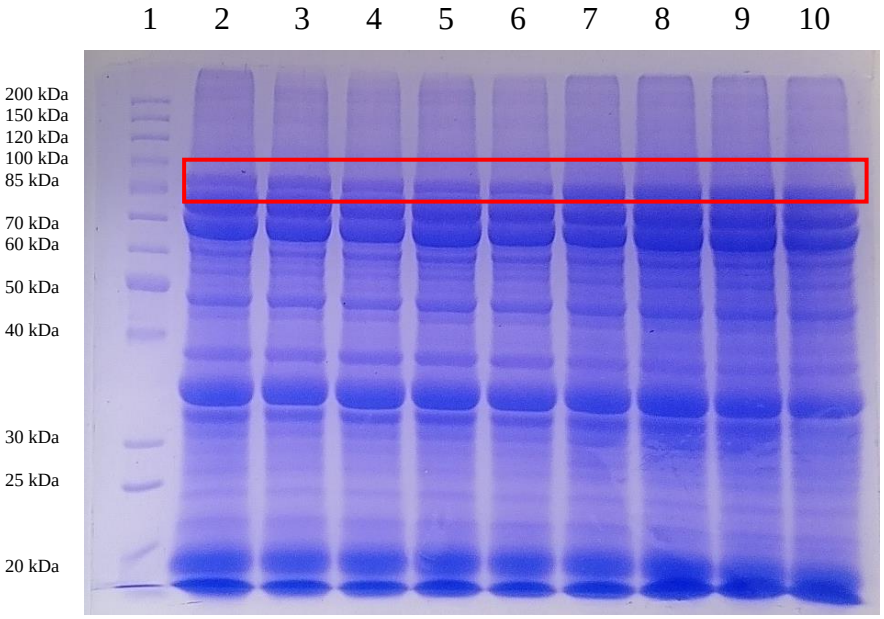


Figure 14: Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) of whole soybean (WS) crude enzyme extraction methods based on boric acid (BA) concentration

Proteins were extracted from WS using either: the osmotic shock buffer (OSB) method with an initial overnight soak with 5 mM sodium metabisulfite ($Na_2S_2O_5$) – hypotonic soak (HS) – followed by a further overnight soak in the presence of 0.5 M sucrose, 10 mM $Na_2S_2O_5$ and varying concentrations of boric acid; or a single over overnight soak of with varying concentrations of boric acid. The red box indicates the region to which lipoxygenase (LOX) proteins should have migrated. Lane 1: Thermo Scientific™ PageRuler™ Unstained Protein Ladder; L2: HS WS; L3: OSB 50 mM BA; L4: OSB 100 mM BA; L5: OSB 150 mM BA; L6: OSB 200 mM BA; L7: 50 mM BA; L8: 100 mM BA; L9: 150 mM BA; L10: 200 mM BA

The original curves determined spectrophotometrically (shown in Appendix 2) were converted to single value bars using the straight-line portion after the initial lag period and before the bleaching had stopped progressing. The orange line indicates the time taken for the lag period to end. Based on the graph (Figure 15) showing the decrease in absorbance at OD_{660} for each buffer's extraction ability of LOX-1 from DS it can be seen that the osmotic shock buffer (OSB) method with 100 mM of boric acid yielded the highest extraction of LOX-1 followed closely thereafter by a single overnight soak with 50 mM boric acid. However, none of the DS samples yielded as much LOX-1 activity as those from WS as the decrease in absorbance did not reach zero in the five-minute timeframe used to determine LOX-1 activity nor did it decrease as far as the WS samples (Figure 16), the original curves can be seen in Appendix . The lag values were excluded from some of the following graphs unless the values were notable. In many cases where the sample exhibited little or no activity the lag period was recorded as 0 and the

entire 5- or 10-minute period was used to determine the rate of reaction. The lack of activity in DS could be attributed to the processing steps employed to extract the oils from the soybeans.

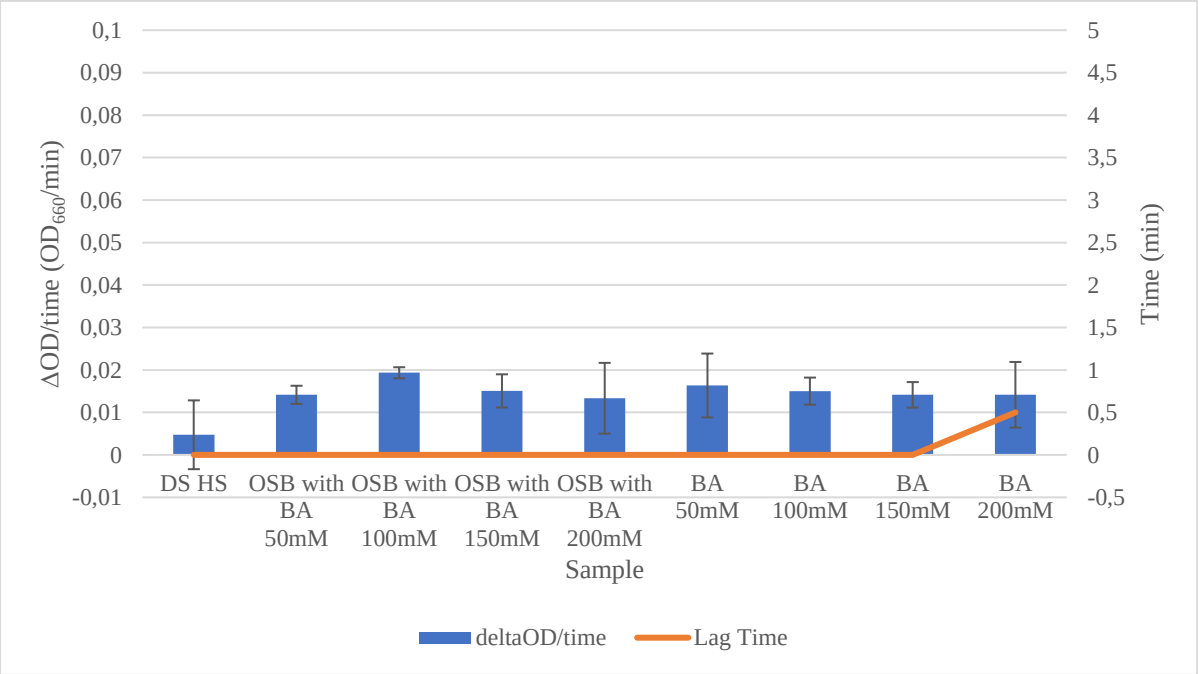


Figure 15: Methylene blue assay to determine lipoxygenase-1 activity in crude enzyme extractions from de-fatted soybeans (DS) based on boric acid (BA) concentration

Proteins were extracted from DS using either: the osmotic shock buffer (OSB) method with an initial overnight soak with 5 mM sodium metabisulfite (Na2S2O5) – hypotonic soak (HS) - followed by a further overnight soak in the presence of 0.5 M sucrose, 10 mM Na2S2O5 and varying concentrations of boric acid; or a single over overnight soak of with varying concentrations of boric acid.

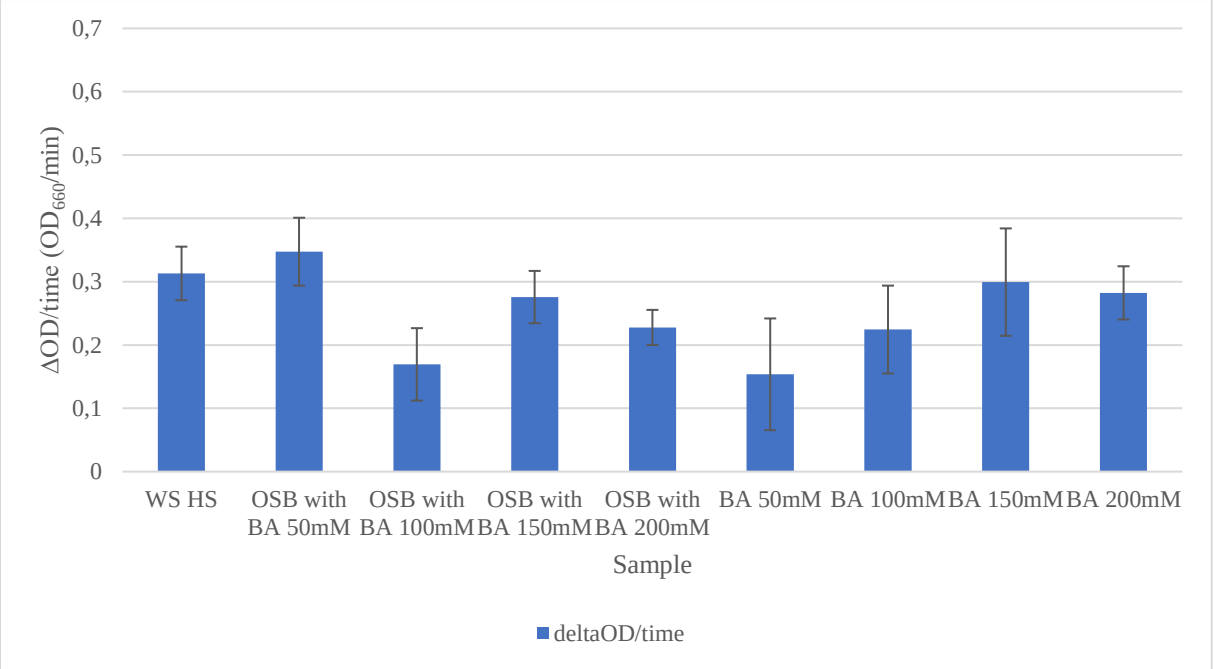


Figure 16: Methylene blue assay to determine lipoxygenase-1 activity in crude enzyme extractions from whole soybeans (WS) based on boric acid (BA) concentration

Proteins were extracted from WS using either: the osmotic shock buffer (OSB) method with an initial overnight soak with 5 mM sodium metabisulfite (Na2S2O5) – hypotonic soak (HS) - followed by a further overnight soak in the presence of 0.5 M sucrose, 10 mM Na2S2O5 and varying concentrations of boric acid; or a single over overnight soak of with varying concentrations of boric acid.

The oils from soybean seeds can be extracted using different methods but most often the soybeans are dried to adjust for moisture content by being heated anywhere from 60 to 88°C which is damaging to the conformation of the proteins within them (Erickson, 2015). This step is followed by "cracking" which helps to remove the hulls of the soybeans in the "dehulling" step. The dehulling step may also be performed under heated conditions whereby the soybeans are heated to approximately 60°C to bring the remaining moisture to the surface of the soybeans before they are rapidly heated to 85°C and dried to loosen the hulls. This step is followed by "conditioning" which once again heats the soybeans to 71°C, but instead of drying to remove moisture the soybeans are steamed to bring the moisture content back up to a level appropriate for the next step – "flaking" – which turns the soybeans into flakes which are finally ready for oil extraction. The oil is removed by the addition of solvents such as hexane which has very little effect on protein structure (Fukushima, 1969). The repeated heating to high temperatures may be responsible for the decrease in enzyme activity if the LOX-1 proteins were denatured

in the process. This phenomenon was also observed by Chedea and co-workers (2008) wherein the defatting process specifically inhibits LOX-1 activity.

The standard deviation between the activity of the replicates however was relatively low as displayed by the standard error bars (Figure 15). The values all lie below 0.1 which indicates that there was very little difference in activity between the biological replicates despite their differences in protein concentration. This could however be due to the fact that with so little activity being displayed at all there was little room for variation between the activity of the samples

The amount of LOX-1 activity between the DS and WS sources is drastically different as is evident by the bleaching effect each set of samples had on the methylene blue in the assay reaction (Figure 15; Figure 16). The sample extracted using the osmotic shock buffer with 50 mM of boric acid yielded the greatest LOX-1 extraction followed by the hypotonic soak which is removed from the bean slurry before the osmotic shock buffer is added. The third highest activity was seen to be extracted during a single overnight soak with 150 mM boric acid and, finally, the fourth highest activity was seen to be from the extract performed by an overnight soak in 200 mM boric acid.

The standard deviation values between the biological replicates are all well below 1.0 which indicates very little difference in activity despite the variation in protein concentration within the WS samples. This indicates that, while the replicated soaks may not have yielded the same amount of total protein within the small samples of beans, the amount of LOX-1 yielded is constant.

The DS showed almost no LOX-2 activity in any of the different extraction buffers (Figure 17) but of the soaks the best activity was observed in the osmotic shock buffer with either 200 mM or 150 mM boric acid, followed by a single overnight soak in 150 mM boric acid or 200 mM boric acid. However, in comparison with the WS investigations (Figure 18) these differences in activity are indiscernible. Once again, these lacklustre enzyme activities could be due to the processing steps used to de-fat the soybeans before they were used in the investigation.

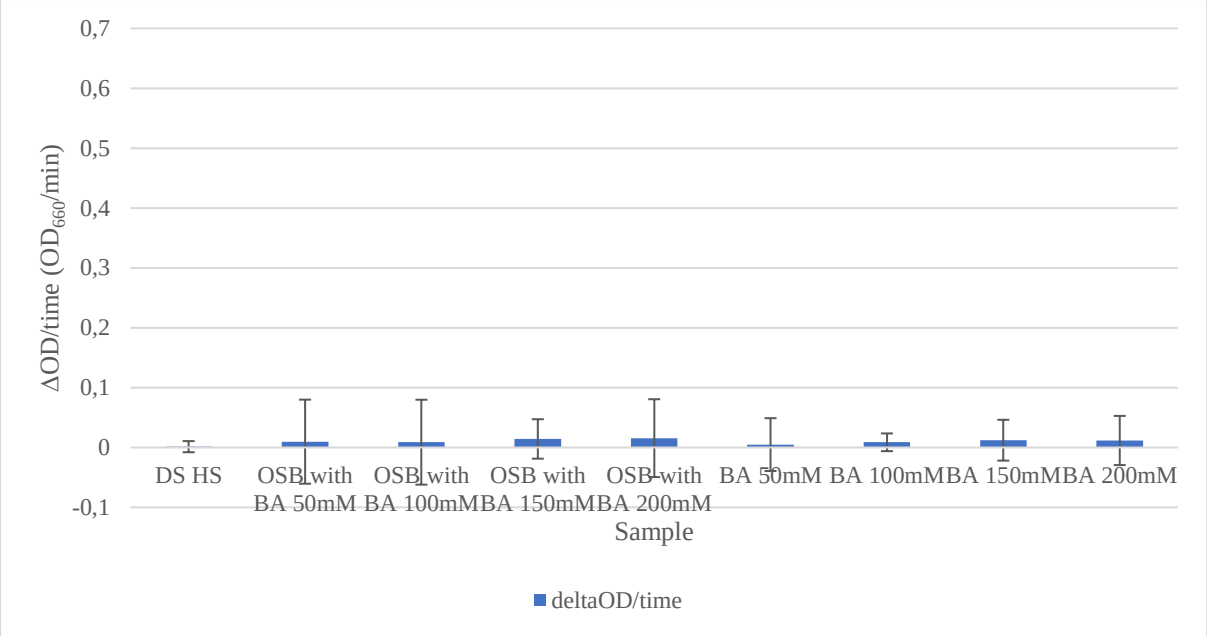


Figure 17: Methylene blue assay to determine lipoxygenase-2 activity in crude enzyme extractions from de-fatted soybeans (DS) based on boric acid (BA) concentration

Proteins were extracted from DS using either: the osmotic shock buffer (OSB) method with an initial overnight soak with 5 mM sodium metabisulfite (Na2S2O5) – hypotonic soak (HS) - followed by a further overnight soak in the presence of 0.5 M sucrose, 10 mM Na2S2O5 and varying concentrations of boric acid; or a single over overnight soak of with varying concentrations of boric acid.

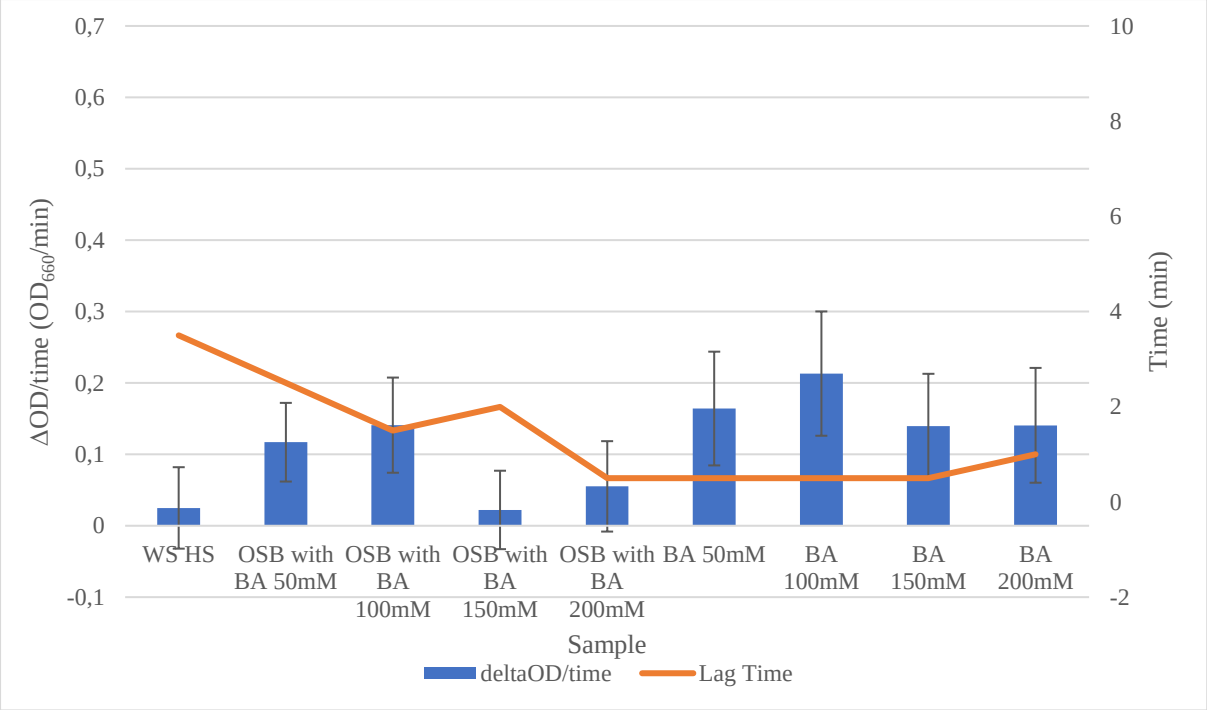


Figure 18: Methylene blue assay to determine lipogygenase-2 activity in crude enzyme extractions from whole soybeans (WS) based on boric acid (BA) concentration

Proteins were extracted from WS using either: the osmotic shock buffer (OSB) method *with an i*nitial overnight soak with 5 mM sodium metabisulfite (Na2S2O5) – hypotonic soak (HS) - followed by a further overnight soak in the presence of 0.5 M sucrose, 10 mM Na2S2O5 and varying concentrations of boric acid; or a single over overnight soak of with varying concentrations of boric acid.

The standard deviations bars are all below 0.1 indicating that the small amount of LOX-2 extracted from the small amounts of beans was similar between replicates as evidenced by the amount of activity displayed during the assay. Due to the scale bar used to quantitate the activity of the samples, however, the standard deviation values look very large in comparison despite the small values they indicate. This variance in activity may be due to the actual amount of the enzyme, within the controlled amount of protein added to the reaction, varying between the biological replicates.

Unlike LOX-1 from WS (Figure 16), where the osmotic shock buffer produced the highest extractions, LOX-2 was extracted best by the single overnight soak with boric acid. A concentration of 100 mM boric acid gave the highest activity as evidenced by the rate of bleaching observed in Figure 18, followed by a concentration of 50 mM boric acid. The rate of reaction of the LOX-2 isoforms is much lower than that of the LOX-1 isoform. The assay was described by Suda and co-workers (1995) to proceed for 10 minutes as opposed to the maximum of 5 minutes for LOX-1. Thus, the slower rate was expected. There is also a longer

lag period shown for these samples than the LOX-1 isoform (where there was no lag). This could be due to the amount of time it takes the LOX-2 isoform to create sufficient peroxy-radicals necessary to begin bleaching the methylene blue. This may be due to the conditions (pH, addition of DTT and acetone, etc.) under which the LOX-2 isoform is tested being less than optimal for highest enzyme activity to prevent the action of LOX-1 interfering and resulting in skewed results.

Due to the lack of activity from the de-fatted soybeans the focus was put on the whole soybean extraction optimum. The time and additional cost of reagents was also taken into account and a single overnight soak in boric acid was chosen going forward. As the two isoforms were extracted better by different concentrations of boric acid – 150 mM boric acid for LOX-1 and 100 mM boric acid for LOX-2 – the intermediate concentration of 125 mM boric acid was chosen for the extraction buffer. Furthermore, the presence of sodium metabisulfite ($Na_2S_2O_5$) in the osmotic shock buffer method was used to preserve the proteins (Barberá *et al.*, 2000) and was carried over to the final buffer for the same purpose. This resulted in a buffer consisting of 125 mM boric acid and 10 mM $Na_2S_2O_5$ to extract both LOX-1 and LOX-2 from DS and WS.

3.2.2. pH of extraction buffer

Proteins can be affected immensely by changes in pH (Pelegrine & Gasparetto, 2005). This can be helpful if it increases the solubility of a protein but can be problematic if the protein is irreversibly denatured in the process (Pelegrine & Gasparetto, 2005). However, more acidic and more basic pH values are greater able to break down tough molecules and release the proteins from the plant material (Cui *et al.*, 2017). The pH of the 125 mM boric acid, 10 mM $Na_2S_2O_5$ was altered to determine which pH value extracted the greatest amount of LOX-1 and LOX-2 from both the DS and WS samples. To avoid the pH of the extraction buffers interfering with the conformation of the enzymes during the activity assay the samples were dialysed overnight against 125 mM boric acid, 10 mM $Na_2S_2O_5$ (pH 7.0), to return the enzymes to their native conformation should they have been affected during extraction.

The bands seen in the SDS-PAGEs are similar which indicates that many of the same proteins are within the DS (Figure 19) and WS (Figure 20) samples. However, as previously discussed, the process of removing the oil from the soybean seeds must have denatured and degraded some of the proteins, as can be seen by the less defined bands in the DS SDS-PAGE gel as well as the lowered activity as compared to the WS extracts.

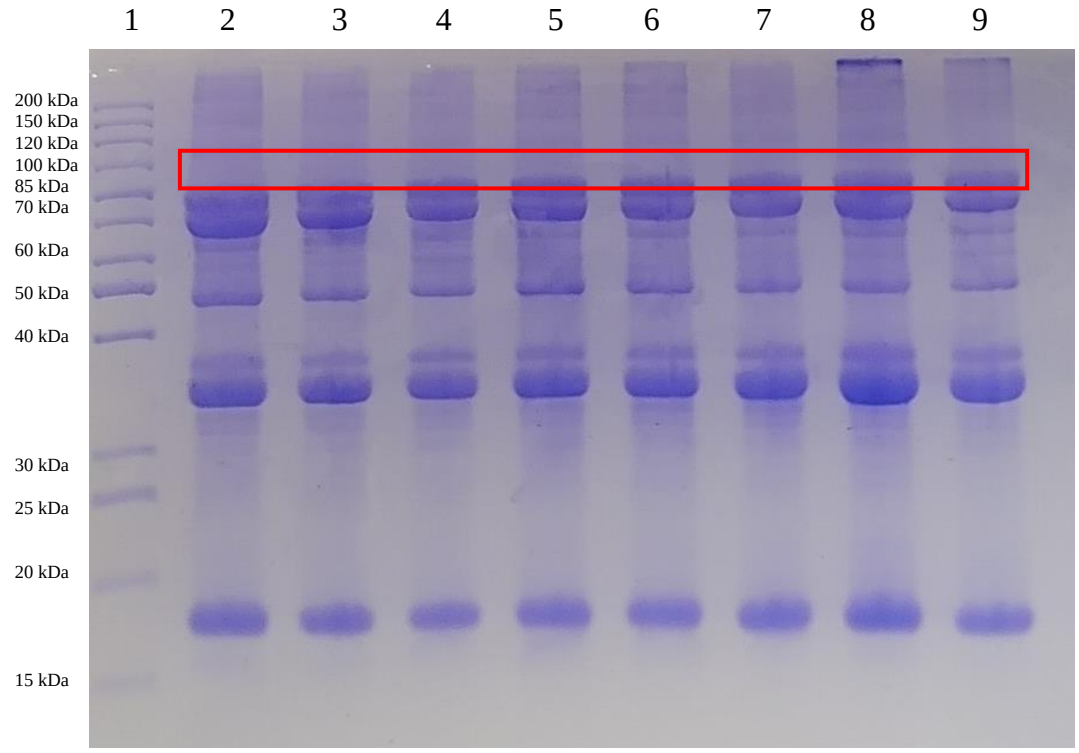


Figure 19: Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) of de-fatted soybean (DS) enzyme extraction methods based on pH of extraction buffer

Proteins were extracted from DS using a single over overnight soak of 125 mM boric acid, 10 mM sodium metabisulfite ($Na_2O_5S_2$) at varying pH values. The red box indicates the region to which LOX proteins should have migrated. Lane 1: Thermo Scientific™ PageRuler™ Unstained Protein Ladder; L2: pH 3; L3: pH 4; L4: pH 5; L5: pH 6; L6: pH 7; L7: pH 8; L8: pH 9; L9: pH 10

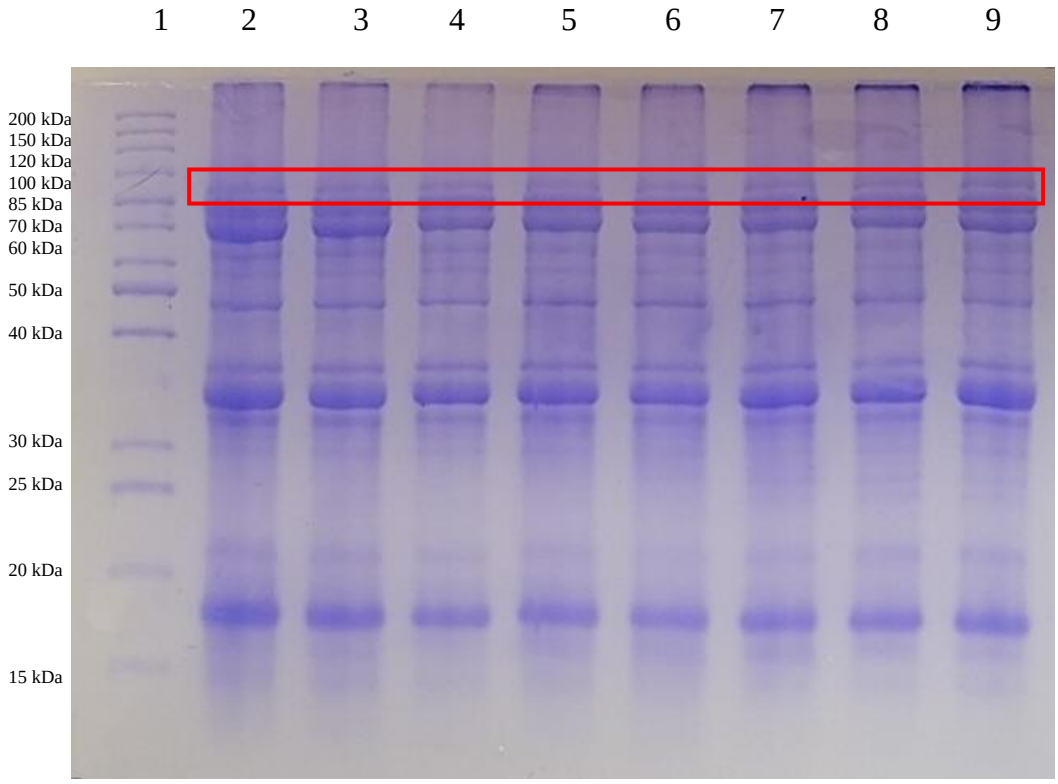


Figure 20: Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) of whole soybean (WS) enzyme extraction methods based on pH of extraction buffer

Proteins were extracted from WS using a single over overnight soak of 125 mM boric acid, 10 mM sodium metabisulfite ($Na_2O_5S_2$) at varying pH values. The red box indicates the region to which LOX proteins should have migrated. Lane 1: Thermo Scientific™ PageRuler™ Unstained Protein Ladder; L2: pH 3; L3: pH 4; L4: pH 5; L5: pH 6; L6: pH 7; L7: pH 8; L8: pH 9; L9: pH 10

The biological replicates of the DS were combined for protein quantification as testing the pH of the extraction buffer was only a formality due to the lack of activity previously observed. Therefore, there was no calculation of standard deviation between the protein extract concentrations. The WS samples, however, once again produced large variations between the replicates in how much protein was extracted. This could be attributed to the small amount of milled beans sampled during each extraction, each sample containing different amounts of LOX enzyme derived from the different parts of the beans, thereby accounting for the observed variance (Appendix 4).

The minimal activity observed from the DS was observed once again during the LOX-1 assay. The highest activity observed was from the extraction buffer at pH 5. The standard deviations observed were very low between replicates, while disproportionately large based on the scale

of the graph, but due to the minimal bleaching of methylene blue in the assay this lack of difference is not unexpected.

The LOX-1 extracted from the WS (Figure 22) showed much greater activity than the DS (Figure 21). The highest activity was observed from the samples subjected to an overnight soak in the buffer altered to pH 7.0. The standard deviation values between replicates are much lower than 0.1 which is a good indication of a consistent amount of LOX-1 enzyme being extracted from the samples of WS.

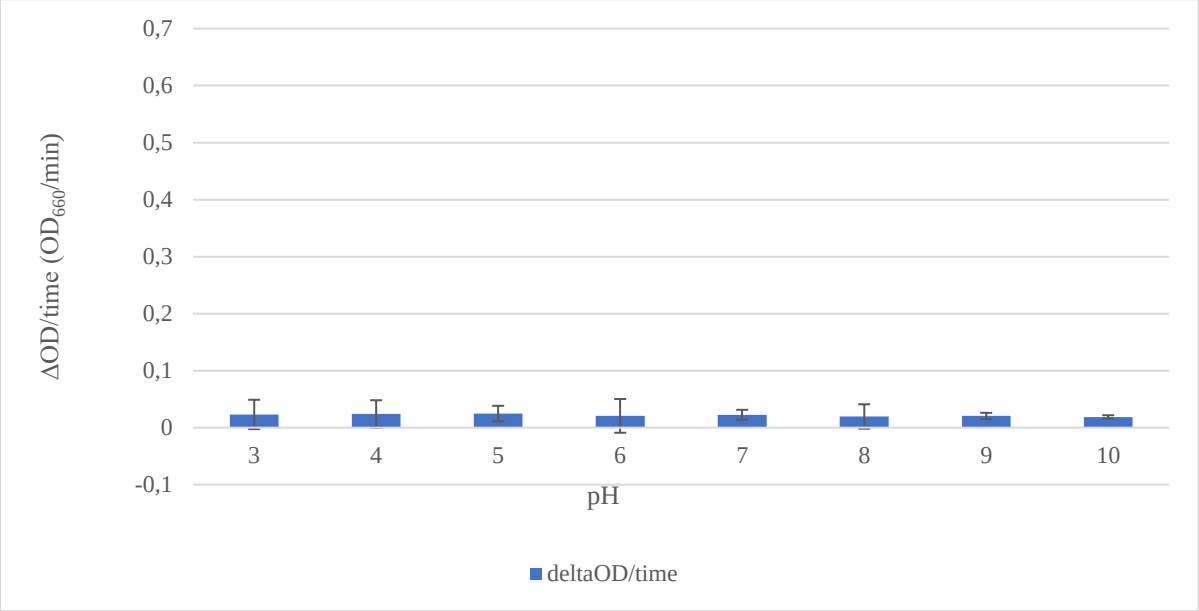


Figure 21: Methylene blue assay to determine lipoxygenase-1 activity in crude enzyme extractions from de-fatted soybeans (DS) based on pH of extraction buffer

Proteins were extracted from DS using a single over overnight soak of 125 mM boric acid, 10 mM sodium metabisulfite ($Na_2O_5S_2$) at varying pH values before being dialysed against the extraction buffer at pH 7 for 24 hours.

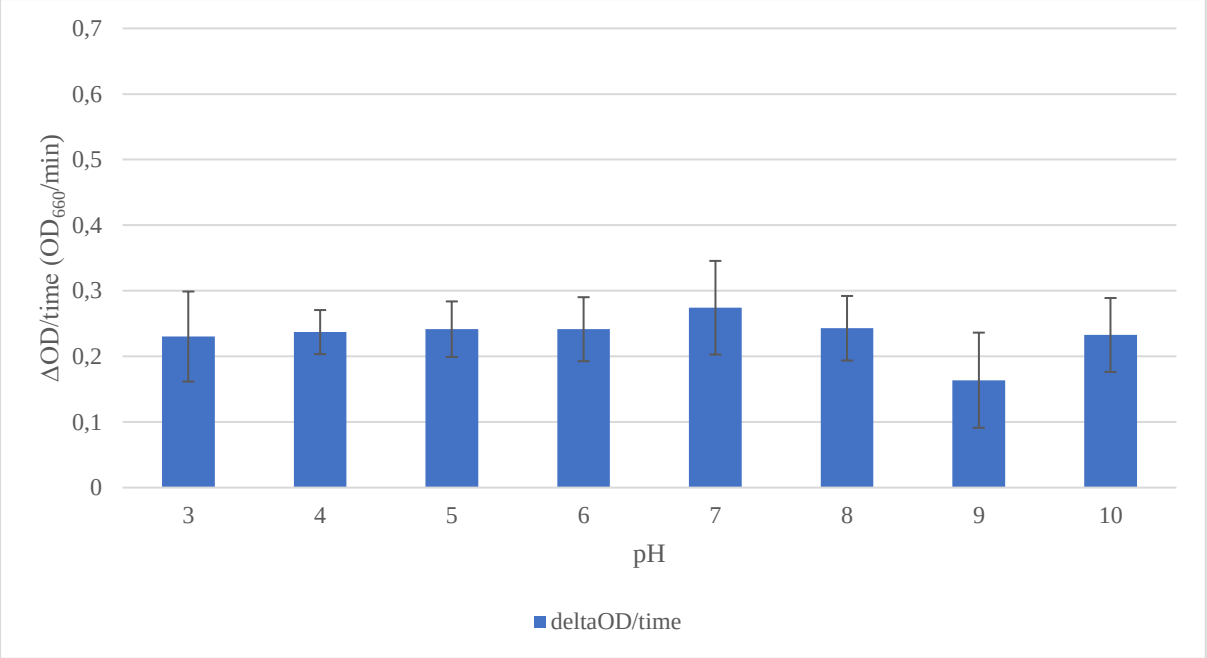


Figure 22: Methylene blue assay to determine lipoxygenase-1 activity in crude enzyme extractions from whole soybeans (WS) based on pH of extraction buffer

Proteins were extracted from WS using a single over overnight soak of 125 mM boric acid, 10 mM sodium metabisulfite ($Na_2O_5S_2$) at varying pH values before being dialysed against the extraction buffer at pH 7 for 24 hours

The assay results for LOX-2 from DS (Figure 23) showed very little activity in any of the different extraction buffers, however the buffers altered to pH 8, 9 and 10 showed the most activity, comparatively. These pH values do not match that seen during the LOX-1 assays for DS. However, the different isoforms may be extracted best under differing conditions. The standard deviations between the biological replicates are less than 0.1 which indicates that the activity between replicates were due to similar amounts of LOX-2 extracted from DS.

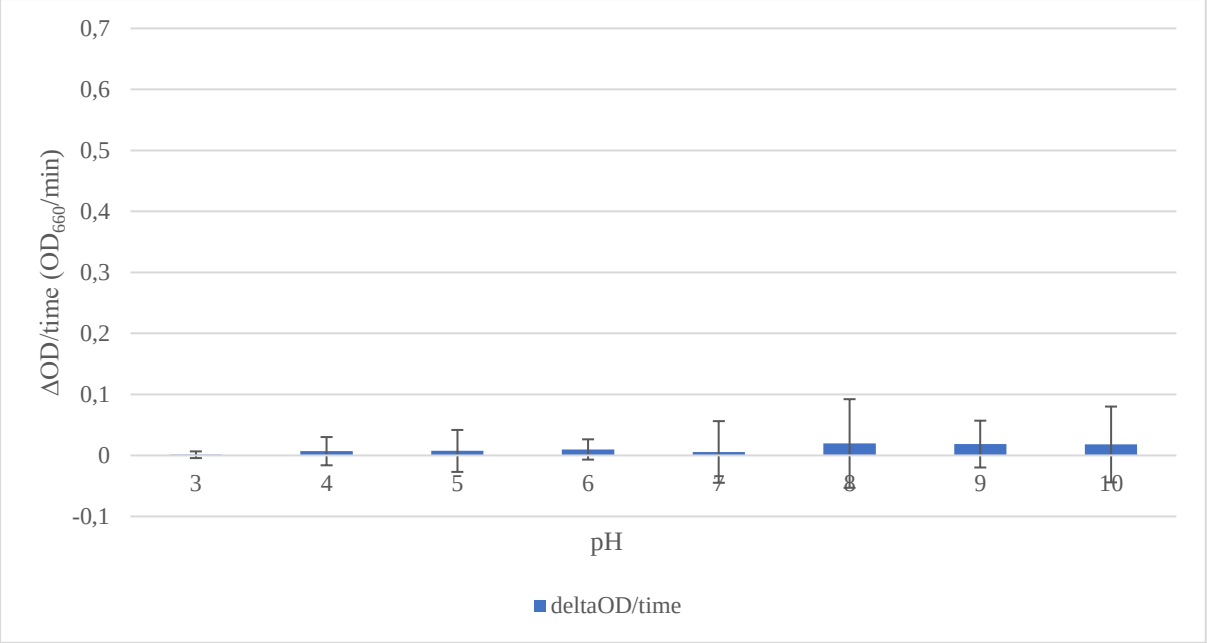


Figure 23: Methylene blue assay to determine lipoxygenase-2 activity in crude enzyme extractions from de-fatted soybeans (DS) based on pH of extraction buffer

Proteins were extracted from DS using a single over overnight soak of 125 mM boric acid, 10 mM sodium metabisulfite ($Na_2O_5S_2$) at varying pH values before being dialysed against the extraction buffer at pH 7 for 24 hours.

The amount of activity displayed from the LOX-2 extracted from WS (Figure 24) is much greater than DS. The methylene blue is completely decolourised in many of the different pH extraction buffers. Unlike the extraction of LOX-1 from WS there was not a clear outlier of which buffer best extracted LOX-2. However, the best extractions appear to have been performed using buffer pH 8, 10 and 7. The rate of bleaching of methylene blue is similar to those of the lower pH values 3 and 4 but the lag time for the other samples extracted using the buffers of higher pH values were lower and thus can be considered to have a greater activity. For this reason, going forward, pH 7 was used for the pH of the common WS extraction buffer. The standard deviations between the replicates are all well below 0.1 and therefore there is very little variation in how much LOX-2 is extracted between samples, despite the variation in total protein extracted.

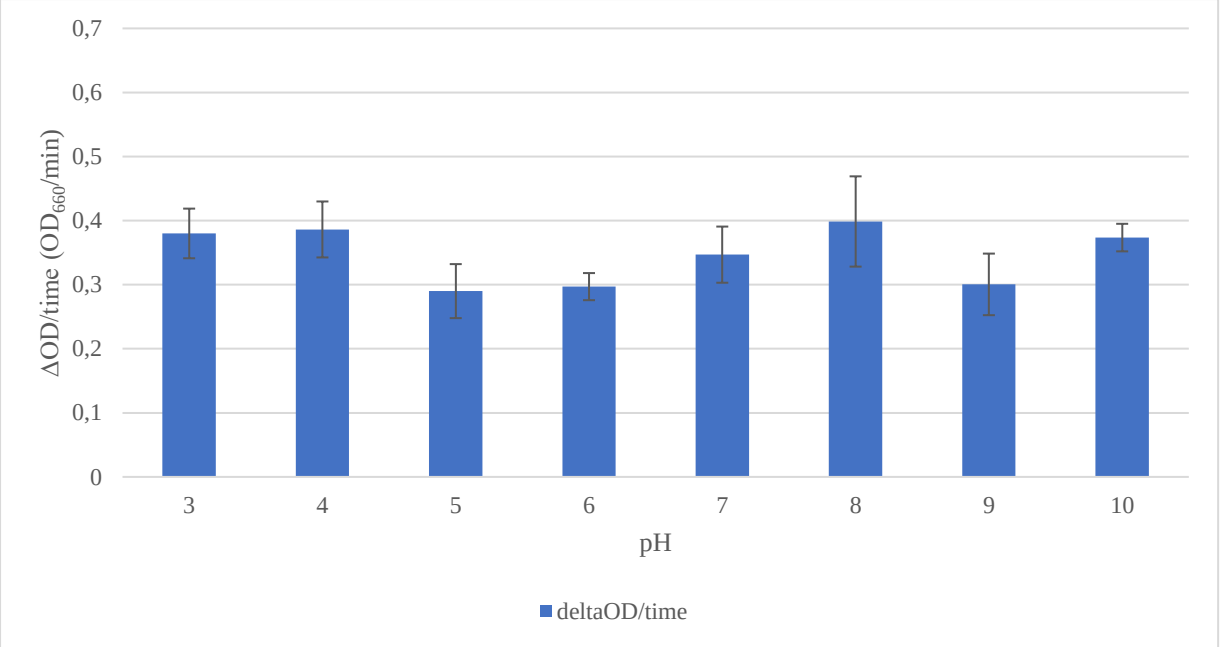


Figure 24: Methylene blue assay to determine lipoxygenase-2 activity in crude enzyme extractions from whole soybeans (WS) based on pH of extraction buffer

Proteins were extracted from WS using a single over overnight soak of 125 mM boric acid, 10 mM sodium metabisulfite ($Na_2O_5S_2$) at varying pH values before being dialysed against the extraction buffer at pH 7 for 24 hours.

From these initial extraction results it was determined that the amount of the LOX-1 and LOX-2 isozymes extracted from DS was not worth continued investigation and only WS were investigated.

3.2.3. Protein precipitation

Unlike when a protein of interest is overexpressed in a host of choice and the protein is found in a much greater proportion to that of the host's endogenous proteins and thus easy to distinguish, the same is not true for a natural host that has not been engineered (Prelich, 2012). The protein of interest will not come attached to a tag for simple purification and may need to be concentrated before it can be isolated from the crude protein collection extracted from the source. The technique of ammonium sulfate precipitation can selectively draw proteins out of solution based on the percentage saturation at which they precipitate (Duong-Ly & Gabelli, 2014).

The literature indicated that at 60% ammonium sulfate saturation nearly all LOX activity was retained (Stevens *et al.*, 1970). This phenomenon was observed in both the assay for LOX-1 (Figure 26) and LOX-2 (Figure 27). The SDS-PAGE (Figure 25) shows how certain proteins extracted from WS are selectively precipitated at different concentrations of ammonium

sulfate. Due to the number of bands continuously visible as the saturation of ammonium sulfate increases, it can be assumed that there are many proteins of similar molecular weight with differing properties. These proteins are removed at each successive jump in ammonium sulfate saturation. The LOX isoforms were removed from the collective set of proteins when the saturation was increased to 60% with solid ammonium sulfate. This was confirmed by the activity of the different fractions in Figure 26 and Figure 27.

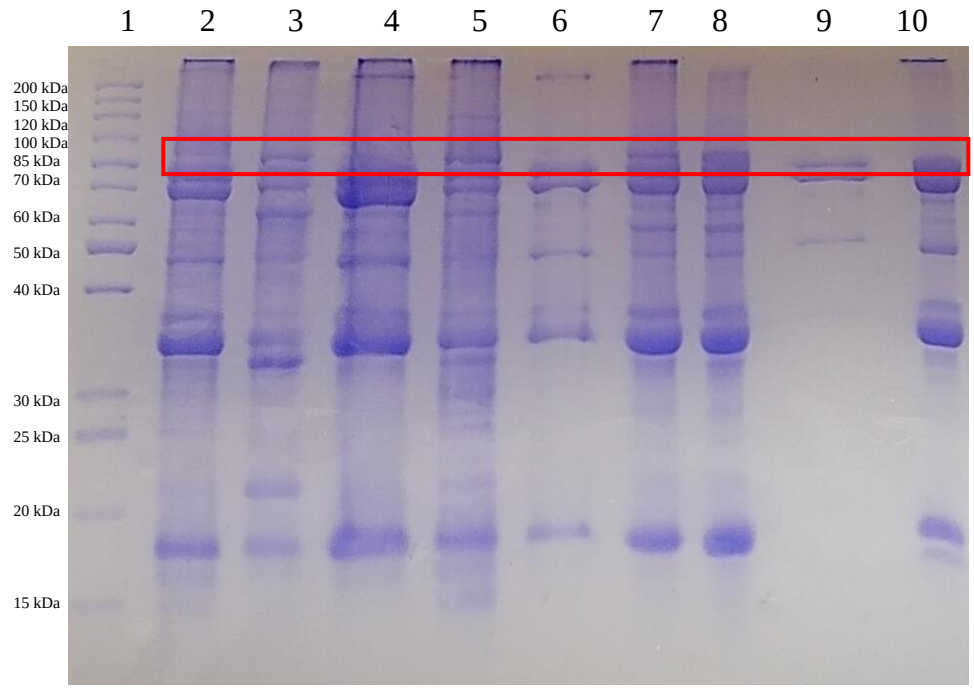


Figure 25: Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) of graded ammonium sulfate (AS) purified whole soybean (WS) enzyme extract

Proteins were extracted from WS using a single over overnight soak of 125 mM boric acid, 10 mM sodium metabisulfite ($Na_2O_5S_2$, pH 7.00). Graded AS precipitation was performed from 20 to 80% saturation, and the 60% AS saturation pellet was dialysed against 125 mM boric acid, 10 mM $Na_2O_5S_2$ (pH 7.00) overnight to remove the AS. The red box indicates the region to which LOX proteins should have migrated. Lane 1: Thermo Scientific™ PageRuler™ Unstained Protein Ladder; L2: 20% AS saturation supernatant; L3: 20% AS saturation pellet; L4: 40% AS saturation supernatant; L5: 40% AS saturation pellet 1; L6: 60% AS saturation supernatant; L7: 60% AS saturation pellet; L8: 60% AS saturation pellet dialysed; L9: 80% AS saturation supernatant; L10: 80% AS saturation pellet

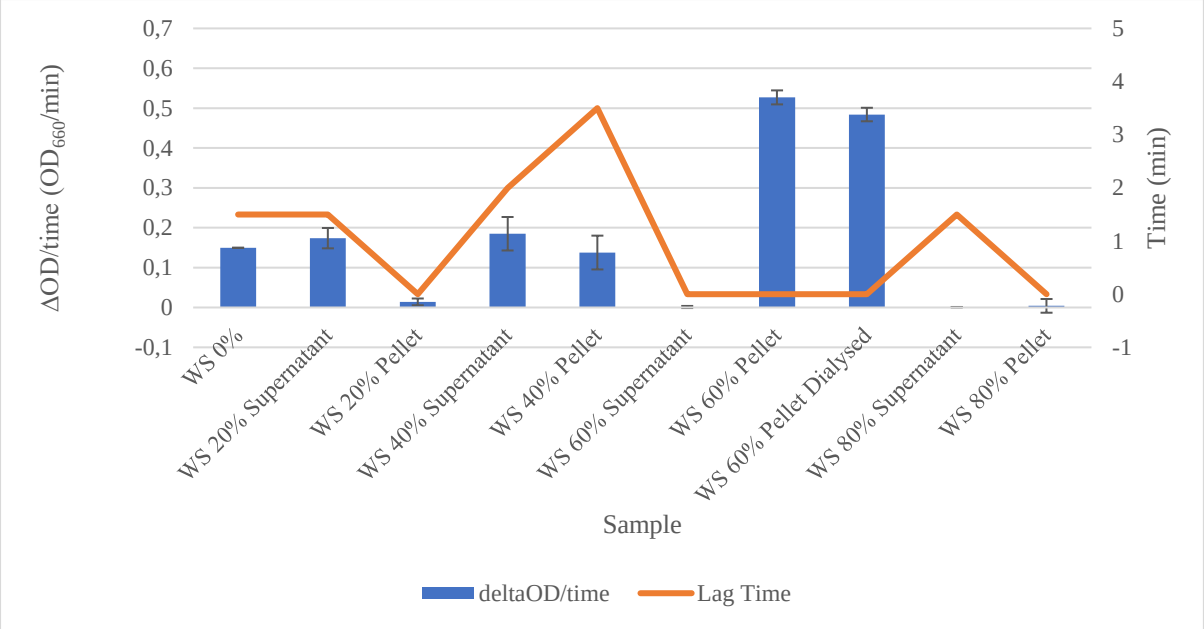


Figure 26: Methylene blue assay to determine lipoxygenase-1 activity in ammonium sulfate (AS) graded precipitation enzyme extractions from whole soybeans (WS)

Proteins were extracted from WS using a single over overnight soak of 125 mM boric acid, 10 mM sodium metabisulfite ($Na_2O_5S_2$, pH 7.00). Graded AS precipitation was performed on 30 ml of extract from 20% to 80% saturation using solid ammonium sulfate

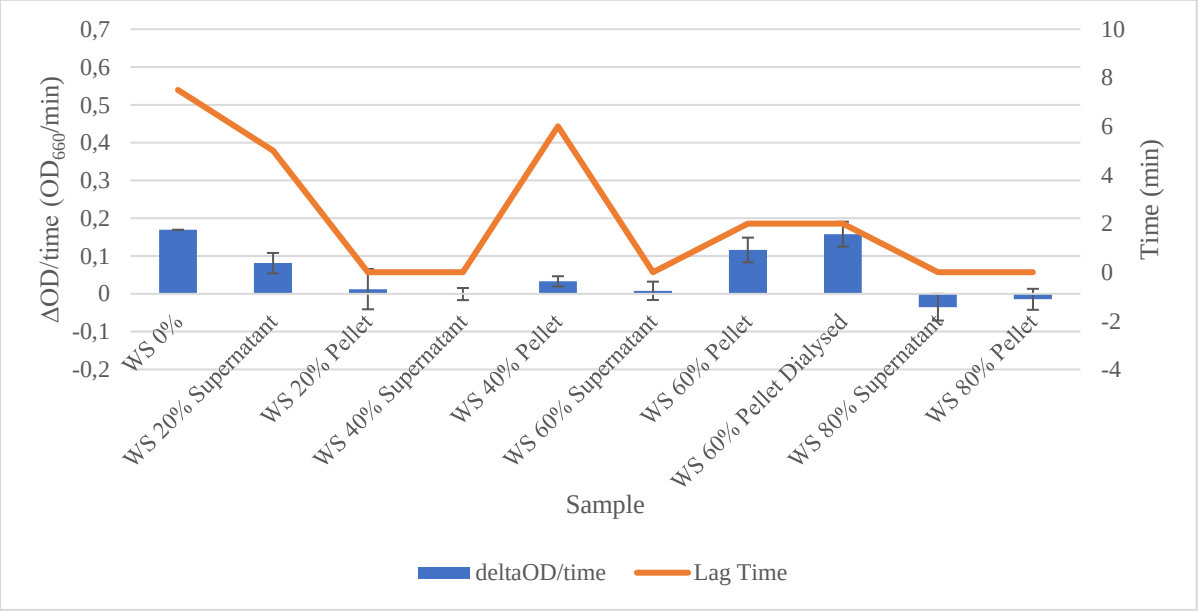


Figure 27: Methylene blue assay to determine lipoxygenase-2 activity in ammonium sulfate (AS) graded precipitation enzyme extractions from whole soybeans (WS)

Proteins were extracted from WS using a single overnight soak of 125 mM boric acid, 10 mM sodium metabisulfite ($Na_2O_5S_2$, pH 7.00). Graded AS precipitation was performed on 30 ml of extract from 20% to 80% saturation using solid ammonium sulfate

The standard error between the protein quantifications (Appendix 5) shows that while there is variation between the samples it is lower than 1.0. As the starting material was the same batch of whole beans using the 125 mM boric acid, 10 mM sodium metabisulfite ($Na_2O_5S_2$, pH 7.00) extraction buffer, each sample should be homogenous in the amount of protein extracted. As the gradient increases the samples do begin to differ in the amount of protein carried through to each successive increase of saturation, however the amount of protein seen in the replicates of the different steps is of a similar concentration. The protein in the pellets was resuspended in a quarter of the volume of the original amount of liquid from the which the proteins were precipitated. This would account for the pellet containing a greater concentration of protein than the remaining supernatant which was carried through to successive precipitation steps. Therefore, one of the advantages of ammonium sulfate precipitation is that the proteins may be concentrated down from the original amount of starting material (Doonan & Cutler, 2004).

As can be seen in Figure 26, the amount of activity observed from the 60% saturated pellet, resuspended in a quarter of the original volume of the liquid extracted from WS, increased drastically from the unprocessed sample. The amount of activity can be seen to increase with each successive round of precipitation as the supernatant left behind after 20% and 40% saturation yields higher activity as more interfering proteins are removed from the samples. Finally at 60% saturation the upper range of interfering proteins that will precipitate out at higher saturations of ammonium sulfate are also removed thus yielding the highest level of activity in the 40-60% range of proteins. A small amount of activity was observed in the 40% saturation sample which indicates that some of the LOX-1 enzyme began to precipitate out during the jump from 20 to 40% saturation. The lag period though (3.5 minutes), as compared to the 60% saturation sample (0 minutes), is much higher along with a lower catalytic rate. This small amount of activity as compared to both the unprocessed sample (which has a lag period of 1.5 minutes) and the 60% saturation sample, does not necessitate the investigation into the exact percentage at which the LOX enzyme begins to precipitate out of solution for this investigation. Future work may involve the investigation thereof.

It can also be observed that while the necessary dialysis step did lower the activity of the sample (Doonan & Cutler, 2004). The salt did not seem to affect the activity of the enzyme in this particular reaction and thus can be assumed to leave the conformation and active site of the enzyme unaltered. However, for other reactions the salt may affect the substrate and product negatively which would be problematic in an industrial setting.

Unlike LOX-1, the 40% ammonium sulfate saturation supernatant did not show activity for LOX-2 (Figure 27). The 20% ammonium sulfate saturation supernatant showed a smaller amount of activity as compared to the unprocessed sample however the lag period before the bleaching began was shorter (5 minutes as compared to 7.5 minutes). While the 40% saturation supernatant results are not expected, the lack of bleaching could be caused by other interfering proteins that have become a larger proportion of the sample than when diluted by other proteins that were removed during the 20% and 40% saturation precipitations (Eisenthal & Danson, 2002). However, the 60% saturated pellet shows much greater activity than the unprocessed sample as seen in Figure 27 when the lowered lag time is taken into account as the bleaching begins to proceed after 2 minutes of incubation rather than 7.5 minutes. The dialysed sample shows an even greater level of activity as seen by a similar rate of bleaching to the unprocessed sample combined with the shortened lag period. This observation for both LOX-1 and LOX-2 indicates that despite the aggregation forced upon the enzymes they do not lose their conformational integrity in the presence of the ammonium sulfate as they are still able to catalyse the production of hydroperoxides from linoleic acid and therefore bleach methylene blue. Thus, their active site must still be unaltered and catalytic activity unhampered.

The method to produce the dialysed pellet that was obtained after the enzyme extract was brought to 60% saturation with solid ammonium sulfate was used for further purification based on the increased activity for both LOX-1 and LOX-2 compared to their unprocessed counterparts.

3.2.4. Ion exchange chromatography

Proteins from a crude extraction can require multiple processing steps to purify the protein of interest from the others found in the initial source material. The protein of interest may not need to be completely separated from all other proteins, in fact it is generally not feasible to do so when the protein is not overexpressed and engineered to have a helpful tag for purification (Doonan & Cutler, 2004). A more attainable goal would be to remove any contaminating molecules and proteins that can interfere with the activity of the enzyme. This can cut down on excessive time and costs involved with multiple rounds of different purification techniques.

The chromatogram produced by the ÄKTA™ Start Unicorn Start Software from the UV Flow cell shows the progress of the proteins binding to and eluting from the DEAE FF 5 ml HiTrap™ column (Figure 28). From approximately 30 to 60 ml the flow through can be seen coming off the column when the protein mixture from the 60% ammonium sulfate precipitation was loaded. From 60 ml to 100 ml one can see further unbound proteins eluting during the wash step with 125 mM boric acid and 10 mM sodium metabisulfite ($Na_2S_2O_5$) binding buffer. At 100 ml the gradient began, using a 1 M NaCl, 125 mM boric acid and 10 mM $Na_2S_2O_5$ eluting buffer. There was almost no elution in the first two 10 ml fractions (consisting of increasing 10% increments of the elution buffer), but at approximately 20% of the elution buffer, proteins began to elute from the column. The intense peak can be seen to continue and finish dropping by the time 60% of the elution buffer had been mixed. Thereafter, there was very little protein elution and once 100% of the elution buffer was running through the column no more proteins were seen to elute.

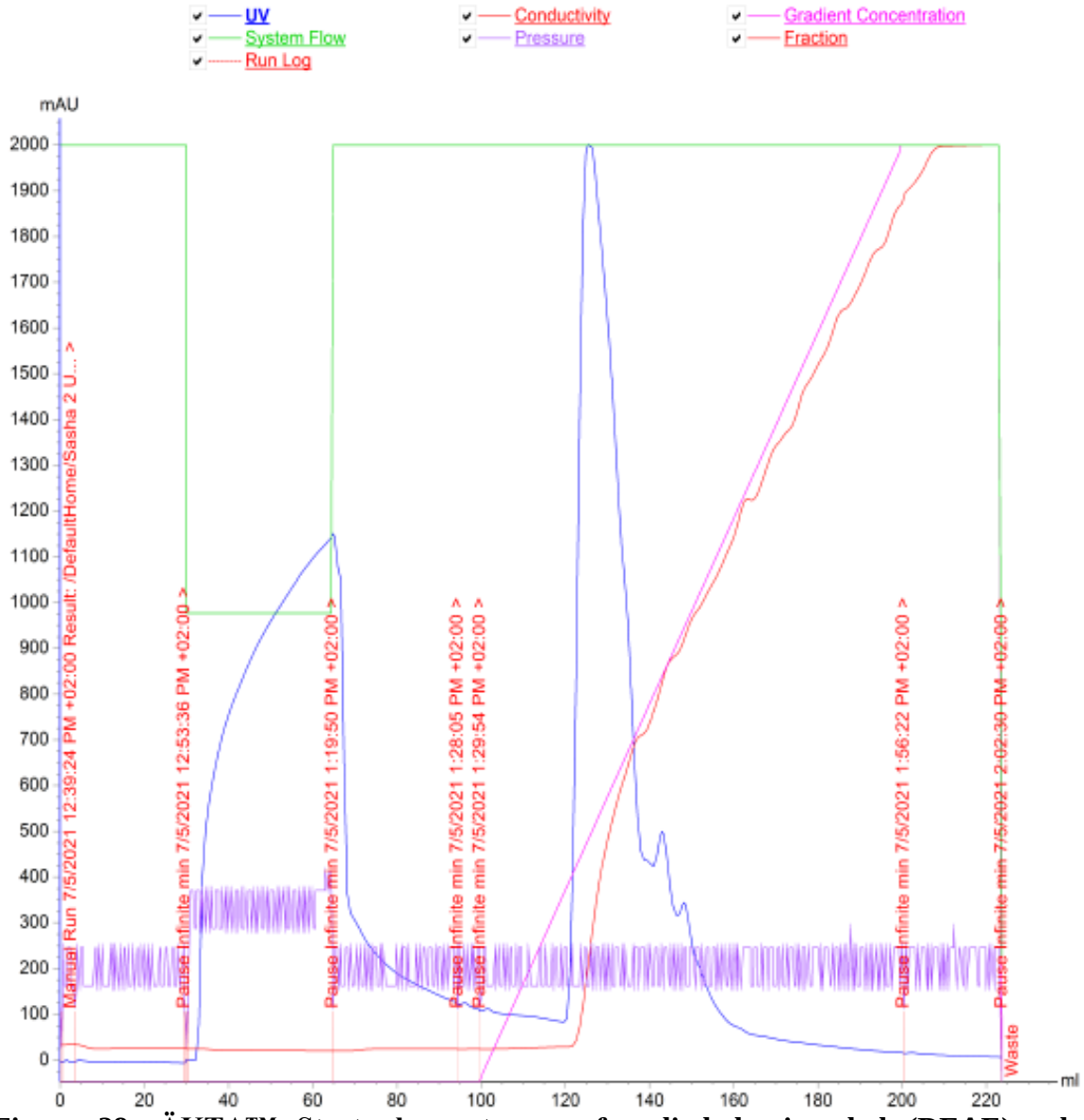


Figure 28: ÄKTA™ Start chromatogram for diethylaminoethyl (DEAE) column chromatography purified whole soybean enzyme extract

Proteins were extracted from WS were partially purified using ammonium sulfate precipitation before being bound to a DEAE FF 5 ml HiTrap™ column and washed with 125 mM boric acid and 10 mM $Na_2S_2O_5$ (pH 7.00) and eluted in ten 10 ml fractions using 125 mM Boric Acid and 10 mM $Na_2S_2O_5$, 1 M NaCl buffer (pH 7.00). The ÄKTA™ start chromatography system (Cytiva, Global Life Sciences Solutions USA LLC, Marlborough, Massachusetts, United States) was used for ion exchange chromatography.

The two biological replicates show differing protein quantifications between the same stages of purification (Table 1) but show a trend in how the purification steps affect the amount of protein carried through from the previous stage in the process. Thus, depending on the starting material and how much volume of liquid was initially strained from the variable amount of soybeans used, the protein concentration will differ, most notably after the concentration step

of ammonium sulfate precipitation. Thus, the extraction of the LOX isoforms using this method is most likely to be repeatable.

As shown in Table 1, the LOX-1 isoform appears to be purified 15.43-fold in the third fraction eluted from the DEAE FF 5 ml HiTrap™ column and 4.59-fold in the fourth fraction while the yield (percent recovery) of these fractions decrease to 44.50 and 9.05% respectively. The LOX-2 isoform is seen to be purified 5.50-fold in fraction 3 and 3.18-fold in fraction 4 and the yield for these fractions are 15.86 and 6.28% respectively. The specific activity, total activity, fold purity and yield values for LOX-1 are much higher than those for LOX-2 due to the smaller amount of total protein required in the reaction (0.1 mg as compared to 1 mg for LOX-2) to show appreciable bleaching.

Table 1: Lipoxygenase purification through diethylaminoethyl (DEAE) column chromatography from whole soybeans (WS)

Sample		Volume (ml)	Protein Concentration (mg/ml)		Total Protein (mg)	Specific Activity (rate/mg)	Total Activity	Fold Purity	Yield (%)
			Replicate 1	Replicate 2					
Unprocessed WS	LOX-1	185	18.2	16	3163.5	1.2375	3914.8	1	100
	LOX-2					0.1104	349.35	1	100
40% ASP Supernatant		180	14.4	14.3					
40% ASP Pellet		45	12.9	18.4					
60% ASP Supernatant		180	13.5	12.4					
60% ASP Pellet	LOX-1	45	15.6	10.7	591.75	5.785	3 423.3	4.6747	87.44
	LOX-2					0.525	310.67	4.7541	88.93
60% ASP Pellet Dialysed		45	7.83	9.65					
0.45 um Filtrate		45	7.81	8.37					
Flow Through		30	5.9	6.49					
Wash		40	3.34	1.57					
Fraction 1		10	<1	<1					
Fraction 2		10	<1	<1					
Fraction 3	LOX-1	10	7.14	11.1	91.2	19.1	1741.9	15.434	44.50
	LOX-2					0.6075	55.404	5.5012	15.86
Fraction 4	LOX-1	10	6.78	5.69	62.35	5.68	354.15	4.5899	9.05
	LOX-2					0.35167	21.927	3.1846	6.28
Fraction 5		10	1.54	1.2					

Proteins were extracted from WS using 125 mM boric acid and 10 mM sodium metabisulfite ($Na_2S_2O_5$). The proteins were subjected to graded ammonium sulfate precipitation (ASP), the 60% saturated ASP pellet (resuspended in a quarter of the original volume of extraction liquid) was dialysed against 125 mM boric acid and 10 mM sodium metabisulfite ($Na_2S_2O_5$, pH 7.0) and filtered through a 0.45 nm filter membrane. The filtrate was bound to a DEAE FF 5 ml HiTrap™ column and washed with 125 mM Boric Acid and 10 mM $Na_2S_2O_5$ (pH 7.00) and eluted in ten 10 ml fractions using 125 mM boric acid and 10 mM $Na_2S_2O_5$, 1 M NaCl buffer (pH 7.00). Protein quantifications were made using the Qubit® 2.0 Fluorometer (485/590 nm; Invitrogen by Life Technologies, Carlsbad, California, United States). Activity of relevant samples of LOX-1 and LOX-2 enzymes were determined using the assays described by Suda and co-workers (1995) and highlighted in white.

The initial purification of the WS consisted of a preliminary precipitation at 40% ammonium sulfate saturation to remove any proteins from the sample that precipitate out at lower salt concentrations. The sample was then brought to 60% saturation and the pellet was re-suspended in a quarter of the original volume of the soak liquid. The sample was then pre-equilibrated at pH 7.0 in the starting buffer (125 mM boric acid and 10 mM $Na_2S_2O_5$) using dialysis with the dual purpose of removing the ammonium sulfate from the sample. This can be seen in the SDS-

PAGE (Figure 29) and Native PAGE (Figure 30) as some of the proteins are removed from the sample. However, the filtration step to ensure that no contaminants that could clog the column were left in the sample, did not appear to affect the proteins as seen when lanes 7 and 8 are compared. The native PAGE is much less distinct than the SDS-PAGE but due to the nature of the technique the proteins will not separate as clearly. There is however, a somewhat visible band at 94-97 kDa (Shibata *et al.*, 1987) on both the SDS-PAGE and the native PAGE, as indicated by the red box, which is the region where the LOX isoforms are expected to be seen. However, a native PAGE is based more so on the shape and charge of proteins rather than purely by size (Bonner, 2007). Therefore, if an appropriate chromogenic substrate is placed upon the gel, after it has completed running, the protein of interest, which will have retained its native conformation and therefore catalytic activity, will ideally react and confirm the presence thereof (Bonner, 2007). The complicated nature of the assay to confirm LOX-1 and LOX-2 activity did not necessarily lend itself to such an endeavour, and the confirmation of enzyme activity by the assay along with protein bands in the correct region on the SDS-PAGEs was deemed confirmation enough of the presence of LOX-1 and LOX-2.

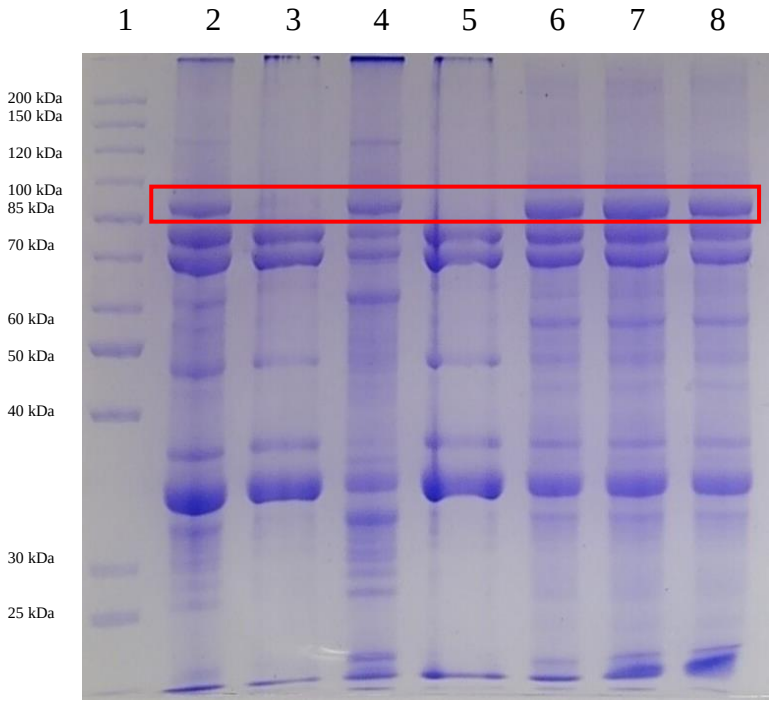


Figure 29: Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) of preparations of whole soybeans (WS) for diethylaminoethyl (DEAE) column chromatography

Proteins were extracted from WS using 125 mM boric acid and 10 mM sodium metabisulfite ($Na_2S_2O_5$). The proteins were subjected to graded ammonium sulfate precipitation (ASP), the 60% saturated ASP pellet was dialysed against 125 mM boric acid and 10 mM $Na_2S_2O_5$ (pH 7.0) and filtered through a 0.45 nm filter membrane. The red box indicates the region to which lipoxygense proteins should have migrated. Lane 1: Thermo Scientific™ PageRuler™ Unstained Protein Ladder; L2: Unprocessed WS; L3: 40% ASP Pellet; L4: 40% ASP Supernatant; L5: 60% ASP Supernatant; L6: 60% ASP Pellet; L7: Dialysed 60% ASP Pellet; L8: 0.45 µm Filtrate

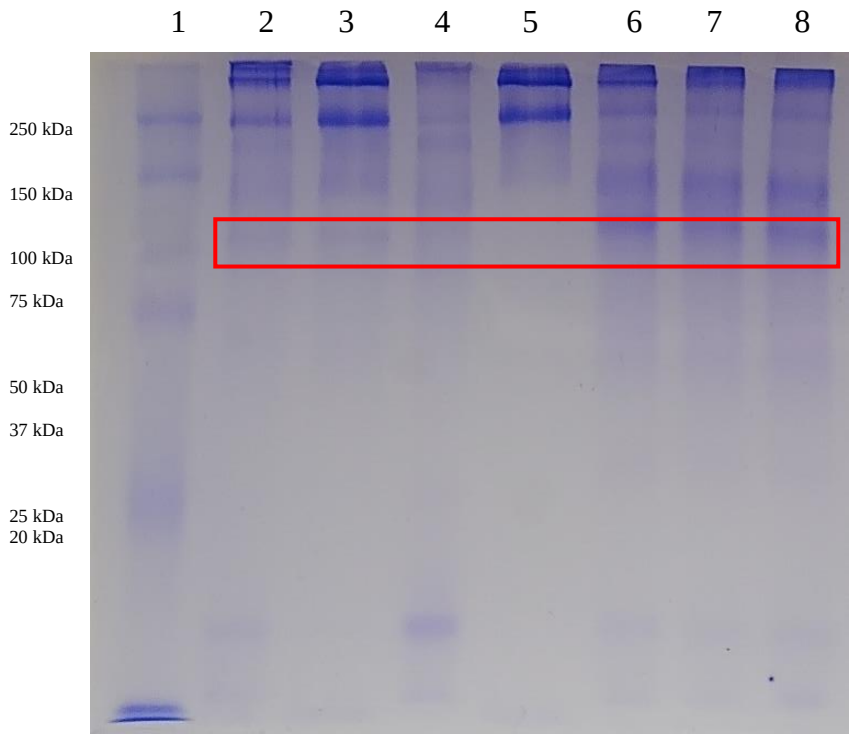


Figure 30: Native polyacrylamide gel electrophoresis (PAGE) of preparations of whole soybeans (WS) for diethylaminoethyl (DEAE) column chromatography

Proteins were extracted from WS using 125 mM boric acid and 10 mM sodium metabisulfite ($Na_2S_2O_5$). The proteins were subjected to graded ammonium sulfate precipitation (ASP), the 60% saturated ASP pellet was dialysed against 125 mM boric acid and 10 mM $Na_2S_2O_5$ (pH 7.0) and filtered through a 0.45 nm filter membrane. The red box indicates the region to which lipoxygenase proteins should have migrated. Lane 1: Precision Plus Protein™ Dual Xtra Standards Ladder; L2: Unprocessed WS; L3: 40% ASP Supernatant; L4: 40% ASP Pellet; L5: 60% ASP Supernatant; L6: 60% ASP Pellet; L7: Dialysed 60% ASP Pellet; L8: 0.45 µm Filtrate

The assays show that the sample with the highest activity from the initial purification steps was the dialysed 60% saturation pellet which had been subjected to filtration through the 45 µm membrane filter. The removal of other contaminating molecules appears to improve the activity of the LOX-1 (Figure 31) and LOX-2 (Figure 32), by a minimal amount. The sample may not consist of pure LOX enzymes, but the removal of some of the other enzymes and components found in the WS appears to have lowered the level of interference on the activity of the LOX-1 and LOX-2 isoforms. The assays also mimic what is seen in the SDS-PAGE whereby no activity is seen from the 60% saturation supernatant however the 40% saturation pellet showed some activity which may be linked to a very faint band on the gel. This follows what was seen during the initial testing of the ammonium sulfate gradient, whereby some of the LOX enzymes begin to precipitate out of solution at 40% saturation (Figure 26; Figure 27).

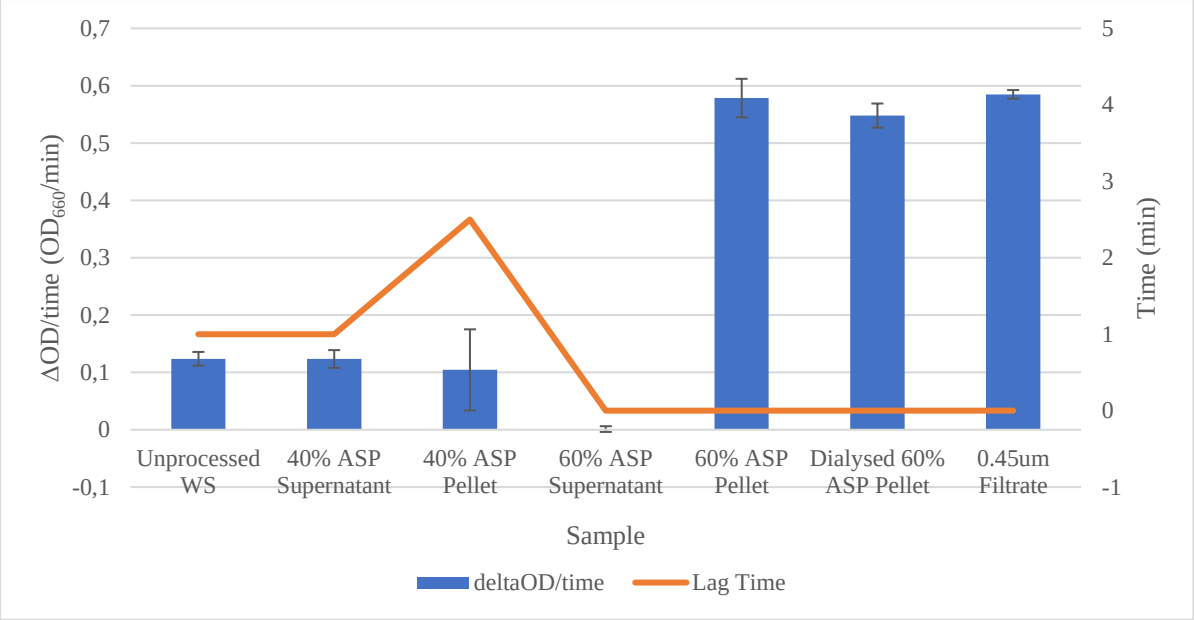


Figure 31: Methylene blue assay to determine lipoxygenase-1 activity in preparations of whole soybeans (WS) for diethylaminoethyl (DEAE) column chromatography

Proteins were extracted from WS using 125 mM boric acid and 10 mM sodium metabisulfite ($Na_2S_2O_5$). The proteins were subjected to graded ammonium sulfate precipitation (ASP), the 60% saturated ASP pellet was dialysed against 125 mM boric acid and 10 mM $Na_2S_2O_5$ (pH 7.0) and filtered through a 0.45 nm filter membrane.

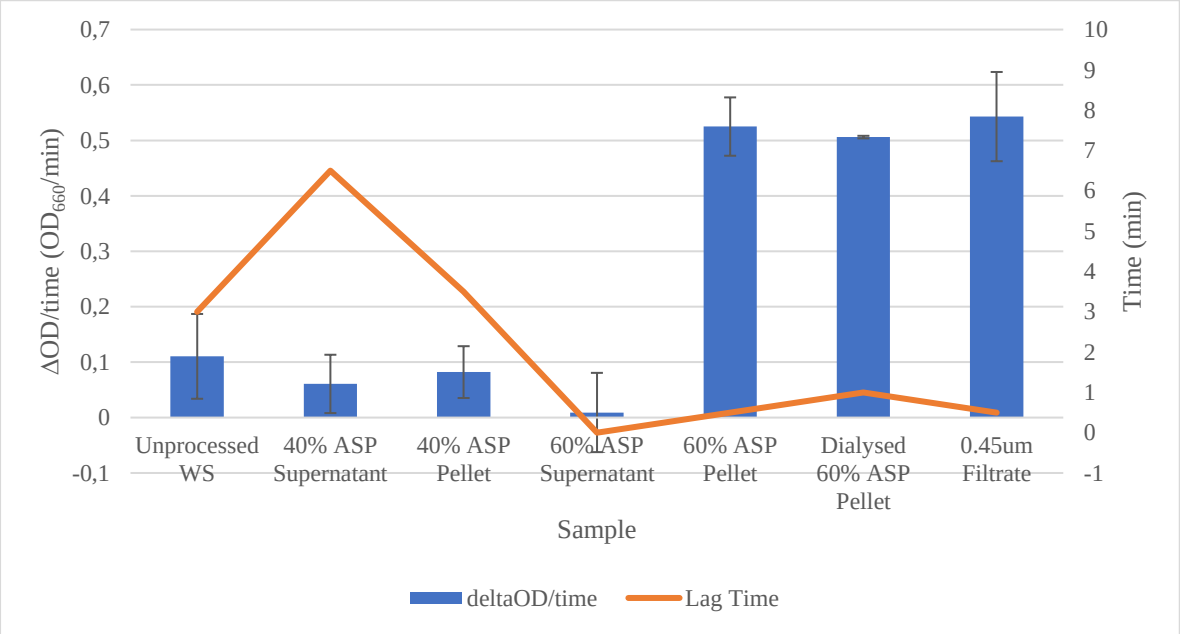


Figure 32: Methylene blue assay to determine lipoxygenase-2 activity in preparations of whole soybeans (WS) for diethylaminoethyl (DEAE) column chromatography

Proteins were extracted from WS using 125 mM boric acid and 10 mM sodium metabisulfite ($Na_2S_2O_5$). The proteins were subjected to graded ammonium sulfate precipitation (ASP), the 60% saturated ASP pellet was dialysed against 125 mM boric acid and 10 mM $Na_2S_2O_5$ (pH 7.0) and filtered through a 0.45 nm filter membrane.

The SDS-PAGE for the ion-exchange chromatography purification (Figure 33), shows some faint bands in the expected region between 85 and 100 kDa, most notably in the fractions showing the most LOX activity in Figure 35, which are less clear in the native PAGE (Figure 34). Thus, lanes 6 and 7 show the bands (Figure 33) most likely indicating the LOX isoforms most clearly, a similarly sized band can also be seen in lane 2 in the flow through. This is unlikely to be the LOX-1 enzymes as the flow through showed no activity in the assay for LOX-1 (Figure 35), but a small amount of activity was seen for LOX-2 (Figure 36) which indicates that it may not have bound to the column as strongly as the LOX-1 isoforms.

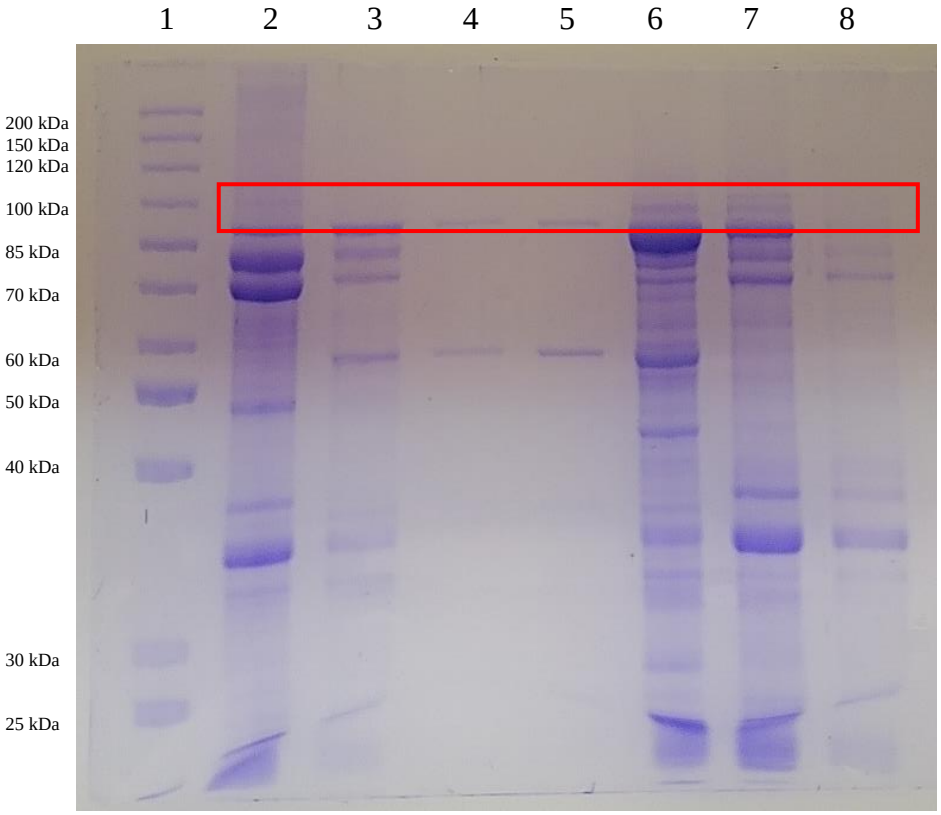


Figure 33: Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) of diethylaminoethyl (DEAE) column chromatography purified whole soybean (WS) enzyme extract

Proteins were extracted from WS using 125 mM boric acid and 10 mM sodium metabisulfite ($Na_2S_2O_5$) and purified before being bound to a DEAE FF 5 ml HiTrap™ column and washed with 125 mM boric acid and 10 mM $Na_2S_2O_5$ (pH 7.0) and eluted in ten 10 ml fractions using 125 mM boric acid and 10 mM $Na_2S_2O_5$, 1 M sodium chloride (NaCl) buffer (pH 7.0). The red box indicates the region to which lipoxygenase proteins should have migrated. Lane 1: Thermo Scientific™ PageRuler™ Unstained Protein Ladder; L2: Flow Through; L3: Wash; L4: Fraction 1 (0-10% NaCl); L5: Fraction 2 (10-20% NaCl); L6: Fraction 3 (20-30% NaCl); L7: Fraction 4 (30-40% NaCl); L8: Fraction 5 (40-50% NaCl)

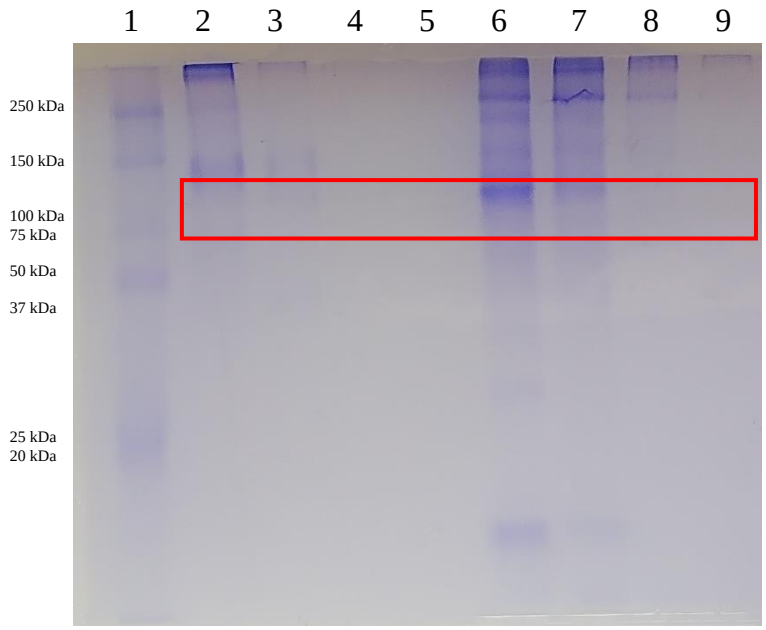


Figure 34: Native-polyacrylamide gel electrophoresis (PAGE) of diethylaminoethyl (DEAE) column chromatography purified whole soybean (WS) enzyme extract

Proteins were extracted from WS using 125 mM boric acid and 10 mM sodium metabisulfite ($Na_2S_2O_5$) and purified before being bound to a DEAE FF 5 ml HiTrap™ column and washed with 125 mM boric acid and 10 mM $Na_2S_2O_5$ (pH 7.0) and eluted in ten 10 ml fractions using 125 mM boric acid and 10 mM $Na_2S_2O_5$, 1 M sodium chloride (NaCl) buffer (pH 7.0). The red box indicates the region to which lipoxygenase proteins should have migrated. Lane 1: Precision Plus Protein™ Dual Xtra Standards Ladder; L2: Flow Through; L3: Wash; L4: Fraction 1 (0-10% NaCl); L5: Fraction 2 (10-20% NaCl); L6: Fraction 3 (20-30% NaCl); L7: Fraction 4 (30-40% NaCl); L8: Fraction 5 (40-50% NaCl); L9: Fraction 6 (50-60% NaCl)

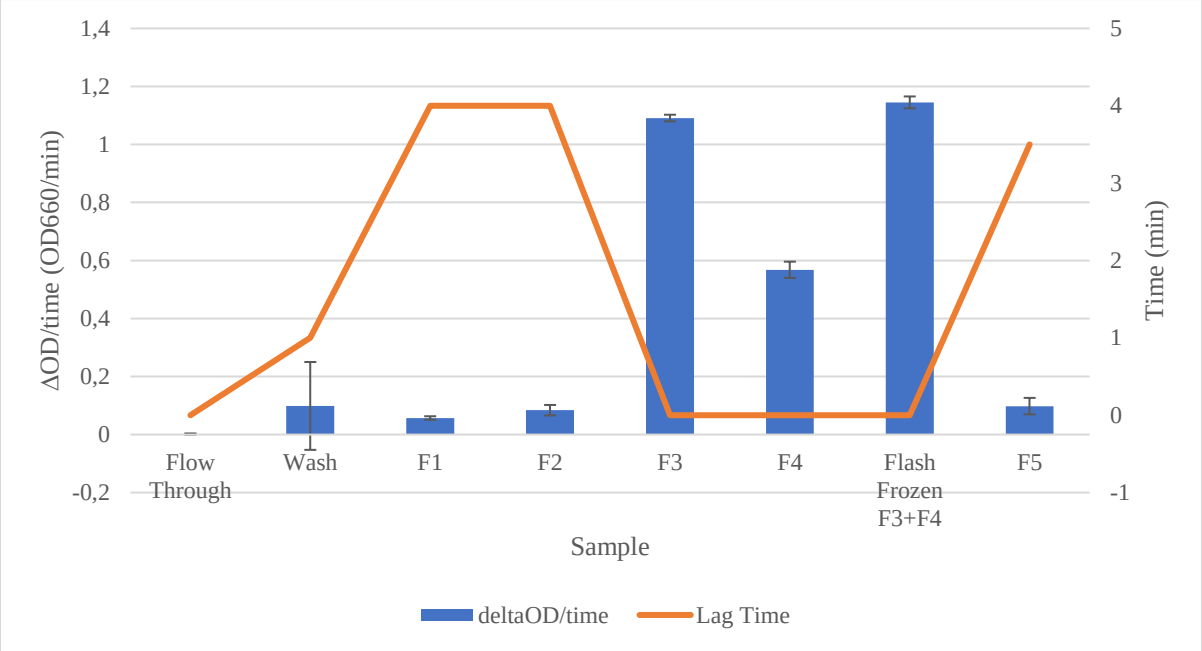


Figure 35: Methylene blue assay to determine lipoxygenase-1 activity in diethylaminoethyl (DEAE) column chromatography purified whole soybean (WS) enzyme extract

Proteins were extracted from WS before being bound to a DEAE FF 5ml HiTrap™ column and washed with 125 mM boric acid and 10 mM sodium metabisulfite ($Na_2S_2O_5$, pH 7.0) and eluted in ten 10 ml fractions (F) using 125 mM boric acid and 10 mM $Na_2S_2O_5$, 1 M sodium chloride (NaCl) buffer (pH 7.00)

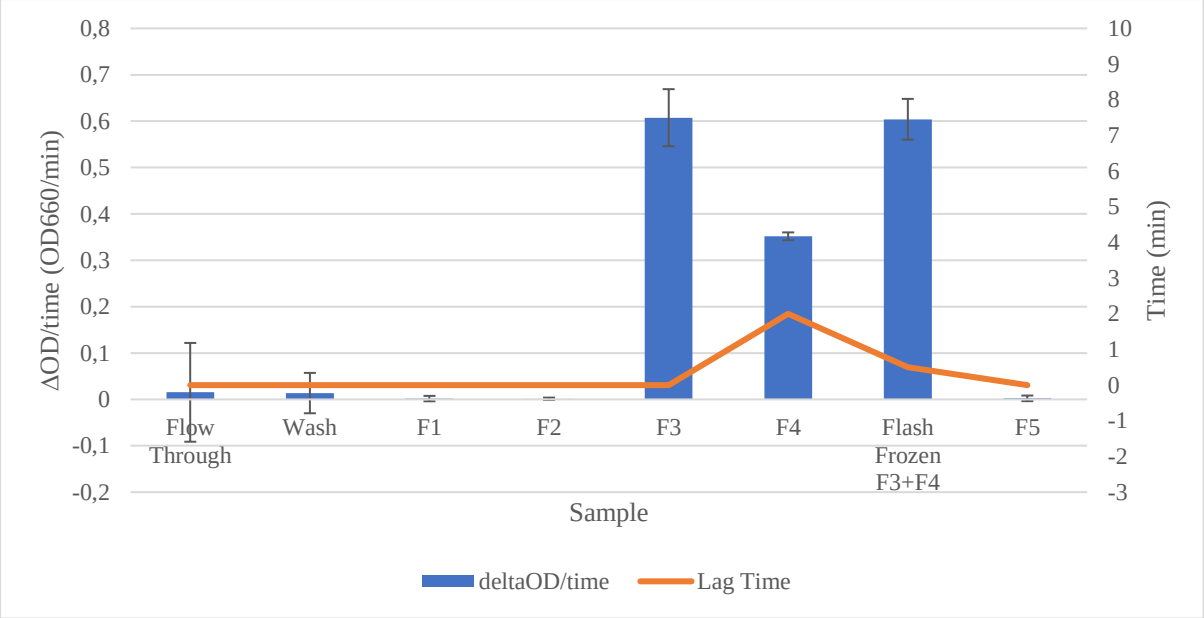


Figure 36: Methylene blue assay to determine lipoxygenase-2 activity in diethylaminoethyl (DEAE) column chromatography purified whole soybean (WS) enzyme extract

Proteins were extracted from WS before being bound to a DEAE FF 5ml HiTrap™ column and washed with 125 mM boric acid and 10 mM sodium metabisulfite ($Na_2S_2O_5$, pH 7.0) and eluted in ten 10 ml fractions using 125 mM boric acid and 10 mM $Na_2S_2O_5$, 1 M sodium chloride (NaCl) buffer (pH 7.0)

This discrepancy could be due to the fact that pH 7.0 was chosen as the pH value above the pI of both LOX isoforms as LOX-2 has a pI of 6.25 and LOX-1 has a pI of 5.68. The binding of proteins to DEAE anion exchange resin works best at a pH value at least one whole pH unit higher than the pI of the protein (Siedow, 1991; Bonner, 2007). Thus, LOX-1 enzymes will bind strongly to the resin as the binding buffer is over one unit higher than the pI, LOX-2, however, is less than one unit away from the pH of the binding buffer and not all enzymes will bind as strongly to the resin. The addition of further binding buffer will therefore wash away these unbound proteins in the flow through step. If the two enzyme isoforms are to be selectively purified separately from one another then the pH of the binding and elution buffers can be altered to better fit the individual enzymes. However, for the purposes of this investigation, the amount of enzyme lost was minimal at pH 7.0 and thus it was not altered going forward.

Due to the concerns raised by the investigations of Cao and co-workers (2003) an aliquot of the combined fractions with the highest LOX-1 and LOX-2 activity was flash frozen using liquid nitrogen and thawed on ice to determine how the process might affect the enzyme

activity before the enzyme was stored at -80°C for later processing. As can be seen in the graphs the combined fractions that were frozen lost minimal LOX-2 activity (Figure 36). The LOX-1 graph (Figure 35) shows a small increase in activity which could be due to combining the two fractions. This revelation would allow processing time to be cut down as fresh enzyme would not need to be prepared for each subsequent experiment if aliquots could be taken from frozen storage as needed.

3.2.5. Controls of activity assays for lipoxygenase

It is imperative to determine that it is indeed the action of the enzyme responsible for the positive reaction of the assay rather than any outside factor. In the case of the methylene blue bleaching assays described by Suda and co-workers (1995) it was necessary to prove that no bleaching of methylene blue occurred without the addition of both enzyme and substrate.

The assays were left to proceed for the full period of time indicated, 5 minutes for the assay measuring LOX-1 activity and 10 minutes for the assay measuring LOX-2 activity. During that time no activity was seen for in the LOX-1 assay wherein no purified enzyme (F3+F4 from the ÄKTA purification, Figure 35 and Figure 36) was added to the reaction system, nor when the substrate was omitted (Figure 37).

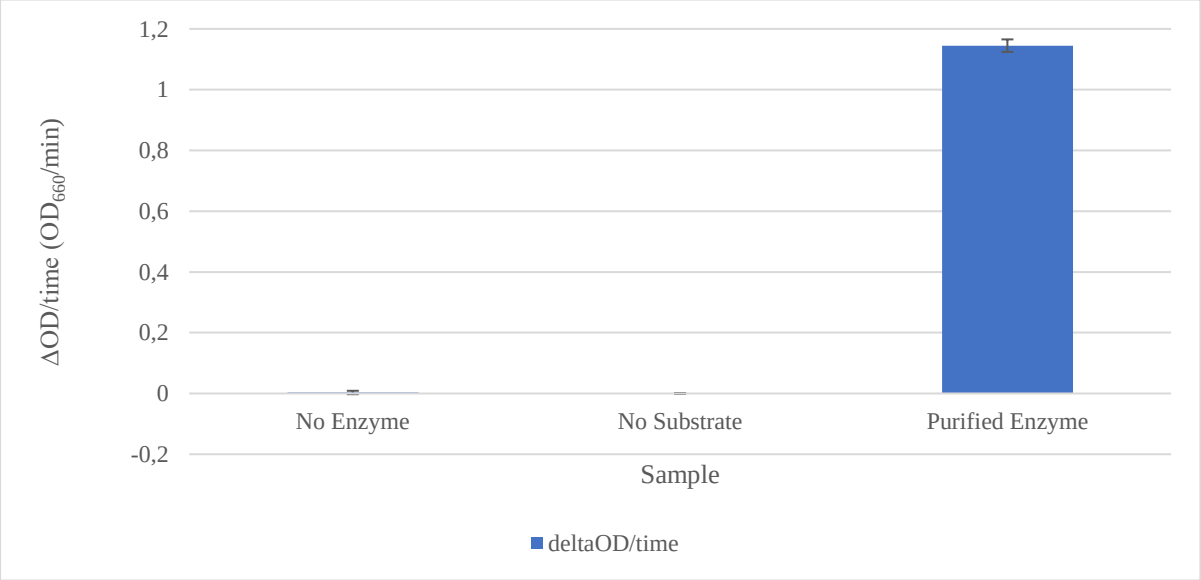


Figure 37: Controls of lipoxygenase-1 assay

Triplicate readings were taken, and averaged, for either no enzyme (purified whole soybean (WS) LOX-1) or no substrate (10 mM sodium linoleate) containing-assay 1 as described by Suda and co-workers (1995). All reaction components other than the enzyme or substrate (which was substituted with ddH_2O) were included in the cuvette to determine whether any other factors were responsible for methylene blue bleaching. The standard deviation bars show variation between the technical replicates. The purified enzyme was used a positive control.

The LOX-2 assay (Figure 38) on the other hand showed minimal bleaching for both the reaction systems missing enzyme and missing substrate. The LOX-2 assay proceeds for 10 minutes, during which time settling could occur as the cuvette is left stationary as the reaction proceeds. However, the bleaching rate seen is over 60 times lower than the purified enzyme containing reaction.

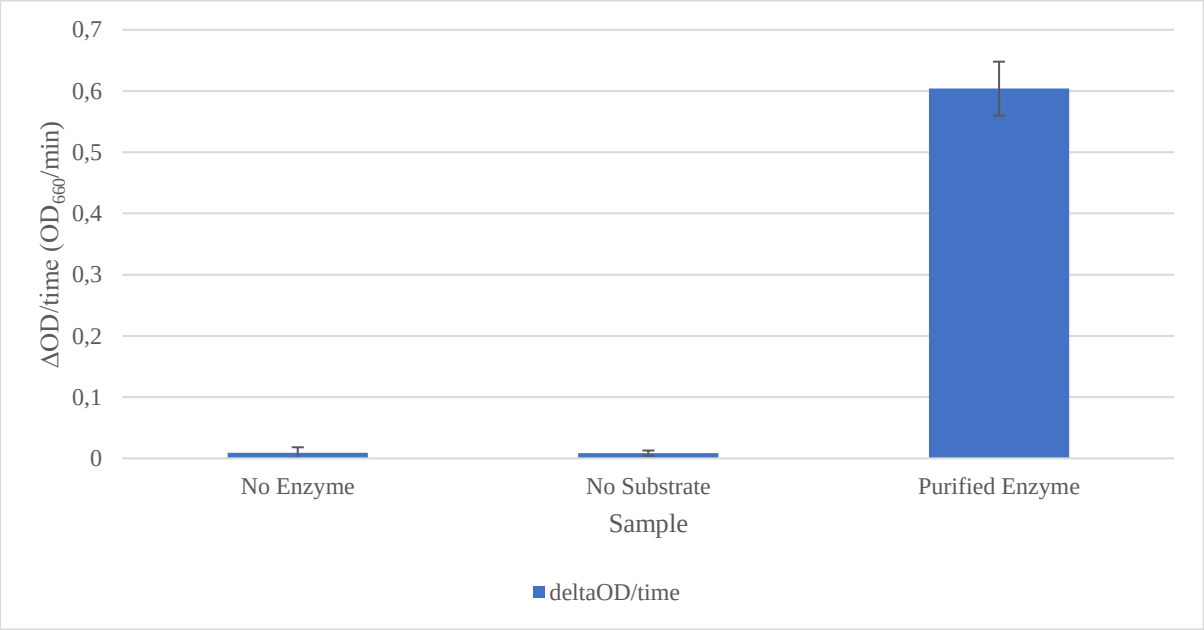


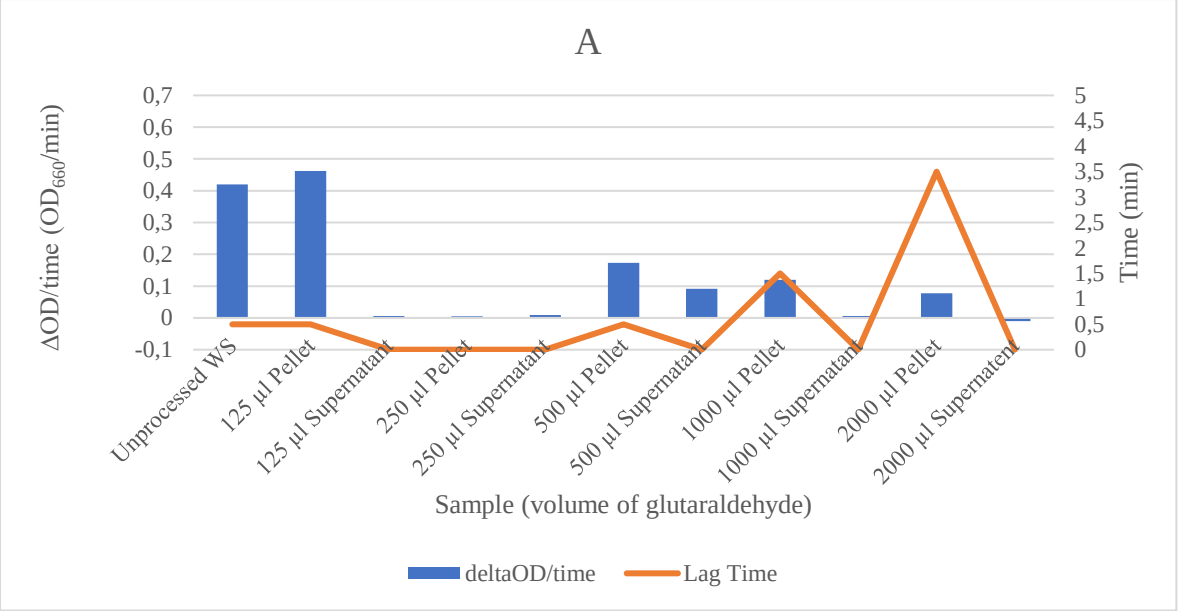
Figure 38: Controls of lipoxygenase-2 assay

Triplicate readings were taken, and averaged, for either no enzyme (purified whole soybean (WS) LOX-1) or no substrate (10 mM sodium linoleate) containing-assay 2 as described by Suda and co-workers (1995). All reaction components other than the enzyme or substrate (which was substituted with ddH_2O) were included in the cuvette to determine whether any other factors were responsible for methylene blue bleaching. The standard deviation bars show variation between the technical replicates. The purified enzyme was used as a positive control.

3.3. Cross-linked enzyme aggregates

An enzyme will be affected by numerous factors in its environment such as pH, temperature, mechanical forces and so on (Sheldon & van Pelt, 2013). A technique which can increase resistance is that of creating cross-linked enzyme aggregates (CLEAs; Sheldon & van Pelt, 2013). Moreover, these CLEAs have been seen to be recyclable which for industrial purposes is an enticing factor. If fresh enzymes are not constantly required, and thus extraction and purification can be avoided, time and costs can be reduced (Velasco-Lozano *et al.*, 2016). The production of CLEAs does not require purified enzymes and they can be made directly from a crude lysate, however, there may be interference from the other proteins in the mixture and thus it may help to use a more purified sample (Sheldon & van Pelt, 2013).

The LOX-1 activity seen in Figure 39, shows that the activity was highest in the sample with the lowest concentration of glutaraldehyde. The unprocessed sample was similar in velocity of methylene blue bleaching, if not slightly lower, to the un-dialysed sample that had been cross-linked with 125 µl glutaraldehyde. Glutaraldehyde is a small dialdehyde molecule capable of cross-linking proteins through lysine residues found on their surfaces (Velasco-Lozano *et al.*, 2016). These preliminary results were sufficient to confirm what the literature indicates wherein lower concentrations of a cross-linking agent will yield better results when the level of cross-linking is lower (van Pelt *et al.*, 2008). When too much cross-linker is present the enzymes will begin to deform due to excessive cross-linking and thus the activity will be lower. Along with the lowered amount of activity, in the samples cross-linked with too much glutaraldehyde, the lag period increases before bleaching begins to occur. This is because the active site of some of the enzymes are hampered and thus unable to perform to the best of their catalytic potential.



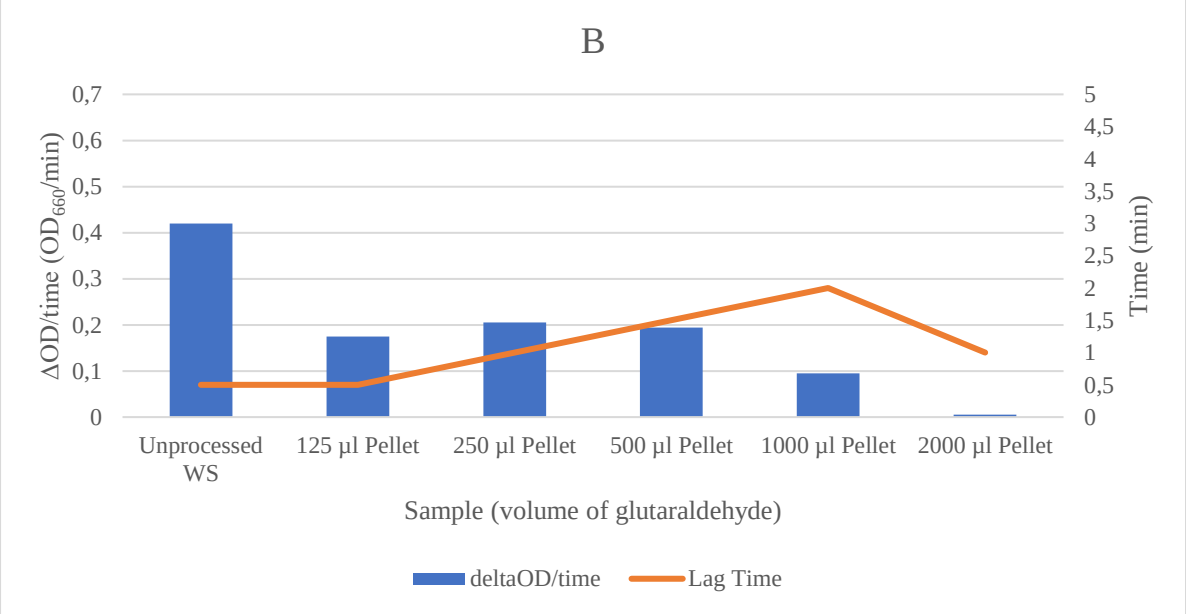


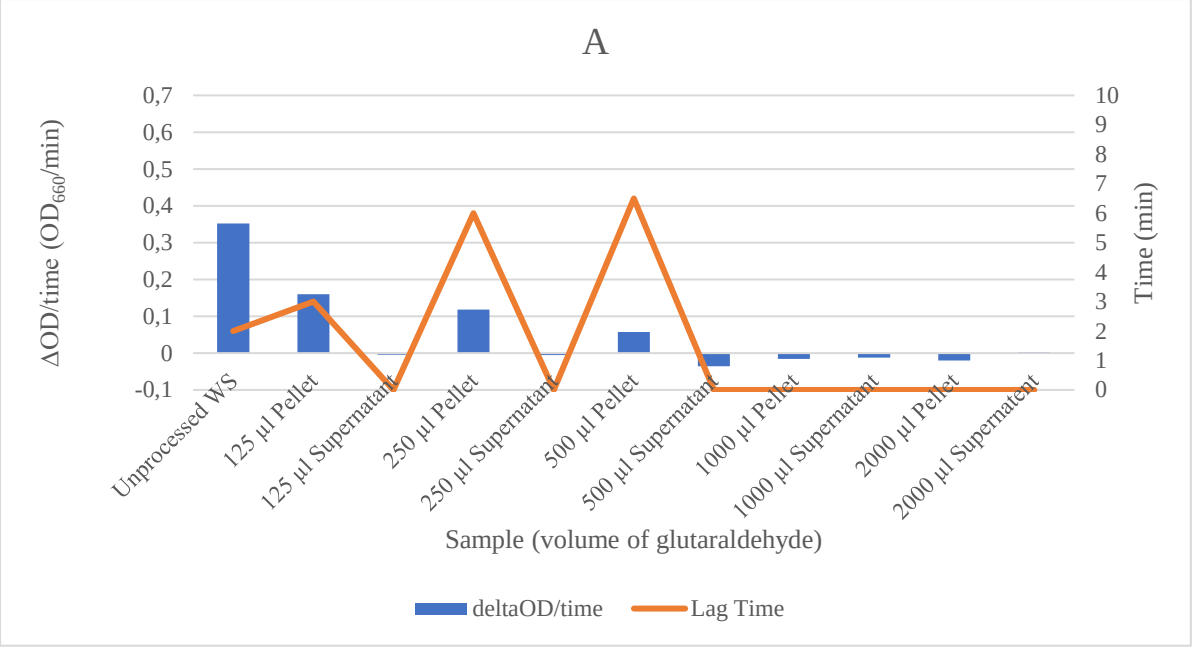
Figure 39: Methylene blue assay to determine lipoxygenase-1 activity in glutaraldehyde crosslinked enzyme aggregates from whole soybeans (WS); **A: original samples; B: dialysed samples**

Cross-linked enzyme aggregates from WS were formed using different concentrations of glutaraldehyde and tested for LOX-1 activity through bleaching of methylene blue before and after dialysis to remove excess glutaraldehyde and contaminating ammonium sulfate.

Before the samples were dialysed the activities were lower, in all cases but the 125 µl glutaraldehyde sample, than they were after excess glutaraldehyde was removed through dialysis. After dialysis the 250 µl glutaraldehyde-containing sample was seen to have activity in a similar range (higher rate of bleaching but a longer lag period) as the dialysed 125 µl

sample whereas before dialysis it appeared to have no activity. Before dialysis the 125 µl sample had activity similar to the unprocessed sample but afterwards had lowered activity which could be accounted for by the loss of enzymes during the dialysis step. As the molecular weight cut-off of the dialysis tubing was low enough to prevent the enzyme from escaping the sample, it is possible that the enzymes that were released from the CLEAs lost their conformational stability and thus had been inactivated. The samples with 1000 and 2000 µl of glutaraldehyde lost activity after dialysis, too high a concentration of cross-linker may not be overcome by dialysis the same way samples with moderate concentrations appear to be.

Similar to the LOX-1 CLEAs (Figure 39) the sample cross-linked with 125 µl glutaraldehyde had the highest LOX-2 activity (Figure 40) before and after dialysis, but appeared to lose activity during the dialysis process. However, the drop in activity was much more intense than for LOX-1 which indicates that LOX-2 may not be bound as well in CLEA form than its counterpart. Furthermore, the 125 µl glutaraldehyde-containing sample had less activity than the unprocessed sample unlike the LOX-1 CLEAs wherein before dialysis the CLEA showed greater activity. It is notable that, unlike the LOX-1 CLEAs where the higher concentration of glutaraldehyde-containing CLEAs gained activity after dialysis, the LOX-2 CLEAs lost activity or did not exhibit any activity before or after the dialysis step. This indicates that LOX-2 is more adversely affected by high levels of cross-linking and loses all activity due to the conformational strain put upon the enzyme under such stresses. It therefore may not be feasible to create and recycle CLEAs using LOX-2 without further fine-tuning.



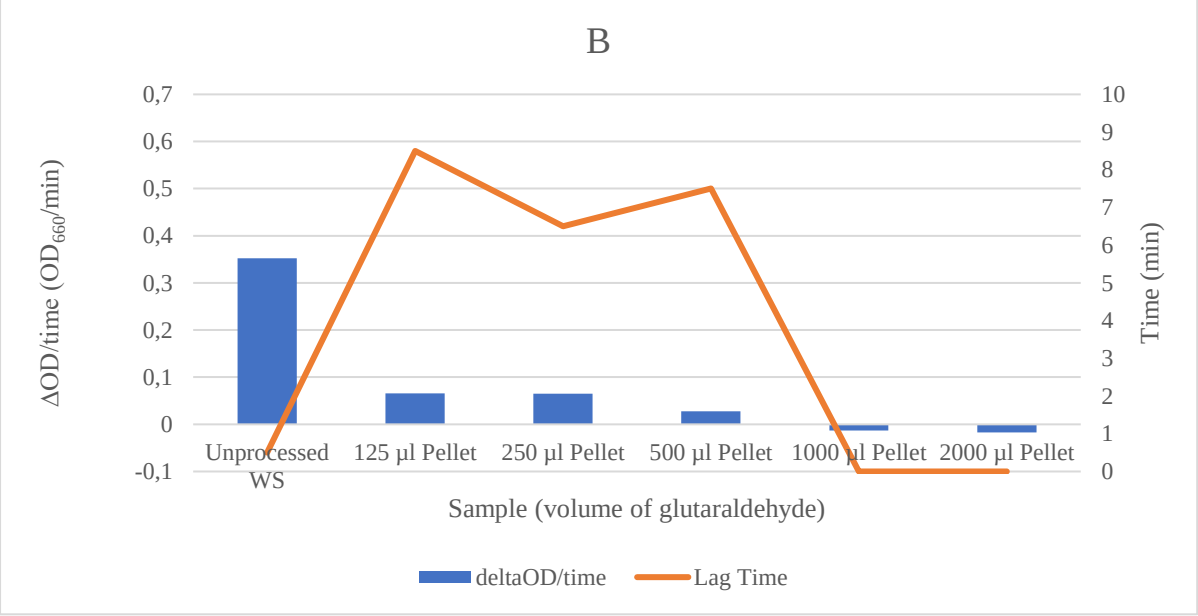


Figure 40: Methylene blue assay to determine lipoxygenase-2 activity in glutaraldehyde crosslinked enzyme aggregates from whole soybeans; A: original samples; B: dialysed samples

Cross-linked enzyme aggregates from WS were formed using different concentrations of glutaraldehyde and tested for LOX-2 activity through bleaching of methylene blue before and after dialysis to remove excess glutaraldehyde and contaminating ammonium sulfate.

3.4. Application of the cross-linked enzyme aggregates in biocatalysis

There are many industrial uses for enzymes, one of which is the conversion of widely available and cheaper terpenes such as valencene into higher value terpenoids such as nootkatone (Figure 9) (Palmerín-Carreño *et al.*, 2015). This conversion in the presence of lipoxygenase and one of its, cheaper, substrates oleic acid is under investigation by APBio. The use of lipoxygenase in CLEA form would be both time and cost effective as it would cut down on production costs for fresh enzyme as well as the time it takes to prepare the LOX enzyme that will be added to the reaction. If the CLEAs are also more robust, as the literature claims (Sheldon & van Pelt, 2013), any change in pH, temperature and substrate concentration may also be less problematic to the action of the enzyme.

As can be seen from the information in Table 2, there are similar amounts of products produced from the valencene substrate with both the un-dialysed and dialysed CLEAs prepared from the crude WS extracts. However, gas chromatography–mass spectrometry (GC-MS) is a semi-quantitative method whereby this is a relative peak area comparison. The compounds detected in the sample are expressed as a percentage of total M/S ions (of both volatile and semi-volatile analytes) counted during the entire GC sample run. No standard curves (in ppm) were used in order to calculate absolute concentrations of the compounds. The production of the hydroperoxides was done using oleic acid rather than the preferred substrate of lipoxygenase, linoleic acid, due to the much lower cost of the oil. There was still the production of hydroperoxides, as is evidenced by the production of the nootkatone from the valencene precursor over the 48-hour period.

Table 2: Gas chromatography-mass spectrophotometry (GC-MS) total concentration (%) of nootkatone produced from valencene using the 125µl glutaraldehyde lipoxygenase (LOX) cross-linked enzyme aggregates (CLEAs) before and after dialysis within a 48-hour period

Sample	Duration (Hours)	Valencene (%)	Nootkatone (%)
LOX CLEA	24	72	2.7
	48	45	24.7
Dialysed LOX CLEA	24	72	1.8
	48	58	7.3

Proteins were extracted from whole soybeans and cross-linked using 125 µl 25% glutaraldehyde The sample, before and after dialysis, was tested in a conversion of valencene to nootkatone reaction and subjected to GC-MS to determine how well the CLEAs converted the valencene to nootkatone.

There is a decrease in nootkatone production between the un-dialysed CLEAs and the dialysed CLEAs (Table 2) which may indicate that LOX-2 plays a larger role in the oxidation of the valencene than LOX-1. This is based on the results seen in Figure 39 and Figure 40 where the LOX-2 CLEAs showed much lower activity after dialysis than before. After 48 hours the un-dialysed sample showed 24.7% total concentration nootkatone and the valencene total concentration had decreased to 45%. In the dialysed sample these values were 7.3 and 58% respectively, which shows that once the CLEAs had been washed their catalytic ability had dropped.

Interestingly, the CLEAs produced using the purified enzymes (Table 3) when compared to the crude enzymes, showed a more consistent production of nootkatone after recycling and dialysis. For the initial reaction using the crude enzyme after 24 hours the nootkatone peak area was 2.7% before and 1.8% after dialysis (recycling) whereas the purified enzymes after 24 hours showed 2.1% before recycling and 2.3% afterwards. As the reaction could not be taken to 48 hours those values cannot be compared, but preliminarily the purified enzyme shows promising results for recyclability. This is industrially favourable as a previous investigation by Smith & Lands (1972) indicated that lipoxygenase is destroyed as it produces hydroperoxides. Their research indicated that the mechanism by which the enzyme is destroyed occurs when the substrate, product and molecular oxygen are all present in the reaction mixture. Thus, over time the lipoxygenase enzymes within a reaction will begin to undergo conformational changes and the production of hydroperoxides will slow and eventually halt, even if there is more of the substrate available for conversion. This conformational change may therefore be reversible if the products seen to be produced after the LOX CLEAs are recycled are in line with those produced before the enzymes had been recycled. Had the amount of nootkatone gone down rather than up (Table 3), it could be possible that the conformational change is permanent but further investigations into the recyclability of LOX may need to be investigated. The improved stability at different temperatures and pHs as well as improved activity that has been reported elsewhere will need to be investigated further if the production of LOX CLEAs is determined to be profitable. The separate LOX isoforms may also need to be tested in the creation of LOX CLEAs to determine how they react separately from one another.

Table 3: Gas chromatography-mass spectrophotometry (GC-MS) total concentration (%) of nootkatone produced from valencene using the 125 µl glutaraldehyde lipoxygenase (LOX) cross-linked enzyme aggregates (CLEAs) within a 24-hour period

Sample	Duration (Hours)	Valencene (%)	Nootkatone (%)
LOX CLEA	24	67	2.1
Recycled LOX CLEA	24	64	2.3

Proteins were extracted from whole soybeans and cross-linked using 125 µl 25% glutaraldehyde. The sample was tested in a conversion of valencene to nootkatone reaction and subjected to GC-MS to determine how well the CLEAs converted the valencene to nootkatone. This conversion was repeated after the sample was recycled by centrifugation and dialysis overnight.

3.5. Optimal enzyme conditions

Enzymes to be used in any capacity need to be investigated for the conditions under which they show their greatest catalytic activity. Once the LOX-1 and LOX-2 isozymes had been purified from the WS source it was feasible to begin investigating the best conditions for their use and storage through the alteration of the assays which test for their activity and buffers and temperatures in which they were stored.

3.5.1. Effect of pH on lipoxygenase activity

It can be seen in Figure 41 that, above and below pH 10, the rate of methylene blue bleaching by LOX-1 decreases. Asbi and co-workers (1989) noted that below pH 7.1 there is a sharp decrease in LOX activity which aligns with the lower rate of methylene blue bleaching in the. Many papers observed that there was maximal LOX-1 activity at pH values around 9.0 (Asbi *et al.*, 1989; Suda *et al.*, 1995). However, the findings of this investigation are that pH 10 produces the highest activity from this particular LOX-1 enzymes which falls in line with the information given by Jacquot and co-workers (2008). It was determined that for further investigations pH 10 would be used in the assay for LOX-1. The p-value between the activity seen using buffer at pH 9 vs pH 10 does not fall below 0.05 (it is 0.149) which means there is not significant difference between the rate of bleaching LOX-1 in the buffers of different pH.

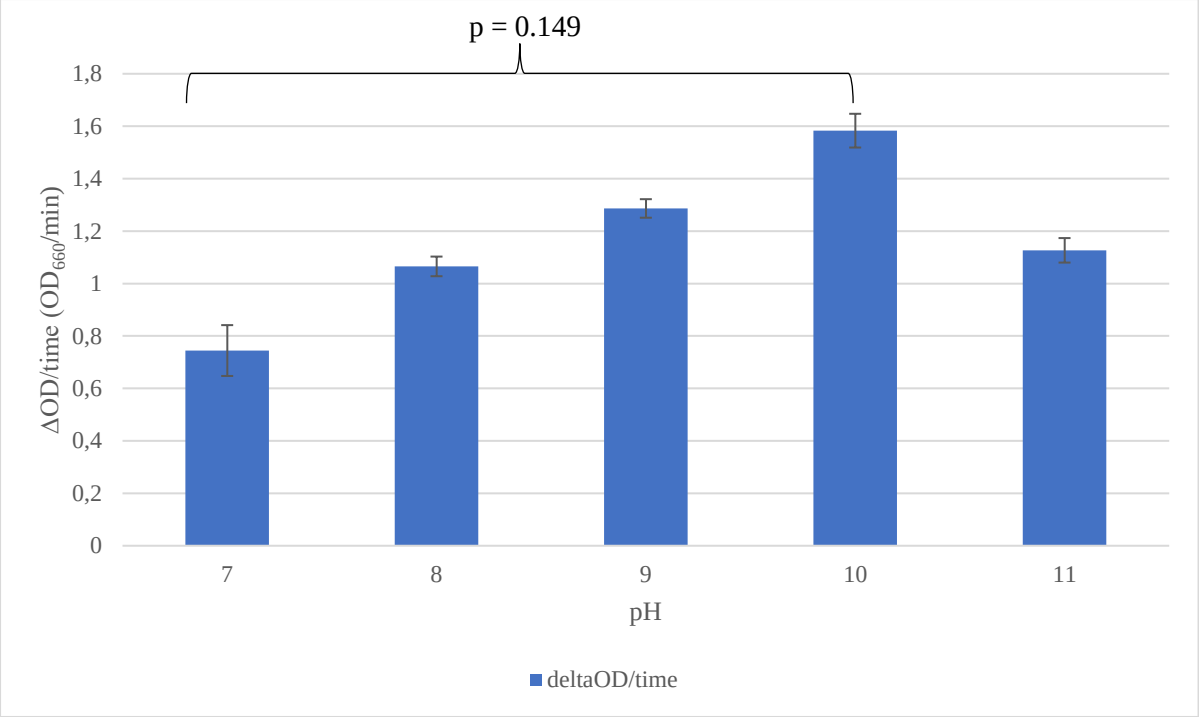


Figure 41: Effect of pH on lipoxygenase-1 activity

Purified enzyme was used to determine the optimal pH of the boric acid buffer used in the methylene blue bleaching assay to determine which pH allowed for optimal enzyme activity. Error bars show variation between technical replicates.

Up until this investigation into pH had taken place, it had been necessary to use 1000 µg of protein from each sample to see complete activity from LOX-2 in the assay described by Suda and co-workers (1995) at pH 6 (Figure 42) during the 10-minute timeframe. Most notably when crude enzyme extract was used rather than the purified enzyme which in turn would not have allowed the preliminary extraction and purification methods to be compared. When the pH was below 6, the reaction mixture became cloudy as the substrate became insoluble in the solution and was therefore no longer optically clear and thus not suitable for spectrophotometry. The speed of the reaction was also much slower than at higher pHs. The lag period decreased as the pH increased. When the pH increased to 7 and 8, with 1000 µg of total protein present, the bleaching proceeded too fast for the spectrophotometer to register the colour change once the enzyme had been added. Therefore, the amount of protein added was lowered by a factor of ten to only 100 µg (the same concentration added to the assay for LOX-1), and the reaction was seen much more clearly.

Upon further investigation, however, Suda and co-workers (1995) discovered that above pH 6.8 LOX-1 began to interfere and start bleaching the methylene blue in conjunction with LOX-2, which could most assuredly account for the drastic increase in the speed of bleaching. Due

to this phenomenon an intermediate range of pH values between 6 and 7 (Figure 43) was investigated to see at which pH the activity was highest without allowing for interference from LOX-1, but still allowing for the use of only 100 µg of protein per reaction to keep conditions in line with the assay for LOX-1. It was necessary to monitor the reaction for much longer than the 10 minutes in order to observe the time it took for 100 µg of protein in the purified enzyme extract to finish bleaching the methylene blue at some of the lower pH values tested. It can be seen that, with an increase of pH, the time taken for bleaching to reach completion became lower with each successive jump of 0.2 pH units as the lag period was overcome faster. The rate of bleaching increased with increasing pH. The best activity was seen at the end of the range before the interference of LOX-1 became problematic and thus, going forward, the investigations into LOX-2 was done with the sodium phosphate buffer pH adjusted to 6.8. The p value (0.001) between the buffers at pH 6.0 and pH 6.8 show significant differences in activity of LOX-2 when the pH is raised.

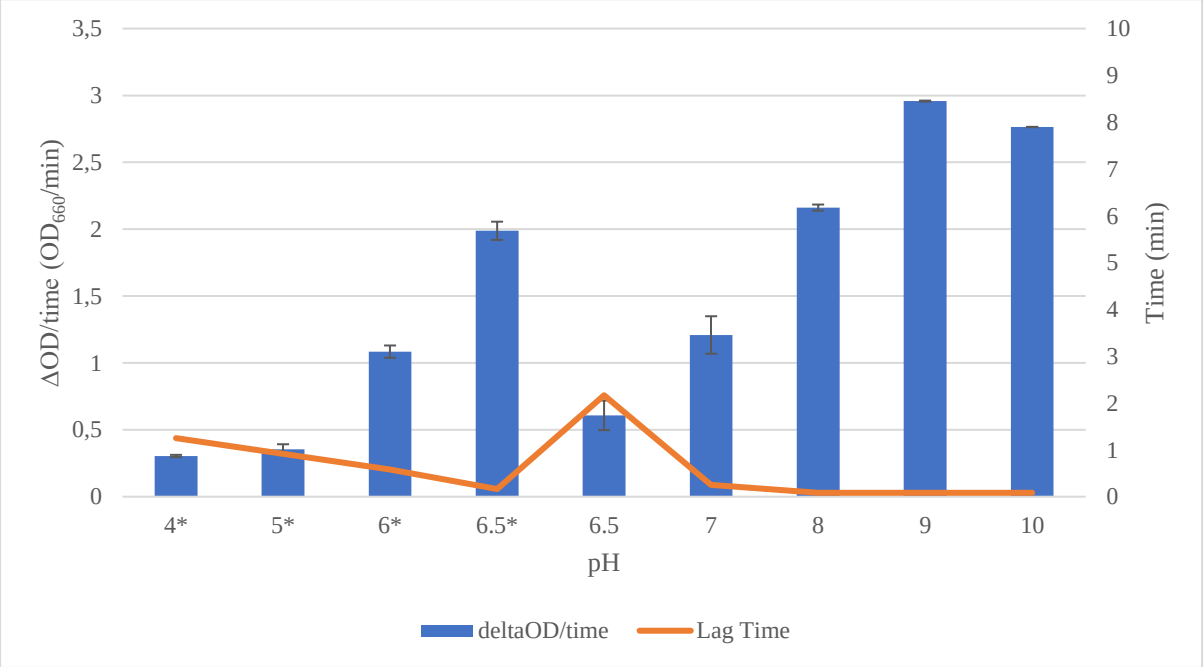


Figure 42: Effect of pH on lipoxygenase-2 activity

Differing amounts of purified enzyme was used to determine the optimal pH of the sodium phosphate buffer used in the methylene blue beaching assay to determine which pH allowed for optimal enzyme activity. Error bars show variation between technical replicates. * Indicates 1000 µg total protein was used per reaction. Otherwise, 100 µg was used

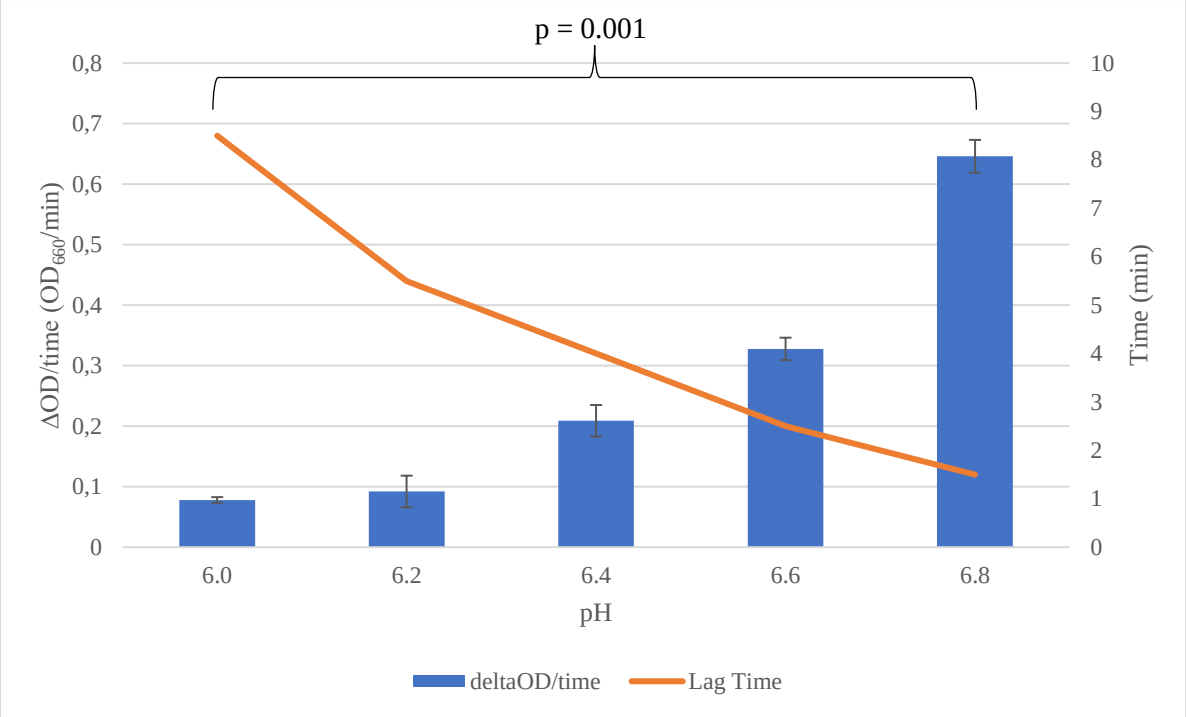


Figure 43: Effect of pH on lipoxygenase-2 activity

Purified enzyme was used to determine the optimal pH of the sodium phosphate buffer used in the methylene blue bleaching assay to determine which pH allowed for optimal enzyme activity. Error bars show variation between technical replicates. 100 µg total protein was used per reaction

3.5.2. Effect of temperature on lipoxygenase activity

Much like pH can affect the rate of a reaction so too can the temperature at which it is performed (Marangoni, 2003). Temperature, however, instead of solubilising the enzyme can confer more kinetic energy to the reaction which in turn brings the enzyme and substrate into contact more often which yields lower lag times and increased rates of reaction (Marangoni, 2003). However, similarly to extreme pH values affecting the conformational stability of the enzyme, causing it to stop working, so too will high temperatures eventually begin to denature the enzyme. It thus becomes prudent to determine the temperature range over which the LOX isoforms are able to continue reacting catalytically as many industrial processes take place at higher temperatures to reduce run-times (Marangoni, 2003).

The temperature of the reaction was maintained by use of a circulating water bath that pumped water around the cuvettes within the spectrophotometer. The buffer and reagents used in the reaction were also prewarmed or cooled to the temperature of the reaction, prior to being aliquoted into the cuvette, to ensure that the enzyme was exposed to the chosen temperature at all times. This guarantees that it only functioned at that temperature, once taken off the ice, which is necessary upon storage to prevent the sample from degrading (at room temperature).

The LOX-1 isoform had a wide range of temperatures at which it still functioned well, as shown in Figure 44. However, the more moderate temperatures of 20, 25 (the temperature indicated by Suda and co-workers (1995)) and 30℃ exhibited a slower bleaching of the methylene blue than the higher temperatures of 40 and 50℃. The highest activity was exhibited at 60 and 70℃. This phenomenon is most likely due to the increased kinetic energy within the sample which will cause the enzyme to interact with the substrate much faster once the enzyme has been introduced into the reaction mixture, compared to the situation at lower temperatures where the molecules in the mixture are less agitated and interact less often (Marangoni, 2003). The lowest temperature tested, i.e. 10℃, showed the slowest bleaching, most likely due to the same phenomenon. However, once the reaction was performed at 80℃, the initial rate of bleaching was akin to the 60 and 70℃ reactions, but soon thereafter stopped bleaching before all the methylene blue had been cleared from the cuvette. This may be due to the high temperature resulting in the inactivation of LOX-1 before it could finish producing sufficient hydroperoxides from the linoleic acid to complete bleaching all the methylene blue. According to the BRENDA database for EC 1.13.11.12: linoleate 13S-lipoxygenase (a.k.a LOX-1) there is no information for the *Glycine max* (soybean) temperature range (BRENDA Enzyme Database, 2022). However, the lowest and highest temperatures at which LOX-1 was seen to work, in multiple organisms, is 5℃ for *Malus domestica* and 75℃ for *Lasiodiplodia theobromae* (Schiller *et al.*, 2015; Patel *et al.*, 2015). This aligns with what was observed in this experimentation whereby the lowest rate of bleaching was observed at 10℃ and the activity began to drop above 70℃. The p-value (0.0003) calculated shows that there is a significant amount of activity increase from the prescribed 25℃ to 60℃ which confirms that the greater kinetic energy conveyed by higher temperatures can increase the rate of LOX-1 catalysis.

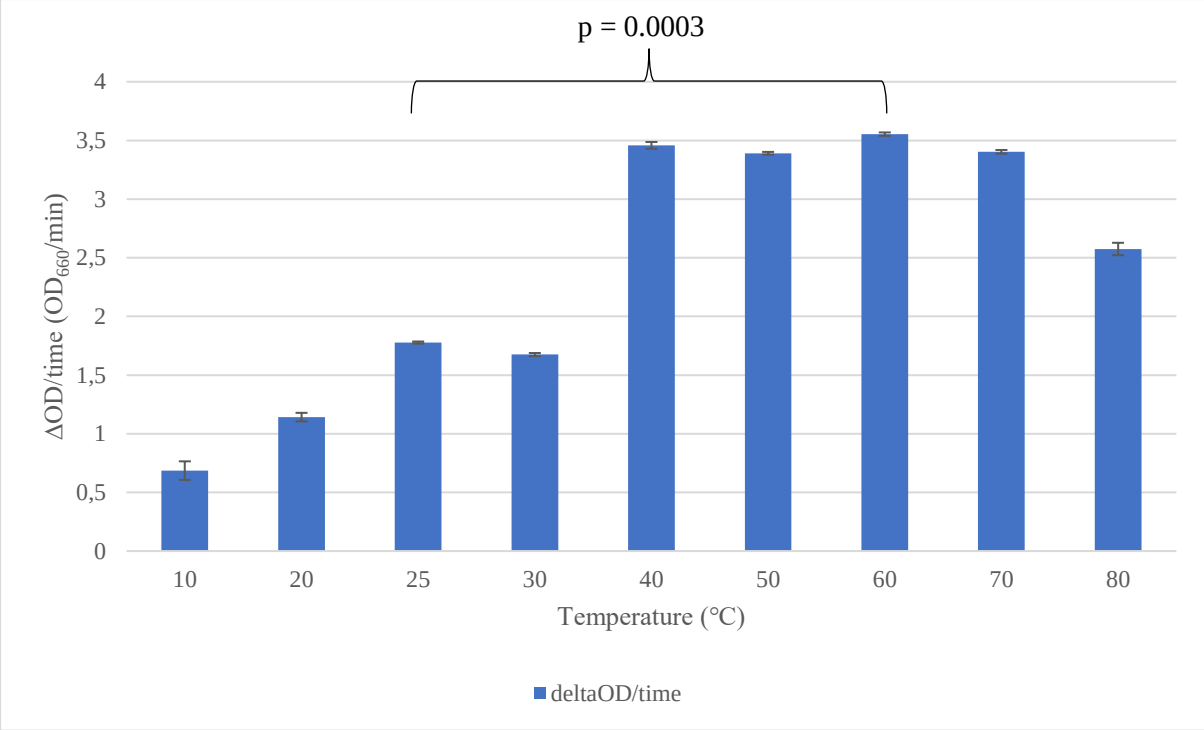


Figure 44: Effect of temperature on lipoxygenase-1 activity

Purified enzyme was used to determine the optimal temperature at which the methylene blue bleaching assay was performed to determine which temperature allowed for optimal enzyme activity. Error bars show variation between technical replicates.

The LOX-2 isoform showed a narrower range of temperatures at which it functioned than LOX-1. The highest activity was exhibited at 40℃, followed by 50℃. The moderate temperature range of 20, 25 and 30℃ lagged behind the two higher temperatures with the increase in temperature accounting for an increased rate of bleaching, which can also most likely be attributed to the increased kinetic energy in the reaction vessel. However, unlike LOX-1, LOX-2 began to lose activity at 60 and 70℃ already rather than at the highest temperature tested for LOX-1 (80 ℃). Similar to LOX-1, the lowest activity was observed in the 10℃ reaction, where the lack of kinetic energy provided by the external heat source resulted in slower bleaching. Unfortunately, LOX-2 has been studied to a lesser extent than LOX-1 and there is no separate page on the BRENDA database as the two isozymes and they have been combined under EC 1.13.11.12: linoleate 13S-lipoxygenase. Therefore, the temperature ranges shown are also not specifically for *Glycine max*. The range for *Olea europaea* is 15 – 55℃ (Padilla *et al.*, 2009), which is similar to the range seen for this LOX-2 whereby the activity increases from 10 until 40℃ and there is a sharp decrease after 50℃. The p-value (0.007)

calculated shows significant difference in the activity displayed by LOX-2 when left to react at 40°C than at the prescribed temperature of 25°C.

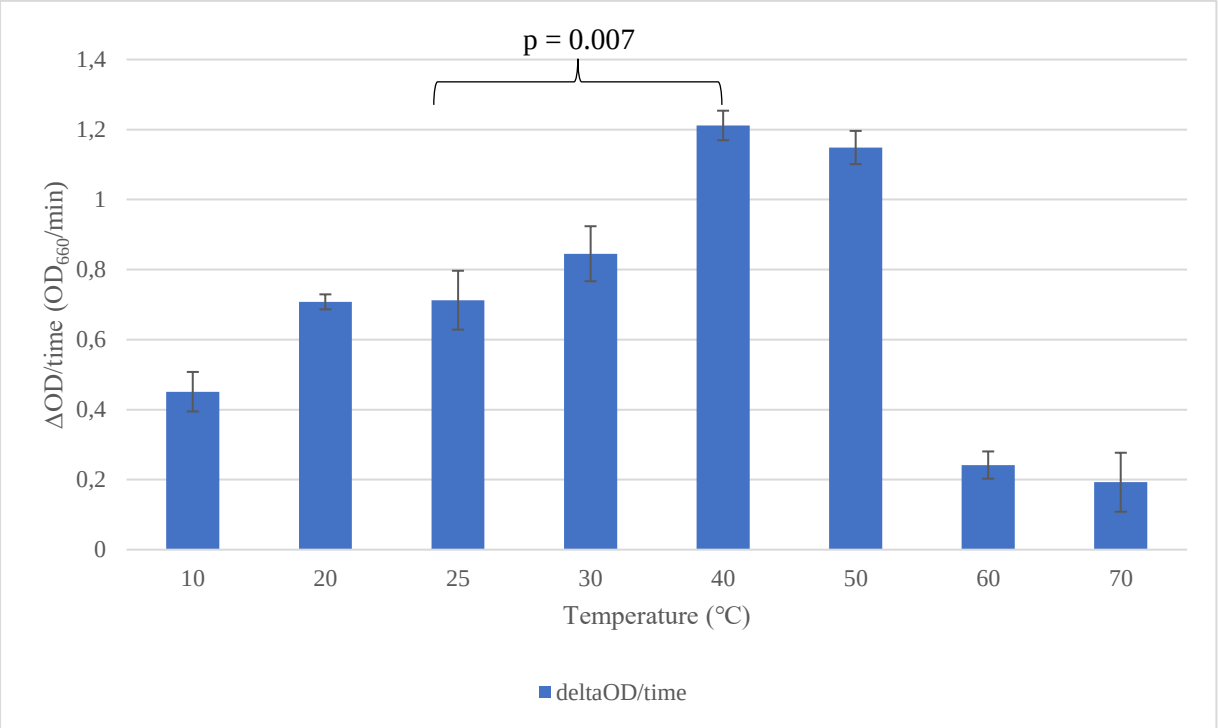


Figure 45: Effect of temperature on lipoxygenase-2 activity

Purified enzyme was used to determine the optimal temperature at which the methylene blue bleaching assay was performed to determine which temperature allowed for optimal enzyme activity. Error bars show variation between technical replicates.

3.5.3. pH of storage buffer

Enzymes are biologically engineered to work under the physiological conditions of the organism from which they come (EMBL, 2022). These are often quite mild in both temperature and pH. Thus, a longer period of exposure to more intense conditions could become de-naturing towards the enzyme and affect the catalytic capability thereof. To determine the long-term effects different storage conditions such as a change in pH will have on the enzyme is necessary as it will not only affect how the enzyme may be stored until use but also the pH under which reactions with long run-times may take place without degrading the enzyme. Enzyme activity was tested after only one hour to determine if there is imminent denaturation at any pH values and then again after 24 hours as many reactions used by APBio run over night and the effect of pH may become problematic if not controlled. Intermediate time-frames can be based on the 24-hour reaction.

After only one hour there is already variation in the activity of the LOX-1 enzyme in the buffers of different pH (Figure 46). It is unlikely that this change in activity could be due to the enzyme's buffer pH affecting the pH of the reaction itself as the amount of enzyme added is minimal in relation to the rest of the volume of reagents. To account for the diluted concentration of protein in the sample (which was initially a concentration of 6.96 mg/ml or 6.96 µg/µl) approximately 28.7 µl of the diluted sample was required to put 100 µg of protein into the cuvette. This was in a total volume of 1000 µl and thus not likely to change the pH of the entire reaction so drastically as to affect activity. Contrary to what may be expected, wherein a very high or low pH will have a denaturing effect on the enzymes, this data indicates that the enzyme bleached the methylene blue faster at the lower and higher ranges of the pH scale, rather than in the neutral range. The lowest rate of bleaching was observed from the samples incubated at pH 6 and pH 7. The slightly more acidic pH of 4 and 5 were the fastest to complete bleaching the methylene blue, followed by the more basic pH 10 followed by a lower rate at pH 11. Though pH 13 was the highest pH value tested it showed the shortest lag period before bleaching began despite having a slower rate of bleaching than many of the lower pH values. The acidic pH of 3 and 2, acted in a similar time frame as was observed for pH 12. And finally, only slightly faster than the pH 6 and pH 7 samples, were pH 9 and pH 8.

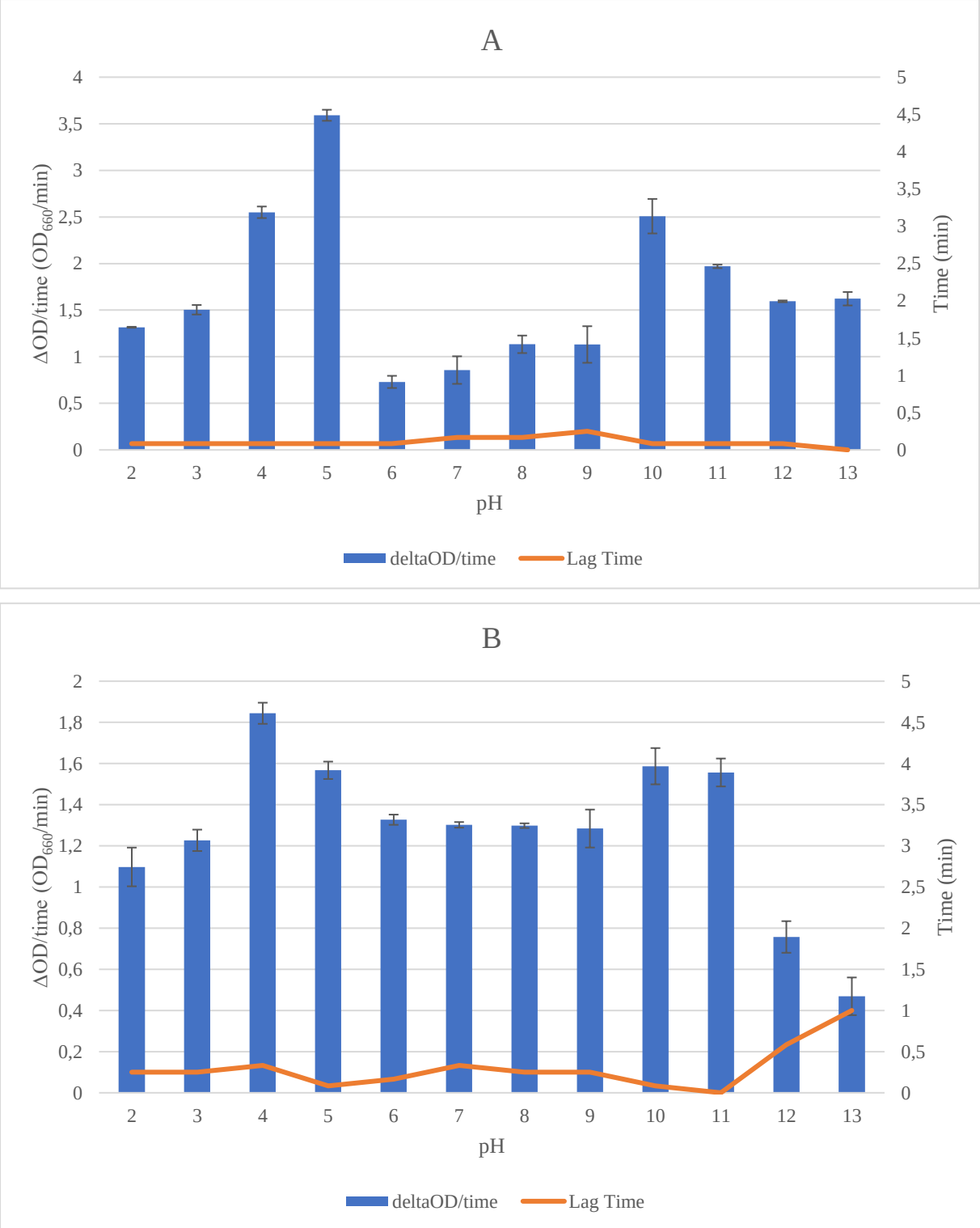


Figure 46: Effect of pH on storage of lipoxygense-1 activity after 1 or 24 hours

Purified enzyme was incubated for 1 or 24 hours with an equal volume of 125 mM boric acid, 10 mM sodium metabisulfite ($Na_2O_5S_2$) at a range of different pH values before being subjected to a methylene blue bleaching assay to determine the optimal storage conditions to maintain optimal enzyme activity. Error bars show variation between technical replicates. A: 1 hour of incubation; B: 24 hours of incubation

Perhaps the reason for these more acidic and more basic pH values allowing for higher activity of the LOX-1 enzyme may be due to their effect on the other proteins that were not cleared from the crude extract during the ammonium sulfate precipitation and DEAE column chromatography purification steps. Generally, many proteins are stable at more neutral pH values. By adding these more acidic or basic buffers to the enzyme samples, a permanently denaturing effect on the other interfering proteins in the sample could have occurred, which did not significantly affect the activity of the LOX-1 enzyme in an adverse way over the course of an hour (O'Brien *et al.*, 2012). Conversely, Baracat-Pereira and co-workers (2001) postulate that multiple forms of LOX within a sample could be responsible for these peaks of enzyme activity in the different ranges of pH values. Their investigation also showed a peak of LOX activity at pH 4.5 when their extract had been incubated for up to 7 days. This peak does not align with any of the well-known LOX isoforms however, and may require future investigation.

After 24 hours (Figure 46), the extremely basic pH values of 12 and 13 were by far the slowest to bleach all the methylene blue and had the greatest lag periods before bleaching began to proceed. This indicates that over a longer period of time, these high pH values will lower the activity of LOX-1. The pH 7 sample on the other hand, while still at the slower end of the tested samples, appears to have gained activity and bleached the methylene blue at a faster rate, as compared to the sample incubated for only one hour. The pH 6 sample reaction increased in speed somewhat compared to its one-hour counterpart. Once again, the basic pH values of 10 and 11 had very high activity and could be due to their inhibition of contaminating proteins from the crude extract. Both pH 4 and 5, the moderately acidic pH values, again showed the highest activity but pH 4 overtook pH 5 for the greatest activity. These pH values may have a stabilising effect on the LOX-1 enzyme, or another isoform (Baracat-Pereira *et al.*, 2001), while inhibiting the other proteins in the sample. In the middle of the bleaching times were the more neutral pHs of 6, 7, 8 and 9, all of which may not have had the same effect that their more acidic or basic counter parts had on the interfering proteins. Interestingly, pH 2 and 3 did not slow (and thus possibly denature) the LOX-1 enzyme the way the very basic pH 12 and 13 seemed to do, as pH 2 and 3 bleached the methylene blue similarly to that observed for the more neutral pH values. Therefore, it may be inferred that LOX-1 remains stable at lower pH values over an extended period of time more so than those that are basic which is contrary to that seen by Asbi and co-workers (1989) who saw an irreversible inactivation of LOX-1 preincubated below pH 3.

According to Asbi and co-workers (1989) the LOX-1 isoform was stable in their experimental procedure after 5 minutes incubation in buffers pH adjusted from 3.2 to 9.2. Below pH 3.2 their findings were that LOX-1 was irreversibly denatured which does not mimic what was seen in this study. Their findings may differ because the LOX source in that case was different (Sigma type V, purified by affinity chromatography) and likely contained a genetically different type of LOX-1 that is less stable at highly acidic pH values. However, as the LOX from the soybeans attained for this investigation (Seeds for Africa, South Africa) has not been studied in such detail it is not possible to say. This may be invested in more detail in the future. The samples stored for 24 hours at pH 2 and 3 still show fast bleaching which indicates that, unlike like the literature would suggest, these low pH values do not completely inhibit this particular LOX-1 isoform extracted from this cultivar of soybeans. Conversely, the actual pH of the buffer in which the enzymes were incubated may have erred slightly more towards the neutral range due to mixing of the enzyme in the elution buffer (pH 7.0) with the buffer altered to the test pH value. Future work may repeat these experiments under more strict conditions.

Unlike LOX-1 (Figure 46), LOX-2 (Figure 47) shows much slower bleaching activity after being left in very basic and very acidic buffers for an hour. The slowest bleaching was observed in the most acidic pH tested (pH 2) followed thereafter by pH 3 based on both rate and increased lag periods before bleaching began. The most basic pH values tested (pH 11, pH 12 and pH 13) did not affect the activity as intensely, but slowed the activity noticeably as compared to the more neutral pH samples, the lag period before bleaching began increased as the pH increased. However, pH 4 and 10 showed greater activity with minimal lag periods. This could suggest that, while LOX-2 is inhibited or denatured when stored at too low or high pH values, it is still active at these slightly more moderate pH values while the other inhibiting proteins are being hampered. The speed of the reaction thereafter remained similar from pH 4 to pH 9, with pH 7 exhibiting the slowest rate of bleaching. This again could be due to the effect that these less neutral pHs have on the other proteins which were carried through from the crude extract.

After 24 hours only the neutral pH values (8, 6, 9, 7) retained the majority of LOX-2 activity, albeit at a much slower rate when compared to one hour. The only other two samples with activity were pH 10 and pH 11, with much greater lag periods than exhibited for the samples tested after only one hour of incubation. All the other pH values showed almost no activity. This is in direct contrast with LOX-1 which retains more activity when stored at more acidic

pH values than those that are basic. This kind of information may help when trying to inhibit a specific isoform for any reason during industrial processing (Chedea et al., 2008).

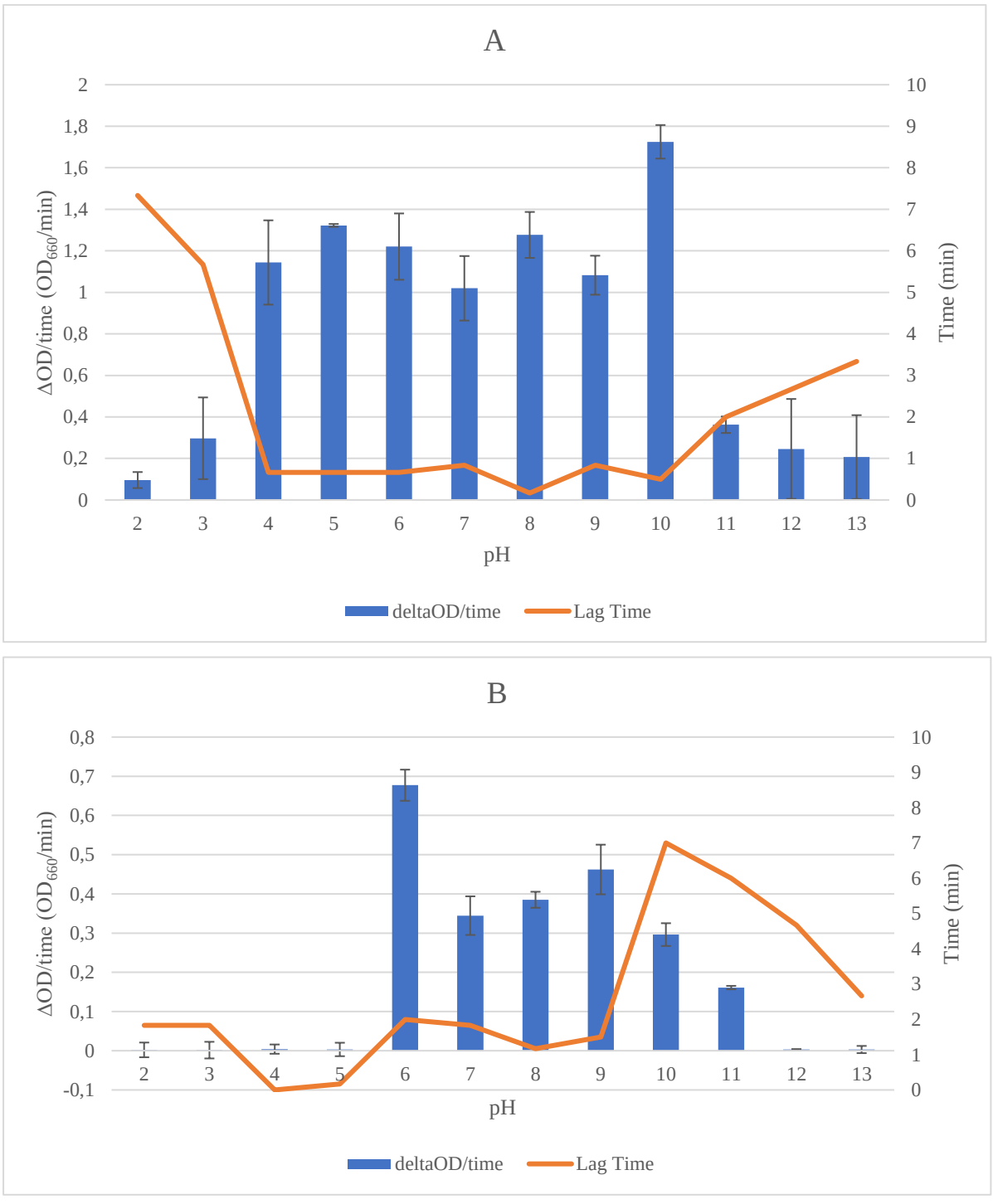


Figure 47: Effect of pH on storage of lipoxygense-2 activity after 1 or 24 hours

Purified enzyme was incubated for 1 or 24 hours with an equal volume of 125 mM boric acid, 10 mM sodium metabisulfite ($Na_2O_5S_2$) at a range of different pH values before being subjected to a methylene blue bleaching assay to determine the optimal storage conditions to maintain optimal enzyme activity. Error bars show variation between technical replicates. A: 1 hour of incubation; B: 24 hours of incubation

3.5.4. Temperature of enzyme storage

It is not often recommended that proteins, let alone enzymes, be stored above 4℃ once removed from their tissue of origin for any length of time if they are not actively part of a reaction (EMBL, 2022). There are proteins that are better stored at ambient temperatures, those known as 'cold-labile', however, it has not been noted that soybean LOX enzymes are affected by such a phenomenon (Simpson, 2010). It is not common to store enzymes at or above room temperature unless they have been lyophilised and rendered inert, however, many reactions in industry take place at higher temperatures as the kinetic interactions will be increased and thus many reactions will proceed to completion faster. This cuts down on time which in turn cuts down on production costs. It thus becomes prudent to determine the effect increasing periods of time at higher temperatures will have on the enzymes to be used in an industrial setting. Enzyme activity was tested after only one hour to determine if there is imminent denaturation at any specific temperature and then again after 24 hours as many reactions used by APBio run over night and the effect of high temperature may become problematic if not controlled. Intermediate time-frames can be based on the 24-hour reaction.

As can be seen in Figure 48, prolonged exposure to different temperatures influences the ability of LOX-1 to bleach methylene blue and therefore its ability to convert linoleic acid to hydroperoxides. After only one hour of incubation at 65℃ there is already a notable decrease in activity as can be seen by how much longer the lag period is for the enzyme to begin bleaching the methylene blue, as compared to the lower temperatures. However, the other slowest enzyme aliquot seen after one hour of incubation is that which was stored at 5℃. This, unlike the 65℃ aliquot, was unlikely to have lost activity due to denaturation but rather the lag time in methylene blue bleaching is likely to be due to the lowered kinetic energy of the enzymes, as they were initially cold before being added to the 25℃ reaction cuvette (Eisenthal & Danson, 2002). The intermediate temperatures showed similar rates of activity after one hour of activity.

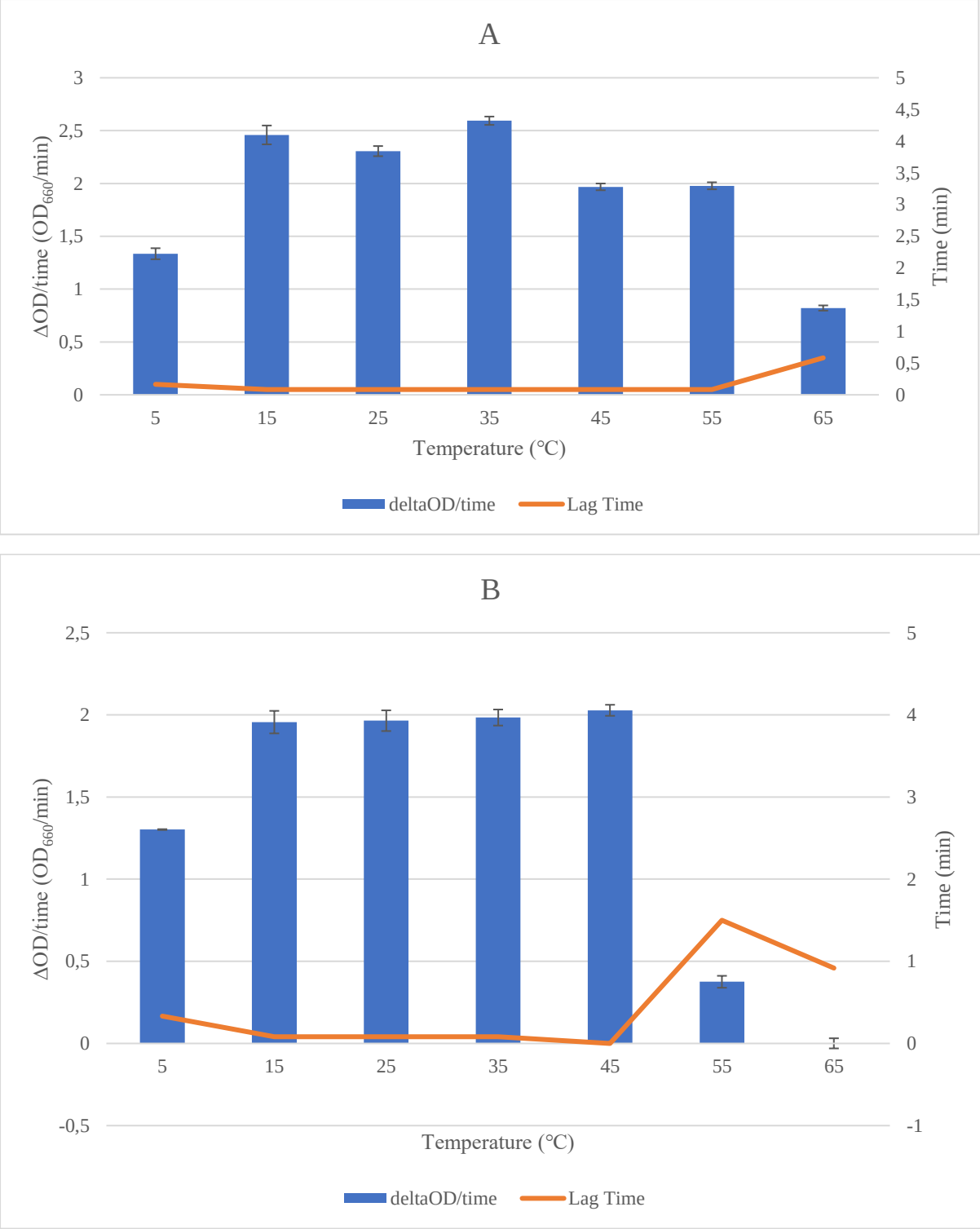


Figure 48: Effect of temperature on storage of lipoxygense-1 activity after 1 or 24 hours

Purified enzyme was incubated for 1 or 24 hours in a range of different temperatures in 125 mM boric acid, 10 mM sodium metabisulfite ($Na_2O_5S_2$) before being subjected to a methylene blue bleaching assay to determine the optimal storage conditions to maintain optimal enzyme activity. Error bars show variation between technical replicates. A: 1 hour of incubation; B: 24 hours of incubation

After 24 hours of storage at the different temperatures the activity in the 65℃ replicates had been lost entirely which indicates that the high temperature caused the inactivation of the enzymes in the solution. Other than the 55℃ samples, which began to lose some activity, all the other temperatures retained a similar amount of activity as they had after only one hour which is very useful information for industrial reactions. Many reactions will proceed faster at higher temperatures (as seen by the speed of the bleaching of the methylene blue in Figure 44 when the reaction was performed at higher temperatures) and thus for the large-scale production of many industrial products faster is most often better. If LOX-1 is stable over long periods of time at these temperatures it opens many avenues to produce products at higher temperatures than room temperature, without loss of activity.

The LOX-1 assay proceeds much faster than that for LOX-2, wherein there is often no lag period before bleaching begins to proceed. However, due to this phenomenon the kinetic energy conferred to the enzyme by the temperature in which it was before being added to the assay has a much greater effect than for LOX-2. When LOX-1 was stored at 5℃ it had a longer lag period than when stored at higher temperatures, due to a lack of kinetic energy in the enzyme aliquot. However, LOX-2 has a longer lag period under most circumstances and thus the sample stored at 5℃ had more time to warm to 25℃ and gain an equal amount of kinetic energy as the other enzyme aliquots. The sample stored at 5℃ seen in Figure 49 showed the highest amount of enzyme activity. The only aliquot that appeared to have lost all activity after one hour of incubation was the 65℃ sample. This lack of stability at higher temperatures falls in line with that described by Christopher and co-workers (1970) and Engeseth and co-workers (1987) whereby LOX-2 was discovered to be much less robust than LOX-1. The other LOX-2 samples progressed much more slowly than the 5℃ sample but still displayed activity, the 35℃ replicates showed faster bleaching than the samples that had been incubated at 15℃, 25℃ and 45℃ and 55℃, however these intermediate temperatures showed a much greater decline in activity than their LOX-1 counterparts. But as LOX-2 was not immediately denatured it indicates that the isozyme may be usable for industrial purposes, however certain additives may be required to protect the enzyme, such as ectoine and diglycerol phosphate (Tao *et al.*, 2011; Lentzen & Schwarz, 2006; Lamosa *et al.*, 2000).

After incubation for 24 hours (Figure 49) the aliquots stored at 5℃ had lost some activity as the speed of the reaction slowed somewhat when compared to those incubated for one hour. The rate of reaction for the samples incubated from 15 to 45℃ had also lost activity when compared to their counterparts incubated for one hour. The lag period before bleaching began

had also increased over the 23-hour period between readings. However, the most important factor is that the enzyme was not denatured and can retain activity at the higher temperatures for industrial use. The samples incubated at 55℃, however, lost all activity through heat inactivation.

There is a large disparity between the stability of LOX-1 and LOX-2 when it comes to the temperature at which they are stored (Christopher *et al.*, 1970). LOX-1 once again proved to be the more stable isoform but if both isoforms are found in the same solution, as is the case in this purification procedure, and both are to be stored for any reason in a liquid form, the best option would have to be 5℃, or more easily accessed 4℃. This is obviously only if a -20℃ or -80℃ freezer is unavailable (Jacquot *et al.*, 2008).

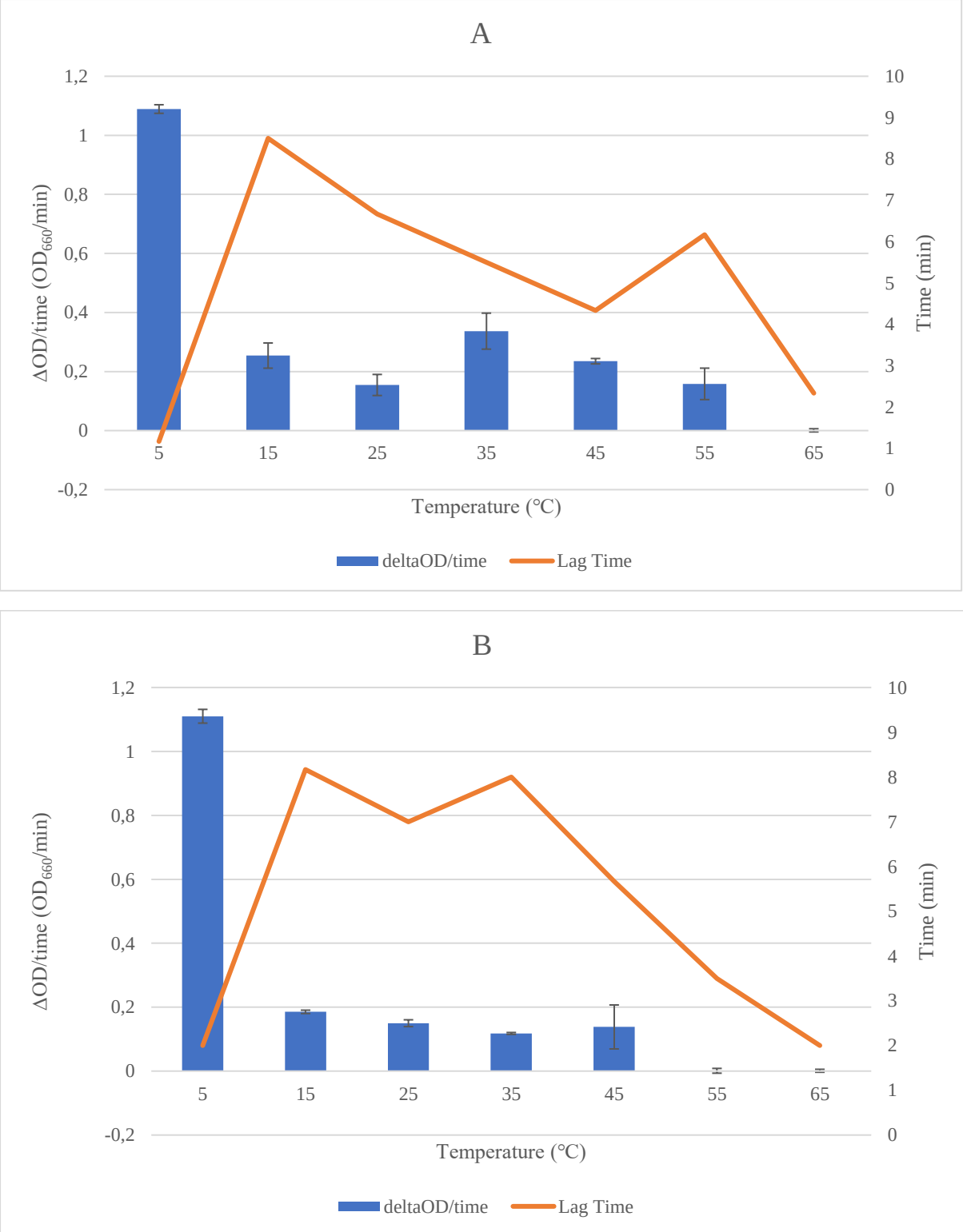


Figure 49: Effect of temperature on storage of lipoxygense-2 activity after 1 or 24 hours

Purified enzyme was incubated for 1 or 24 hours in a range of different temperatures in 125 mM boric acid, 10 mM sodium metabisulfite ($Na_2O_5S_2$) before being subjected to a methylene blue bleaching assay to determine the optimal storage conditions to maintain optimal enzyme activity. Error bars show variation between technical replicates. A: 1 hour of incubation; B: 24 hours of incubation

3.5.5. Long-term enzyme storage

An important factor in the manufacture and packaging of an enzyme for industrial use, or even resale, is the long-term storage thereof (Hartmann & Asch, 2019). If the enzyme does not need to be transported over long distances, it may be feasible to keep the enzyme cool until it is used – either frozen or in the fridge – however the cold-chain may be broken if any problems arise, and thus further storage options must be investigated. For the long-term storage of an enzyme in unpredictable storage conditions, lyophilisation is an alternative as it allows the enzymes to be stored ambiently for extended periods of time (Day & Stacey, 2007).

LOX-1 (Figure 50) shows a slow decrease in activity over the 75 days it was stored at 4°C. This is without the addition of co-solvents or metal ions, the only preservative added was the initial 10mM sodium metabisulfite ($Na_2S_2O_5$), which was part of the initial extraction buffer and the subsequent buffers in which the enzyme was suspended. Sodium metabisulfite is a preservative used in many food products to extend shelf-life. The substance contains sulfur which inhibits oxidation processes in any microorganisms and therefore halts their reproduction and protects the food product from degradation (Han, 2020). Thus, if the shelf-life of the enzyme in liquid form is to be increased further the addition of these types of protective agents can be investigated. There is a significant decrease in activity seen between day 0 and day 75 of storage at 4°C ($p = 0.007$) however activity is retained and the enzyme may continue to be used in reactions.

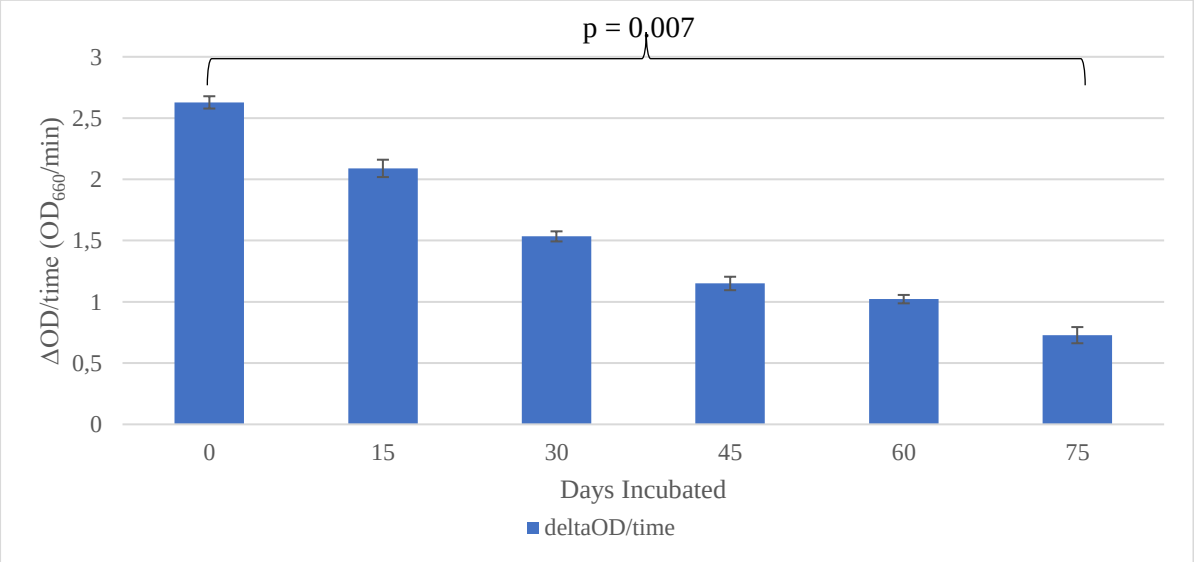


Figure 50: Effect of storage in aqueous form at 4°C on lipoxygenase-1 activity after 15-day increments

Purified enzyme containing 10 mM sodium metabisulfite ($Na_2O_5S_2$) was stored for 75 days. The enzyme was subjected to a methylene blue bleaching assay to determine the shelf life of the enzyme. Error bars show variation between technical replicates.

As with the pH and temperature stability tests, LOX-2 has shown itself to be less robust than LOX-1 and does not display as long a shelf-life at 4°C. While LOX-1 was still showing a fair amount of activity at 75 days (Figure 50), LOX-2 had lost all activity after 60 days (Figure 51). LOX-1 showed a gradual decrease in activity each time it was tested and could most likely be stored at 4°C for much longer than 75 days, however, future work can include such an investigation. The limited self-life of LOX-2 could possibly be delayed with the addition of the additives mentioned previously (Gray, 1988), but it would depend on the requirements for the enzyme as to whether this would be necessary, as up to 45 days the loss of activity was fairly consistent, the rate stayed the same up to 30 days with an increase in lag time before the bleaching began. After 45 days the lag time continued to increase, and rate began to slow until finally at 60 days there was minimal activity observed.

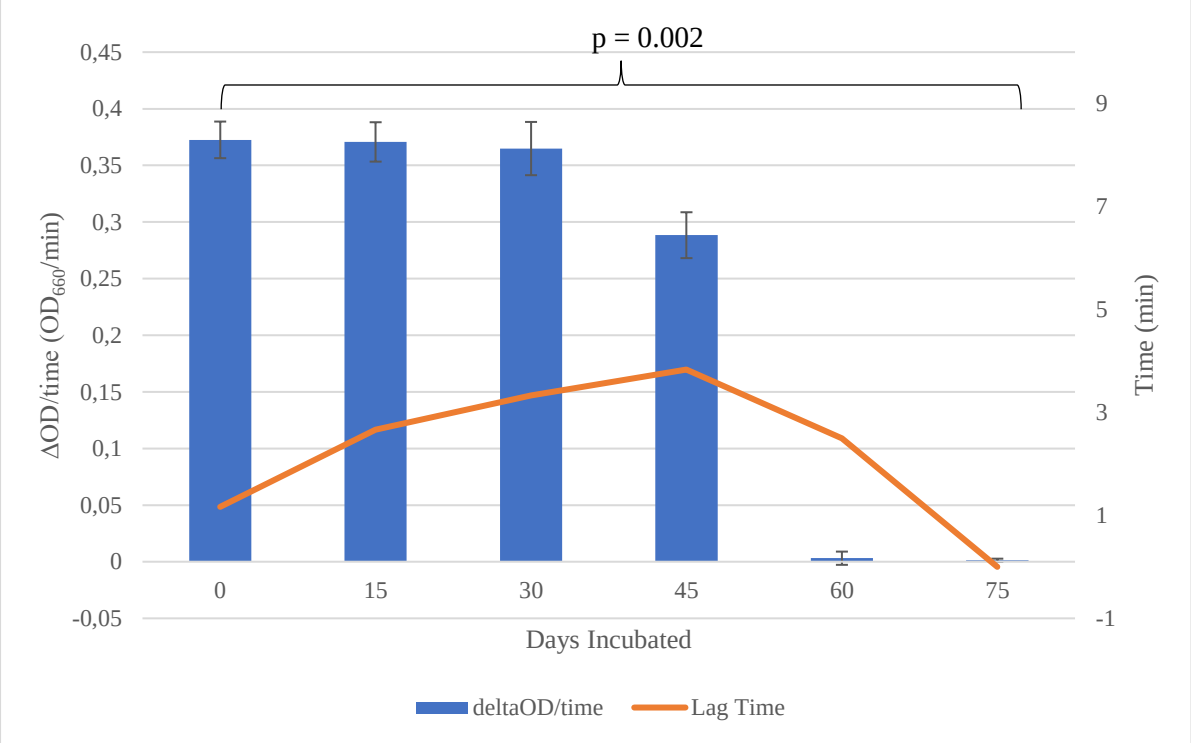


Figure 51: Effect of storage in aqueous form at 4°C on lipoxygenase-2 activity after 15-day increments

Purified enzyme containing 10 mM sodium metabisulfite ($Na_2O_5S_2$) was stored for 75 days. The enzyme was subjected to a methylene blue bleaching assay to determine the shelf life of the enzyme. Error bars show variation between technical replicates.

Another way that enzymes can be stored for long periods of time is through lyophilisation. LOX-1 (Figure 52) showed a small decrease in activity and increase in lag time from immediately after the sample had been freeze dried to after the 16 weeks of storage at room temperature in its lyophilised state. Not nearly as much activity was lost over the course of 112

days (16 weeks) at room temperature than over 75 days at 4℃ in a liquid state. Once again, this was without the addition of cryopreservatives which could further protect the enzyme during the lyophilisation process. However, if the enzyme is to be packaged and sold as is, with the minimal processing and purification described above, the lyophilisation step is a major boost in both shelf-life and transportability. If the enzyme does not need to be stored in a fridge or freezer, the ability to get the enzyme to the consumer becomes much less complicated and ensures the enzyme maintains its activity through the process giving the customer a longer window of opportunity in which to use the enzyme. There is not a significant loss in LOX-1 activity over the 16-week storage period as evidenced by the p value between the test being greater than 0.05 (p = 0.3).

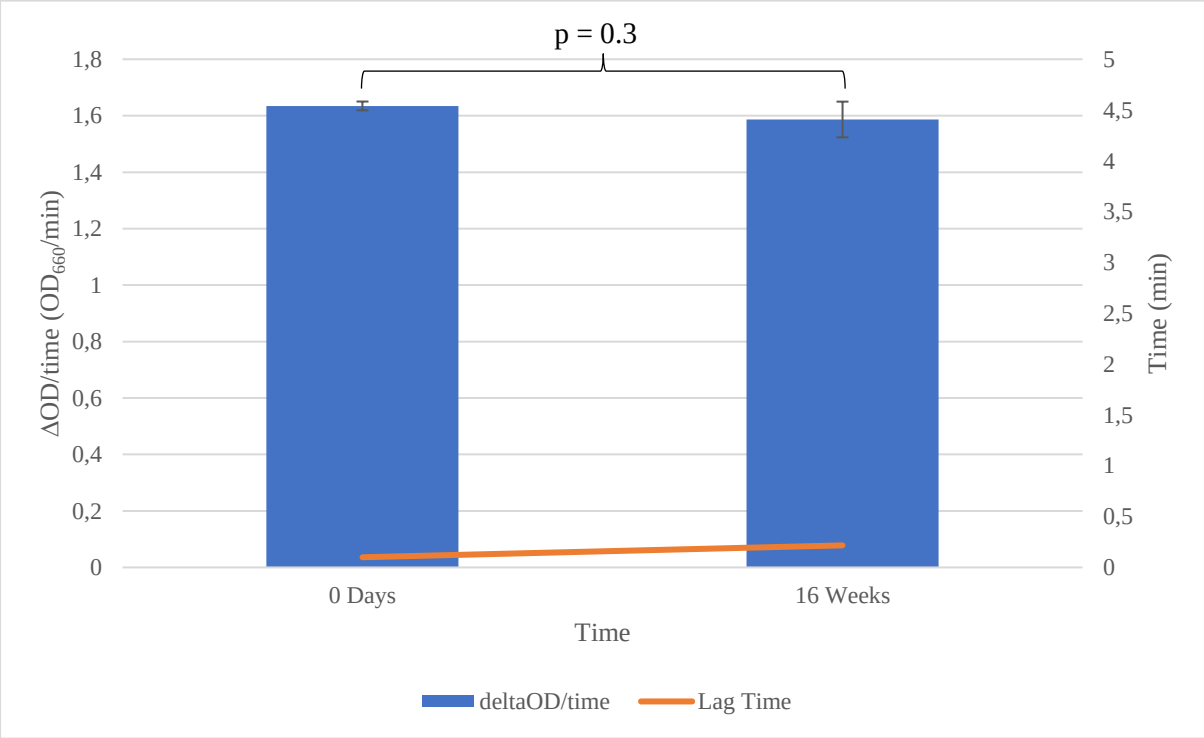


Figure 52: Effect of lyophilisation on lipoxygenase-1 activity after 0 days to 16 weeks of storage at room temperature

Purified enzyme containing 10 mM sodium metabisulfite ($Na_2O_5S_2$) was stored for 16 weeks after lyophilisation. The enzyme was subjected to a methylene blue bleaching assay to determine the shelf life of the enzyme. Error bars show variation between technical replicates.

While the freeze drying itself had little effect on the activity of LOX-2 (Figure 53), after 16 weeks at room temperature the activity had been lost almost entirely. As can be seen in Figure 53 there is a very small decrease in absorbance over the 10 minutes for which the assay was monitored in the 16-week old sample but the enzyme did not retain its activity as well as LOX-1 (Figure 52) as shown by the significant loss in activity (p = 0.001). The LOX-2 isoform would

therefore benefit more from protective additives than the LOX-1 isoform (Gray, 1988). Future investigations can determine the factors that affect the long-term storage of LOX-2 along with more periodic testing of lyophilised samples to determine an accurate shelf-life. If the isozyme were separated from LOX-1 these effects may be more easily elucidated.

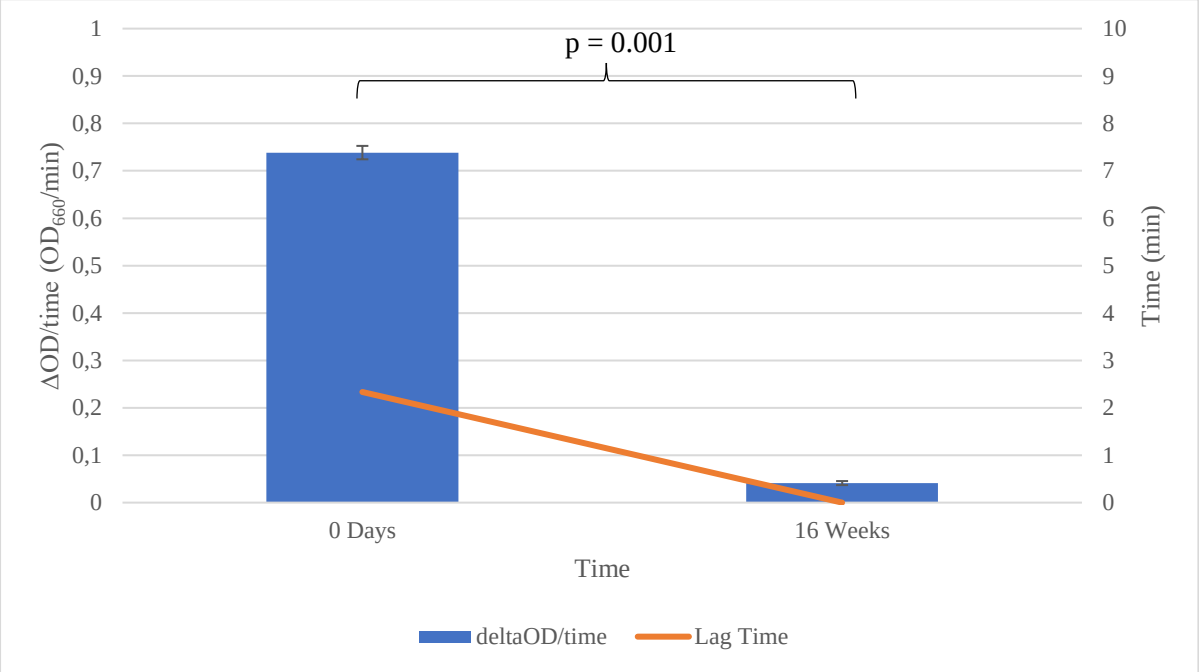


Figure 53: Effect of lyophilisation on lipoxygenase-2 activity after 0 days to 16 weeks of storage at room temperature

Purified enzyme containing 10 mM sodium metabisulfite ($Na_2O_5S_2$) was stored for 16 weeks after lyophilisation. The enzyme was subjected to a methylene blue bleaching assay to determine the shelf life of the enzyme. Error bars show variation between technical replicates.

3.6. Enzyme kinetics

All enzymes exhibit activity under the correct conditions and in the presence of the correct substrate (Marangoni, 2003). The greatest amount of activity an enzyme can exhibit will depend on the type of substrate and amount present in the reaction vessel. The velocity of a reaction will be affected by numerous factors such as the temperature of the reaction, the pH at which the reaction takes place, as well as the substrate and in what form it is added (Marangoni, 2003). It is important therefore, to ensure that when determining the kinetic values associated with an enzyme that these factors are closely monitored and kept consistent to ensure that the rate of the reaction is affected purely on the basis of substrate concentration (Robinson, 2015).

As can be seen in Figure 56 (Appendix 6), at too low a substrate concentration (0.2 µmol) there is no bleaching of the methylene blue. It is most likely that LOX-1 is unable to encounter

enough linoleic acid (in sodium linoleate form) to produce sufficient hydroperoxides and peroxyl radicals to bleach the methylene blue. However, from 0.4 µmol onwards the bleaching reaction increases in rate until approximately 0.8 µmol where the bleaching peaks and is completed in 14 seconds. At higher concentrations of sodium linoleate, the rate of bleaching slows and by 2 µmol of sodium linoleate the reaction is the slowest which indicates that too high a concentration of substrate may actually be inhibitory. This phenomenon was also observed by Egmond and co-workers (1976) whereby they determined that linoleic acid acts as a hyperbolic competitive inhibitor on the LOX-1 enzyme. Smith & Lands (1972) also noted that when the concentrations of linoleic acid and other fatty acid substrates of lipoxygenase were increased, the lag period increased prior to the activity being spectrophotometrically notable, and the total activity itself was reduced. They speculated that when present in too high a concentration the substrate binds to the product site inhibiting the formation of further product which could account for what was observed in this investigation.

Table 4: Kinetic values for soybean lipoxygenase-1 and lipoxygenase-2

Isozyme	**Calculated Vmax (µmol/sec/mg protein)**	**Literature Vmax (µmol/sec/mg protein)**	**Calculated Km (µmol)**	**Literature Km (µmol)**
LOX-1	0.1137 linoleic acid in 0.2 M borate (pH 10.0)	0.1719 linoleic acid in 0.1 M borate (pH 9.0) (Keereetaweep *et al.*, 2010)	1.313 linoleic acid in 0.2 M borate (pH 10.0)	0.021 linoleic acid in 0.1 M borate (pH 9.0) (Keereetaweep *et al.*, 2010)
LOX-2	0.0183 linoleic acid in 0.2 M sodium phosphate (pH 6.8)	0.0433 linoleic acid in 0.2 M sodium phosphate (pH 6.8) (Mellor *et al.*, 2010)	2.335 linoleic acid in 0.2 M sodium phosphate (pH 6.8)	0.019 linoleic acid in 0.2 M sodium phosphate (pH 6.8) (Mandal *et al.*, 2013)

Kinetic values Vmax (maximum rate of reaction) and Km (enzyme affinity for substrate) were calculated for both LOX-1 and LOX-2 using differing substrate concentrations of sodium linoleate and compared to data obtained from the literature

Only the exponential phase of each of the different tests conducted to construct Figure 56 were used to create straight line equations in Microsoft Excel (Microsoft Corporation, Redmond, Washington, United States). The equations followed the y = mx + c format wherein the m value refers to the gradient of the line and could thus be used as the rate of the reaction. These values were plotted on the graph seen in Figure 57 to determine the Vmax (the maximum rate of the reaction) and Km (enzyme affinity for the substrate) values of LOX-1 in the reaction (Cornish-

Bowden, 1979). The Vmax value was clearly seen to be when 0.8 µmol sodium linoleate was used in the reaction. The Michaelis-Menten plot however, is not the most accurate plot for the determination of kinetic values, instead the double-reciprocal plot, or Lineweaver-Burk plot (Figure 58), is a more accurate determinant of activity. However, as previously discussed, LOX-1 was not fully purified from WS, and enzyme kinetics studies are best performed on pure enzymes. Furthermore, LOX-1 becomes inhibited at higher concentrations of substrate (Egmond *et al.*, 1976). Thus, when the rate of activity begins to dip with higher concentrations of substrate the Lineweaver-Burk plot does not show great correlation for increasing concentrations of substrate. Thus, to achieve an R^2 value of 0.81, concentrations above 1.2 µmol were excluded in the calculation of Vmax and Km kinetic values. However, the values calculated are a useful starting point and in future, once the enzymes have been fully purified these tests may be performed again. The Vmax value was determined from the linear equation fit to the data points as 0.1137 µmol/s/mg protein in 0.2 M boric acid (pH 10.0) at 25℃. Thereafter the Km value was determined to be 1.313 µmol sodium linoleate.

According the BRENDA comprehensive enzyme information system (BRENDA Enzyme Database, 2022) for the linoleate 13S-lipoxygenase (EC 1.13.11.12), the Km value for LOX-1 from *Glycine max* (soybean) is 0.021 µmol at 0.1 M boric acid (pH 9.0) in the investigation by Keereetaweep and co-workers (2010). This is a much lower concentration of substrate than discovered in this investigation (1.313 µmol) but the value was calculated from an assay similar to that described by Axelrod and co-workers (1981) wherein the enzyme's activity is directly monitored by the production of conjugated double bonds at 234 nm. The methylene blue assay described by Suda and co-workers (1995) is an indirect method of measuring hydroperoxide formation by monitoring the bleaching of the blue colour from the reaction. For this reason, the concentration of linoleic acid required to bleach the methylene blue may be higher than that which is required for conjugated double bonds to be formed and monitored spectrophotometrically.

Research by Keereetaweep and co-workers (2010) went on to give a Vmax value of 0.1719 µmol/s/mg protein which is approximately three times higher than the maximum rate of this reaction which was 0.1137 µmol/s/mg protein. The methylene blue assay was bleached to completion in 14 seconds as opposed to being monitored for hours as is indicated by the units in which the Vmax value was given by Keereetaweep and co-workers (2010). The greater amount of substrate required for the methylene blue reaction to proceed therefore may account for this decrease in reaction velocity as well as the purer source of LOX-1 used by

Keereetaweep and co-workers (2010) which was purchased from Cayman Chemicals (Michigan).

Unlike the LOX-1 substrate gradient (Figure 56), which very clearly increased in rate with a similar amount of lag time, the LOX-2 gradient (Figure 59) did not begin bleaching the methylene blue within a similar time frame. The rate of bleaching after the lag period had ended was not proportional to how long it took for the bleaching to begin. As can be seen in Figure 60 the rate of bleaching increases from 0.2 µmol up until it plateaus at 1.0 µmol. The reaction that was performed with 1.2 µmol and 1.4 µmol of sodium linoleate had a much shorter lag period than the 1.0 µmol sample but similar rates of reaction whereby the 1.0 µmol and 1.2 µmol reactions bleached the methylene blue at a velocity of 0.0061 µmol/sec/mg protein whereas the 1.4 µmol reaction was bleached at 0.006 µmol/sec/mg protein (Figure 60). The higher substrate concentrations in these reactions must have lowered the amount of time required to produce sufficient hydroperoxides to bleach the methylene blue but was overcome when the substrate concentration began to inhibit LOX-2. Therefore, while LOX-2 does not react exactly as LOX-1 does, it can be seen to be inhibited by higher concentrations of substrate which is in line with the LOX-1 isoform.

As was done for LOX-1, only the exponential phase of each of the different assays in Figure 59 showing LOX-2 activity was used to create straight line equations in Microsoft Excel (Microsoft Corporation, Redmond, Washington, United States). These equations followed the $y = mx + c$ wherein the m value refers to the gradient of the line and could thus be used as the rate of the reaction. These values were plotted on the graph seen in Figure 60 to show the Michaelis Menten plot for LOX-2, once again the greater concentrations (>1.4 µmol) of substrate were excluded to ensure that the correlation was more significant ($R^2 = 0.85$). The Vmax value was theoretically seen when the substrate concentration reached 1 µmol in the reaction and was maintained at 1.2 µmol but had a much shorter lag time. The rate of reaction began to decrease when 1.4 µmol was added to the reaction. The Vmax value was thereafter determined using the Lineweaver-Burk plot (Figure 61) to be 0.0183 µmol/s/mg protein which is much lower than that for LOX-1 due to the 420 seconds required for the methylene blue to be completely bleached rather than the 14 seconds in which LOX-1 was able to do so.

The conditions of the assay were obviously different as the LOX-2 reaction takes place in 0.2 M sodium phosphate buffer (pH 6.8) at 25°C with the addition of acetone and dithiothreitol to inhibit the activity of LOX-1 while maintaining that of LOX-2. Very little research has been

done upon soybean LOX-2 and thus there is no reference information for the *Glycine max* LOX-2 isoform on the BRENDA database (BRENDA Enzyme Database, 2022) to which to compare the kinetic values. However, Bild and co-workers (1977) reported the Vmax value for LOX-2 to be 0.0433 µmol/s/mg protein in sodium phosphate buffer (pH 6.8) at 15℃ (Bild *et al.*, 1977). The Vmax value (0.0183 µmol/s/mg) in this investigation is approximately 2.3 times lower than that reported in literature. This can most likely be attributed to the difference in the assay used to acquire the kinetic parameter. Much more protein was required in this investigation, due to minimal purification, and this could also account for the lower relative activity.

Using the linear equation derived from the Lineweaver-Burk plot (Figure 61) it was possible to determine the Km value for LOX-2. The Km value was determined to be 2.335 µmol sodium linoleate in 0.2 M sodium phosphate (pH 6.8) at 25℃ which is higher than the Km value for LOX-1 which once again falls in line with what the data has shown for the two isoforms throughout this investigation. An investigation by Mandal and co-workers (2013) determined the Km value for LOX-2 from Indian soybean (*Glycine max* (L.) Merrill) was 0.019 µmol linoleic acid in an assay that measured the increase of absorbance at 234 nm at 25°C in a buffer of 0.2 M sodium phosphate buffer (pH 6.8). Similar to the issue seen for LOX-1 where the Km value was much higher in the literature this value may have been skewed by the use of the methylene blue assay requiring more substrate to bleach the methylene blue. Furthermore, Mandal and co-workers (2013) were using a recombinant, overexpressed enzyme and thus may have used a much purer form of LOX-2 which would have allowed less substrate to be used per mg of total protein in the reaction.

The LOX-1 and LOX-2 enzymes used in this investigation both show kinetic values (Table 4) that are different from that shown in the literature. Both the Km values (affinity for the substrate) for the two isoforms are higher than that observed in other experiments while the Vmax values (maximum rate of reaction) are lower, which could be attributed to the use of different assay procedures as well as the amount and source of lipoxygenase enzymes. The enzymes used in this investigation were not purified to completion but were rather in a protein mixture. For this reason, a higher concentration of total protein had to be added to the reaction system to see the bleaching of the methylene blue at a reasonable rate. Furthermore, greater concentrations of substrate (the Km values reflect this, more substrate available would result in a higher-seeming affinity for the enzyme) were required in the indirect assay to detect lipoxygenase activity through the bleaching of methylene blue rather than directly by the

production of conjugated double bonds. The lower Vmax values are most likely due to these higher enzyme and substrate concentrations resulting in comparatively lower rates of reaction than those reported elsewhere. Thus, if the assays described by Suda and co-workers (1995) are to be used for further kinetic testing of the LOX enzymes, there is further fine tuning that may be needed to bring these values into the range of other reported kinetic values. This could be achieved by a greater level of purification of the two isozymes, from the initial crude extract and from one another, than was attempted during the course of this investigation. Furthermore, once purified further the LOX-1 and LOX-2 isoforms could be used to calculate kinetic values such as Kcat (turnover rate) and Kcat/Km (catalytic efficiency).

4. Conclusion

Two assays described by Suda and co-workers (1995) were chosen to quantify the activity of lipoxygenase as one assay showed the activity of LOX-1 and the other LOX-2, whereas many other assays described in the literature showed only the activity for LOX-1 or a combined activity of all the soybean lipoxygenase isoforms. The assays showed marked differences between the LOX-1 and LOX-2 isoforms extracted from de-fatted and whole soybeans. Due to the much lower activity observed in the de-fatted soybean during the initial assays, it was determined that they were not worth continued investigation. Investigation determined that milled whole soybeans subjected to a single overnight soak in 125 mM boric acid, 10 mM sodium metabisulfite yielded the greatest LOX-1 and LOX-2 activity. It was further determined that pH 7 best extracted the two isoforms in conjunction.

The crude protein extracts were subjected to an ammonium sulfate gradient precipitation to remove the lower end of proteins that precipitate out of solution at 40% saturation, and then brought to 60% saturation to precipitate out the majority of the LOX enzymes. There was no activity observed in the 80% saturation fraction indicating that all lipoxygenase enzymes were removed from the soybean extract. Future work may include an investigation into the specific range of ammonium sulfate concentrations at which all the LOX-1 and LOX-2 isozymes can be completely precipitated out of the crude enzyme extract. To remove the salt the protein pellet was subjected to dialysis which did not appear to lower the activity of the LOX isoforms. Ion-exchange chromatography further purified the LOX-1 and LOX-2 isoforms which yielded an increase in activity as compared to the crude enzyme extract. Storage at -80°C, after flash-freezing with liquid nitrogen, showed minimal loss in activity. Further purification using

additional chromatographic processes may be investigated to purify the isozymes from the crude extract more thoroughly as well as from one another.

The crude enzyme extracts, as well as the ion-exchange chromatography purified enzymes, were cross-linked using 25% (v/v) glutaraldehyde (0.0125% total enzyme volume) to create cross-linked enzyme aggregates (CLEAs). Both LOX-1 and LOX-2 activity was observed through the assays, as well as a preliminary indication that enzyme recyclability was feasible through a conversion of valencene to nootkatone reaction. Further investigations into the improved stability that can be attained by producing cross-linked enzyme aggregates of LOX enzymes will need to take place to determine whether the production of these CLEAs is economically viable.

The assays used to detect LOX-1 and LOX-2 activity were then altered to show maximum activity using 100 µg of total protein. It was determined that despite the indication in the literature that LOX-1 works most optimally in 0.2 M borate buffer altered to pH 9, it was instead pH 10 that gave the highest activity. Furthermore, it was observed that at pH 6.8 (0.2 M sodium phosphate buffer), LOX-2 showed a much higher level of activity over the pH 6 buffer which had been described by the experimenters who had designed the assay. The range of temperatures at which the enzymes retained activity were tested by altering the temperature at which the assays were performed. LOX-1 showed increasing activity with increasing temperature up to 70℃ but lost activity while working at 80℃. LOX-2 on the other hand only increased in activity up to 50℃ and lost activity from 60℃ upwards.

The stability of the enzymes under different storage conditions was investigated and it was found that LOX-1 was stable over a greater range of pH values than LOX-2 over a 24-hour period. However, both isoforms retained activity between pH 6 to 9. LOX-1 showed itself to be more robust than LOX-2 when incubated at higher temperatures for 24 hours but both retained some activity at 45℃. The stability of the LOX isoforms at these higher temperatures is useful for industrial processing as many reactions will proceed faster when the temperature is higher and thus will reduce production time and costs.

LOX-1 showed a greater shelf-life in aqueous form at 4 ℃ than LOX-2 whereby there was a gradual loss of activity over the 75 days of storage. LOX-2 only retained activity up to 45 days of storage with gradual loss of activity up to this point. These timeframes may be increased with the addition of certain additives which should be investigated in future if the LOX enzymes are to be stored under such conditions for extended periods of time. The LOX-1

isoform was seen to retain almost all activity after 16 weeks of storage at room temperature in lyophilised form. This could be a more viable option for long-term enzyme storage and will ensure that the enzyme may be transported without loss of activity. LOX-2 unfortunately, had lost all activity after this time even though the lyophilisation process itself had not affected the enzyme's activity adversely. Thus, LOX-2 may benefit more from cryoprotective substances if it is to be preserved by freeze-drying. Further investigation will be required to determine the effect such substances will have on the activity of the enzyme as well as its long-term stability under such circumstances. The lyophilisation process may require repetition to determine the exact timeframe in which LOX-1 and LOX-2 are still active.

Finally, the enzyme kinetics of the two LOX isoforms was investigated. LOX-1 showed peak activity when 0.8 µmol sodium linoleate was available for catalysing the reaction but at higher concentrations the speed of the reaction began to slow. This is due to linoleic acid, the preferred substrate, acting as a hyperbolic inhibitor of LOX-1. The Km value was calculated to be 1.313 µmol sodium linoleate in 0.2 M boric acid (pH 10.0) at 25℃ while the Vmax value was 0.1137 µmol/s/mg protein. The Km value was higher than indicated in the literature while the Vmax value was lower, both of which could be attributed to the different assay used along with use of an impure source of enzyme. The methylene blue may require more substrate and enzyme to become fully bleached and measured than in the other assays which measure conjugated double bond formation.

LOX-2 required more sodium linoleate than LOX-1 to reach maximum velocity (1.0 µmol) but only began to be inhibited at higher concentrations of substrate (1.4 µmol). The velocity of the LOX-2 reactions was much lower than for LOX-1 as it took much longer for bleaching to begin and the rate of bleaching was slower (0.0511 µmol/sec/mg protein for LOX-1 and 0.0061 µmol/sec/mg protein for LOX-2 was seen in the Michaelis-Menten plots). The Vmax value was calculated to be 0.0183 µmol/s/mg protein in 0.2 M sodium phosphate buffer (pH 6.8) at 25 ℃ with the addition of acetone and dithiothreitol to inhibit the activity of LOX-1 while maintaining that of LOX-2. The Km value was seen to be 2.335 µmol sodium linoleate. Once again, the Km value was higher, and the Vmax value was lower than that in the literature which could again be attributed to the greater amount of substrate and enzyme required to bleach methylene blue than required for the more direct spectrophotometric assays. Future work may elucidate a more thorough procedure for enzyme purification that could lower the amount of enzyme required for the reaction to take place by removing further contaminants in the enzyme sample which will help determine more reliable kinetic parameters and calculate both catalytic

efficiency and turnover rates for the two LOX isozymes. Another more direct assay may also show results similar to the literature and alternative assays that measure the activity of the separate isoforms should be investigated.

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6. Appendices

6.1. Appendix 1

Table 5: Protein concentrations of crude enzyme extractions from de-fatted (DS) and whole soybeans (WS) based on boric acid concentration

Sample and Buffer	**Protein Concentration (mg/ml)**				
	Replicate 1	Replicate 2	Replicate 3	Average	Standard Deviation
DS HS	4.92	2.23	2.05	3.07	1.6076
DS OSB 50 mM Boric Acid	10.4	13.6	14.1	12.7	2.0075
DS OSB 100 mM Boric Acid	11.0	13.6	20.7	15.1	5.0210
DS OSB 150 mM Boric Acid	10.6	10.6	16	12.4	3.1177
DS OSB 200 mM Boric Acid	16.9	8.28	12.6	12.6	4.3100
DS 50 mM Boric Acid	7.05	7.98	12.8	9.28	3.0865
DS 100 mM Boric Acid	6.40	10	9.75	8.72	2.0102
DS 150 mM Boric Acid	8.06	11	10	9.69	1.4948
DS 200 mM Boric Acid	7.23	7.76	6.13	7.04	0.8314
WS HS	11.8	5.17	4.89	7.29	3.9112
WS OSB 50 mM Boric Acid	10.3	16.9	12.6	13.27	3.3501
WS OSB 100 mM Boric Acid	11.67	14.7	12.0	12.79	1.6623
WS OSB 150 mM Boric Acid	15.7	13.2	13.6	14.17	1.3429
WS OSB 200 mM Boric Acid	10.3	12.3	13.3	11.97	1.5275

WS 50 mM Boric Acid	11.9	12.3	13	12.4	0.5568
WS 100 mM Boric Acid	12.2	11.8	13.4	12.47	0.8327
WS 150 mM Boric Acid	8.23	8.63	11.7	9.52	1.8985
WS 200 mM Boric Acid	11.9	9.11	11.9	10.97	1.6108

Proteins were extracted from DS and WS using either: the osmotic shock buffer *(OSB)* method, an initial overnight soak with 5 mM sodium metabisulfite (Na2S2O5) – hypotonic soak (HS) followed by a further overnight soak in the presence of 0.5 M sucrose, 10 mM Na2S2O5 and varying concentrations of boric; or a single over overnight soak of with varying concentrations of boric acid. Protein quantifications were made using the Qubit® 2.0 Fluorometer (485/590 nm; Invitrogen by Life Technologies, Carlsbad, California, United States).

6.2. Appendix 2

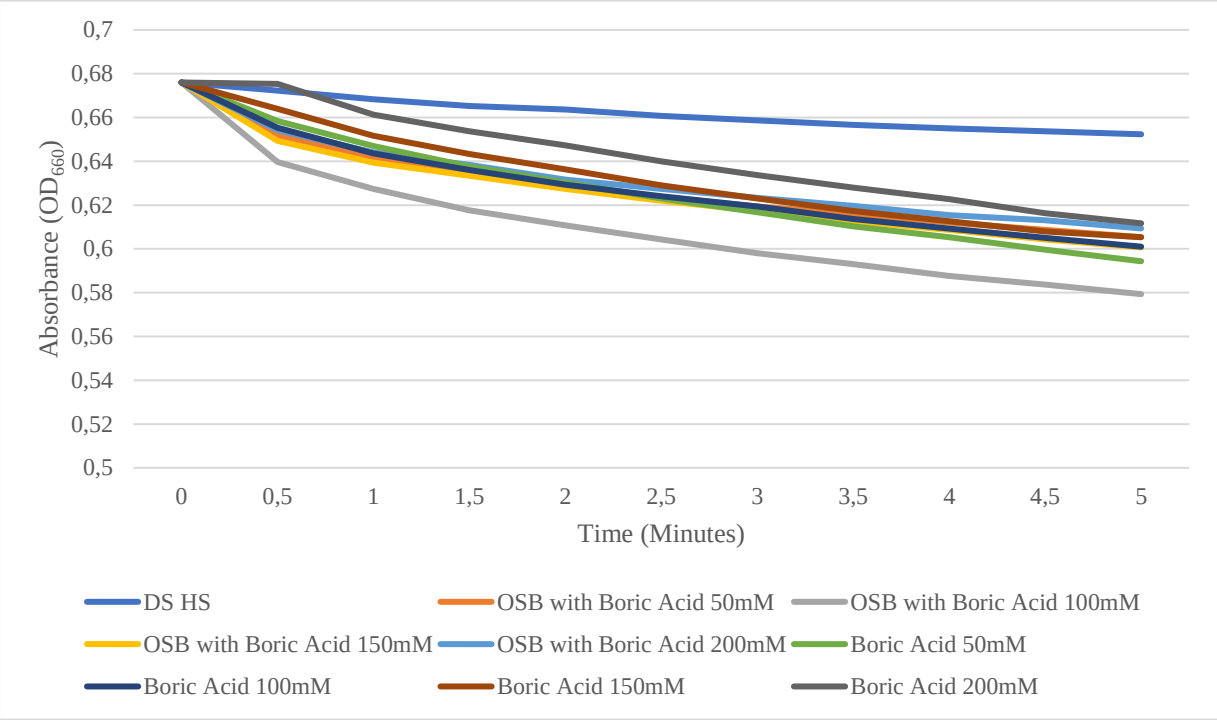

Figure 54: Effect of boric acid concentration on amount of lipoxygenase-1 extracted from de-fatted soybeans (DS)

Proteins were extracted from DS using either: the osmotic shock buffer (OSB) method with an initial overnight soak with 5 mM sodium metabisulfite (Na2S2O5) – hypotonic soak (HS) - followed by a further overnight soak in the presence of 0.5 M sucrose, 10 mM Na2S2O5 and varying concentrations of boric acid; or a single over overnight soak of with varying concentrations of boric acid.

Table 6: Standard deviation of triplicate results of methylene blue assay to determine lipoxygenase-1 activity in crude enzyme extractions from de-fatted soybeans (DS) based on boric acid concentration

	Standard Deviation								
Time (Minutes)	**DS HS**	**OSB with BA 50 mM**	**OSB with BA 100 mM**	**OSB with BA 150 mM**	**OSB with BA 200 mM**	**BA 50 mM**	**BA 100 mM**	**BA 150 mM**	**BA 200 mM**
0	0	0	0	0	0	0	0	0	0
0,5	0.0078	0.0045	0.0074	0.0074	0.0115	0.0309	0.0076	0.0201	0.0339
1	0.0129	0.0061	0.0083	0.0085	0.0166	0.0318	0.0107	0.0167	0.0366
1,5	0.0170	0.0074	0.0073	0.0093	0.0197	0.0320	0.0112	0.0148	0.0369
2	0.0193	0.0078	0.0074	0.0105	0.0229	0.0316	0.0116	0.0147	0.0352
2,5	0.0235	0.0085	0.0071	0.0113	0.0258	0.0318	0.0120	0.0142	0.0351
3	0.0256	0.0076	0.0073	0.0125	0.0270	0.0315	0.0116	0.0134	0.0351
3,5	0.0284	0.0079	0.0059	0.0130	0.0296	0.0317	0.0123	0.0132	0.0343
4	0.0307	0.0085	0.0057	0.0137	0.0306	0.0309	0.0127	0.0137	0.0339
4,5	0.0320	0.0090	0.0054	0.0147	0.0321	0.0311	0.0127	0.0134	0.0339
5	0.0331	0.0087	0.0053	0.0159	0.0340	0.0307	0.0131	0.0123	0.0327

Proteins were extracted from DS using either: the osmotic shock buffer (OSB) method with an initial overnight soak with 5 mM sodium metabisulfite (Na2S2O5) – hypotonic soak (HS) - followed by a further overnight soak in the presence of 0.5 M sucrose, 10 mM Na2S2O5 and varying concentrations of boric acid; or a single over overnight soak of with varying concentrations of boric acid. Biological triplicates were performed.

6.3. Appendix 3

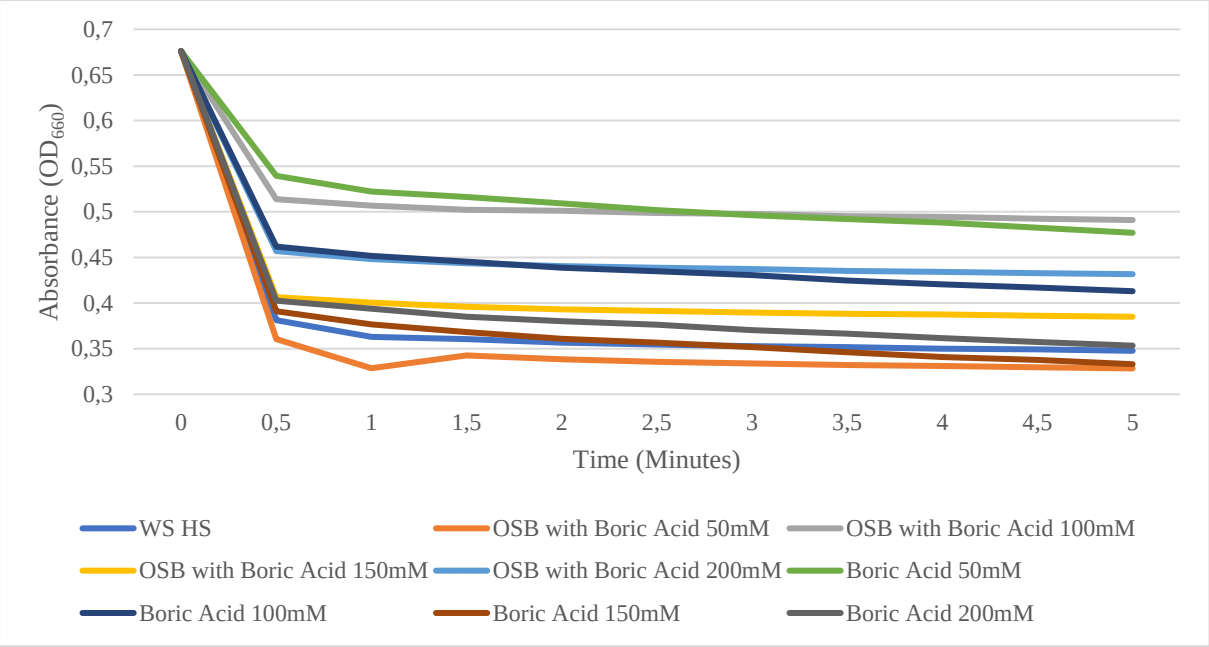

Figure 55: Methylene blue assay to determine lipoxygenase-1 activity in crude enzyme extractions from whole soybeans (WS) based on boric acid concentration

Proteins were extracted from WS using either: the osmotic shock buffer (OSB) method with an initial overnight soak with 5 mM sodium metabisulfite (Na2S2O5) – hypotonic soak (HS) - followed by a further overnight soak in the presence of 0.5 M sucrose, 10 mM Na2S2O5 and varying concentrations of boric acid; or a single over overnight soak of with varying concentrations of boric acid.

Table 7: Standard deviation of triplicate results of methylene blue assay to determine LOX-1 activity in crude enzyme extractions from whole soybeans based on boric acid concentration

	Standard Deviation								
Time (Minutes)	**WS HS**	**OSB with BA 50 mM**	**OSB with BA 100 mM**	**OSB with BA 150 mM**	**OSB with BA 200 mM**	**BA 50 mM**	**BA 100 mM**	**BA 150 mM**	**BA 200 mM**
0	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
0.5	0.0452	0.0340	0.1063	0.0229	0.0235	0.0762	0.0578	0.0691	0.0947
1	0.0435	0.0514	0.1091	0.0333	0.0243	0.0762	0.0579	0.0694	0.0966
1.5	0.0423	0.0380	0.1094	0.0331	0.0246	0.0749	0.0586	0.0693	0.0991
2	0.0408	0.0399	0.1097	0.0340	0.0241	0.0737	0.0576	0.0707	0.0988
2.5	0.0395	0.0405	0.1093	0.0339	0.0245	0.0738	0.0563	0.0703	0.0984
3	0.0381	0.0410	0.1093	0.0335	0.0233	0.0730	0.0558	0.0692	0.0992
3.5	0.0366	0.0424	0.1088	0.0339	0.0233	0.0726	0.0577	0.0695	0.0981
4	0.0372	0.0432	0.1088	0.0342	0.0229	0.0727	0.0570	0.0685	0.0978
4.5	0.0352	0.0435	0.1088	0.0346	0.0230	0.0729	0.0564	0.0691	0.0966
5	0.0346	0.0438	0.1083	0.0337	0.0227	0.0720	0.0567	0.0692	0.0963

Proteins were extracted from WS using either: the osmotic shock buffer (OSB) method with an initial overnight soak with 5 mM sodium metabisulfite (Na2S2O5) – hypotonic soak (HS) - followed by a further overnight soak in the presence of 0.5 M sucrose, 10 mM Na2S2O5 and varying concentrations of boric acid; or a single over overnight soak of with varying concentrations of boric acid. Biological triplicates were performed.

6.4. Appendix 4

Table 8: Protein concentrations of crude enzyme extractions from de-fatted (DS) and whole soybeans (WS) based on pH of buffer

Sample and pH	**Protein Concentration (mg/ml)**				
	Replicate 1	Replicate 2	Replicate 3	Average	Standard Deviation
DS pH 3				13.1	
DS pH 4				14.5	
DS pH 5				13.2	
DS pH 6				14.2	
DS pH 7				14.4	
DS pH 8				17.3	
DS pH 9				15.7	
DS pH 10				17.7	
WS pH 3	13.1	10.7	9.62	11.14	1.7812
WS pH 4	12.1	11.8	13.6	12.5	0.9644
WS pH 5	11.6	11.4	10.8	11.27	0.4163
WS pH 6	12.3	14.3	10.9	12.5	1.7088
WS pH 7	10.3	11.2	10.4	10.63	0.4933
WS pH 8	10.7	12.3	11.1	11.37	0.8327
WS pH 9	11.8	14.1	10.8	12.23	1.6921
WS pH 10	12.3	12.2	11.7	12.07	0.3215

Proteins were extracted from DS (replicates combined before protein quantification) and WS using a single over overnight soak of 125 mM boric acid, 10 mM sodium metabisulfite *(*$Na_2O_5S_2$*)* at varying pH values before being dialysed against the extraction buffer at pH 7 for 24 hours. Protein quantifications were made using the Qubit® 2.0 Fluorometer (485/590 nm; Invitrogen by Life Technologies, Carlsbad, California, United States).

6.5. Appendix 5

Table 9: Protein concentrations of graded ammonium sulfate (AS) purified whole soybean (WS) enzyme extract

Sample	**Protein Quantification (mg/ml)**				
	Replicate 1	Replicate 2	Replicate 3	Average	Standard Error
Unprocessed				24.1	
20% ASP Supernatant	14.9	15.6	15.6	15.37	0.2333
20% ASP Pellet	11.8	10.32	11.9	11.34	0.5108
40% ASP Supernatant	14.4	13.4	15.4	14.4	0.5774
40% ASP Pellet	18.6	16.7	18.6	17.97	0.6333
60% ASP Supernatant	13	15.2	13.6	13.93	0.6566
60% ASP Pellet	14.2	15.1	14.5	14.6	0.2646
60% ASP Pellet Dialysed	12.5	13.3	12.2	12.67	0.3283
80% ASP Supernatant	5.16	5.08	5.28	5.17	0.0581
80% ASP Pellet	12.2	14.3	13.4	13.3	0.6083

Proteins were extracted from WS using a single over overnight soak of 125 mM boric acid. 10 mM $Na_2O_5S_2$ (pH 7.00). Graded ammonium sulfate precipitation *(ASP)* was performed on 30 ml of extract from 20% to 80% saturation using solid ammonium sulfate (AS) Protein quantifications were made using the Qubit® 2.0 Fluorometer (485/590 nm; Invitrogen by Life Technologies. Carlsbad. California. United States)

6.6. Appendix 6

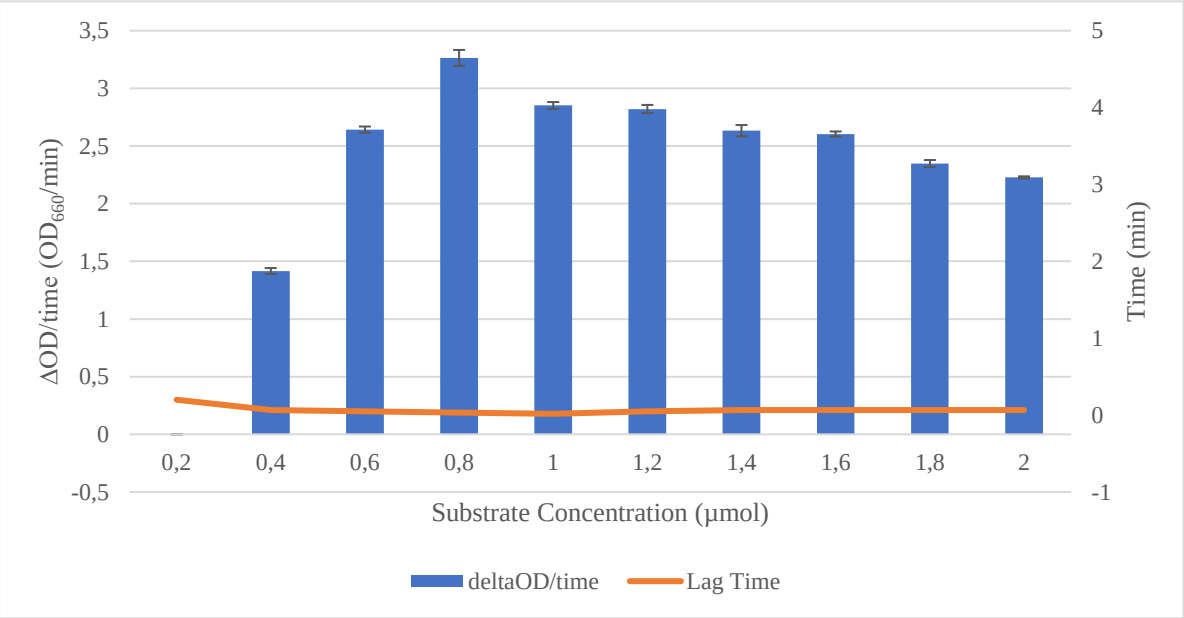

Figure 56: Effect of substrate concentration on lipoxygenase-1 activity

Partially purified enzyme was tested for changes in speed of methylene blue bleaching when incubated with increasing concentrations of sodium linoleate substrate. Substrate concentrations are shown on the axis as the total concentration in the 1 ml assay volume. Error bars show variation between technical replicates.

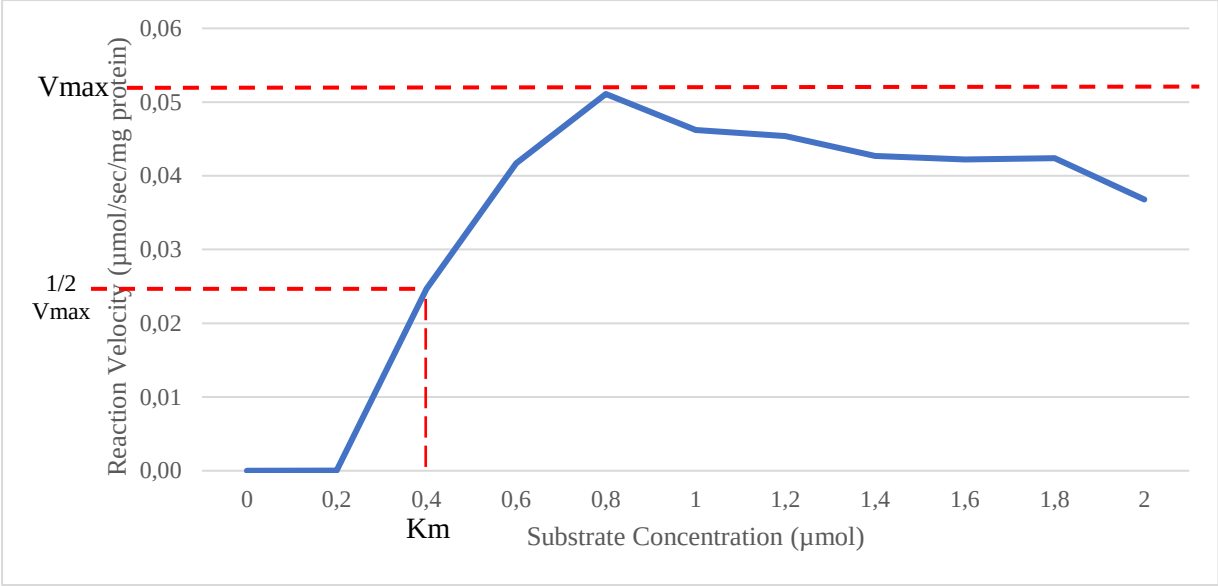

Figure 57: Michaelis-Menten plot of lipoxygenase-1 activity

Partially purified enzyme was tested for changes in speed of methylene blue bleaching when incubated with increasing concentrations of sodium linoleate substrate. Substrate concentrations are shown on the axis as the total concentration in the 1 ml assay volume. The velocity of each reaction was determined using the gradient value in the straight-line equation applied to the exponential portion of the curve. The greatest activity was observed with 0.8 µmol concentration of sodium linoleate with a velocity of 0.0511 ΔOD_{660}/sec.

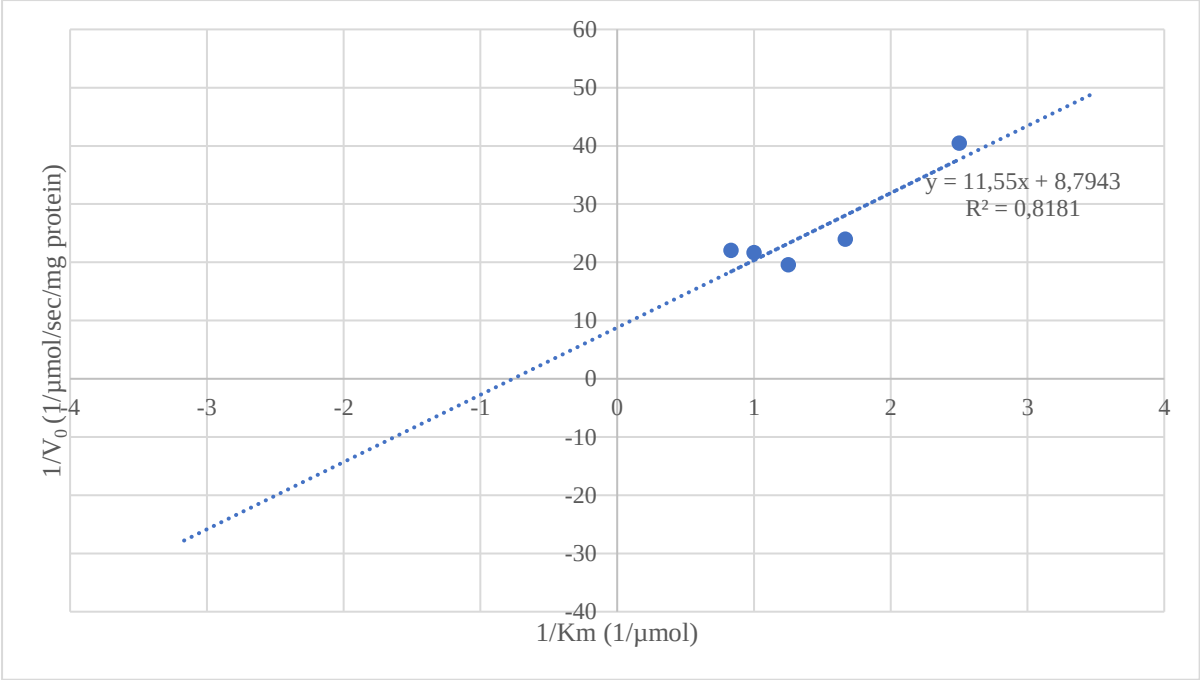

Figure 58: Lineweaver-Burk plot of lipoxygenase-1 activity

Partially purified enzyme was tested for changes in speed of methylene blue bleaching when incubated with increasing concentrations of sodium linoleate substrate.

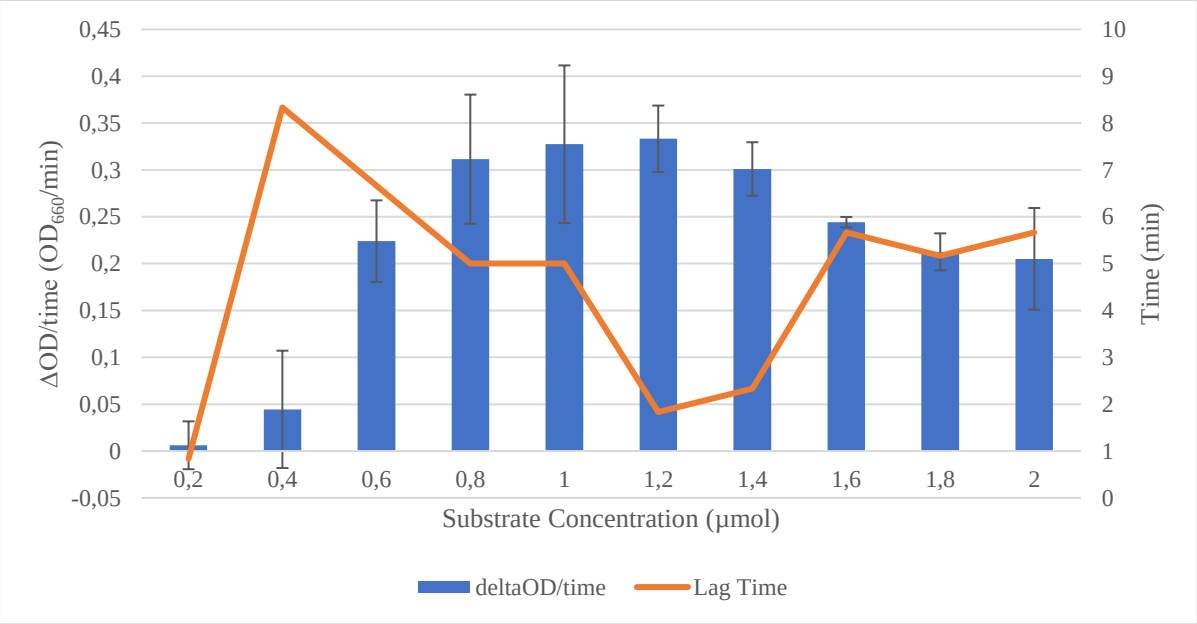

Figure 59: Effect of substrate concentration on lipoxygenase-2 activity

Partially purified enzyme was tested for changes in speed of methylene blue bleaching when incubated with increasing concentrations of sodium linoleate substrate. Substrate concentrations are shown on the axis as the total concentration in the 1 ml assay volume. Error bars show variation between technical replicates.

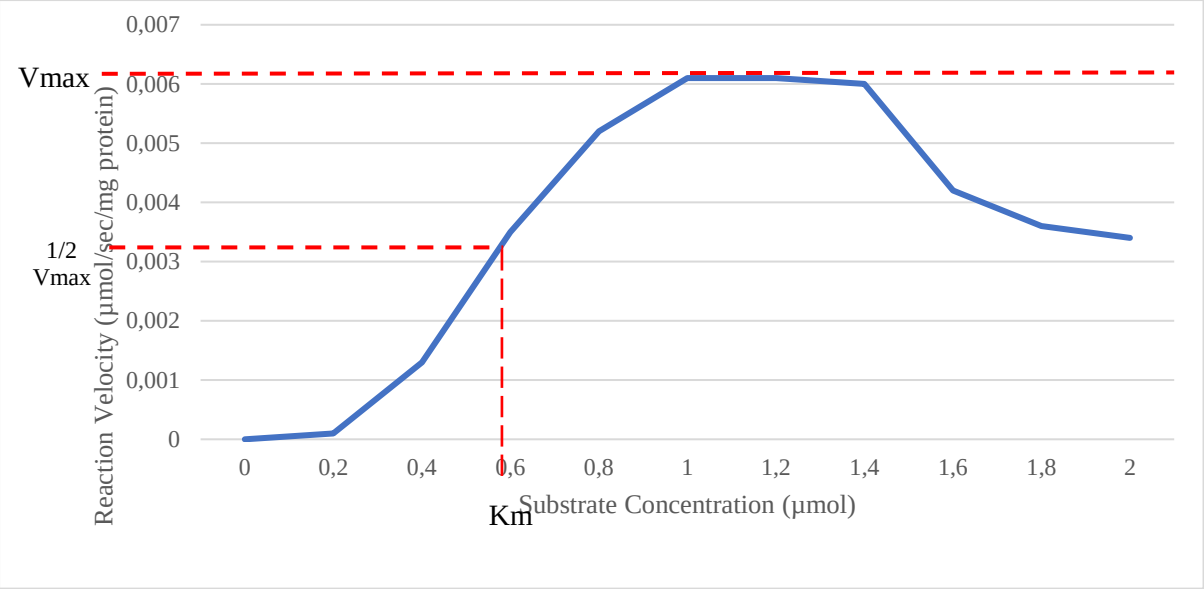

Figure 60: Michaelis-Menten plot of lipoxygenase-2 activity

Partially purified enzyme was tested for changes in speed of methylene blue bleaching when incubated with increasing concentrations of sodium linoleate substrate. Substrate concentrations are shown on the axis as the total concentration in the 1 ml assay volume. The velocity of each reaction was determined using the gradient value in the straight-line equation applied to the exponential portion of the curve. The greatest activity was observed with 1 µmol and 1.2 µmol concentration of sodium linoleate with a velocity of 0.0061 ΔOD_{660}/sec and second highest at 1.4 µmol with a velocity of 0.006 ΔOD_{660}/sec.

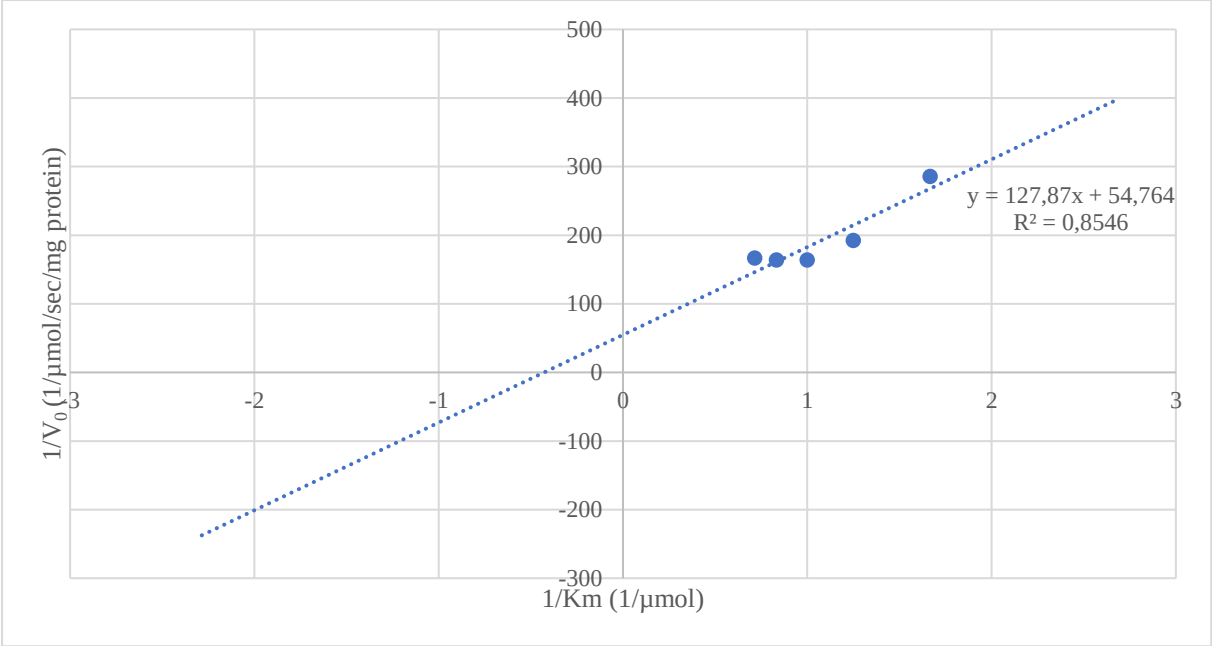

Figure 61: Lineweaver-Burk plot of lipoxygenase-2 activity

Purified enzyme was tested for changes in speed of methylene blue bleaching when incubated with increasing concentrations of sodium linoleate substrate. Substrate concentrations were converted to µM to better mimic the literature.

6.7. Appendix 7

Table 10: Amount of solid ammonium sulfate required to bring 100 ml of solution to desired saturation (%) at 0°C

	Percentage (%) Ammonium Sulfate Saturation at 0°C				
Initial concentration of ammonium sulfate (% saturation at 0°C)	20	40	60	80	100
	Solid ammonium sulfate (grams) to be added to 100ml of solution				
0	10.6	22.6	36.1	51.6	69.7
20	0	11.3	24.1	38.7	55.7
40		0	12	25.8	41.8
60			0	12.9	27.6
80				0	13.9
100					0

Table adapted from Duong-Ly & Gabelli (2014); volume of crude protein extract should be used to determine how much solid ammonium sulfate will be required to bring to desired percentage saturation. Mixture must be stirred at 4°C for 30 minutes before centrifugation at 20,000 x g for 30 minutes at 4°C. The resulting supernatant can be brought to a higher saturation using the correct amount of solid ammonium sulfate.