Discovery of New Enzymes for Degrading Wood Extractives and Bleaching

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A Thesis

In

The Department

of

Biology

Presented in Partial Fulfillment of the Requirements

for the Degree of Master of Science at

Concordia University

Montreal, Quebec, Canada

November 2006

Your file Votre référence
ISBN: 978-0-494-28876-4
Our file Notre référence
ISBN: 978-0-494-28876-4

ABSTRACT

Discovery of New Enzymes for Degrading Wood Extractives and Bleaching

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This thesis focuses on discovering new enzymes for applications in the control of extractives in pulp and paper manufacturing and high efficiency biobleaching. The goal was to better understand the potential of soybean lipoxygenase for removal of detrimental pulp extractives from thermomechanical pulp (TMP). Lipoxygenase treatment at both a 1% and 12% pulp consistency resulted in a 15% reduction in total extractives. The enzyme showed a high specificity towards lipophilic fractions primarily consisting of fatty acids and their esters. Several extractive compounds such as resin acids and lignans inhibited lipoxygenase-catalyzed reactions with fatty acids. In proving the concept of lipoxygenase for extractives degradation, a 96-well UV microplate assay was developed to screen enzymes from fungal species with high reduction oxidases.

A second contribution from this thesis was to determine the potential of accessory enzymes in pulp bleaching. A commercial lipase, lipase A "Amano" 12® was used since it exhibited high levels of accessory enzymes. Enzymatic treatment of hardwood and softwood kraft pulps resulted in a notable reduction of kappa number and hexenuronic acid contents. Two enzyme fractions containing high accessory enzyme activities were obtained. There fractions exhibited high bleaching efficiency and a high selectivity as was demonstrated which led to a notable reduction in the amount of sugar released compared to present enzyme bleaching with the commercial xylanase produced by Iogen.

ACKNOWLEDGEMENTS

I would like to thank Dr. Xiao Zhang who made this work possible through his unfailing support, ever-patient willingness to offer guidance during my more than frequent unannounced visits to his office, always believing in me, and accepting me as both his student and friend. I would also like to thank Drs. Adrian Tsang and Mike Paice for their kind and patience guidance as well for having given me the opportunity to have a collaborative research project between the Centre for Structural and Functional Genomics at Concordia University, and the Pulp and Paper Research Institute of Canada (Paprican). Without these individuals, this opportunity would not have been possible. I will cherish this experience for the rest of my life. Many thanks to Drs. Paul Joyce, Reginald Storms and Vincent Martin for accepting to be members of my committee, and for their valuable suggestions on this thesis.

I am indebted to all the staff at Paprican and colleagues at Concordia University for their invaluable input and support throughout my project.

I am grateful to my family, Phuoc Nguyen, Van-Tri Nguyen, and Xuan-Chi Nguyen for their love, help and support.

A special thank-you to Diana Le for having encouraged, supported, and having unconditional patience for me over the past two years, I know it must not have been easy at times.

Last, but certainly not least, I would like to offer my fellow students, Vineet Dua, a warm thank you for his companionship throughout these years who sense of humour has always been a source of encouragement, Bonny Choy and Amy Wong for their close friendships since the beginning of our "wild" university journey.

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LIST OF ABBREVIATIONS

A. niger	*Aspergillus niger*
A. pullulans	*Aspergillus pullulans*
T. reseii	*Trichoderma reesei*
PVC	Polyvinyl acetate
LCC	Lignin-carbohydrate complex
DCS	Dissolved and colloidal substances
PM	Paper machine
TMP	Thermomechanical pulp
LOX	Lipoxygenase
UBHW	Unbleached hardwood kraft pulp
O2HW	Oxygen delignified hardwood kraft pulp
UBSW	Unbleached softwood kraft pulp
O2SW	Oxygen delignified softwood kraft pulp
o.d. pulp	Oven dried pulp
Enz-D-E	Enzyme-chlorine dioxide-alkaline extraction
ND	Not detected

Chapter 1

Introduction

Paper manufacture is one of the largest industries in Canada employing about 60000 Canadians, and contributing over 16 billions of dollars to Canada's trade balance [1]. In 2004, paper production in this country reached over 16 million tonnes with exports accounting for over 80% of this value, making Canada the world's largest exporter of pulp and paper [1]. A simplified pulp and paper process is represented in Figure 1.1., indicating that this industry is a large consumer of resources mainly: woodchips, chemicals, water, and energy. Annually, this industrial sector consumes 47-53 million tonnes of forest biomass, 4.5 million tonnes in chemicals, 225 billion kilowatt-hours in energy, and 2000 million tonnes of water [2]. Clearly, this industry is in need of innovative methods to use our resources more efficiently and with fewer environmental consequences.

1.1. Overview of applications of biotechnology in pulp and paper manufacture

The pulp and paper industry is utilising biotechnological research to reduce its environmental impact and improve its economic performance. Main technologies currently under investigation involve modifications to virgin and recycled wood fibres, biobleaching, and mill effluent remediation [3]. Biological treatments offer many processing and environmental advantages, as compared to chemical reagents. These advantages include, ease of degradation and disposal, as well as very low, if any, toxic or

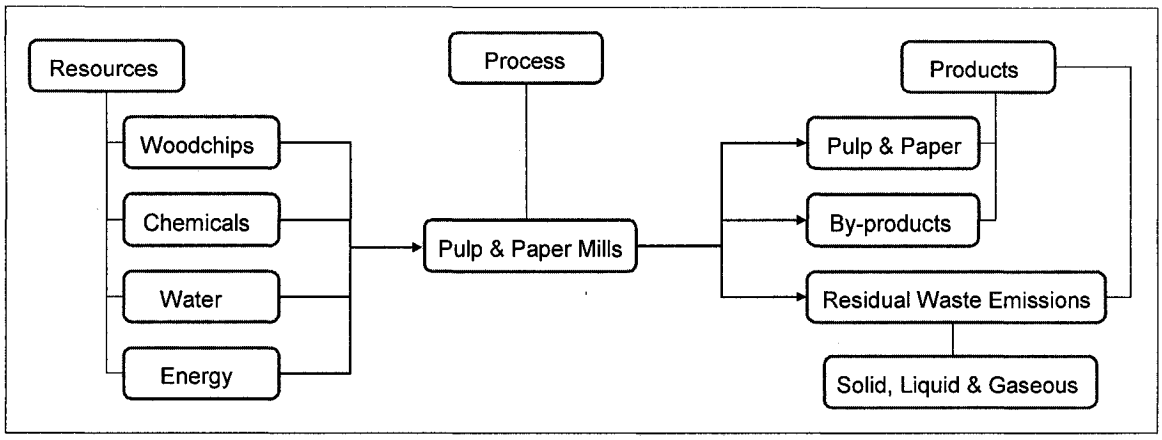

Figure 1.1. A cartoon illustrating a simplified pulp and paper process with three main areas: resources, process and products. The resources constitute woodchips, chemicals, water and energy where they are process in the pulp and paper mills into paper products, by-products, and residual waste emissions in solid, liquid, and gaseous forms [4].

corrosive properties. There advantages can potentially eliminate the need for expensive specialized equipment (alloys, polymers, etc.). Enzyme treatment operations, more importantly, can be easily implemented and do not need sophisticated technology [5]. With rapid advancements in the enzyme production industry, the price of enzymes has and continues to diminish, making these attractive technologies more economically viable. Table 1.1., provides an overview of some current and potential fungal enzyme applications in the pulp and paper industry. These enzymes are primarily produced in *Aspergillus niger* and *Trichoderma reesei* [6, 7]. Presently, interdisciplinary research in microbiology, molecular biology and enzymology is being performed with the goal of enhancing enzyme production efficiencies [7].

1.1.1. Biopulping/Altering refining energy

Traditional wood pulping/refining processes are costly and energy intensive. A possible solution to reducing cost and energy use is through a biotechnological approach known as biopulping that involves the direct application of fungal cultures onto wood chip piles prior to mechanical pulping [8]. Biopulping reduces refining energy demands [9], and improves pulp properties [10, 11]. The enzymes secreted by biopulping fungi have been postulated to be components of the cellulolytic enzyme system [5-7, 12]. Other enzymes for this application have also been explored, and suggested that xylanases could also be used to decrease the energy consumed during the refining of chemical pulps [6, 7, 13, 14].

1.1.2. Retention

Papermakers use cationic polymers to improve the retention of fines and filler particles in the paper sheet. The paper stock components that constitute paper products

Table 1.1. A list of some current and potential enzymes used in the pulp and paper industry to reduce environmental impact and improve economic performance.

Pulp & Paper Sector	Application	Industrial Enzyme	Potential Enzyme	References
Mechanical pulping	Reducing refining energy		Cellobio-hydrolases, Xylanases, Cellulases	[5-7, 12-14]
Kraft pulping	Prebleaching	Xylanases	Laccases, Peroxidases	[5-7, 15]
Papermaking	Retention	Pectinases		[5, 6, 16, 17]
	Extractives control	Lipases	Laccases	[5, 6, 18-20]
	Enhancing drainage	Cellulases, Amylases, Hemicellulases		[5-7, 21, 22]
	Whitewater Treatment		Lipases, Laccases, Pectinases, Hemicellulases	[17, 23]
	Deinking	Cellulases		[5-7, 24]
	Stickies control	Esterases		[5]
Process maintenance	Boil-outs & Slime control	Amylase, Lipase, Protease		[5, 7, 25]

are negatively charged, and have to be neutralised by a positive charge. This is known as cationic demand [26]. Retention aids in the form of chemical coagulants carry a very high charge density are used for this purpose, thus helping retention. Chemical coagulants are effective; however, cost is an issue amongst these chemicals [26]. Enzymatic treatment with pectinases can be used as an alternative to reduce cationic demand for certain mills that use alkaline peroxide bleached pulp [5, 6, 16, 17].

1.1.3. Enhancing Pulp Drainage and Paper Machine Speed

The efficient drainage of pulp furnishes on the wire of paper machines is a desired attribute to maximise machine speed. The results from past studies have indicated that xylanases and cellulases, particularly endoglucanases, yield the largest increases in freeness, which is defined as quality of pulp stock that determines rate at which it parts with water when being formed into sheet on wire screen or perforated plate [5-7, 21, 22].

1.1.4. Whitewater treatment

Growing economic and environmental concerns have resulted in an effort to reduce and recycle fresh water utilisation in mechanical pulp and paper mills. One attractive method is water reuse and system closure: however, major problems such as the accumulation of dissolved and colloidal substances (DCS) [27] lead to lower paper quality, increasing rates of corrosion, and reduced paper machine runnability are associated with this approach. Biotechnological treatment with hemicellulase, pectinase, lipase and oxidative enzyme can target the reduction of DCS in the mill whitewater [17, 23].

1.1.5. Deinking

The pulp and paper industry recognises that it is vital to utilise recovered fibre for a more environmentally friendly process [28]. The use of industrial enzymes for deinking secondary fibres such as waste paper serves this purpose and also reduces the large amounts of chemicals employed in conventional deinking operations. Cellulase treatments appear to have the ability of releasing ink particles bound to the fines and separately from the fibre, enhancing the removal of ink by flotation [5-7, 24].

1.1.6. Stickies Control

Recycled fibres often include sticky contaminants, called stickies [29]. These compounds such as polyvinyl acetate (PVC) reduce paper quality and cause paper machine downtime. They originate from additives that increase the bonding capability after paper manufacture [30]. Current methods of dealing with stickies do not completely solve the problem [29]. However, fibres treatment with esterases may hold great potential [5]. This biocatalyst converts PVC to the less sticky polyvinyl alcohol [5].

1.1.7. Slime control and Boil-outs

The formation of slime by microorganisms in the process water in pulp and paper mill systems are another problem because they can cause fouling, plugging and corrosion [25]. The enzymatic combination of amylase, lipase and protease can remove slime and control the growth of bacteria in paper machine systems [5, 7, 25]. An advantage of these biocatalysts is that they are environmentally benign compared to their chemical counterpart.

1.2. New opportunities for enzyme applications

In the newsprint industry, problematic extractives cause paper machine runnability problems that include low friction and strength impairment [31]. Bleaching chemicals are also of concern for the pulp and paper industry because they are environmentally harzardous due to their chlorine nature. There are new potential enzymes that could alleviate these problems. Enzyme applications for these two purposes will be discussed in detail in sections 1.3., 1.4., and 1.5.

1.2.1 Wood extractives or pitch control

Wood contains a considerable amount of organic compounds which can be extracted by means of polar or non-polar solvents. These compounds termed wood extractives are a heterogenous group of substances with variable chemical behaviour. In principle, they can be grouped into two main classes: lipophilic (fatty) and hydrophilic substances depending on their hydrophobicity and solubility in mill process waters [32]. In most wood species, the extractives content varies between 2% and 5% but can be as high as 15% in some tropical species. The main physiological functions of these extractives in a living tree include storage of nutrients, preservation of wood from microbial and insects attack, and formation of colour and odour. However, the presence of wood extractives in pulp and papermaking processes has generated a series of problems, particularly in mechanical pulping processes. Mechanical pulping primarily uses mechanical friction force and compressive force to separate fibers from wood or wood chips. During this process, most wood extractives are released from fiber lumens and resin canals and accumulate in the process streams. Fatty acids and their esters are the major constituents of wood extractives in most wood species and are known to cause a range of detrimental problems in paper products and paper machine operations,

including "pitch" deposition, decreased paper machine runnability and impairment of paper strength and optical properties.

In recent years, the slippage problems caused by deposition of fatty acids and their esters on the paper surface have become a major issue in the newsprint industry [33]. The slippery paper problem or low coefficient of friction is related to the measurement of how easily one surface slides over another. The deposition of lipophilic extractives on paper surfaces alters its coefficient of friction. In a paper sheet formed by pure cellulose, the hydroxy groups on the cellulose would create a strong attraction to the cellulose of another paper sheet [34-36]. This strong attraction allows a certain degree of friction between the two paper sheets. It can be said, because of surface contamination by lipophilic extractives, that there is a reduction in the coefficient of friction dramatically [34-36]. Once lipophilic extractives such as fatty acids are deposited on paper surfaces, the lipophilic/hydrophobic fatty acids tails would reduce the attraction force between the cellulose molecules which results in a low coefficient of friction and causes the slippery paper problem.

So far, there is a lack of an economically viable solution to the extractives problem. Mills have chemical treatments to increase the coefficient of friction, but high cost follows in the use of these chemicals [37]. Lipase and esterase treatments such as the Resinase® approach have been used to reduce "pitch problems" with some success [38]. However, these enzymes only hydrolyze ester-bonded extractives (e.g., glycerides and steryl esters) and release fatty acids which can still cause papermaking problems [5]. High reduction potential laccase has been demonstrated to react with a broad range of wood extractives including phenolic compounds, unsaturated fatty acids and their esters

[39, 40]. Treatments of mechanical pulp by laccase were shown to notably reduce both lipophilic and hydrophilic extractives content [20]. However, one problem that remains to be resolved is the decrease in brightness that occurs after laccase treatment of mechanical pulp [27].

1.2.2. Improved selective biobleaching

The majority of the colour remaining in pulp before the papermaking process can be attributed to lignin [41]. The chromophoric lignin is dissolved or modified in bleaching, with a multistage process of alternating oxidizing and extractive treatments with chemicals and interstage washing operations [42]. In most commercial processes chlorination is still the first bleaching step, and known as the pre-bleaching stage. As chlorination produces the largest number of environmentally hazardous chemical in the bleach plant effluents [43], attempts have been made to replace chlorine or to reduce the amounts of chlorine used.

Biobleaching of kraft pulp using xylanase has provided pulp mills with advantages such as improving environmental performance, reducing the cost of bleaching chemicals, increasing productivity and enhancing pulp properties over their chemical equivalents. This technology has been well adopted worldwide. In North America, approximately 2.5 million tonnes of kraft pulp produced annually employ xylanase as a pre-bleaching step. However, several negative effects from xylanase bleaching recognized in the last few years have hindered the further application of xylanase bleaching. In the industrial practice, xylanase prebleaching can typically cause a pulp yield loss of up to 1% based on dry pulp [44]. This loss is mainly due to the excessive hydrolysis of pulp hemicellulose by the enzymes. The solubilized hemicelluloses also

lead to a noteworthy increase in the amount of COD and BOD in the ensuing bleaching effluent system. Mills with limited effluent treatment capacity were forced to stop using xylanase bleaching technology. Mechanistic studies have shown that xylanase treatment enhances kraft pulp bleaching by either removing re-deposited xylans on pulp fiber surfaces or cleaving the linkages between xylan and residual lignin [45]. It should be noted that the major effects of xylanase treatments are only apparent after subsequent bleaching [46], meaning that the enzyme works by improving the reaction mechanisms of other bleaching chemicals. A hypothesis suggests that the enzymes partially hydrolyse the reprecipitated xylan on the pulp during the pulping process without degrading the cellulose portion [45]. As solubilisation of the hemicellulose proceeds, a large increase in fibre porosity allows accessibility to the remaining lignin molecules, and in subsequent bleaching stages more lignin can be extracted from the fibres [47]. This reduces the amount of bleaching chemicals needed to obtain the desired brightness [48]. Another common proposition is that the xylan backbone has lignin moieties bound to it via lignin-carbohydrate complexes (LCC), which when hydrolysed by the action of the enzymes are made water-soluble. Consequently, the increased solubility allows for enhanced diffusion away from the pulp fibres [47].

Conventional kraft pulping produces unbleached pulp with 3-5% residual lignin content. Due to its close interactions with carbohydrates in the pulp, the residual lignin is difficult to remove without negative effects such as degrading pulp strength and viscosity. A recent study has shown that the presence of hexenuronic acids in kraft pulp is another major factor contributing to high bleaching chemical consumption, decreased brightness and increased brightness reversion [49]. Hexenuronic acids are derived from

xylan based hemicellulose and formed during the kraft pulping process. It is postulated that the presence of hexenuronic acids promotes the formation of lignin-carbohydrate complexes, as the hexenuronic acid moiety may be a site for lignin-carbohydrate linkage [50, 51]. Therefore, efficient biobleaching protocol could entail a more selective removal of xylans that interact with residual lignin or hexenuronic acids in order to minimize the formation of lignin-carbohydrate complexes, excessive hydrolysis and BOD release.

1.2.3. Research Rationale

The present solutions to improve upon natural resource management with the exception of biotechnological application requires that more resources such as chemicals, water, and energy are needed to reach this end [52]. Moreover, instead of decreasing the environmental impact from pulp and paper mills, this adds to the large amounts of natural resources already used to produce the initial paper products.

The application of biotechnology solutions or more specifically the use of enzymes from fungi and bacteria have been evaluated for a broad range of applications, and consequent benefits for the pulp and paper industry. Biotechnological manipulations increased the quality and supply of feedstocks for pulp and paper while concomitantly decreasing the requirements of other manufacturing resources and lowering costs [7]. Enzyme applications complement and/or enhance conventional wood processing operations, and may provide many economic and environmental benefits through waste reduction, energy savings and reductions in toxic chemicals released into the environment. The research presented in this thesis focused on discovering new enzymes for extractives control and high efficiency biobleaching.

Extractives, also known as pitch, constitute over 60% of wood fatty acids and triglycerides derived from linoleic acids and other unsaturated fatty acids [53, 54]. The degradation of lipophilic extractives is the key solution to resolve extractives problems. Lipoxygenase has the capability of oxidizing unsaturated fatty acids and their esters providing an opportunity to alleviate extractives related problems. Lipoxygenase was first discovered in soybean [55] and soybean lipoxygenase-1 was the first to be sequenced and characterized [56]. Soybean lipoxygenase-1 is relatively easy to purify, can be obtained in large quantities and is stable. This enzyme has been used as a model system for understanding all lipoxygenases. Therefore, in this study, soybean lipoxygenase were chosen to evaluate its application to degrade the extractives present in mechanical pulp.

In high efficiency biobleaching, several naturally enzymes, loosely described as accessory enzymes, have attracted increasing attention due to their specific activity at the interfaces between lignin and carbohydrates. For instance, feruloyl esterases can selectively remove ferulic acid, an analog to the monomer of lignin from arabinoxylan present in the plant cell wall. Accessory enzymes activities have been found in several commercial enzyme preparations [57]. A commercial lipase from the fungus *Aspergillus niger*, lipase A "Amano" 12, produced by Amano Enzyme Inc. (Nagoya, Japan) was shown to exhibit a high level of feruloyl esterase activity. In this study, enzymatic treatment of both hardwood and softwood kraft pulp with the Amano lipase preparation before and after oxygen delignification was examined for bleaching activity.

Chapter 2

Materials and Methods

2.1. Chemicals

Linoleic acids were purchased from Sigma-Aldrich (Oakville, ON) with a purity of ≥ 99% and only samples from freshly opened bottles were used in this study. Abietic acid (ABA) and pimaric acid (PA) were purchased from Helix Biotech Co. (New Westminster, BC) with purities of ≥ 99%. *Alfa*-conidendrin was a gift from Dr. Norman Lewis at Washington State University, Pullman, WA.

2.2. Enzyme

Three lipoxygenases designated as LOX A, B, and C were used in this study. Both LOX A and B are soybean lipoxygenases (LOX A; L-7395 and LOX B: 62340) obtained from Sigma-Aldrich (Oakville, ON) with pH optima at 9.0. LOX C is a potato lipoxygenase acquired from Cayman Chemical (Michigan, USA) with a pH optima at 6.3. Moreover, LOX A is the main enzyme used for the degradation of wood extractives in thermomechanical pulp, and in this thesis is synonymous with soybean lipoxygenase (type 1-B). Several other types of oxidative enzymes were used including two laccases (NS 51002 from *Trametes villosa* and NS 51003 from *Myceliophthora thermophila*) obtained from Novozymes North America Inc. (Franklinton, N.C.) and a manganese peroxidase purified in the lab according to a protocol described previously [58]. The pH optima of these other oxidative enzymes are around 5.0. Hydrolytic enzymes were also tested: cellulase *Trichoderma reesei* (C-2730) from Sigma-Aldrich (Oakville, ON) and

xylanase *Trichoderma reesei* obtained from Iogen (Ottawa, ON) with pH optima both at 6.0. Lipase A "Amano" 12 was purchased from Amano Enzyme Inc. (Nagoya, Japan). A purified feruloyl esterase and alpha-glucuronidase from *Aspergillus pullulans* was a gift from Dr. Bernard Prior at the University of Stellenbosch, Matieland, South Africa. Two other purified feruloyl esterases, and an alpha-glucuronidase from *Aspergillus niger* was also a kind contribution from Dr. Emma Masters at Concordia University, Montréal, Québec.

2.2.1. Enzyme solutions preparation

The preparation of soybean lipoxygenase (LOX A) stock solution was performed under a stream of nitrogen gas where the desired amount of enzyme was added to a 20 mL liquid scintillation vial. This enzyme was dissolved to homogeneity in a 0.1 M sodium borate buffer (pH 9.0) with constant stirring at 150 rpm. The vials were capped under nitrogen gas, and then left at room temperature for 30 minutes before experiments were performed. LOX B stock solution was prepared using the same procedure as for LOX A. The final concentration was adjusted to have a similar enzyme activity on linoleic acid substrate as the LOX A stock solution based on the developed bench assay. LOX C was obtained in liquid form containing ammonium sulphate as a stabilizer. This enzyme solution was de-salted, and concentrated by Amicon ultrafiltration device through a regenerated cellulose YM membrane (molecular mass cut-off 1 kDa; Millipore, Bedford, Massachusetts, U.S.A) using 0.1 M sodium phosphate buffer (pH 6.3) under nitrogen gas. The final enzyme solution was adjusted to a similar activity as the LOX A stock solution in the same manner as that of LOX B. A crude lipase A enzyme solution

was prepared by dissolving an appropriate amount of the lyophilized powder in 100 ml of a 50 mM phosphate buffer (pH 6.0). This crude enzyme solution was then filtered through a 250 ml Nalgene CN filter unit (0.2 μm pore size and 50 mm diameter) for sterility and to remove particulates. This sample was then desalted and concentrated against a 50 mM phosphate buffer at pH 6.0 with the same method described above for the LOX C. This enzyme stock solution had lipase and xylanase specific activities of 3.69, and 60.38 U/mg respectively at pH 6.0 with a protein content of 2.6 mg/ml. A series of enzyme dosages, 0 to 13 mg of protein from the lipase A "Amano" 12 was used for pulp treatment.

2.2.2. Enzyme assays

In the standard lipoxygenase bench assay, the final reaction mixture contained 0.5 mM linoleic acid with 0.1 ml of a 7.4 mg/ml soybean lipoxygenase solution in a pH 9 0.1 M sodium borate buffer for a total volume of 3.0 mL. This amount of substrate used here is the optimal linoleic acid concentration that has been chosen by previous studies for reaction with lipoxygenases. In the latter method, lower linoleic acid concentrations gave the same initial background absorption but did not provide the enzyme with sufficient substrate to determine an accurate enzyme activity, whereas higher concentration increased the initial background absorption that could potentially interfere with the assay, and there was no gain in enzyme activity as compared to 0.5 mM. Oxidation of linoleic acid to hydroperoxide dienes during lipoxygenase treatment was monitored by the increase in absorption at 234 nm (A_{234}) on a CARY 1 UV-Visible Spectrophotometer (Varian, Australia) over thirty minutes at room temperature. The amount of hydroperoxide dienes produced was calculated based on the molar extinction

coefficient of 24000 $M^{-1}cm^{-1}$ [59]. Lipoxygenase activity was expressed as units per milligram of enzyme preparation (U/mg), where one unit increases A_{234} by 0.001 per min at pH 9.0 at 25°C when linoleic acid is the substrate in a 3.0 mL volume. In the initial microplate assay, the conditions and reaction components were identical to the standard assay except that the volumes were ten times less. The optimal reaction contained 0.65 mM linoleic acid with 0.01 mL of a 7.4 mg/ml soybean lipoxygenase solution in a pH 9 sodium borate buffer for a total volume of 0.3 mL, and was performed in a 96-well Costar UV transparent flat bottom plate with absorption change at 234 nm monitored by a Bio-Tek PowerWave HT Microplate Spectrophotometer. Arachidonic acid and trilinolein were also used as substrates, following the same procedure described for the standard assay. The lipase assay was followed by a method previously described using *p*-nitrophenol palmitate (PNPP) as a substrate [60]. Feruloyl esterase activities were determined by the method in Mastihuba et al. [57]. α-L-Arabinofuranosidase activities were determined by the method published in Biely et al. [61]. Xylanase activities were based on the method of Miller [62]. The protein content of each enzyme fraction was determined as described in Bradford [63].

2.3. Lipoxygenase treatments of thermomechanical pulp (TMP)

Lipoxygenase treatments of TMP were carried out at 30°C for 2 h. Two levels of enzyme dosage were applied, 1 and 10 mg of enzyme per g of oven-dried pulp (mg/g). The enzyme treatments of 1% consistency pulp were performed in 2 L flasks, with 10 g of pulp in 1 L of 100 mM sodium borate buffer (pH 9) shaken at 250 RPM. A 250 mL Amicon stirred cell device was used during lipoxygenase treatment of TMP at 12% pulp

consistency. In the Amicon chamber 20 g of pulp was added in 200 mL of borate buffer and a constant stream of air was supplied from the bottom of the chamber at approximately 20 mL per minute. The Amicon device was placed in a water bath to maintain the temperature at 30°C during the treatment. Both control and enzyme treated pulp samples were washed with deionized water after the treatments before subsequent analysis.

2.3.1. Wood extractives analysis

Both control and enzyme treated TMP samples were freeze-dried for 2 days to remove water prior to the subsequent Soxhlet extraction step. The Soxhlet extraction was carried out according to Tappi standard procedure (T 204 cm-97) using acetone as a solvent. The acetone containing extractives was evaporated to 25 mL and collected in a 25 mL volumetric flask. Two 1 mL samples were taken for gas chromatography analysis while the remaining 23 mL was transferred to a pre-weighed desiccated alumina weighing dish and placed in an the oven at 105°C to determine dry weight.

Extractives groups, including resin and fatty acids, lignans and sterols, and triglycerides were measured as trimethylsilyl derivatives by gas chromatography according to the method described by Orsa and Holmbom [64].

2.3.2. Wood extractives isolation from TMP for oxygen uptake measurement

To prepare extractives for oxygen uptake measurements, the following procedure was used to minimize any potential oxidation that may occur on extractive compounds. Ten grams of freeze-dried TMP was placed in a 1L Erlenmeyer flask followed by the addition of 800 mL of 95% acetone. The flask was then placed in an incubator shaker and mixed at 300 rpm for 48 h at room temperature. The flask was flushed with nitrogen

before incubation to minimize autooxidation during the extraction. After 48 h of extraction, the solvent fraction was separated from pulp fibre by filtering through a 0.45 mm medium coarse glass filtration funnel and collected in a 1L round bottom flask. The amount of solvent was then reduced to about 18 mL by rotary evaporation. Samples (2 × 1.5 mL) were taken for GC analysis. To the remaining concentrated extractives, 15 mL of 0.1 M sodium borate buffer (pH 9.0) was added to form an emulsion. The emulsion was further evaporated to remove residual acetone to a final volume of 15 mL.

2.3.3. Comparing oxygen consumption rates during the lipoxygenase reactions with linoleic acids and wood extractives

Oxygen consumption rates during the reactions were determined in a Rank oxygen cell equipped with a Clark electrode (Rank Brothers Limited, Cambridge, UK). A HAAKE A80 water bath was used to maintain the temperature in the cell at 30 ± 1°C during the reaction. The concentration of oxygen was assumed to be 187.5 μM under atmospheric air saturation. The 3 mL reaction mixture contained about 2.8 mM of linoleic acids and 70 μg of enzyme in pH 9 sodium borate buffer in the control experiment. The oxygen consumption rate during lipoxygenase reaction with wood extractives was measured under the same conditions except that wood extractives (9 mg) were used instead of linoleic acids. The oxygen uptake rate was taken within the first minute after enzyme addition to obtain the maximum reaction rate.

2.3.4. Determination of oxygen consumption rate during lipoxygenase reaction with linoleic acids in the presence of different inhibitors

The determination of the oxygen uptake during lipoxygenase-catalyzed reaction of linoleic acid with or without different inhibitors follows a similar procedure described

in section 2.3.3 except 100 μL of 95% ethanol or inhibitor (abietic acid, pimaric acid and *Alfa*-conidendrin) solutions was added to the reaction chamber before the addition of enzyme. To test the inhibition effects, three model extractive compounds were dissolved independently in 95% ethanol at concentrations ranging from 30 μM to 40 mM. A volume of 100 μL of extractive solutions was added to the cell and mixed with linoleic acids before adding the lipoxygenase to obtain oxygen consumption rates during reaction in the presence of inhibitors. The percentage of inhibition of lipoxygenase activity was calculated by dividing oxygen uptake rate of the inhibited reaction by that of the control reaction. The LC_{50} value represents the amount of inhibitor required to inhibit 50% of the original lipoxygenase activity.

2.4. Biobleaching of kraft pulps

All pulp treatments were initially performed in a stainless steel Hobart mixer, where the pulp, at a 10% consistency, was mixed at 150 rpm with the required enzyme dosage and pre-heated (50°C) in 50 mM phosphate buffer at pH 6.0. This pulp mixture was then transferred to a small plastic bag and incubated for 2 hours in a water bath at 50°C. The pulp mixture was then filtered so that only the enzyme treated pulp remained. This pulp then was washed with 2 liters of deionized water, and made into handsheets for kappa number and hexenuronic acid determination. The control samples were treated in the same manner except there was no enzyme addition.

2.4.1. Protein purification of lipase A "Amano" 12

Desalting of crude lipase A "Amano" 12 mixture was carried out using Bio-Rad disposable desalting columns: Econo-Pac™ 10DG (732-2010) at pH 6.0 as per the

manufacturer's instructions. The desalted enzyme was then purified through a Superdex 200 HR 10/30 column (17-1088-01) using an eluent containing 50 mM phosphate buffer with 0.15 M sodium chloride. The resulting protein fractions were then individually concentrated in an Amicon ultrafiltration device through a regenerated cellulose YM membrane (molecular mass cut-off 1 kDa) (Millipore, Bedford, Massachusetts, U.S.A) against a 50 mM phosphate buffer at pH 6.0.

2.4.2. Handsheet preparation

Handsheets were prepared according to the Canadian Pulp and Paper Technical Association Standard Testing Methods [65].

2.4.3. Kappa number measurement and hexenuronic acids measurement

The automated kappa number procedure was used for kappa number measurement [66]. The hexenuronic acid content for the pulp samples was determined by an ion-chromatograph method described previously [67].

2.4.4. Carbohydrate analysis

Concentration of arabinose, galactose, glucose, xylose, and mannose were measured using high performance anion exchange chromatography with pulsed amperometric detection on a Dionex DX 500 ion chromatography system according to Reid et al [17].

2.4.5. Bleaching of kraft pulp following enzyme-chlorine dioxide-alkaline extraction (Enz-D-E) sequence

In the enzyme treatment (Enz) stage, the pulps were treated as described in section 2.4 with the exception that enzyme treated pulps were not made into handsheets, but rather used in the subsequent chlorine dioxide (D) stage. In D stage, pulps at 4%

consistency were incubated at 45°C for 50 minutes. Three different dosages of chlorine dioxide were applied at 0.26, 0.21, and 0.16 active chlorine multiples (ACM). The chloride dioxide (ClO_2) was pre-heated to 45°C before adjusting the pH of the pulp with sulfuric acid to between 2.2 and 2.6. For the final alkaline extraction (E) stage, D bleached pulps at 10% consistency were incubated at 75°C for 60 minutes after mixing with a percentage of pre-heated sodium hydroxide dependant on the initial kappa number of the various pulps. In the hardwood and softwood kraft pulp, the sodium hydroxide amounts were respectively 1.25%, and 2.0%, while in the oxygen delignified hardwood and softwood kraft pulps these amounts were 1.0%, and 1.25%. After 60 minutes, the pulps were washed with deionized water, and made into handsheets for kappa and hexenuronic acid determination.

Chapter 3

Degradation of Wood Extractives in Thermomechanical Pulp by Soybean Lipoxygenase

This chapter investigated the potential of using soybean lipoxygenase enzyme to degrade the wood extractives in thermomechanical pulp (TMP).

3.1. Results

3.1.1. Composition of extractives in mechanical pulp

A black spruce mechanical pulp sample (TMP) collected from an eastern Canadian pulp mill in January 2005 was used in this analysis. The extractives present in TMP were obtained by extracting the pulp with acetone in a Soxhlet apparatus. The total extractives content of TMP was about 1.78% as measured by the dry weight of the solvent fraction. The different extractives groups were determined by GC/FID analysis (Table 3.1.), where 51.4% of the pulp extractives are related to steryl esters and triglycerides, while the resin and fatty acids represent approximately 25% of the total extractives through eight replicates.

3.1.2. Degradation of pulp extractives by lipoxygenase treatment

The soybean lipoxygenase-1 obtained from Sigma was used for pulp treatments. In the initial experiment, lipoxygenase treatments were carried out in 1% pulp solutions. Extractive contents of TMP after enzyme treatments were analyzed and compared with those of control TMP treated under the same conditions without enzyme addition over eight replicates.

Table 3.1. The extractives content present in the black spruce thermomechanical pulp sample (TMP) was obtained by extracting the pulp with acetone in a Soxhlet apparatus. The different extractives groups were then determined by GC/FID analysis, where 51.4% of the pulp extractives are related to steryl esters and triglycerides, while the resin and fatty acids represent 24.3%, and lignan and sterols constitute 18.9% of the total extractives through eight replicates.

	Total extractives	Resin and fatty acids	Lignans and sterols	Steryl ester and triglycerides
Weight based on oven dry pulp (mg/g)	17.7 ± 0.8	4.3 ± 0.24	3.35 ± 0.14	9.1 ± 0.45

As shown in Figure 3.1., a two-hour lipoxygenase treatment decreased the extractive content by approximately 10 and 15% at enzyme dosages of 1 mg/g and 10 mg/g, respectively.

A number of previous studies have demonstrated that lipophilic extractives are the major cause of various problems encountered in the papermaking process [68, 69]. It was shown that about 40% of fatty acids and their esters in mechanical pulp (i.e. steryl esters and triglycerides) are derived from linoleic acids [53]. By using GC/FID, the amount of lipophilic and hydrophilic extractives in control and enzyme treated TMP were determined. In Figure 3.1., lipoxygenase treatment selectively reduced the amount of lipophilic extractives. About 30% of the lipophilic extractives were removed by enzyme treatment at 10 mg of lipoxygenase/gram of pulp. Lipoxygenase had little effect on hydrophilic extractives fraction. The amount of lignan and sterols remained unchanged after the two-hour enzyme treatment.

In a typical mechanical pulping process, the pulp consistency can change from less than 1% to as high as 50%. It is of interest to know whether the enzyme treatment can achieve the same effect on extractives removal at a higher pulp consistency. Treatment of TMP pulp at 12% consistency was then carried out. To ensure enough oxygen supply, the treatment was performed in a reaction chamber with a constant airflow supplied from the bottom of the chamber. This reaction chamber mimics the high and medium consistency pulp storage towers typically used in mill processes. The treatment of TMP at 12% consistency with air supplementation gave a similar reduction of both total and specific extractives contents in TMP as the treatment at 1% consistency.

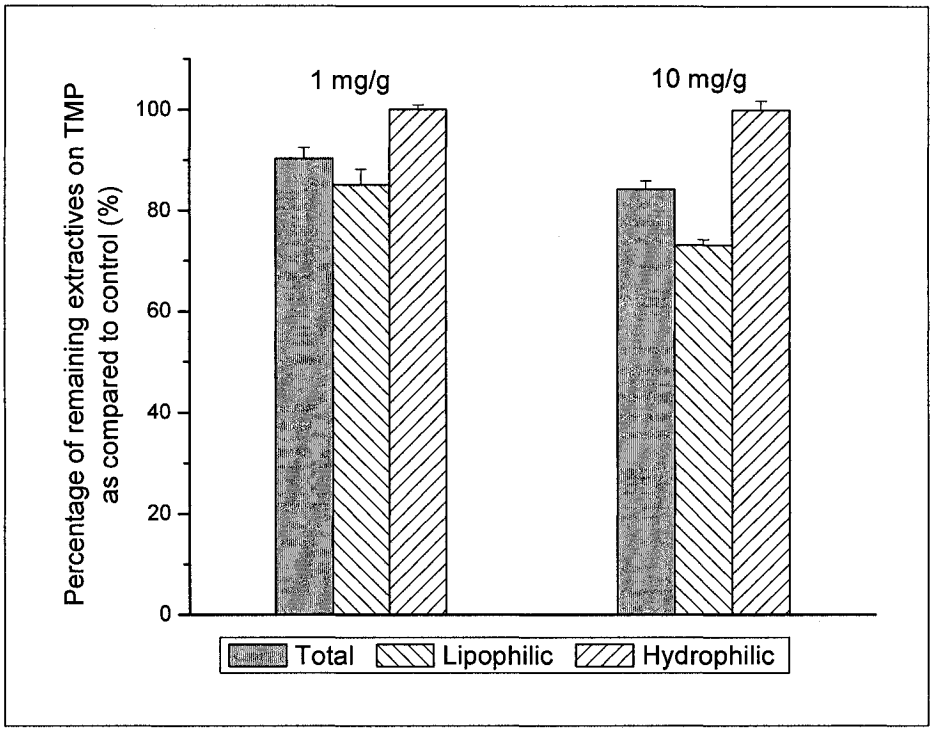

Figure 3.1. Removal of wood extractives, total and their individual groups of lipophilic and hydrophilic extractives by lipoxygenase treatment at 1mg/g of oven-dried pulp and 10 mg/g of oven dried pulp. Extractive contents of TMP after enzyme treatments were analyzed by GC/FID and compared with those of control TMP treated under the same conditions without enzyme addition over eight replicates. A two-hour lipoxygenase treatment decreased the extractive content by approximately 10% and 15% at enzyme dosages of 1 mg and 10 mg of lipoxygenase respectively. Moreover, this enzyme treatment selectively reduced the amount of lipophilic extractives, where the largest removal was at 30% of a 10 mg lipoxygenase dosage. Lipoxygenase had little effect on the hydrophilic extractive fraction, where the amount remained unchanged after the two-hour enzyme treatment.

3.1.3. Lipoxygenase reacts with wood extractives

This study provides direct evidence that lipoxygenases can reduce extractives content in pulp. Earlier work has also suggested the possibility of using lipoxygenases to reduce model wood "pitch" content in pulp and paper [70]. To confirm this observation, lipoxygenase reactivity towards actual wood extractives obtained from TMP was determined. Acetone extractives were obtained from the same TMP. In order to minimize any potential oxidation, the extraction was performed at room temperature and flushed with nitrogen. The extractives fraction was re-dissolved in ethanol and dispersed in pH 9 buffer to a final volume of 18 mL. The extractives content of the final emulsion was determined as shown in Table 3.2. The oxygen uptake rate was measured within the first minute of the reaction. In a parallel experiment, the same amount of enzyme was used to react with linoleic acid to obtain the reference oxygen uptake rate for the enzyme at this dosage. As the extractives obtained from pulp is a mixture of compounds, the molar concentration of this fraction is impossible to estimate. Therefore, a comparison between the reaction rates of the two substrates, linoleic acids and extractives, on a weight basis was made. An oxygen uptake rate (0.028 ± 0.007 μmole O_2/minute/mg of extractives) was recorded during lipoxygenase reaction with extractive mixture. However, this rate was lower roughly a ten-fold difference (0.25 ± 0.019 μmole O_2/minute/mg of linoleic acid) than expected compared with the reference linoleic acid, as lipophilic extractives represent more than 80% of the total extractives in the mixture used. Due to the complexity of this mixture, a simple conclusion cannot be drawn just by comparing the reaction rates based on substrate weight.

Table 3.2. Extractives present in the isolation of extractives from TMP* by acetone extraction for oxygen uptake measurements were determined by GC/FID analysis, where 38.0% of the pulp extractives are related to steryl esters and triglycerides, while the resin and fatty acids represent 25.3%, and lignan and sterols constitute 12.8% of the total extractives.

	Total extractives	Resin and fatty acids	Lignans and sterols	Steryl ester and triglycerides
Concentration (mg/mL)	7.5 ± 0.25	1.9 ± 0.13	0.96 ± 0.2	2.85 ± 0.13

*TMP stirred with acetone at room temperature for 48 h

3.1.4. Inhibitors of lipoxygenase activity

Results from the above experiment suggest the presence of compounds that interfere with the lipoxygenase activity. Many compounds including phenols and abietic acids inhibit lipoxygenase activity. However, little information is available on the effects of wood extractives on lipoxygenase activity. As a considerable amount of phenolic compounds and resin acids were found in the extractives mixture, the determination of the effects of several representative extractives on lipoxygenase activity was studied.

Resin acids are the predominant nonvolatile terpenoids found in most softwood species. They are divided into two groups, pimarane and abietane, depending on whether they contain or do not contain a conjugated double bond (or contain either isoprenyl/isopropyl or methyl/vinyl at the C-13 position). Abietic acid and pimaric acid were chosen in this study to represent the respective abietane and pimarane groups. Lignans are a group of compounds consisting of two phenylpropane units linked in different manners [71]. Although some of these compounds represent the dimeric structure which may also be found in lignin molecules, the biosynthetic pathway of these two groups are distinct and no interaction occurs between these components in the living tree [72]. Measurement of lipoxygenase activity was initially attempted based on absorption change at 234 nm after reacting with linoleic acid. Both lignan and resin acids caused interference in the absorption in the low UV range. Therefore, the spectroscopy method is not a suitable method for this type of study.

Oxygen uptake rate was used to measure the inhibition effects of these chemicals on lipoxygenase during reaction with linoleic acids. A Rank oxygen cell equipped with a Clark electrode was used in this analysis. As shown in Figure 3.2., all three model

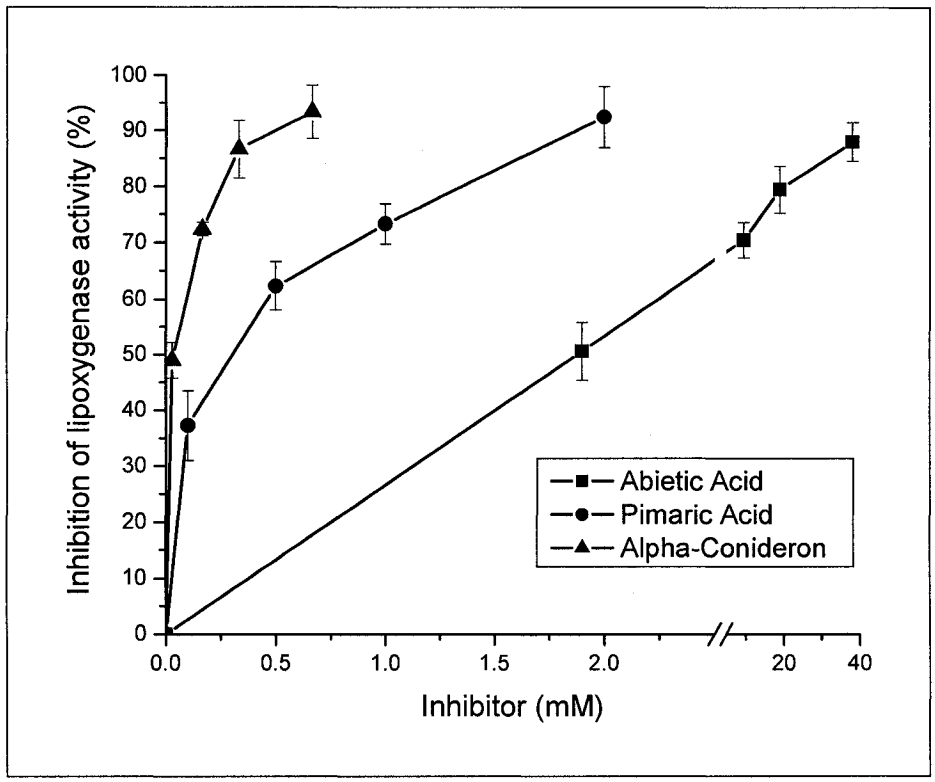

Figure 3.2. The oxygen uptake rate of lipoxygenase was used to measure the inhibition effects of abietic acid, pimaric acid, and *alfa*-conidendrin during reaction with linoleic acid. A Rank oxygen cell equipped with a Clark electrode was used in this analysis. These compounds displayed inhibition of lipoxygenase activity towards linoleic acid. The percentage inhibition was dependent on the concentration of each respective inhibitor. *Alfa*-conidendrin caused the strongest inhibition of lipoxygenase activity. The concentration of linoleic acid approximated the fatty acid content in typical mechanical pulp mill process streams. The amount of the three inhibitors in the oxygen cell covers the possible ranges of extractives concentrations in the mill process.

compounds displayed inhibition of lipoxygenase activity towards linoleic acids. The percentage inhibition was dependent on the concentration of the inhibitors. The LC_{50} values of each inhibitor were estimated from the graph. The LC_{50} of abietic acid is about 1.9 mM compared to a LC_{50} of pimaric acid around 0.4 mM. Alpha-conidendrin had a much more notable impact on inhibiting lipoxygenase with a LC_{50} of less than 30 μM.

3.2. Discussion

Lipoxygenases are non-heme dioxygenases with specific ability to catalyze the oxidation of polyunsaturated fatty acids to hydroperoxides. The specific ability of lipoxygenases to degrade linoleic acids leads to a potential application of lipoxygenase in papermaking processes to degrade wood extractives that have detrimental effects on pulp and paper properties. The putative effects of lipoxygenase to react with a model "pitch" mixture were observed in a previous patent study [70], where lipoxygenase treatment of a model "pitch" containing 60% of linoleic with abietic acid, oleic acid and olive oil was carried out. Although the method used to determine extractives reduction after enzymatic treatment was indirect and tentative, this was the first attempt to explore the opportunity of using lipoxygenase in papermaking.

The results presented here provide direct evidence that lipoxygenase can reduce the amount of wood extractives, specifically the lipophilic extractive fraction, present in pulp. Most of the extractives present in TMP retain the same properties and structures that they have in wood. This is particularly true when the pulp samples were collected in winter when the natural degradation of wood extractives caused by microorganisms and

autooxidation is at a minimum. The observed 25% reduction of lipophilic extractives content in TMP may bring about some changes in paper surface properties [33].

The temperature and pH range were chosen to provide optimum conditions for the enzyme. The pulp consistency varies at different steps in the pulping process, usually between 4 – 12%, while the pulp consistency is lower than 1% at the paper machine headbox. Lipoxygenase is a dioxygenase which requires sufficient molecular oxygen to maintain its reaction with substrates. The different pulp consistencies are expected to affect dissolved oxygen concentration in the solution. High solid content may reduce the level of dissolved oxygen available for the enzyme treatment. The ability of lipoxygenase to remove extractives at high pulp consistency determines whether it is applicable to be implemented in the mill process. When the 12% pulp slurry was treated by lipoxygenase without oxygen supplementation, there was a small reduction in pulp extractives after the treatment. However, when the same treatment was carried out under aeration, the effects of enzyme on extractives removal was similar to that was obtained from treating 1% consistency pulp. The treatment of TMP in an Amicon ultrafiltration device mimics the pulp storage towers in most pulp and paper mills, where air or oxygen can be supplied.

The oxygen uptake rate during lipoxygenase reaction with wood extractives confirmed the effects of lipoxygenase on TMP extractive degradation. The observed reactivity of lipoxygenase towards wood extractives is likely due to the reaction with unsaturated fatty acids and their esters (e.g. steryl esters and triglycerides). The mechanism of the lipoxygenase catalyzed reaction of unsaturated fatty acids and triglycerides has been well studied. Linoleic acids and trilinolein are the typical substrates used in these mechanism studies. The reaction starts with abstraction of a hydrogen atom

from the pentadiene structure on the linoleic acid to form a radical species. In the presence of molecular oxygen, a hydroperoxide diene is subsequently formed following the addition of oxygen to the radical. This species can undergo either inter or intra molecular attack to either continue the radical reaction on another fatty acid chain or break down to degradation products. In plants and animals, the fragmentation of hydroperoxides typically requires several other enzymes such as hydroperoxide lyases [59]. However, non-enzymatic degradation of fatty acid hydroperoxides by transition metals has also been proposed. For example, iron and copper are able to decompose fatty acid hydroperoxides to smaller fragments [73, 74]. TMP typically contains a variety of transition metals including Cu, Fe and Mn [75]. The content of Cu, Fe, and Mn in this TMP was analyzed by ICP spectroscopy. An amount of Fe and Mn, 113 mg and 59.2 mg respectively per kilogram of dry pulp, was found in the pulp with low Cu ion content at 2.1 mg/kilogram of dry pulp. It has been shown that the presence of 10^{-5} M iron in the reaction mixture is sufficient to catalyze the decomposition of linoleic acid hydroperoxide. Although there is a debate as to whether the binding of Fe by lignin in pulp restricts the oxidation potential of Fe [76, 77], the high level of Mn content is likely to play an important role in the degradation of hydroperoxide derivatives. The fate of wood lipophilic extractives after reacting with lipoxygenase is a subject worthy of future investigations.

Lipoxygenases exist in nature in several different forms. The enzyme used in this study is a type I lipoxygenase (LOX 1) from soybean. Besides LOX 1, there are at least two other lipoxygenase isozymes from soybean, LOX 2 and LOX 3. LOX 1 has been shown to be most effective in oxidizing free or polar fatty acids, while LOX 2 and 3 are

more effective in oxidizing fatty acid esters. The pH optima and specific activities of LOX 2 and 3 are also different from LOX 1. LOX 2 has the highest specific activity and works best at pH 6.5, while LOX 3 has a broader pH optimum range from 4.5 to 9. Lipoxygenases are also abundant in human and fungal species. The recent discovery of fungal manganese lipoxygenases has revealed another group of lipoxygenases which may have its own unique ability to oxidize fatty acids [78-80]. The manganese lipoxygenase produced from *Gaeumanomyces graminis* has a broad pH range from 5 to 11 and good heat stability [78]. The enzyme remains active at temperatures up to 63°C and can be stored at 4°C for several months without losing activity. Some of these enzymes will bring a greater potential to degrade wood extractives under mill process conditions.

Despite the high percentage of the lipophilic extractive fraction in the wood extractive mixture prepared from pulp, the activity of lipoxygenase on a wood extractive mixture only represents approximately 15% of its activity on pure linoleic acid based on substrate weight. While it is recognized that a quantitative comparison cannot be drawn when a mixture is used, the postulate is that some extractive components in the mixture hindered the reactivity of lipoxygenase towards fatty acids and their esters. Besides fatty acids and esters, resin acids and lignans, a group of phenolic compounds, are the two other predominant extractive groups in wood. Finding effective means to deactivate or inhibit lipoxygenases has been the subject of extensive research during the past few decades, with a primary focus to identify compounds that can hinder lipid peroxidation. Extracts from several natural plants have shown potent effects in inhibiting lipoxygenase activity [81]. The inhibition effects of phenols and phenolic compounds on lipoxygenase have been demonstrated previously. One study [82] has observed the inhibition effects of

abietic acids, a type of resin acid in wood, during the lipoxygenase reaction with linoleic acids. This study is the first attempt to determine the effects of different types of wood extractives on lipoxygenase activity. The three substrates used are the predominant resin acids and lignans found in most wood species.

A high linoleic acid concentration was used in the oxygen cell to ensure a maximum initial velocity, and the oxygen consumption rate was measured within the first minute to avoid any inhibitory effects from the reaction products. The concentration of linoleic acid also approximated the fatty acid content in typical mechanical pulp mill process streams [53]. The amount of the three inhibitors in the oxygen cell covers the possible ranges of extractives concentrations in the mill process. As shown in Figure 3.2., α-conidendrin apparently caused the strongest inhibition of lipoxygenase activity. Many phenolic compounds have high radical scavenging activity and can effectively prevent lipid peroxidation [81, 83]. The LC_{50} values of most phenolic compounds are between 1 to 50 μM which is similar to the value this study obtained for α-conidendrin. Although the lignan content in wood extractives is relatively low, they had an impact on the efficiency of lipoxygenase for removal of extractives. Both types of resin acids also have an inhibitory effect on lipoxygenase-catalyzed reaction with linoleic acids. Resin acids are a group of extractives exclusive to several softwood species. The reason pimaric acid exhibits a higher inhibitory effect than abietic acid is unclear; there is little information on the reactivity between lipoxygenase and resin acids in the literature.

3.3. Conclusion

The results obtained from this study demonstrate a potential application of lipoxygenases to degrade wood extractives that have detrimental effects on paper product qualities and mill operations. Lipoxygenase treatment of TMP resulted in a 15% reduction of total extractives in pulp. The activity of lipoxygenases is specific towards the lipophilic extractive fraction in pulp which primarily consists of fatty acids and their esters. The lipophilic extractives content was reduced by more than 25%, while there was little change in pulp hydrophilic extractive content after 2 hours of enzyme treatment. The presence of transition metal ions in the TMP, manganese in particular, may contribute to the further fragmentation of fatty acid hydroperoxides produced by lipoxygenase reaction with wood extractives. Lipoxygenase exhibited a high activity towards pure wood extractives obtained from TMP. However, some of the extractives such as resin acids and lignans may have inhibitory effects on lipoxygenase-catalyzed reactions with linoleic acid. Further work is needed to identify the effects of the lipoxygenase treatment on paper surface properties and to determine whether it is economical to implement this enzyme treatment in a TMP/newsprint mill.

Chapter 4

Microplate Enzyme Assay for Screening Lipoxygenases to Degrade Wood Extractives

Successful testing has shown that lipoxygenases have the potential to degrade problematic wood extractives in the papermaking process. However, commercially available lipoxygenase is currently too expensive for this application. This chapter investigated the development of a 96-well UV microplate assay to screen enzymes from fungal species for a more cost-effective alternative lipoxygenase.

4.1. Results and Discussion

4.1.1. Microplate assay platform to screen for enzymes degrading wood extractives

The first approach was to establish a suitable substrate for microplate assay development. A characteristic of substrates for lipoxygenase is a 1,4-pentadiene moiety in compounds such as unsaturated fatty acids and esters [84, 85]. Arachidonic acid, trilinolein, and linoleic acid are the most common substrates for lipoxygenase-catalyzed reactions [86]. Linoleic acid has been used as a substrate for the study of lipid peroxidation involving lipoxygenase [87]. Arachidonic acid is a common substrate for examining mammalian lipoxygenase [78, 88] and it has shown a lower reactivity with some of the soybean lipoxygenases. Trilinolein is a frequent substrate used for studying plant lipoxygenases [78, 80, 86]. The reaction rates of lipoxygenase with these substrates were determined based on the formation of hydroperoxide diene derivatives. Soybean

lipoxygenase designated LOX A was chosen as the representative enzyme for lipoxygenases due to its abundance.

The three substrate solutions were prepared separately at concentrations of 5 mM, and 0.074 mg/ml of LOX A was used to react with these substrates. The amount of hydroperoxide dienes produced after 10 minutes of each reaction was determined and used for comparing the reactivity amongst the three substrates. It was found that, after 10 minutes of the reaction with lipoxygenase, 144.6 μM, 32.4 μM and 0.05 μM of diene products were produced from linoleic acid, arachidonic acid and trilinolein substrate solution respectively. Soybean lipoxygenase demonstrated the highest reactivity towards linoleic acid, and therefore was used here to develop a microplate assay. Additional benefits of using linoleic acid are that it is a model substrate for lipoxygenases and that it is a representative constituent of wood extractives.

4.1.2. Microplate assay development

The development of a microplate assay format allows a rapid screening of a large number of fungal enzymes from different species which gives a cost benefit per assay. However, a simple scale down from standard assays may lead to a low sensitivity and accuracy. In the initial microplate assay mixture, 0.02 mL of the linoleic acid stock solution, 0.01 mL lipoxygenase solution, and 0.27 ml of buffer were used. A comparison of the reaction volumes for the two assay methods is listed in Table 4.1.

A LOX A activity curve obtained at different enzyme concentrations was determined at bench and microplate assay scales. The enzyme concentrations varied between 7.4×10^{-4}, 1.5×10^{-3}, 3.7×10^{-3}, 7.4×10^{-3}, and 1.5×10^{-2} mg/mL obtained from diluting LOX A stock solution with pH 9 sodium borate buffer from which aliquots were

Table 4.1. Comparison of enzyme quantities and chemical reagents needed between the developed microplate assay and that of the standard bench assay. An approximate ten-fold reduction in both these elements is noted for the microplate assay.

Reaction Mixture: Components	Bench Scale Assay	96-Well Microplate Assay
Total, μL	3000	300
Linoleic acid Colloidal Substrate, μL	200	20
Buffer, μL	2700	270
Enzyme, μL	100	10
Number of Assays/Hour	2 (in duplicate)	96 (in duplicate)

taken for the respective assays. The trend for both bench and microplate methods was similar, with optimum enzyme dosage for both in determining LOX A activity found at about 3.7 x 10^{-3} mg/mL. A typical enzyme activity versus concentration curve of the bench and microplate scale spectroscopy assay is shown in Figure 4.1.

These initial results indicate that the bench assay has some potential to be transferred to the 96-well microplate system. The kinetics of LOX A reaction with linoleic acid at optimum enzyme concentration was then examined. When the time-dependent enzyme kinetics were compared (Figure 4.2.), it was evident that there was a difference in hydroperoxide diene product formation between these two methods with progressing reaction time. The rate in the first five minutes was similar for both assays, but the concentration of hydroperoxide dienes determined at 30 minutes on the microplate assay is approximately 25% lower than obtained from the standard bench assay. In terms of product formation this correlates to a difference of 27.0 μM.

Two factors were considered for the lower enzyme activity in microplate assays: substrate activation and molecular oxygen, since both the amount of dissolved oxygen and substrate can notably affect the reaction rates of lipoxygenase. The addition of oxygen to the reactions by intermittent shaking of the microplate at intervals of five minutes before each reading was tried. No notable change in hydroperoxide dienes formation occurred, therefore, the non-agitated system provided the LOX A with sufficient oxygen. At an initial linoleic acid concentration of 0.5 mM, the initial product formation kinetics of both methods were comparable, however, as the reaction proceeded there was a decreased formation of dienes with the microplate assay (Figure 4.2.). This could be attributed to an insufficient absolute amount

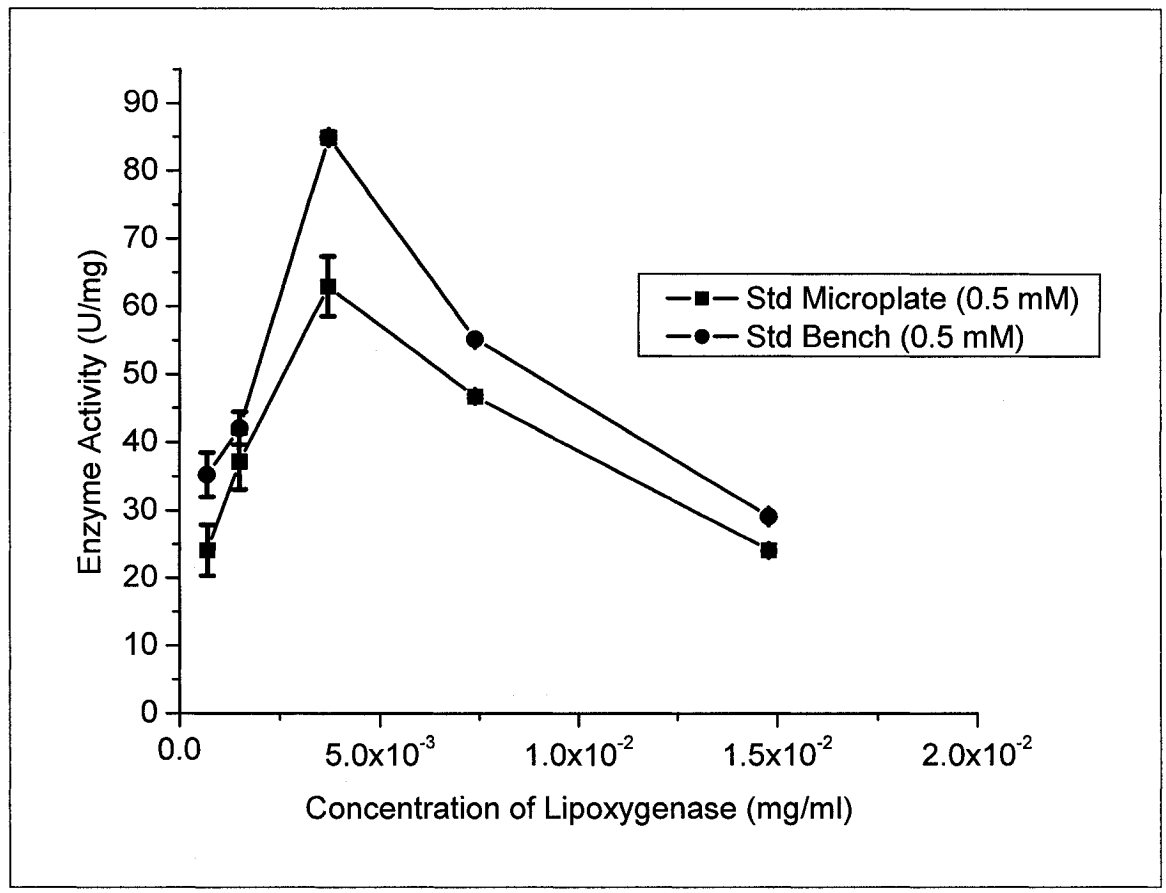

Figure 4.1. A comparison of enzyme activity profiles between the initial conditions on the developed microplate method versus the standard bench assay at different concentrations of lipoxygenase. A LOX A activity curve obtained with enzyme concentrations varying between 7.4 x 10^{-4}, 1.5 x 10^{-3}, 3.7 x 10^{-3}, 7.4 x 10^{-3}, and 1.5 x 10^{-2} mg/mL was determined for both these assays. The trend for both bench and microplate methods was similar, with optimum enzyme dosage for both in determining LOX A activity found at about 3.7 x 10^{-3} mg/mL.

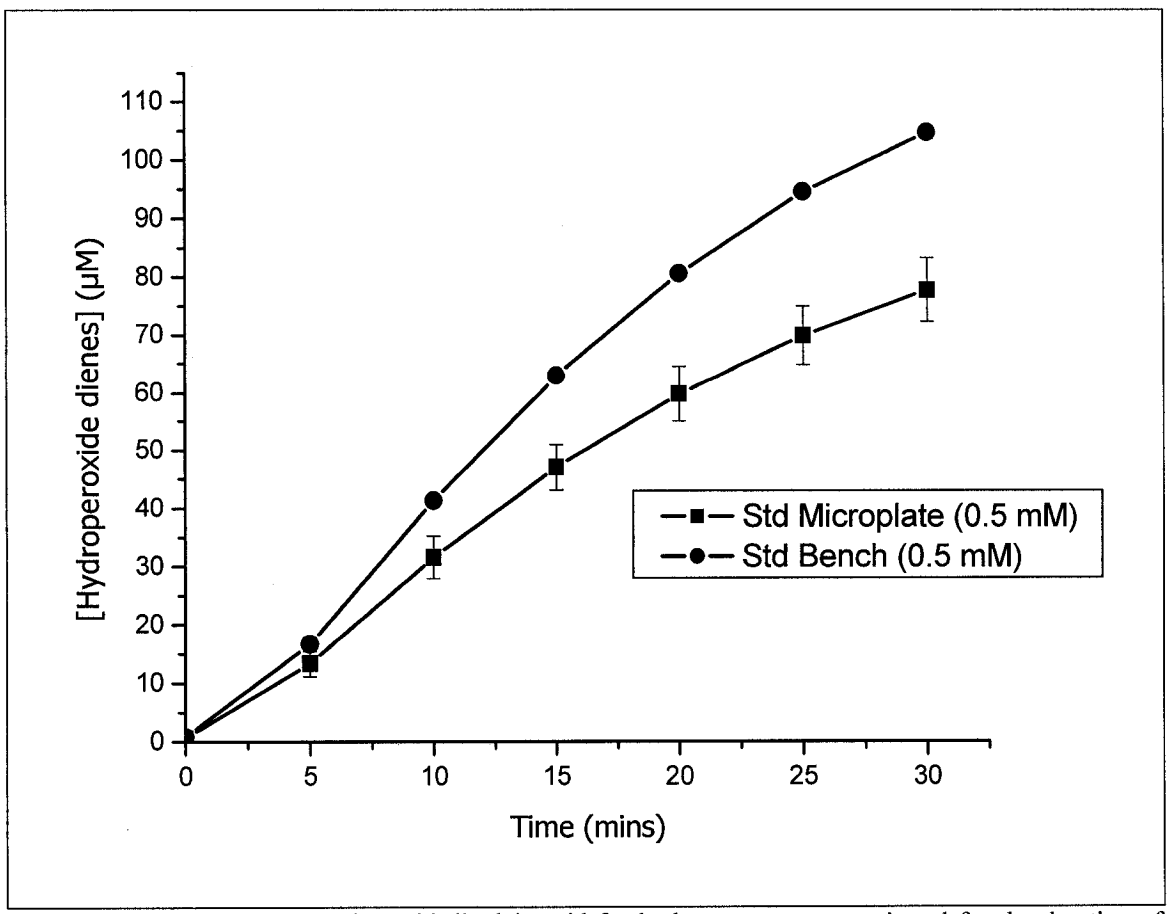

Figure 4.2. Lipoxygenase reacting with linoleic acid for both assays were monitored for the duration of thirty minutes at a wavelength of 234 nm, a characteristic wavelength for their product; hydroperoxide dienes. Quantitative examination of the reaction kinetics in terms of enzyme activity between the microplate and bench assay indicates that, the microplate assay has a slightly lower enzyme activity than that of the bench assay. The reaction rate in the first five minutes seems similar for both assays, but the concentration of hydroperoxide dienes determined at thirty minutes on the microplate assay is approximately 25% lower than obtained from the standard bench assay.

of substrate present in the reaction mixture for optimal functioning of LOX A. It is known that this enzyme does not follow typical Michaelis-Menten kinetics [59, 89]. The original microplate assay included linoleic acid substrate at a concentration of 0.5 mM in the reaction mixture. The concentration of hydroperoxide dienes at 15 minutes of reaction (time chosen arbitrarily) was approximately 47.1 μM when carried out over 8 replicates. At this same time interval, an increase in substrate concentration to 0.65 and 0.9 mM increased product formation to around 61.0 and 72.5 μM, respectively (Figure 4.3.) in the microplate assay. The 0.65 mM substrate loading, comparable to the bench assay product formation of circa 63.0 μM at this same time interval, indicates a similar level of sensitivity. These results are further confirmed by statistical analysis (Table 4.2.).

The t-values at 10, 20, and 30 minutes for 0.65 mM substrate were below that of the critical t-value indicating similarity to the bench assay, while for the 0.5 and 0.9 mM concentrations the t-values were significantly higher. These results indicate that the microplate assay developed for lipoxygenases at an optimized substrate concentration of 0.65 mM can replace the bench lipoxygenases assay.

4.1.3. Specificity of microplate assay in identifying lipoxygenases

In order to ascertain that the assay is specific to lipoxygenases, a cellulase and xylanase from *Trichoderma reesei* were chosen as negative controls. These enzyme controls also served another purpose in that this microplate assay is designed to screen fungal culture filtrates for secreted lipoxygenases. Fungal species are known to secrete large amounts of cellulases and hemicellulases. Therefore, the substrate for this assay must not react with these enzymes. The enzyme controls reacting with linoleic acid over 8 replicates were negatives, both t-tests were not statistically significant at the 95%

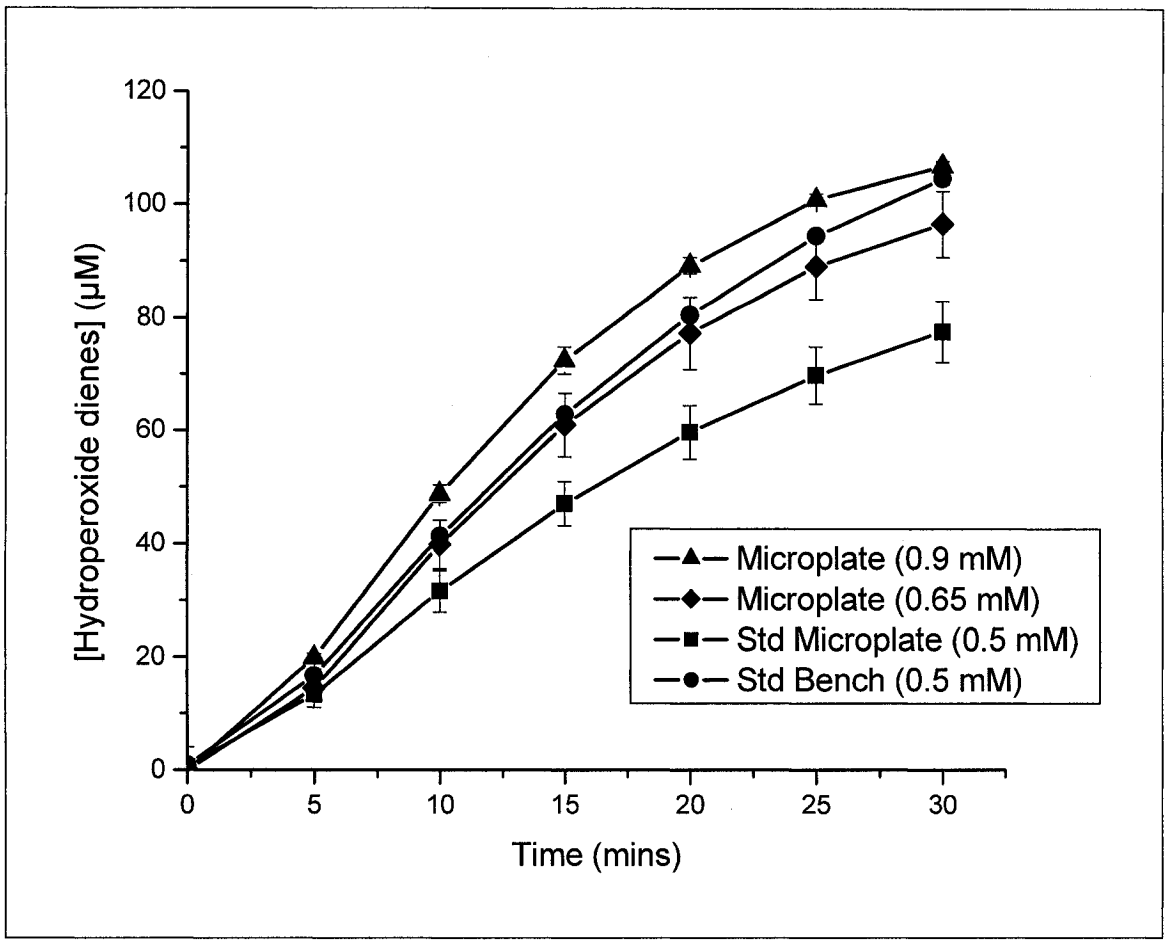

Figure 4.3. The microplate assay was optimized by increasing the concentration of linoleic acid to improve the sensitivity level of the microplate assay to that of the bench assay. The increase to 0.65 mM lead to a reaction kinetic profile similar to that of the bench assay, where each substrate plot was based on eight replicates determinations at the various substrate concentrations. A statistical kinetic analysis of these data on the microplate assay as compared to that of the bench assay was performed. It was determined that the 0.65 mM substrate loading of the microplate assay is statistically identical to that of the bench assay.

Table 4.2. A statistical analysis comparing the various substrate loadings on the microplate assay to those conditions on the standard bench assay for identification of the optimal substrate concentration needed for the microplate method to be similar. The t-values at 10, 20, and 30 minutes for 0.65 mM substrate were below that of the critical t-value indicating similarity to the bench assay, while for the 0.5 and 0.9 mM concentrations the t-values were significantly higher. These results indicate that at a 95% confidence interval the microplate assay developed for lipoxygenases at an optimized substrate concentration of 0.65 mM can replace the bench lipoxygenases assay.

Substrate Concentration (mM)	T-value (95% confidence interval)			N
	10 minutes	20 minutes	30 minutes	
0.5	7.43	12.4	13.8	8
0.65	***1.11**	***1.29**	***1.91**	8
0.9	13.6	19.7	6.79	8
t-test: *calculated t = 1.11, 1.29 & 1.91 <critical t value = 2.14, at P = 0.05, DF = 14				

confidence level (Table 4.3.). To further validate this screening process towards the goal of discovering lipoxygenases, other commercial lipoxygenases from soybean (LOX B), and potato (LOX C) were tested with this method. In 8 replicates both enzymes could be assayed by the microplate method with a statistical significance ($p<0.0001$) (Table 4.3.). Thus the developed microplate assay allows a rapid screening of potential lipoxygenases for their ability to degrade linoleic acid. This microplate assay is likely to be applicable for discovering other oxygenases than lipoxygenases that have the potential to degrade lipophilic wood extractives.

4.1.4. Testing for other oxidative enzymes in degrading linoleic acid

It was apparent that this assay is able to differentiate between lipoxygenases and hydrolytic enzymes which are potentially produced in fungal cultures. However, previous work has shown that some other oxidative enzymes can also cause lipid peroxidation. For example, fungal laccase was demonstrated to degrade trilinolein and manganese peroxidase was shown to promote the peroxidation of unsaturated fatty acids [39, 90, 91]. The examination of whether this microplate assay is capable of detecting these oxidative enzymes was performed. Three oxidative enzyme preparations were evaluated with this microplate assay: a *Trametes villosa* laccase (NS 51002), a *Myceliophthora thermophila* laccase (NS 51003) and a *Trametes versicolor* manganese peroxidase (MnP). In the testing of these oxidative enzymes, *T. villosa* laccase activity with linoleic acid was detected over 8 replicates with a statistical significance ($p<0.0001$) (Table 4.3.). However, the laccase from *M. thermophila*, and the MnP did not show any activity under these conditions (Table 4.3.). A previous study showed that the activity of laccase towards the degradation of trilinolein depends on the reduction potential of the specific

Table 4.3. To further validate this screening process towards the goal of discovering lipoxygenases, other commercial lipoxygenases from soybean (LOX B), and potato (LOX C) were tested with this method. In eight replicates both enzymes could be assayed by the microplate method with a statistical significance ($p<0.0001$). To verify the specificity of the microplate assay under optimized conditions, a cellulase and xylanase were chosen as negative controls. The enzyme controls did not react with linoleic acid over eight replicates ($p>0.05$). In the testing of other oxidative enzymes capability in degrading the substrate of this assay, three oxidative enzyme preparations were evaluated with this microplate assay: a *Trametes villosa* laccase, a *Myceliophthora thermophila* laccase and a *Trametes versicolor* manganese peroxidase (MnP). In the testing of these oxidative enzymes, *T. villosa* laccase activity with linoleic acid was detected over 8 replicates with a statistical significance ($p<0.0001$). However, the laccase from *M. thermophila*, and the MnP did not show any activity under these conditions ($p>0.05$).

Enzyme	Supplier	Optimum pH	P-value (n = 8)
LOX A (Soybean)	Sigma	9.0	Statistically Significant ($p<0.0001$)
LOX B (Soybean)	Sigma	9.0	Statistically Significant ($p<0.0001$)
LOX C (Potato)	Cayman	6.3	Statistically Significant ($p<0.0001$)
Cellulase (*Trichoderma reesei*)	Sigma	6.0	Not Statistically Significant ($p>0.05$)
Xylanase (*Trichoderma reesei*)	Iogen	6.0	Not Statistically Significant ($p>0.05$)
Laccase (*Trametes villosa*)	Novozyme	5.0	Statistically Significant ($p<0.0001$)
Laccase (*Myceliophthora thermophila*)	Novozyme	5.0	Not Statistically Significant ($p>0.05$)
Manganese Peroxidase (*Trametes versicolor*)	Paprican	5.0	Not Statistically Significant ($p>0.05$)

laccase [39]. *T. villosa* has a value of 780 mV, and *M. thermophila* is around 470 mV versus normal hydrogen electrode, respectively [92, 93]. The undetected absorption change during *M. thermophila* laccase reaction with linoleic acid substrate on the microplate assay format suggested a low activity of this enzyme owing to its low reduction potential. MnP also showed negative reaction by the assay, possibly due to the absence of hydrogen peroxide to initiate the reaction.

The ability to detect fungal laccases by this microplate assay extends its application to enzyme candidates other than lipoxygenases. However, the sensitivity of the assay towards lipoxygenases is significantly higher than those of laccases. In a comparison of enzyme activity per μg of protein over an initial two minutes of reaction, the LOX A had an absorption change of 0.343 compared to only 0.0261 for the *T. villosa* laccase.

4.2. Conclusion

The development of a microplate assay for determining lipoxygenase activity has been successful based on quantifying at 234 nm the hydroperoxide dienes derived from linoleic acid. The microplate assay can measure 96 samples simultaneously with a 10-fold reduction in reagents compared to the bench scale spectroscopy assay. The assay provided a quantitative measurement of lipoxygenases with a comparable sensitivity to the conventional assay. This assay may also be used to detect other types of oxidases capable of degrading extractives. The microplate assay therefore has the potential to allow high throughput screening of a number of oxygenases for their applications in pulp and paper manufacturing.

Chapter 5

Bleaching of Kraft Pulp by a Commercial Lipase, Lipase A "Amano" 12 ®

-- Accessory enzymes demonstrate specific activities toward degrading hexenuronic acids

In this chapter, a commercial lipase containing accessory enzymes was investigated for its potential to bleach kraft pulp to allow for high selectivity with a notable reduction in sugar released compared to commercial xylanase bleaching.

5.1. Results

5.1.1. Kappa number & hexenuronic acids measurements of kraft pulps

The kappa number is a value indicating relative lignin content. It determines the amount of bleach required in order to obtain a given degree of whiteness during digestion of wood pulp as part of the process of papermaking. The number is established by measuring the amount of a standard potassium permanganate solution that is absorbed by the respective pulp. Four different pulp samples were used in the study including unbleached hardwood kraft pulp (UBHW), oxygen delignified hardwood kraft pulp (O2HW), unbleached softwood kraft pulp (UBSW), oxygen delignified softwood kraft pulp (O2SW). These samples have different kappa numbers as well as hexenuronic acid contents (Table 5.1.). The kappa number of the four pulp samples varied from 7.6 to 31.8, while the hexenuronic acid content ranged from 18.5 to 34.9 millimole per kilogram of o.d. pulp (mmol/kg).

5.1.2. Optimum lipase A dosage for pre-bleaching stage

To find an optimum enzyme dosage for pulp treatment, a series of dosages were

Table 5.1. Four different pulp samples: unbleached hardwood kraft pulp (UBHW), oxygen delignified hardwood kraft pulp (O2HW), unbleached softwood kraft pulp (UBSW), and oxygen delignified softwood kraft pulp (O2SW) were used in the study. The kappa number of the four pulp samples varied from 7.6 to 31.8, and the hexenuronic acid content ranged from 18.5 to 34.9 millimole per kilogram of o.d. pulp (mmol/kg).

Type of Kraft Pulp	Kappa Number	Hexenuronic Acid (mmol/kg)
Unbleached Hardwood KP (UBHW)	14.5	34.9
Unbleached Softwood KP (UBSW)	31.8	21.8
Hardwood-O_2 Delignification (O2HW)	7.6	33.5
Softwood-O_2 Delignification (O2SW)	14.5	18.5

tested from 0 to 13 milligrams of protein from lipase A per gram of oven dried pulp (mg/g). The residual kappa number and hexenuronic acid content were determined after two hour treatments. As shown in Figure 5.1, a 2-hour lipase treatment of UBHW resulted in a notable reduction of both kappa number and hexenuronic acid content at all enzyme dosages tested. A high enzyme loading of 13 mg reduced kappa number by about 2.5 points and hexenuronic acid content by almost 9 mmol/kg, while a low enzyme addition 0.26 mg reduced kappa number by 1.3 and hexenuronic acid content by 2.3 mmol/kg. The most notable reduction occurred at an enzyme dosage of 1.3 mg and higher, therefore this dosage (1.3 mg) was selected for the subsequent experiments. The ability of lipase A to reduce kappa and hexenuronic acid in the four pulp samples was then determined (Table 5.2.). It is evident that the lipase A treatment had a positive bleaching effect on these kraft pulps compared to control experiments. The percent reduction in kappa number and hexenuronic acid content is shown in bracket in the table. Comparing hardwood and softwood kraft pulps over eight replicates, the lipase A is more effective to reduce the kappa number of hardwood kraft pulp. The kappa removal after one-step lipase A treatment for hardwood and softwood kraft pulps is between 11.8-12% and 2-10% respectively. The enzyme exhibited a high reactivity towards hexenuronic acid in all four pulps with reduction ranging from 13% to 24.4%. It is apparent that the enzyme is more reactive towards oxygen delignified pulps. Lipase A treatment degraded 16.7 % and 24.4% of hexenuronic acid present in O2HW and O2SW, respectively compared to a reduction between 13% and 16% in brownstock pulps (UBSW and UBHW).

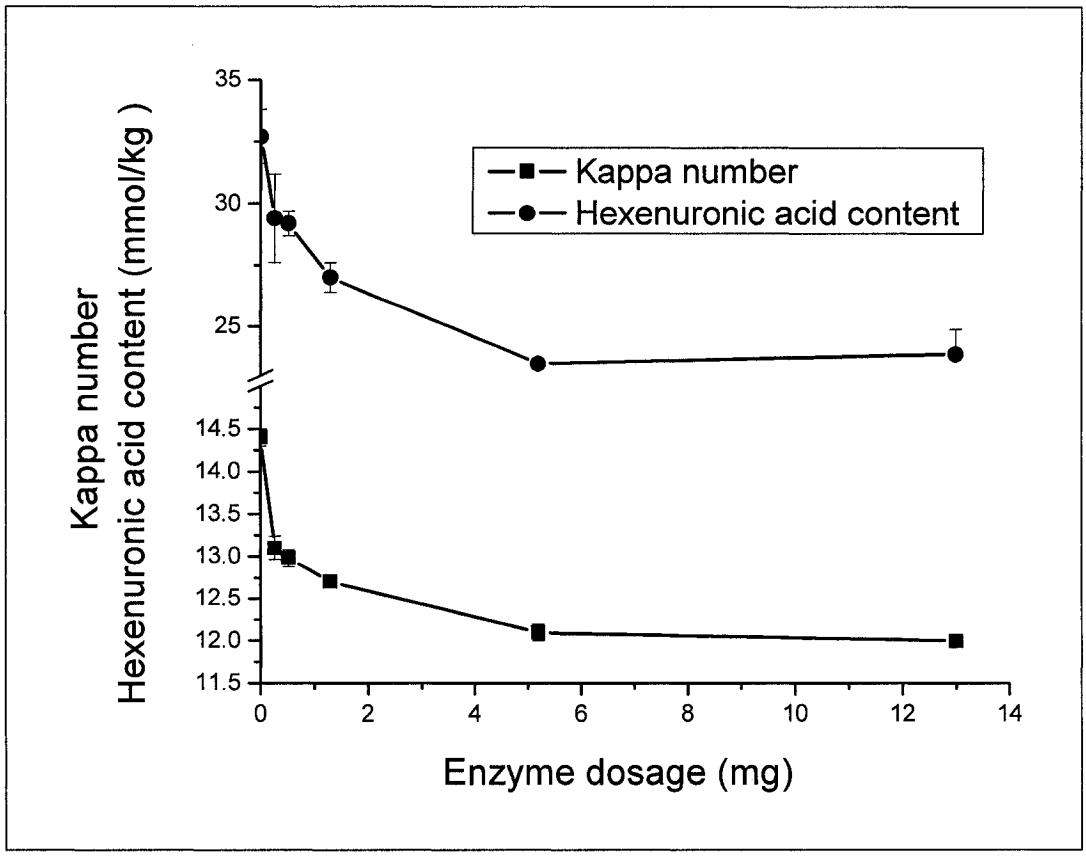

Figure 5.1. A series of enzyme dosages were tested from 0 to 13 milligrams of protein from lipase A per gram of oven dried pulp (mg/g) to find an optimum enzyme dosage for pulp treatment, The residual kappa number and hexenuronic acid content were determined after two hour treatments of UBHW. There was a notable reduction of both kappa number and hexenuronic acid content at all enzyme dosages tested. A high enzyme loading of 13 mg reduced kappa number by about 2.5 points and hexenuronic acid content by almost 9 mmol/kg, while a low enzyme addition 0.26 mg reduced kappa number by 1.3 and hexenuronic acid content by 2.3 mmol/kg. The most notable reduction occurred at an enzyme dosage of 1.3 mg therefore this dosage was selected for subsequent experiments.

Table 5.2. At a lipase A dosage of 1.3 mg, the ability of lipase A to reduce kappa and hexenuronic acid in the four pulp samples was determined. The percent reduction in kappa number and hexenuronic acid content is shown in bracket in the table. Comparing hardwood and softwood kraft pulps over eight replicates, the lipase A is more effective at reducing the kappa number of hardwood kraft pulp. The kappa removal after one-step lipase A treatment for hardwood and softwood kraft pulps is between 11.8-12% and 2-10% respectively. The enzyme exhibited a high reactivity towards hexenuronic acid in all four pulps with reduction ranging from 13% to 24.4%. Lipase A treatment degraded 16.7 % and 24.4% of hexenuronic acid present in O2HW and O2SW, respectively compared to a reduction between 13% and 16% in brownstock pulps (UBSW and UBHW). The enzyme is more reactive towards oxygen delignified pulps.

Type of Kraft Pulp	Treatment	Kappa Number (percent decreased)	Hexenuronic Acid (mmol/kg) (percent decreased)
UBHW	Control	14.4	32.7
	Lipase	12.7 (11.8 %)	27.5 (15.9 %)
UBSW	Control	31.7	19.3
	Lipase	31.1 (1.9 %)	16.8 (13 %)
O2HW	Control	7.5	32.3
	Lipase	6.6 (12 %)	26.9 (16.7 %)
O2SW	Control	14.3	19.7
	Lipase	12.8 (10.5 %)	14.9 (24.4 %)

5.1.3. Purified accessory enzymes for pre-bleaching stage

In parallel experiments, several purified accessory enzymes including feruloyl esterases and arabinofuranosidases obtained from *A. pullulans* and *A. niger* were used for pulp or cooked xylan (model substrate: xylan containing hexenuronic acid group) treatment to determine their efficiency in lignin reduction and/or degradation of hexenuronic acid. The enzyme treatments lead to no decrease in either of these compounds (Table 5.3.). A number of reasons for this lack of bleaching activity could be because of the protein purification process not functioning, the limiting amount of enzymes where the dosage is not optimum, or perhaps synergism is needed between two or more of these accessory enzymes with xylanase for optimal bleaching performance. This consequently led to further studies on the lipase A preparation.

5.1.4. Comparison of commercial lipase and commercial xylanase bleaching potency

A comparison between the lipase A with a xylanase, which is commercially used for pulp bleaching was next. The xylanase was obtained from Iogen and has an optimum enzyme loading for kraft pulp bleaching previously determined at 1.3 xylanase units per gram of oven dried pulp (XU/g). A two-hour treatment of UBHW by Iogen xylanase did not bring any changes to either pulp kappa number or hexenuronic acid content with only a slight reduction of 0.6 kappa and 1.6 mmol/kg respectively compared to the control sample. However, the same treatment with lipase A at an equivalent xylanase activity had a superior bleaching ability where the kappa number was decreased by 1.5 kappa and hexenuronic acid content by 5.2 mmol/kg from the same control. The xylanase treatment was also carried out on the other three pulp samples, O2HW, UBSW and O2SW; no

Table 5.3. Purified accessory enzymes of feruloyl esterases and arabinofuranosidases obtained from *A. pullulans* and *A. niger* were used for pulp or cooked xylan treatment to determine their efficiency in lignin reduction and/or degradation of hexenuronic acid. These enzyme treatments lead to no decrease in either of these compounds.

	Pulp	Cooked Xylan
Feruloyl Esterase 1 *A. pullulans*	No effect	--
Feruloyl Esterase 2 *A. niger*	No effect	--
Feruloyl Esterase 3 *A. niger*	No effect	--
Arabinofuranosidase 1 *A. pullulans*	No effect	--
Arabinofuranosidase 2 *A. niger*	--	No effect

reductions in kappa number and hexenuronic acid content were observed.

To determine the effects of lipase A and xylanase treatment on pulp bleachability, a DE bleaching sequence was carried out following enzyme treatments of UBHW. Three levels of active chlorine multiples, 0.16, 0.21 and 0.26, were used in D stage. As shown in Figures 5.2, and 5.3, both lipase A and xylanase pretreatments resulted in bleached pulps with lower kappa number and hexenuronic acid content compared to those obtained from control experiments. Lipase A was apparently more effective than the xylanase towards bleaching kraft pulp at all ACM levels. Bleaching of lipase A pretreated UBHW using 0.16 ACM resulted in similar kappa number and hexenuronic acid content to those obtained from control bleaching using 0.26 ACM. Lipase A exhibited a superior bleaching ability to commercial xylanase with lower kappa number and hexenuronic acid content obtained after DE bleaching at all three ACM levels. However, the crude lipase A solution contains several enzyme activities. To further determine the active enzyme components that contribute to this superior bleaching effect, the crude lipase mixture was fractionated by size exclusion chromatography and showed three major protein fractions. The majority of the protein (about 90% based on protein content) was collected as fraction 1 which showed predominantly lipase activity with minor xylanase activity. Two other fractions, fractions 2 and 3, were collected later in the column separation. The enzyme activities present in the three fractions were determined and compared with Iogen xylanase (Table 5.4). Both fraction 2 and 3 demonstrated xylanase activity when birch xylan was used as substrate. The specific xylanase activities in either fraction 2 or 3 were considerably lower than that of Iogen xylanase. There is feruloyl esterase activity detected in fraction 2 (7.5 U per milligram of enzyme (U/mg)) while the same enzyme

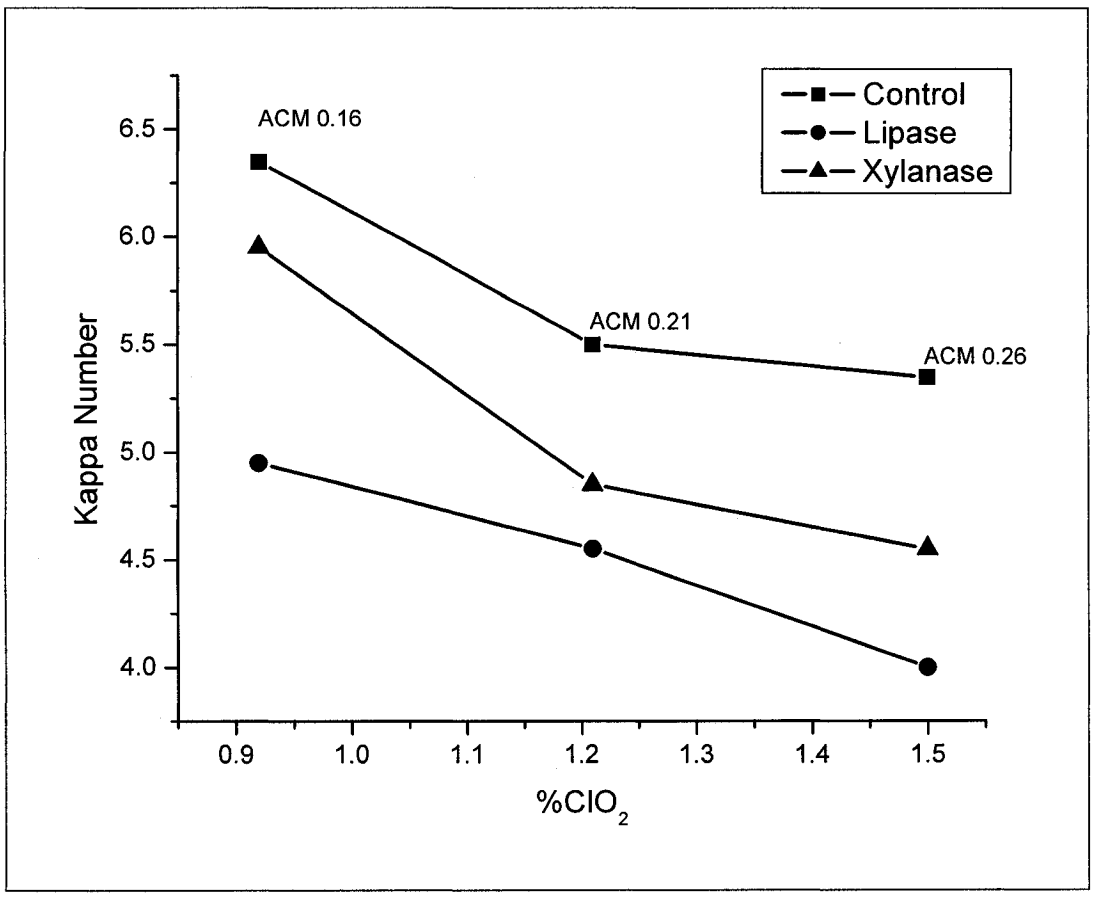

Figure 5.2. A DE bleaching sequence was carried out following enzyme treatments of UBHW to determine the effects of lipase A and xylanase treatment on pulp bleachability. Three levels of active chlorine multiples, 0.16, 0.21 and 0.26, were used in D stage. Lipase A exhibited a superior bleaching ability to commercial xylanase with lower kappa number obtained after DE bleaching at all three ACM levels compared to those obtained from control experiments.

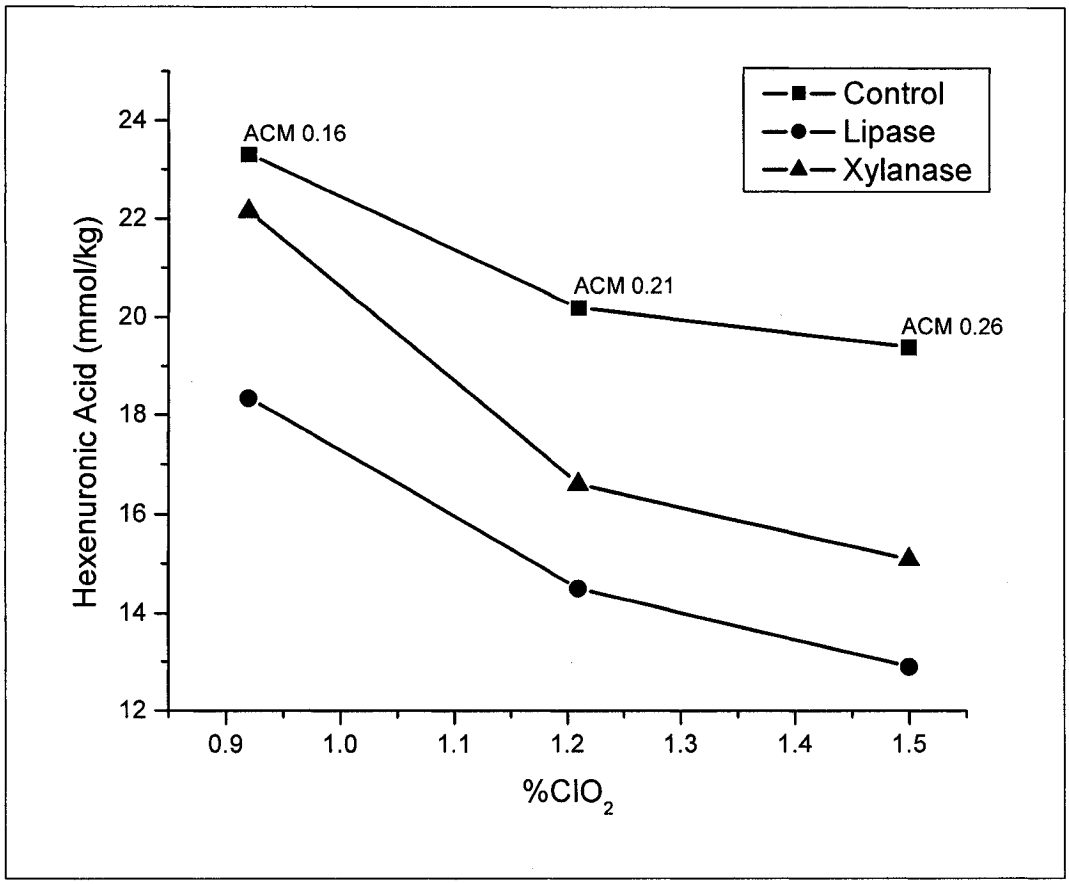

Figure 5.3. A DE bleaching sequence was carried out following enzyme treatments of UBHW to determine the effects of lipase A and xylanase treatment on pulp bleachability. Three levels of active chlorine multiples, 0.16, 0.21 and 0.26, were used in D stage. Lipase A exhibited a superior bleaching ability to commercial xylanase with lower hexenuronic acid content obtained after DE bleaching at all three ACM levels compared to those obtained from control experiments.

Table 5.4. To determine the active enzyme components in Lipase A that contribute to this bleaching effect, the crude lipase mixture was fractionated by size exclusion chromatography and showed three major protein fractions. The majority of the protein constituting about 90% based on protein content was collected as fraction 1 that contained lipase activity (5.8 U per milligram of enzyme (U/mg)) with minor xylanase activity. Two other fractions, fractions 2 and 3, were collected. Both fraction 2 and 3 had xylanase activity when birch xylan was used as substrate, where the specific xylanase activities in either fraction 2 (21 U/mg) or 3 (72 U/mg) was lower than that of Iogen xylanase (670 U/mg). There is feruloyl esterase activity detected in fraction 2 (7.5 U/mg)) while the same enzyme activity in fraction 3 is 1.6 U/mg. Feruloyl esterase activity was not detected in Iogen xylanase. Fraction 2 also exhibited a higher arabinofuranosidase activity (8.7 mU/mg) than either lipase A fraction 3 (2.9 mU/mg) or Iogen xylanase (4.0 mU/mg). There was little accessory activity detected in lipase A fraction 1.

Sample	Lipase pH 6.5 (U/mg)	Feruloyl Esterase pH 6.5 (U/mg)	Arabino-furanosidase pH 6.5 (mU/mg)	Xylanase pH 6.5 (U/mg)
Lip Fraction 1	5.8	ND	ND	ND
Lip Fraction 2	0.04	7.5	8.7	21
Lip Fraction 3	0.2	1.6	2.9	72
Iogen Xylanase	ND	ND	4.0	670

*Not detected

activity in fraction 3 is 1.6 U/mg. Feruloyl esterase activity was not detected in Iogen xylanase. Fraction 2 also exhibited a higher arabinofuranosidase activity that either lipase A fraction 3 or Iogen xylanase. There was little accessory activity detected in lipase A fraction 1.

To compare the bleaching efficiency of the three lipase A fractions with Iogen xylanase, a treatment of the UBHW with these four enzymes fractions based on the same protein content was performed. Table 5.5, clearly demonstrated a bleaching effect for lipase fraction 2 and 3 treatments. At the same enzyme protein content, fraction 2 displayed the highest capability to remove hexenuronic acid and reduce pulp kappa number. Fraction 3 was also effective for removing of hexenuronic acid and reducing kappa number. Neither fraction 1 nor Iogen xylanase has a bleaching effect.

5.1.5. Selectivity of commercial lipase fractions

The selectivity of these enzymes or enzyme fractions for biobleaching was also tested by determining the amount and type of sugars released after enzyme treatment of UBHW. As seen in Table 5.6., a two-hour xylanase treatment released about 1.2 g/L of sugars from pulp during bleaching of 10 g of UBHW at 10% consistency into the filtrate. There was also a considerable amount of sugar hydrolyzed after lipase A fraction 2 treatment about 1.1 g/L, while only a small amount, 0.27 g/L, was released from pulp after fraction 3 treatments. The results indicate that highly selective enzymes for pulp bleaching are likely present in lipase A fractions 2 and 3.

5.2. Discussion

Finding a more selective chemical or enzyme treatments for kraft pulp bleaching has been a major challenge for pulp and paper manufacturing. The ability of accessory

Table 5.5. To compare the bleaching efficiency of the three lipase A fractions with Iogen xylanase, a treatment of the UBHW with these four enzymes fractions based on the same protein content was performed. At the same enzyme protein content, fraction 2 displayed the highest capability to remove hexenuronic acid and reduce pulp kappa number. Fraction 3 was also effective for removing of hexenuronic acid and reducing kappa number. Neither fraction 1 nor Iogen xylanase has a bleaching effect.

Sample	Kappa Number	Hexenuronic Acid (mmol/kg)
Control	14.4	32.7
Xylanase	13.8	31.1
Fraction 1	14.4	32.1
Fraction 2	12.3	26.5
Fraction 3	12.7	27.8

Table 5.6. The selectivity of these enzyme fractions for biobleaching was also tested by determining the amount and type of sugars released after enzyme treatment of UBHW. A two-hour xylanase treatment released about 1.2 g/L of sugars from pulp during bleaching of 10 g of UBHW at 10% consistency into the filtrate. A considerable amount of sugar hydrolyzed after lipase A fraction 2 treatment about 1.1 g/L, while only a small amount, 0.27 g/L, was released from pulp after fraction 3 treatments. The results indicate that highly selective enzymes for pulp bleaching are likely present in lipase A fractions 2 and 3.

	Sugars released mg/L				
Sample	Arabinose	Galactose	Glucose	Xylose	Mannose
Lipase Fraction 2	ND	27	50	1080	50
Lipase Fraction 3	ND	ND	ND	270	ND
Iogen Xylanase	ND	ND	140	1200	ND

enzymes to debranch hemicellulose side groups that may interact with residual lignin promises selective delignification [94-96]. A recent study tried to demonstrate the potential of a feruloyl esterase from *Aspergillus niger* for bleaching wheat straw Soda AQ pulp [96]. However, the results presented in the paper were not sufficient to conclude that this enzyme had a direct bleaching capability. The authors compared the bleaching effects by calculating the kappa reduction obtained after alkaline extraction (E stage) versus original pulp kappa prior to E stage. However, when the kappa numbers of different samples were compared after E stage, there was only a marginal difference between control pulp and feruloyl esterase treated pulp. A number of other previous attempts using pure accessory enzymes originating from genetically modified microorganisms did not lead to any major breakthrough finding to confirm the bleaching effects of these enzymes on wood pulp. Moreover, in the current study purified alpha-glucuronidases and feruloyl esterases from *A. pullulans* and *A. niger* were used for bleaching experiments (Table 5.3.). These accessory enzymes on their own did not have the ability to decrease lignin or hexenuronic acid content. However, past studies have indicated that synergism is needed amongst this group of enzyme along with xylanase for bleaching [97]. The latter amount of purified accessory enzymes were not sufficient for a synergistic analysis, therefore in the present research, a commercial lipase enzyme was chosen that has been previously determined to contain a high feruloyl esterase activity [57]. The notable reduction of kappa number and hexenuronic acid content after lipase A treatment of different kraft pulps suggested that feruloyl esterase or other accessory enzymes may have a direct bleaching effect. The presence of diferulic acid bridges between polysaccharides and lignin in cell wall has been demonstrated in many plants.

The presence of linkages between lignin and glucuronic acid attached to the xylan backbone was also identified in wood [98]. The kappa number was originally proposed to represent the amount of residual lignin in pulp. Recent studies have indicated that hexenuronic acid also contributes to kappa number. Hexenuronic acid is formed from 4-methyl-glucuronic acid residues present on xylan during kraft pulping [7]. This acid group has been found to be as reactive as lignin towards electrophilic bleaching chemicals [49], contributing to an increase in bleaching chemical consumption, decreased brightness, increased brightness reversion and poor metal removal. Previous work has proposed that the hexenuronic acid moiety is likely to be a site in kraft pulps where lignin-carbohydrate linkages are formed [50, 51]. Using an empirical equation previously proposed [99] where 11.6 mmol hexenuronic acid/kg corresponds to 1 kappa number unit, it became apparent that the hexenuronic acids represent between 5%-37% of the kappa measured on the four kraft pulp samples. The role of hexenuronic acids in kappa number measurement is much less in softwood pulp than those in hardwood pulp. It was also clear that oxygen delignification did not degrade hexenuronic acids but rather leads to an increase percentage of hexenuronic acids contribution to the pulp kappa number.

As mentioned, lipase A treatments led to a reduction of hexenuronic acids in a range of 13% to 24.4% in four kraft pulps. When the reduction in hexenuronic acids is calculated based on its percentage of kappa number, it was very interesting to find that the reduction in hexenuronic acids represents 26%, 36%, 51% and 27% of the kappa number removal in respective UBHW, UBSW, O2HW and O2SW. The ratio of molar consumption of permanganate between one hexenuronic acid and phenylpropane unit is about 1 to 1.5 [100], which means one mole hexenuronic acid can contribute up to 40%

of the total kappa number when linking with one mole of lignin molecule. The presence of hexenuronic acid and lignin complex has been proposed previously [51], and such a structure has been thought to be the main reason for the recalcitrance of residual lignin. Of course, in this complex, one hexenuronic acid group will be attached to more than one lignin molecule. Therefore, the results of hexenuronic acid reduction implied that the lipase A specifically acted on degrading hexenuronic acids which in consequence remove the lignin attached on these acids. This is the first evidence showing that an enzyme can specifically remove hexenuronic acids. These results also substantiate the early theory that hexenuronic acid is a site for the formation of the LCC complex [50, 51].

While, a one-step enzyme treatment can notably decrease kappa number of UBHW, O2HW and O2SW, lipase A also demonstrated a better bleachability than the commercial xylanase following subsequent chemical bleaching processes. Bleaching lipase A pretreated UBHW by a DE sequence resulted in a chlorine dioxide saving of up to 38 % compared to control. This saving is much more than can be obtained by xylanase prebleaching. Fractionation of lipase A into its sub-fractions enabled us to further identify the active enzyme constituents present in the crude enzyme solution. Three main fractions were obtained after size exclusion chromatography separation. The first fraction (FRC1) contains the majority of the protein from the enzyme and showed a higher lipase activity with a trace of xylanase activity (Table 5.4.). Two subsequent fractions (FRC2 and FRC3) obtained showed negligible lipase activity, while containing several accessory activities. There is also an appreciable amount of xylanase activity detected in both fractions, but much lower than in Iogen xylanase.

The feruloyl esterase activity present in Amano lipase A enzyme was previously determined by using either 4-nitrophenyl ferulate (4NPF) or ethyl ferulate as substrate. The specific activity of lipase A on 4NPF was about 1.1U/mg, while its activity on ethyl ferulate was 0.411U/mg. Fractions 2 and 3 from lipase A exhibited high feruloyl esterase activities of 7.5 and 1.7 U/mg respectively using 4 NPF as substrate. A similar bleaching effect was obtained from fraction 2 and 3 treatment of UBHW. This result further substantiated that feruloyl esterase or its related enzymes may help enhance wood pulp bleachability. The lack of feruloyl esterase activity in Iogen xylanase is the most probable reason for its relative low bleaching efficiency. A noticeable amount of arabinofuranosidase activity was detected in both fractions 2 and 3 as well as in Iogen xylanase. The arabinofuranosidase has been shown to act synergistically with xylanase in bleaching of wood pulp [101, 102]. However, its reaction with hexenuronic acid deserves further study.

Bleaching selectivity is considered as one of the most important criteria for a superior bleaching enzyme. To determine the selectivity of each enzyme and enzyme fraction, the amount of sugar release in the filtrates was measured after enzymatic treatment of UBHW. As shown in Table 5.6, Iogen xylanase treatment released a considerable amount of xylose with some glucose. It is not clear whether the glucose is hydrolyzed from cellulose or glucomannan. It is known that this xylanase contains some endoglucanase activity as measured by CMC assay. Lipase fraction 2 treatment also solubilized an appreciable amount of xylose with some galactose, glucose and mannose. It deserves to be mentioned that these enzyme fractions are not pure after one step column separation, several protein bands were detected in all three fractions by

polyacrylamide gel electrophoresis. The release of galactose, glucose and mannose suggest that fraction 2 also acted on glucomannan present in kraft pulp. The lipase fraction 3 gave the most promising results in terms of sugar degradation. Although, this fraction appears to have a higher xylanase activity based on the DNS assay, it only released a small amount of xylose. The DNS assay using soluble birch xylan as a substrate is a well accepted method for measuring xylanase activity, however, it does not necessarily predict the true hydrolytic potential of the enzyme on isolatable substrate. [103-105]. The ability of lipase fraction 3 to bleach kraft pulp with minimum hemicellulose degradation reaffirms the presence of a highly specific enzyme in lipase A for removing xyloses associated with either residual lignin and/or hexenuronic acids. Examining the effects of these fractions on pulp bleaching will be included in our future work. Further study is required to identify the key enzyme components present in the Lipase A mixture and to elucidate the mechanism of these enzyme reactions on LCC and hexenuronic acid-xylan model compounds.

5.3. Conclusion

A commercial lipase, lipase A "Amano" 12 ® enzyme can be used to bleach kraft pulp and has a superior bleaching efficiency to commercial xylanase. The enzyme demonstrated a specific activity toward degrading hexenuronic acids and subsequent release of lignin that are attached on these acid groups. The presence of accessory enzyme activities including feruloyl esterase and arabinofuranosidase are likely to be the major factors contributing to its superior bleaching. The results from this study present the first evidence to demonstrate that accessory enzymes can have a direct bleaching

effect, removing hexenuronic acids and reducing kappa number present in wood pulp. The application of accessory enzymes promises a selective biobleaching strategy for pulp and paper industry.

Chapter 6

Summary and Conclusion

The pulp and paper industry is a large consumer of resources mainly in wood chips, chemicals, water, and energy. Innovative methods are needed to use these resources more efficiently and with fewer environmental consequences. Currently, biotechnology has the potential to offer a wide range of effective, sustainable processes for the pulp and paper industry to complement and/or enhance their conventional operations. My thesis research has explored and assessed the application of enzymes in this area. Soybean lipoxygenase two hour pulp treatments at both 1% and 12% consistency of TMP resulted in a 15% reduction in total wood extractives with specificity towards lipophilic extractives primarily consisting of fatty acids and their esters. This discovery could have a potential direct impact on pulp and paper manufacturing. However, during this study it was found that several extractive compounds such as resin acids and lignans inhibited lipoxygenase catalyzed reactions. It would be of interest therefore to further investigate similar enzymes that are not inhibited in this area to allow for a higher degree of reactivity with problematic wood extractives. Another area worthy of further investigation is in the effects of transition metal ions in the TMP. The presence of manganese in particular, may potentially contribute to a more rapid reaction by the enzyme due to the fragmentation of fatty acid hydroperoxides, its products from reactions with wood extractives. In the successful application of the latter enzyme, a part of this thesis was devoted to developing a 96-well UV microplate assay to screen enzymes from fungal species for a better and more cost-effective alternative lipoxygenase. A number of

hydrolytic enzymes, and other types of oxidases were also tested by this protocol to examine the specificity of the assay. The results indicate that the developed microplate assay may aid genomic researchers in providing an inexpensive method for accelerated screening of a large number of enzymes to identify potential lipoxygenases and other oxidative enzymes with specific action in degrading wood extractives. An area that needs further exploration on this method would be in the identification of novel fungal oxidative enzymes through respective culture filtrates. These potential biocatalysts would be more accessible than their commercially available counterparts, and it follows with a higher probability of directly impacting treatment of wood extractives at mills. The microplate assay has the potential for reducing costs by discovering new, more efficient enzymes. In xylolytic/accessory enzymes for a more efficient and environmentally conscious bleaching process, a commercial lipase, lipase A "Amano" 12® was investigated for its potential to bleach kraft pulp. Enzymatic treatment of a variety of hardwood and softwood kraft pulps resulted in a notable reduction of kappa number and hexenuronic acids content lending to a considerable savings of chlorine dioxide compared to a control bleaching sequence. Two enzyme fractions containing high accessory enzymes activities were obtained after size exclusion chromatography, and demonstrated a high bleaching efficiency as well as a high selectivity with a notable reduction in sugar released compared to commercial xylanase bleaching. Further experiments that would be worth investigating are the isolation of the enzyme component(s) in these two fractions involved in the selective biobleaching. This could be done through an improved purification scheme, where each enzyme could be identified through LC-MS, and thereafter each enzyme or various combinations could be tested

with pulps to determine the mechanism of selective biobleaching. This may lead to the discovery of a novel and improved enzyme(s) to replace conventional xylanase for biobleaching in the pulp and paper industry.

Research in this area has not reached its fullest potential, biotechnology remains an expanding, exciting, and promising field of study for both academic research and industrial development in pulp and paper. It is evident that this industry has the potential to take advantage of these opportunities to improve the efficiency of pulp and paper processes.

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Appendix

Sections of this thesis are in the following publications:

1. Zhang, X., **Nguyen, D.**, Paice, M.G., Tsang, A. and Renaud, S. Degradation of wood extractives in thermo-mechanical pulp by soybean lipoxygenase. Paprican Research Report (PRR 1775) and journal Enzyme and Microbial Technology, in press (available online 4 July 2006).

2. **Nguyen, D.,** Zhang, X., Paice, M.G., Tsang, A. and Renaud, S. Microplate enzyme assay for screening lipoxygenases to degrade wood extractives. Paprican University Report (PUR 891) and journal Biocatalysis and Biotransformation, acceptance.

3. Zhang, X., **Nguyen, D.,** Jiang, Z-H., Audet, A., Paice, M.G., Renaud, S. and Tsang, A. Bleaching of kraft pulp by a commercial lipase: accessory enzymes degrade hexenuronic acids. Paprican Research Report (PRR 1819).

4. Zhang, X., **Nguyen, D.,** Paice, M.G., Tsang, A. and Renaud, S. Application of lipoxygenase to degrade wood extractives in thermomechanical pulp. *Proceedings* of the 60th Annual Pacific Coast Branch Pulp and Paper Technical Association of Canada Conference. Parksville, British Columbia, Canada, April 21-22, 2006.

5. **Nguyen, D.,** Zhang, X., Paice, M.G., Tsang, A. and Renaud, S. Microplate enzyme assay for screening lipoxygenases to degrade wood extractives.

Proceedings of the 5th International Non-Wood Fiber Pulping and Papermaking Conference (5th INWFPPC) incorporating 3rd International Symposium on Emerging Technologies of Pulping and Papermaking (3rd ISETPP). Guangzhou, China, State Key Laboratory of Pulp and Paper Engineering and South China University of Technology, November 8-10, 2006.