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A BIOMIMICKING APPROACH FOR HEMICELLULOSE PROCESSING

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"If I had asked my customers what they wanted, they would have told me ´faster horses´."

H. Ford

ABSTRACT

Lignocellulose can become the best opportunity for the society to reduce its dependency on the harmful petroleum based products as well as to produce clean energy. In each part of the production cycle, biomass based products have a better environmental profiles than their petroleum based counterparts. Woody biomass has a vast availability, but it suffers from recalcitrance that is mostly caused by lignin that is functioning as a matrix, surrounding and binding the carbohydrates that are currently the most valuable of the wood components.

Lignin-carbohydrate (LC) bonds are believed to be a key element in this recalcitrance and research has shown that these types of bonds are common in wood. These bonds are important in an economical point of view as well, as e.g. residual lignin structures in pulp (lignins bonded to the cellulose and hemicelluloses) require expensive bleaching sequences for their removal.

The LC-structures can also be exploited technically as we now have demonstrated. We developed a method that utilizes phenolic end groups that are bonded to different hemicelluloses for cross-linking. The enzyme laccase was used for the cross-linking to create a cost-efficient processing scheme to both isolate and increase the molecular weight of the hemicelluloses. Membrane filtration was used as the key separation technique, which enables the establishment of industrial scale production. The final product had improved mechanical and thermal properties and could be used e.g. as barrier film component in renewable packaging. Nanocomposite formation with nanofibrillated cellulose was also studied. This improved the film properties further. The complexes are also possible to use as model compounds for lignin-carbohydrate complexes in wood.

This technique can also be seen to mimic the lignification and lignin-carbohydrate network formation phenomena in plants enabling the formation of entire networks of wood components. Our results suggests that the side chains of hemicellulose might play an important role in network formation and that hemicellulose molecules can carry more than one lignin phenolic end group to fulfill this capability.

Sammanfattning

Lignocellulosa kan bli den bästa möjligheten för samhället att minska beroendet av skadliga petroleumbaserade produkter, liksom för att producera miljövänlig energi. I varje del av produktionscykeln har biomassebaserade produkter en bättre miljömässig profil än deras petroleumbaserade motsvarigheter. Vedbiomassa finns i mycket stor mängd, men är svårbearbetat för vissa processer till stor del beroende på ligninet som fungerar som en matrix, som omger och sammanbinder de kolhydrater som för närvarande betraktas som mest värdefulla av vedkomponenterna.

Kovalenta bindningar mellan lignin och kolhydrater (LC) tros vara en nyckelfaktor för vedens motståndskraft, och forskning visar att dessa typer av bindningar är vanliga i ved. Bindningarna är vidare viktiga ur ett ekonomiskt perspektiv, då ligninstrukturer i vedmassa (ligninet bundet till cellulosa och hemicellulosa) kräver dyra bleksekvenser för att avlägsnas.

LC-strukturerna kan också användas tekniskt, som visas i detta arbete. Vi har utvecklat en metod for att använda fenoliska ändgrupper bundna till olika hemicellulosor för att tvärbinda dessa. Enzymet lackas användes för tvärbindningen i ett kostnadseffektivt processchema för att isolera och öka molekylvikten på hemicellulosa. Membranfiltrering utgör här ett nyckelsteg, som är tillämpbart i industriell skala. Produkten hade förbättrade mekaniska och termiska egenskaper, och visades kunna användas för barriärfilmer i förpackningar gjorda av förnyelsebara material, inklusive kompositer med nanocellulosa, där kompositbildningen ytterligare förbättrade egenskaperna. Komplexen kan också användas som modelkomponenter för lignin-kolhydratnätverk i ved.

Tekniken kan också ses som en modell för hur lignifiering och bildning av lignin-kolhydratnätverk sker i växter, vilket leder till bildning av stora nätverk av olika vedkomponenter. Våra resultat föreslår att sidogrupperna på hemicellulosor spelar en viktig roll för detta, och att hemicellulosamolekyler kan bära mer än en fenolisk ligninändgrupp, som kan delta i nätverksbildandet.

LIST OF PAPERS
This thesis is a summary of the following papers:

I. Oinonen P., Areskogh D. and Henriksson G. (2013) Enzyme catalyzed cross-linking of spruce galactoglucomannan improves its applicability in barrier films. Carbohydrate Polymers, 95, 690-696.

II. Oinonen P., Zhang L., Lawoko M. and Henriksson G. (2014) On the formation of lignin-polysaccharide networks in Norway spruce. Manuscript

III. Bi R., Oinonen P., Wang Y. and Henriksson G. (2014) A method for studying effects on lignin-polysaccharide networks during degradation ad technical processing of wood. Manuscript

IV. Oinonen P., Zhang L., Lawoko M. and Henriksson G. (2014) Structural studies of an enzymatically produced lignin-carbohydrate complex. Manuscript

V. Oinonen P., Moriana R., Krawczyk H. and Henriksson G. (2014) The composite formation of cross-linked galactoglucomannan-lignin networks and cellulose nanoparticles as defined by thermal and mechanical testing. Manuscript

VI. Krawczyk H., Oinonen P. and Jönsson A-S. (2013) Combined membrane filtration and enzymatic treatment for recovery of high molecular mass hemicelluloses from chemithermomechanical pulp process water. Chemical Engineering Journal, 225, 292-299

Author´s contribution to the appended papers:

I. Principal author. Designed the experiments and performed most of the experimental work and most of the preparation of the manuscript.

II. Principal author. Designed the experiments and performed all of the experimental work and had a major part in preparation of the manuscript.

III. Second author. Took part in outlining the experiments, performed some of the experimental work and took part in the preparation of the manuscript.

IV. Principal author. Designed the experiments and performed all experimental work and had a major part in preparation of the manuscript.

V.   Principal author. Designed some of the experiments and performed all experimental work and had a major part in preparation of the manuscript.

VI.   Second author. Took part in outlining the experiments, performed some of the experimental work and took part in the preparation of the manuscript.

This thesis also contains unpublished results.

For information about materials and methods, the reader is referred to Papers I-VI.

Other related material:

Wallenberg Wood Science Center, Theme 1 (2014/2015): Demonstrator, Manuscript.

Patent publication: PCT/WO2012071004, (2012) A method to increase the molecular weight of wood mannans and xylans comprising aromatic moieties.

Oinonen P, Areskogh D. and Henriksson G. (2011) The processing and upgrading of hemicellulose mixtures. Proceedings in the 16th ISWFPC, Tianjing, China

LIST OF ABBREVIATIONS

TMP	Thermomechanical pulping
CTMP	Chemithermomechanical pulping
TGA	Thermogravimetric analysis
DSC	Differential scanning calorimetry
OTR	Oxygen transmission rate
LCC	Lignin-carbohydrate-complex
SEC	Size exclusion chromatography
NMR	Nuclear magnetic resonance
In vivo	In a living organism
In vitro	In a test tube
AcGGM	Acetylated galactoglucomannan
MWL	Milled wood lignin
Mn	Number average molecular weight
Mw	Weight average molecular weight
Mp	Peak molecular weight
Me	Molecular weight between entanglements
Tg	Glass transition temperature
CMC	Carboxymethyl cellulose
UV	Ultraviolet
RI	Refractive index

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1 INTRODUCTION

1.1 WOOD STRUCTURE

1.1.1 Lignocellulose

Lignocellulosic biomass is considered to be one of the greatest possibilities for building a sustainable society regarding its energy and material needs. In comparison to petroleum-based products, each stage of the life cycle of biomass derived polymers and chemicals gives better environmental profiles than their petrochemical counterparts (Shen and Patel, 2008). The utilization of biomass-derived polymers to produce e.g. renewable and easily degradable packaging films or chemical precursors can result in significant decrease of the carbon footprint of pulp-, paper- and packaging industries as well as improve their profitability (Shen and Patel, 2008; Farrell et al., 2006).

When considering lignocellulosic sources for energy and fuel production, the principal technical selection criteria for biomass species are growth rate, ease of management, harvesting and intrinsic material properties, such as moisture/ash/alkali content (McKendry, 2001). For materials production, similar principles apply but material properties obviously gain a higher importance. Wood based sources has proven to be superior for many material production applications regarding most of the above mentioned aspects and the new and emerging biorefinery concepts broaden the application areas towards energy, fuel and chemical production as well (da Costa Souza et al., 2009).

For biofuel production, the currently used technologies use plants storage sugars such as starches as a feedstock (1st generation biofuels). They are used because of the sugars are readily available without high energy

demanding processing methods. These sugar sources however in most cases is a part of the food production cycle and should thus be avoided. For energy and material applications, it would be preferable to use biomass sources that are outside the food production scheme (FAO, 2009).

Mature wood consists of different layers starting from the outernmost layer of bark which can be divided into inner- and outer bark (phloem), primary and secondary xylem and pith. Most of the wood volume consists of secondary xylem, which is mostly lignified dead wood cells (although some metabolic activity exists) and has the function of water transportation (Daniel G., 2009).

To use structural polymers of plants (such as cellulose) as a feedstock for biofuel (2nd generation) and e.g. material and specialty chemical production, the recalcitrance of biomass (caused mainly by cellulose crystallinity and lignin matrix) needs to be circumvented. This is often done by pre-treatment, which is often the most costly part of the processing (Himmel et al., 2007; da Costa Souza et al., 2009). Therefore there is a lot of interest in achieving more understanding of the structures that are responsible of the recalcitrance to be able to improve and make the processes more cost-effective.

1.1.2   Wood components and their availability for processing

Wood biomass contributes the largest amount of organic matter in the world (FAO, 2006). It consists mainly of three main types of polymers: carbohydrates (cellulose and hemicelluloses) and the aromatic polymer lignin (Sjöström 1993). Cellulose is a rigid straight chain polymer of glucose, arranged into amorphous and crystalline structures that form fibrillar structures. The fibrils are arranged into fibrillar bundles and further into macrofibres (Daniels, 2009).

Wood hemicelluloses are amorphous, branched polymers. The main hemicellulose of Norway spruce is galactoglucomannan that has a main chain consisting of 1-4-linked β-D-mannopyranosyl and β-D-glucopyranosyl residues with the ratio of glucopyranosyl and mannopyranosyl of 1:3-4. It has varying contents of 1-6-linked α-D-

galactopyranosyl units linked to mannose as sidechains and the mannose residues are often acetylated in the 2- or 3-positions (Teleman, 2009).
About 5-10 % (w/w) of the softwood consists of arabinoglucuronoxylan which has a backbone of β-(1-4)-D-xylopyranosyl residues and is substituted with 4-O-methylglucuronic acid residues as well as α-L-arabinofuranose substituents (Teleman, 2009). Lignin is synthesized in all vascular plants (Weng and Chapple, 2010). It consists of phenyl propanoid units that are polymerized in a web shaped polymer (Henriksson, 2009).

The three components exist in matrixes forming hierarchial structures with a three-dimensional structure and provide physical and chemical resistance that protects the wood integrity (Himmel et al., 2007). This natural recalcitrance of wood affects the availability of the wood polymers to enzymes (Fukuzawa et al., 1982; Zhu et al., 2009) and chemicals used in the biomass utilizing processes.

Usually the first step of wood processing into fibres and other components is the reduction of the particle size that will increase the surface area and the efficiency of the following chemical or physical treatments (da Costa Souza et al., 2009). Mechanical grinding or pre-extraction of soluble polymers is often used. Increased knowledge of the hierarchical and molecular structure of wood has led to some new developments on the pulp production field. These new approaches can utilize the combination of physical, chemical and enzymatic treatments for opening up the wood structure for improved pulping processes (Wang et al., 2013).

1.1.3   Biosynthesis of wood polymers and lignification

Heteropolysaccharides (including hemicelluloses) as all cell wall polysaccharides are formed from activated nucleotide monosaccharides (either uridine or guanidine diphosphate –forms) that undergo glycosylation by glycosyltransferases (Pauly et al., 2013). Glycosyltransferases are highly specific enzymes that catalyze the formation of the specific linkages between monosugars and form the polymer structures. Hemicelluloses are synthesized in the Golgi apparatus and excreted from the cell by exocytosis in oligomeric form (Dhugga, 2001), while cellulose is synthesized directly on the plasma membrane by the rosette terminal complexes (Perrin, 2001). The reason for this is that

directly after cellulose is synthesized, it is assembled into crystalline structures. The formation of lignin differs from the formation of polysaccharides in that the formation of the bulk of the lignin structures after the synthesis of monolignols seems to be more random because it is formed by radical coupling. Chemically lignin is a polymer of phenyl propanoid units (mainly coniferyl alcohol, sinapyl alcohol and *p*-coumaryl alcohol), which are connected with different types of ethers and carbon-carbon bonds (Boerjan et al, 2003). The monolignols are synthesized in the phenylpropanoid pathway from an entry compound of phenylalanine that acts as the carbon skeleton for the different hydroxycinnamic acids (monolignols) (Lewis, 1999).
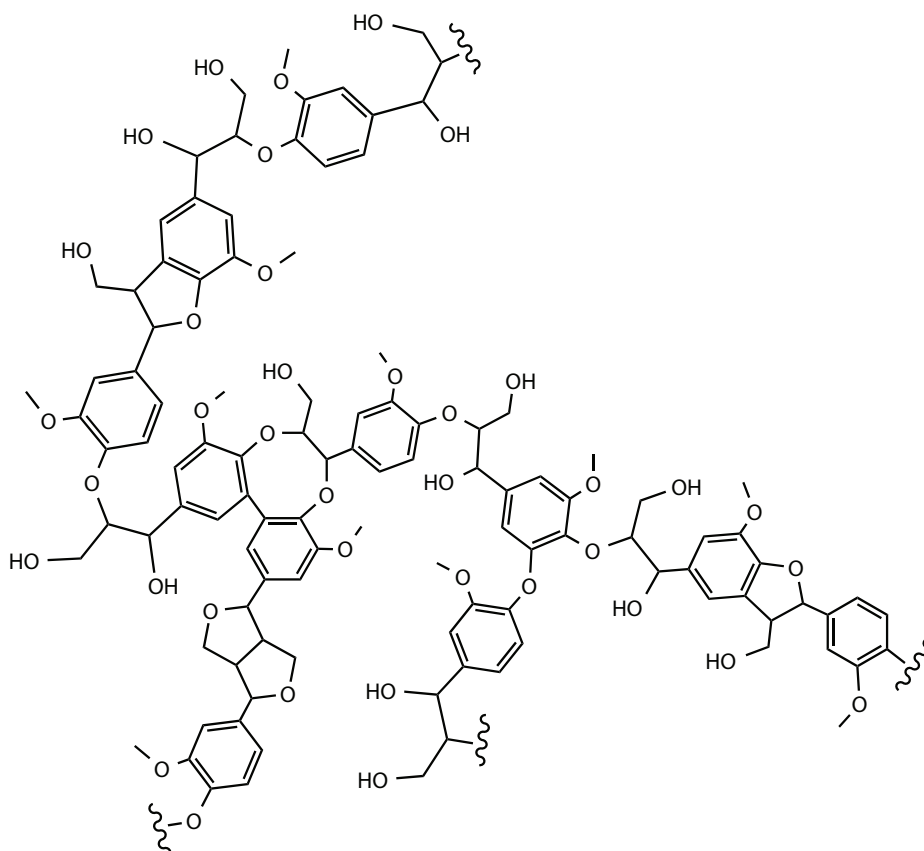
Figure 1-1 Structure of lignin

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There are a few different theories as to how lignification occurs and how the three-dimensional structure of lignin is formed. Most researchers claim that lignin is formed by an uncatalyzed polymerization of enzymatically generated radicals (Adler, 1977), rather than an enzyme-catalysed condensation of activated intermediates, as is the case for most other biopolymers (Nelson and Cox, 2005). It has been demonstrated that cell wall-associated oxidase activity is expressed in developing xylem of a wide taxonomic range of trees (Richardson et al, 2000), supporting the theory that enzymes such as laccases and/or peroxidases are involved in lignification. In addition to enzymatic oxidation, low molecular weight redox shuttles might be involved in lignification presenting yet another hypothesis as to how monolignol radicals are generated (Önnerud et al, 2002; Hatfield et al, 2008).

What we can observe in the structure of lignin in the lignified xylem of wood (that consists the most of the wood tissue mass) is that lignin contains a wide variety condensed carbon-carbon and ether bonds with the major lignin substructures being β-aryl ether, phenylcoumaran, dibenzodioxocin, resinol and spirodienone (Kim and Ralph, 2010).

Oxidative enzymes are believed to take part in lignification by catalyzing the activation of monolignols to radicals. Several enzymes have been thought to participate such as peroxidases (Harkin and Obst, 1973), laccases (Richardson et al., 2000) and other phenoloxidases such as coniferyl alcohol oxidase (Savidge and Udagama-Randeniya, 1992). Some researchers also suggest that oxidases and peroxidases act in different stages of lignification because of the differences in their oxidation potential and more importantly their requirements for substrates. Oxidases (such as laccase) require only oxygen whereas peroxidases require hydrogen peroxide as the second substrate, which could be toxic and harmful for the plant during early stages of xylem development (Sterjiades et al., 1993).

These enzymes often exist in a multitude of isoenzymes and have broad substrate specificity. Because of the lack of specificity and the difficulties arising from the large amount of isoenzymes, straightforward and conclusive ways to explain the role of laccases in lignification has been difficult. To obtain more concise evidence, researchers have recently constructed transgenic plants with multiple disrupted laccase genes

resulting in sever plant growth arrest, increased phenolics amount and even almost complete abolished lignin deposition in some parts of the plant, thus indicating the importance of laccases during lignification (Zhao et al., 2013; Ranoucha et al., 2002)

1.1.4 Network structure of wood

Wood has been used for a wide variety of applications in the society for hundreds of years ranging from wooden boats and houses to the new and arising high end applications with nanocelluloses and lignin carbon fibres (Eichhorn et al., 2010; Norberg et al., 2012). The reason for the large variety of products that can be obtained from wood, can perhaps be related to the chemical character of the main wood polymers, where the carbohydrates exhibit hydrophilic and the aromatic structures and some extractives hydrophobic nature, thus creating possibilities to create products for different applications. Some parts of wood, such as parenchyma cells, should also be considered as living tissues that includes cellular machinery structures with proteins and biological membranes that can end up in some products derived from wood matrix. Bulk of the wood is however considered as dead tissue and consists mainly of the three main polymers cellulose, lignin and hemicellulose, which determine the overall behavior of the wood biomass in different processes and applications (Sjöström, 1993). However the molecular structure of these molecules put together does not account for all of the excellent properties of wood, which has raised questions about if the different polymers could be covalently connected.

1.1.5 Lignin-carbohydrate complexes

1.1.5.1 Evidence and the history

Already early observations suggested that lignin and carbohydrates could be connected because of the difficulties in their separation from one another (Erdmann, 1866; Björkman, 1956). Later research has indicated that lignin is linked through covalent bonds to all the major polysaccharides in the wood cell wall. It is also shown to be evident that the lignin polymer cross-links various polysaccharides to each other (Lawoko et al., 2006). As the separation of lignin and carbohydrates is

essential in especially pulping processes, this subject has been of great importance. The residual lignin structures in pulp for example require expensive bleaching processes, which have created interest in how these structures are connected to carbohydrates. The structures have been isolated enzymatically from spruce and pine pulp (Laine et al., 2004). Lately, more and more structural data regarding the existence of the lignin-carbohydrate complexes has been achieved, making their importance acknowledged in the scientific community (Watanabe et al., 1988, 1989; Laine et al., 2004; Lawoko et al., 2005, 2006, 2013, Balakshin et al., 2011, Du et al., 2014).

1.1.5.2 LCC-types and their analysis

The following lignin-carbohydrate linkages are suggested in the literature: α-ether, α-ester (Freudenberg and Grion 1959; Freudenberg and Harkin 1960), and phenyl glycosidic linkages (Kosikova et al. 1972, Balakshin et al., 2011) (see figure 1-2). They can be investigated e.g. with 2D-NMR techniques (Blakshin et al., 2011; Du et al., 2014; Ralph et al). Bonds between lignin and carbohydrates are however difficult to distinguish because of their low abundance and because they give cross peaks in 2D-NMR close to the carbohydrates, that often produce high intensity peaks. One way to overcome this problem is to use hydrolytic enzymes to reduce the amount of carbohydrates in the samples (Du et al., 2014).
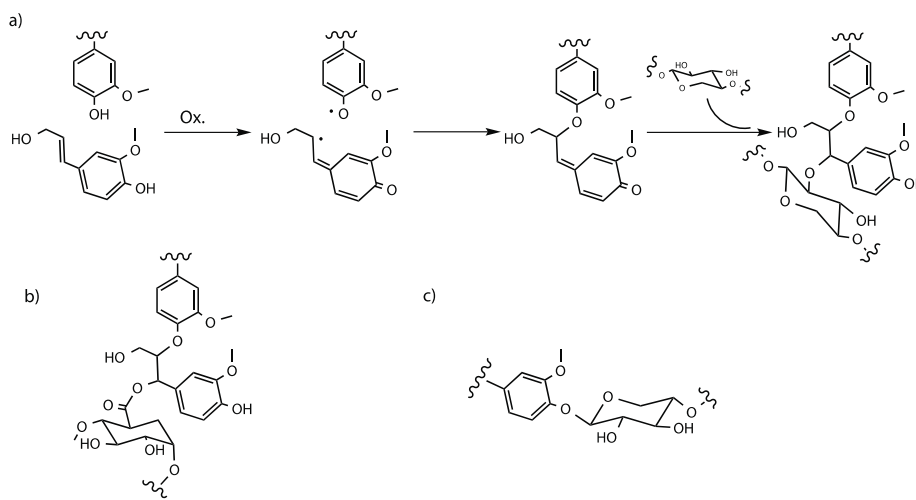
Figure 1-2 LCC-types. a) the formation of α–ether b) α-ester structure c) phenyl glucoside structure

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1.1.5.3 LCCs affecting wood properties and its processing

As previously discussed, lignin-carbohydrate bonds have been shown to exist in wood. Some researchers have also reported residual lignin structures that are connected to the carbohydrates after pulping. This was shown e.g. by Laine and coworkers who reported residual lignin carbohydrate complexes from spruce and pine pulp (Laine et al., 2004). The residual lignin structures are difficult to bleach and are responsible for a major cost in pulping. It has been shown that lignins bound to all different carbohydrates can be found as very resistant structures in pulp (Lawoko et al., 2004, 2005).

LCC-structures are believed to be important in other processes as well. Bioethanol production is hindered by lignin as lignin protects the polysaccharides. The recalcitrance of woody tissues caused by lignin that protects the carbohydrates is obvious, but also the unspecific binding of cellulases to different lignin structures causes problems for fermentation processes (Palonen et al., 2004).

1.2 WOOD AS A RESOURCE

1.2.1 Hemicelluloses from mechanical and pre-hydrolysis pulping

Pulping concepts aims in the liberation of fibers fixed in the wood or plant matrix in either mechanical or chemical ways or with combinations (Bränvall, 2009). The more modern biorefinery concepts that aims in utilizing all of the wood compounds usually depend on efficient separation of the three components of wood; cellulose, hemicellulose and lignin. Water is used in most pulping processes (except organosolv processes) and can be seen as a general solvent. In different conditions regarding pH, temperature and pressure, different wood components can be dissolved. These components goes either to waste water treatment (mechanical pulping) or to a closing system where the pulping chemicals can be recycled (chemical pulping) (Bränvall, 2009). New biorefinery concepts aim in utilizing all of the dissolved components and not only the remaining solid cellulose product (Sixta and Schild 2009). Some of the more important application areas for lignocellulosic components include various applications for nanoscale cellulose fibrils, as well as lignins and already well established lignosulphonates. Kraft lignins has been shown to be a promising feedstock for e.g. carbon fibre production (Norberg et al., 2012) and lignosulphonates have commercial applications in the areas of concrete admixtures, animal food, gypsum board production, pigment dispersion, complexing agents, sludge containers, wastewater treatment, emulsion stabilizers and lead acid batteries. (Gargulak and Lebo, 2000) Hemicelluloses have been of lesser interest for applications because they often suffer from low molecular weight and co-extraction with other wood components even if lately the interest has increased with new research (Willför et al., 2008). They also consist of mixtures of pentose and hexose sugars, that creates problems in fermentation applications (Roca and Olsson, 2002; Zaldivar et al., 2001).

The more efficient utilization of all fractions derived from the processes can reduce the environmental effect of the factories by reducing the amount of organic matter that is released back to the nature. Creating products of the dissolved molecules can however be challenging because

of the heterogenic nature of the fractions and the expenses related to the separation and concentration of the different components (Persson et al., 2007).

1.2.1.1   TMP and CTMP processes

High-yield pulping of wood (i.e. thermomechanical pulping, TMP and chemothermomechanical pulping, CTMP) retains most the wood material in the end product pulp. The yield of the TMP process for softwoods is approximately 97 % and for CTMP between 80-95 % (Sundholm, 1999). The yield- losses of these processes consist mainly of hemicelluloses and their degradation products, but also of lignin degradation products and to a lesser extend components of extractives (Sjöström, 1993). These components are dissolved to the processing liquids and discarded to the wastewater treatment. The total production of paper in the world was about 10.8 million tons in 2013 and mechanical pulping took 26 % of this. Mechanical pulping is in stagnating because of the decreased consumption of printing paper (Skogsindustrierna, 2013). However especially CTMP fibers are used in the packaging industry which is one of the more rapidly growing markets in the pulp and paper industry with 3-4 % annual growth rate (Brolin, 2013).

1.2.1.2   Pre-hydrolysis and dissolving pulp production

Dissolving pulp production has undergone renaissance in the last few years. This is due to the increased awareness to the impacts of oil based products, but mostly because of the increased price of cotton due to problems related to irrigation in the cotton production fields (Gillham et al., 1996). The pulp and paper companies has reacted to the new market situation by establishing new dissolving pulp facilities to match the growing demand of the dissolving pulp products such as clothes textiles.
Novel types of processes for the production of dissolving pulp have been developed for the new markets. A good example is the Austrian company Lenzing´s VisCBC® processes (Sixta and Schild 2009). The process was recently established in a mill run by the company SAPPI (Lenzing 2012). The process uses a pre-extraction step where hemicelluloses are dissolved in alkali and separated before the pulping stage, which creates possibilities for establishing interesting biorefinery concepts (Sixta and Schild, 2009).

1.2.2   Waste water treatment in mechanical pulping processes

Effluent streams from pulp and paper mills are mainly treated on-site in external treatment plants. Great care is being taken to minimize the impact of the mills to the surrounding environment. The levels of emissions are strictly regulated by the officials. This means that a great amount of resources in terms of investments, energy and chemicals are being used to treat the waste material. The pulp and paper industry is ranked as the third world´s largest consumers of water and is consequently producing high amounts of wastewaters (Sevimli, 2005).

1.2.2.1   Products from hydrothermal treatments

Processes relying on hydrothermal treatments have been shown to have potential as a vast hemicellulose source. Because of their branching and often highly substituted structure, they are soluble in neutral or nearly neutral water. Increased pressure and temperature usually improves the extraction yields but also creates hydrolysis reactions from cleaved acetyl groups (Song et al., 2008). Especially pulping processes with pre-hydrolysis or pre-extraction unit processes (Sixta and Schild 2009) and mechanical pulping processes have been studied (Willför et al., 2008). In these conditions, the hemicelluloses are co-extracted with a multitude of other molecules. To be able to achieve a purified hemicellulose product e.g. membrane filtration or ethanol precipitation can be used (Willför et al., 2008). To create an industrially viable process, membrane filtration has shown to be a cost-effective alternative (Persson et al., 2007).

1.2.3   Hemicellulose applications

Because of their heterogenic nature, co-extraction with other molecules and often low molecular weight after processing, there are currently no commercial hemicellulose products. Their availability and potential for various applications has brought up a lot of interest for hemicelluloses lately. Hemicelluloses have been studied in e.g. the pharmaceutical industry for drug delivery agents, anti-microbials, adhesives and stabilizers (Alonso-Sande et al., 2009). For food industry, emulsifiers, gelling agents, stabilizers and thickeners has been studied (Willför et al., 2008). Food and pharmaceutical industrial applications however often require highly

purified products in the applications, which may hinder the applicability of especially wood derived hemicelluloses for these purposes. Therefore less valued application areas with higher volumes have been of interest such as packaging films (Gröndahl et al., 2004). Packaging of food is very important for preserving food quality, reducing the amount of preservatives in food and minimizing food wastage. Roughly one third of the food produced in the world today gets lost or wasted (FAO, 2011) which emphasizes the importance of packaging industry in the world. As the industry has been showing interest towards bio-materials for replacing petroleum-based materials (Shen and Patel, 2008), this creates great opportunities for the use of wood hemicelluloses.

2   AIM OF THE WORK

The aim of the work in one hand was to find out what kind of LCC-structures exists in hotwater extracted hemicelluloses with the emphasis on Norway spruce galactoglucomannans. How these structures could be used in the processing of hemicelluloses in a simple and scalable process that could output a hemicellulose product with improved properties. Inspiration was taken from the natural phenomenon of lignification where oxidoreductase-enzymes functions *in vivo* as catalysts for the polymerization of monolignols thus creating the polymeric lignin structure. The working hypothesis was that low molecular weight aromatic structures were likely linked to hemicelluloses that would enable their use as handles for crosslinking the hemicelluloses. This would enable the increasing of the molecular weight of the hemicelluloses that would improve the material properties. After finding out about the potential of the crosslinking technique and its mechanism, the patented technique was to be taken into large scale to prove industrial applicability.

3 EXPERIMENTAL

This is an overview of the methods that were used. For more details, see the associated publications.

3.1 MATERIALS

Norway spruce hemicelluloses were obtained from the different hydrothermal treatments of industrial wood chips or alternatively sawdust from a sawmill of the company Setra in Heby, Sweden. Eucalypt hemicelluloses were obtained from the treatment of industrial chips of *Eucalyptus urograndis*. TMP and CTMP process waters were obtained from Swedish pulp mills. The *Trametes versicolor*-laccase was purchased from the company Sigma. Two other laccase enzymes were provided by the company Novozymes (Bagsvaerd, Denmark) by the denotations NS51002 and NS51003. The enzymes originated from *Trametes villosa* and *Myceliphtora thermophila*, respectively. The properties of the two enzymes differ because of their origin. The basidiomycete *Trametes villosa* laccase has a higher redox potential (E°780 mV) and lower pH optimum (pH 5) whereas the laccase from *Myceliphtora thermophila* has a redox potential of (E°480 mV) and pH optimum of 7.5.

3.2 LACCASE CROSS-LINKING OF HEMICELLULOSES

Laccase oxidation was extensively used in this work for the oxidation of aromatic moieties attached to the hemicelluloses. The crosslinking technique consists of the following parts: hemicellulose extraction, hemicellulose isolation, laccase oxidation and product fractionation. Hemicelluloses that are suitable for this technique can be extracted in various processes employing water in neutral or nearly neutral pH. Elevated temperature and pressure increases the extraction yield. Isolation can be done e.g. with membrane filtration or solvent precipitation which will increase the purity of the sample regarding the hemicelluloses and

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decrease the amount of extractives and small molecular weight lignins. Laccase oxidation is done in pH and temperature that is optimal for the used enzyme, usually in pH ~4,5 and ~40°C with basidiomycete-derived laccases and pH ~7.0 and 50°C with ascomycete-derived laccases. Oxygen is introduced by e.g. bubbling or in a pressurized vessel. Fractionation of the cross-linked hemicelluloses is done with membrane filtration.

3.3   CARBOHYDRATE AND LIGNIN ANALYSIS

3.3.1   Chemical analyses

Carbohydrate composition analysis performed by hydrolyzing the polymeric material in acid hydrolysis and then analyzing the released monomeric sugars in anion-exchange chromatography. Lignin was quantified as Klason lignin. The analyses were performed according to the SCAN-CM 71:09 and Tappi 222 om-02 standard methods.

3.3.2   Size-exclusion chromatography

Size-exclusion chromatography was performed to analyze the molecular weight distribution of the samples and especially to follow how the cross-linking affected the molecular weight of the hemicelluloses and lignin moieties. It was also used to indirectly show how the carbohydrates (that gives mostly RI-signal) and aromatic molecules (that absorbs UV) are connected and how the signal profiles change according to different enzymatic treatments. The analyses were performed with a HPLC-system equipped with an RI and a diode array UV-detector and pullulan standards were used for size quantification.

3.3.3   Linkage analysis

Linkage analysis of the carbohydrate polymers was used to find out about how the different sugars in the polymers are connected to one another. We studied both the bulk of the Norway spruce galactoglucomannans as well as the carbohydrate structures that remain in the proximity of the aromatic molecules after enzymatic hydrolysis. For the analysis the samples were permethylated, depolymerised and acetylated: and the resultant partially

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methylated alditol acetates (PMAAs) were analysed by gas chromatography-mass spectrometry (GC-MS) as described by York et al (1985). The service of Complex Carbohydrate Research Center at the University of Georgia was used for the analysis.

3.3.4   NMR techniques

^{13}C and HSQC-NMR -techniques were used to analyze mostly the aromatic moieties that were attached to the hemicelluloses. HSQC crosspeaks were used to assign the signals derived from the aromatic structures. ^{31}P-NMR after phosphite derivatization of the samples was used to analyze how the enzyme oxidizes the phenolic moieties and enables the crosslinking phenomenon. The ^{31}P-NMR analysis was made according to Granata and Argyropoulos (1995). The peak assignment for the HSQC was made according to Ralph (1988).

3.4   ANALYSIS OF MATERIAL PROPERTIES

3.4.1   Mechanical properties

The mechanical strength of the produced polymers in the form of freestanding films was studied by the means of mechanical testing. We wanted to find out how the increased molecular weight would affect the mechanical strength and flexibility of films made with the produced materials. The stress–strain behavior of the films was determined with Instron 5944-machine according to the ASTM D882-09 standard (ASTM, 2009).

3.4.2   TGA and DSC

Thermogravimetric analysis (TGA) and Differential scanning calorimetry (DSC) were used to analyze the thermal behavior of the samples. The effect of the crosslinking as well as composite formation was emphasized. DSC measurements were performed on a Mettler Toledo DSC 822e (Columbus, OH) previously calibrated with indium and zinc standards. The following thermal scans were used: i) First heating scan from -50 ºC to 200 ºC, hold at 200 ºC for 3 minutes; ii) Cooling scan from 200 ºC to -50 ºC,

hold at -50 ºC for 3 minutes and iii) Second heating scan from -50 ºC to 450 ºC. The heating and cooling rates were set at 10 ºC/min. The thermogravimetric data were obtained from a TGA Mettler-Toledo 851 module (Columbus, OH) under nitrogen atmosphere (50 ml/min), at 10 ºC/min in a temperature range between 25 and 750 ºC. A deconvolution method based on an asymmetrical model that was successfully applied in previous works (Moriana et al., 2010) was employed in this work to separate the main thermal decomposition processes.

3.4.3   Oxygen transmission rate

Oxygen transmission rate was measured to finally find out about the potential of these hemicelluloses in packaging applications and more specifically as barrier films. The analysis was performed using a Systech Instruments, 8001 oxygen permeation analyzer. Testing was performed at a temperature of 23∘C, 50% relative humidity and one atmosphere oxygen pressure according to ASTM D3985 standard (ASTM, 2010).

4 RESULTS AND DISCUSSION

4.1 HEMICELLULOSE CROSS-LINKING BY LACCASE VIA AROMATIC LINKAGES (PAPERS I-II)

As described earlier, hemicelluloses are widely available in different biomass processes in large quantities. One of the main challenges for their utilization is however their low molecular weight (because of hydrolysis during processing) and that they are often dissolved together with an abundance of other compounds making their isolation costly.

During this work, a novel method for cross-linking hemicelluloses that bear aromatic moieties was developed. This was done to improve the properties of the hemicelluloses in material applications by increasing the molecular weight. The increased molecular weight increased entanglement of the hemicelluloses to each other as well as additives (polymers and nanoparticles), improving their properties and makes it possible for them to be used for making e.g. packaging film products.

4.1.1 Isolation of the hemicelluloses

To study the crosslinking of different hemicelluloses, hydrothermal treatment by using different techniques was used for the extraction. In laboratory scale, hotwater extraction was the main means of treatment method. Norway spruce (*Picea abies*) chips and sawdust were used for the most part as the source material, but also Eucalypt (*Eucalyptus urograndis*) and wheat straw (*Triticum aestivum*) were used to demonstrate the wide applicability of the technique. Also thermomechanical (TMP) and chemithermomechanical (CTMP) pulping process waters were used as sources for Norway spruce hemicelluloses. The extracts underwent enrichment of the hemicelluloses from other molecules by either ultrafiltration or ethanol precipitation. The crosslinking reaction was thereafter performed for the hemicelluloses that contained several types of hemicelluloses with and without aromatic moieties. The cross-linked

samples (of Norway spruce) were thereafter fractionated to high and low Mw fractions by ultrafiltration. Figure 4-1 describes a typical crosslinking as visualized by size exclusion chromatography. The composition of the extracts and crosslinking products are described in the figure 4-2.
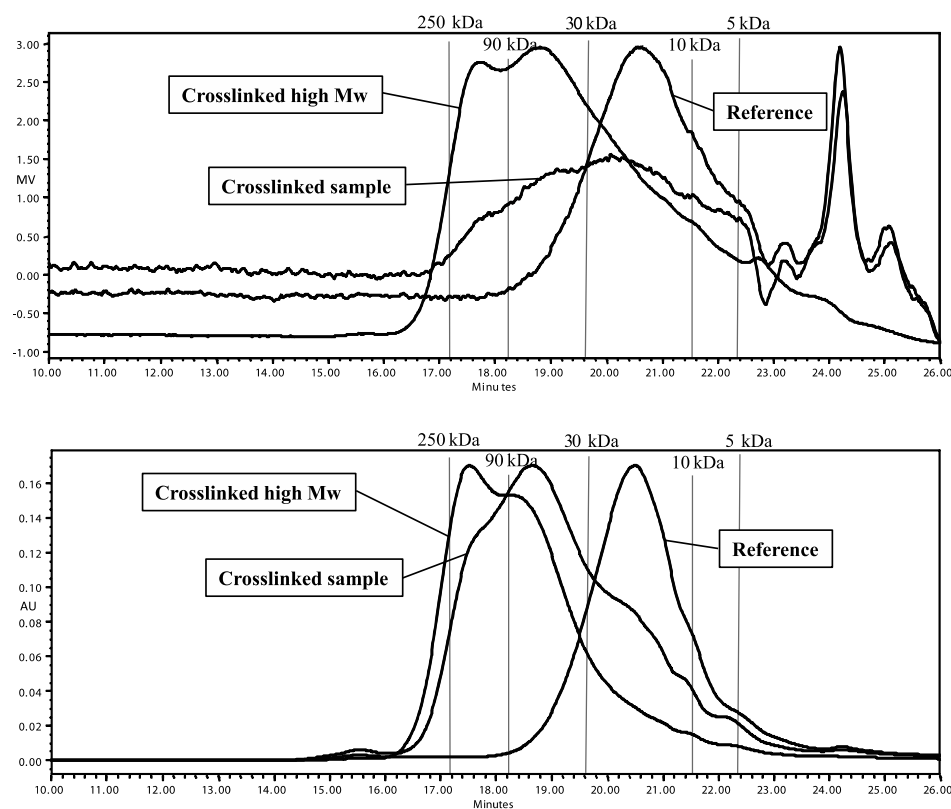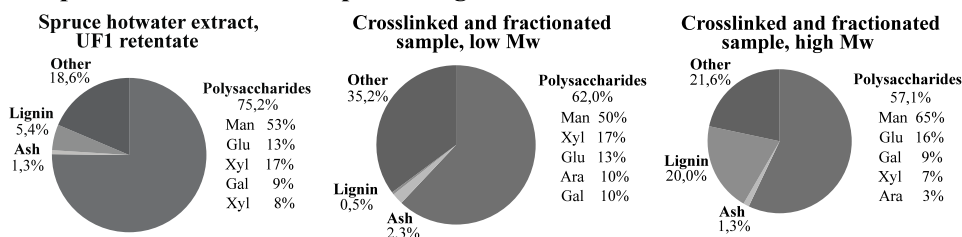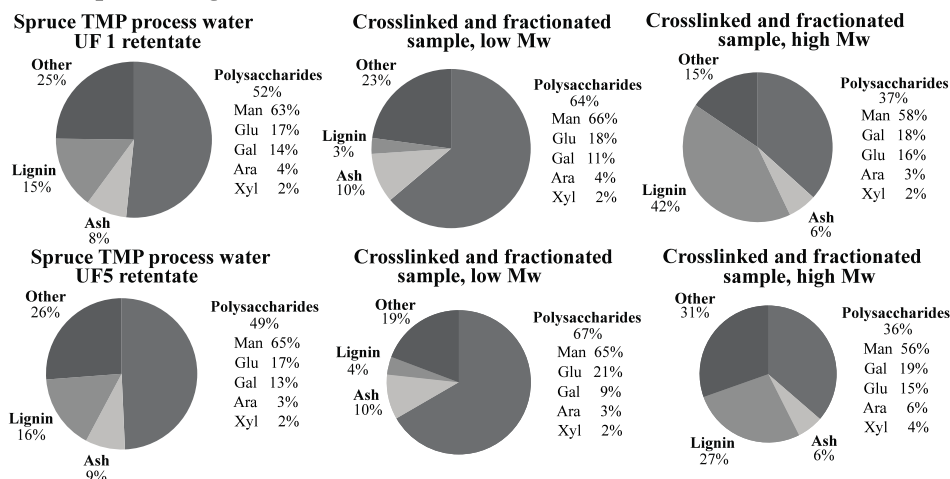

Figure 4-1 Crosslinking and fractionation of Norway spruce galactoglucomannan analyzed by Size exclusion chromatography. Upper panel represents the graph of the RI-detector and lower panel the UV (280 nm)-detector.

A. Spruce hotwater extract processing

Spruce hotwater extract, UF1 retentate

Other 18,6%
Lignin 5,4%
Ash 1,3%
Polysaccharides 75,2%
Man 53%
Glu 13%
Xyl 17%
Gal 9%
Xyl 8%

Crosslinked and fractionated sample, low Mw

Other 35,2%
Lignin 0,5%
Ash 2,3%
Polysaccharides 62,0%
Man 50%
Xyl 17%
Glu 13%
Ara 10%
Gal 10%

Crosslinked and fractionated sample, high Mw

Other 21,6%
Lignin 20,0%
Ash 1,3%
Polysaccharides 57,1%
Man 65%
Glu 16%
Gal 9%
Xyl 7%
Ara 3%

B. TMP processing

Spruce TMP process water UF 1 retentate

Other 25%
Lignin 15%
Ash 8%
Polysaccharides 52%
Man 63%
Glu 17%
Gal 14%
Ara 4%
Xyl 2%

Crosslinked and fractionated sample, low Mw

Other 23%
Lignin 3%
Ash 10%
Polysaccharides 64%
Man 66%
Glu 18%
Gal 11%
Ara 4%
Xyl 2%

Crosslinked and fractionated sample, high Mw

Other 15%
Lignin 42%
Ash 6%
Polysaccharides 37%
Man 58%
Gal 18%
Glu 16%
Ara 3%
Xyl 2%

Spruce TMP process water UF5 retentate

Other 26%
Lignin 16%
Ash 9%
Polysaccharides 49%
Man 65%
Glu 17%
Gal 13%
Ara 3%
Xyl 2%

Crosslinked and fractionated sample, low Mw

Other 19%
Lignin 4%
Ash 10%
Polysaccharides 67%
Man 65%
Glu 21%
Gal 9%
Ara 3%
Xyl 2%

Crosslinked and fractionated sample, high Mw

Other 31%
Lignin 27%
Ash 6%
Polysaccharides 36%
Man 56%
Gal 19%
Glu 15%
Ara 6%
Xyl 4%

C. Eucalyptus and wheat straw hydrolyzates

Eucalyptus hydrolyzate

Glucose 19%
Arabinose 2%
Xylose 51%
Galactose 20%
Mannose 8%

Wheat straw hydrolyzate

Glucose 26%
Arabinose 14%
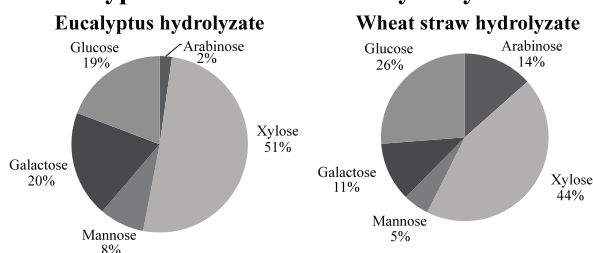Xylose 44%
Galactose 11%
Mannose 5%

Figure 4-2 Chemical composition of the studied samples. A. The composition of the spruce hotwater extract ultra filtrated with 1 kDa MWCO membrane as well as the fractions after the enzymatic cross-linking and fractionation by ultra filtration with a 30 kDa MWCO membrane. B. The composition of the process water from the TMP process ultrafiltrated with either 1 kDa or 5 kDa MWCO membrane as well as the fractions after the enzymatic cross-linking and fractionation by ultra filtration with a 30 kDa MWCO membrane. C. The sugar composition of eucalypt and wheat straw hotwater extracts purified by ethanol precipitation.

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4.1.2   The cross-linking phenomenon

Different lignin structures have been cross-linked by laccase treatment by a variety of researchers (Hatakka et al., 1993; Viikari et al., 1999) Crosslinking of lignosulfonates was extensively studied by Areskogh (2011). The cross-linking of model compounds suggested that sulfonated and non-sulfonated lignin structures behave differently during laccase treatment and that the sulfonyl groups direct the lignin reactions towards polymerization reactions (Areskogh et al., 2009).

In this work, we performed the cross-linking reactions in a matrix of carbohydrates, with predominantly lower molecular weight lignin molecules that were attached to the carbohydrates. The cross-linking phenomenon was further studied for Norway spruce hemicelluloses that were fractionated by ultrafiltration to contain molecular weights between approximately 5-10 kDa. This was done to study how these oligomer-sized complexes are cross-linked by the enzymatic treatment on a molecular level and concentrating on the phenolic moieties and the amount of increased molecular weight. The weight average molecular weight (Mw) of the produced complexes were typically about 2.4 times higher after crosslinking and fractionation when observing the results obtained with the RI-detector (figure 4-3). When observing the peak molecular weight (Mp) obtained by the UV-detector, specific mostly to aromatic structures the increase in weight average molecular weight was much higher, being about 4.8 times (figure 4-3). This suggested that the hemicellulose-lignin complexes contained at least two aromatic moieties thus in theory making them capable of forming endless lignin carbohydrate networks *in vitro*. Hypotheses on how this phenomenon could occur *in vivo* in Norway spruce were made according to the results from the ^{31}P-NMR and research done on this field (sections 4.1.3 and 4.1.4).

By using ^{31}P-NMR, we were able to confirm that the hemicellulose-lignin complexes contained both condensed guaiacyl C5-substituted units and non-condensed guaiacyl type phenolic end groups in the untreated sample (figure 4-4). The disappearance of the signal assigned to the non-condensed guaiacyl type units after the enzymatic treatment indicated the radical crosslinking reaction while the condensed guaiacyl C5-substituted molecules remains unchanged. That the disappearance of the non-condensed units was a result of polymerization and not degradation was confirmed by the size exclusion chromatography results showing a significantly   increased   molecular   weight   profile   (figure   4-3).
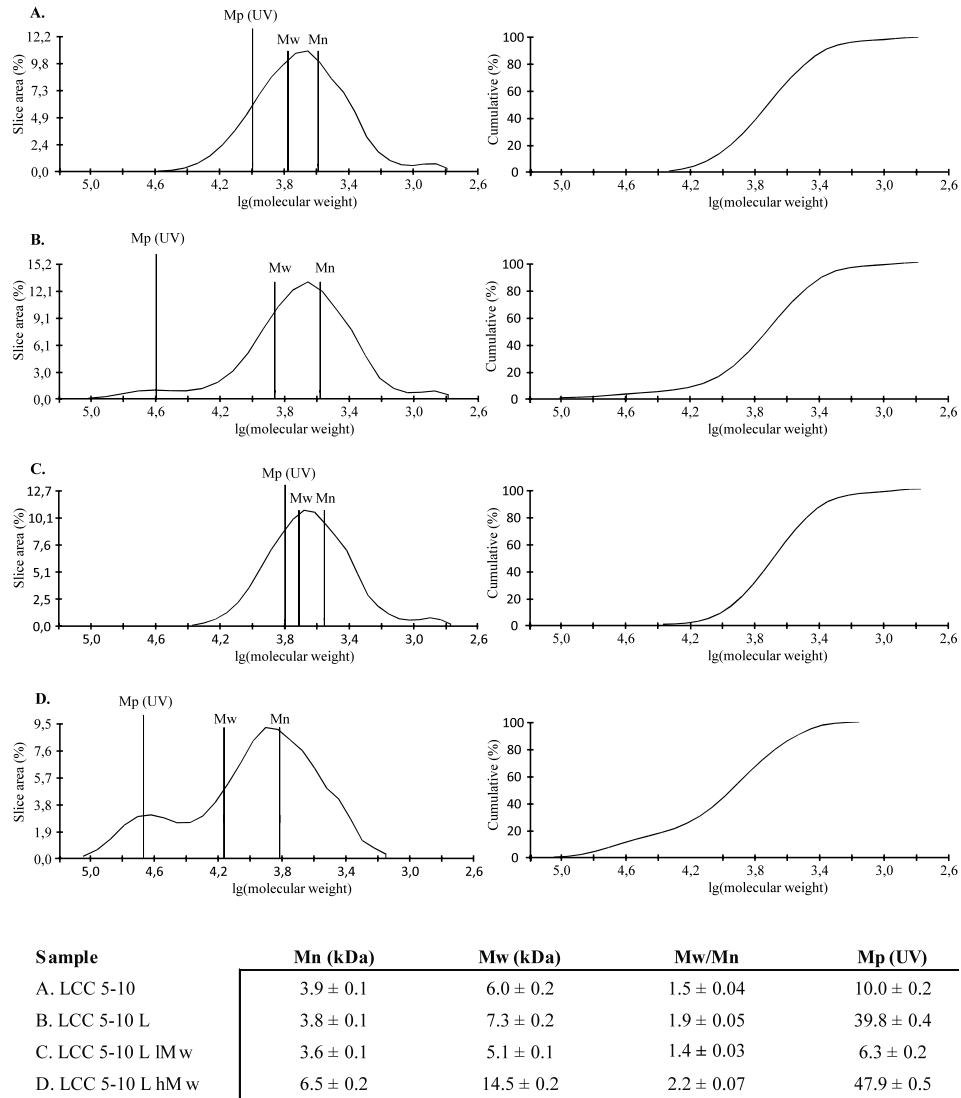
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Sample	Mn (kDa)	Mw (kDa)	Mw/Mn	Mp (UV)
A. LCC 5-10	3.9 ± 0.1	6.0 ± 0.2	1.5 ± 0.04	10.0 ± 0.2
B. LCC 5-10 L	3.8 ± 0.1	7.3 ± 0.2	1.9 ± 0.05	39.8 ± 0.4
C. LCC 5-10 L lM w	3.6 ± 0.1	5.1 ± 0.1	1.4 ± 0.03	6.3 ± 0.2
D. LCC 5-10 L hM w	6.5 ± 0.2	14.5 ± 0.2	2.2 ± 0.07	47.9 ± 0.5

Figure 4-3. Size exclusion chromatography (SEC) of the samples before and after laccase treatment (A and B, respectively) and fractionation (C and D). Number (Mn) and weight (Mw) average molecular weight as well as polydispersity (Mw/Mn) is based on the results from the RI-detector response and the peak molecular weight (Mp) is based on the UV-detector response. The molar masses are measured relative to pullulan standards. The Briggs logarithm (lg) of the molecular weights are presented both as slice areas (left column) and as cumulative molecular weights (right column).
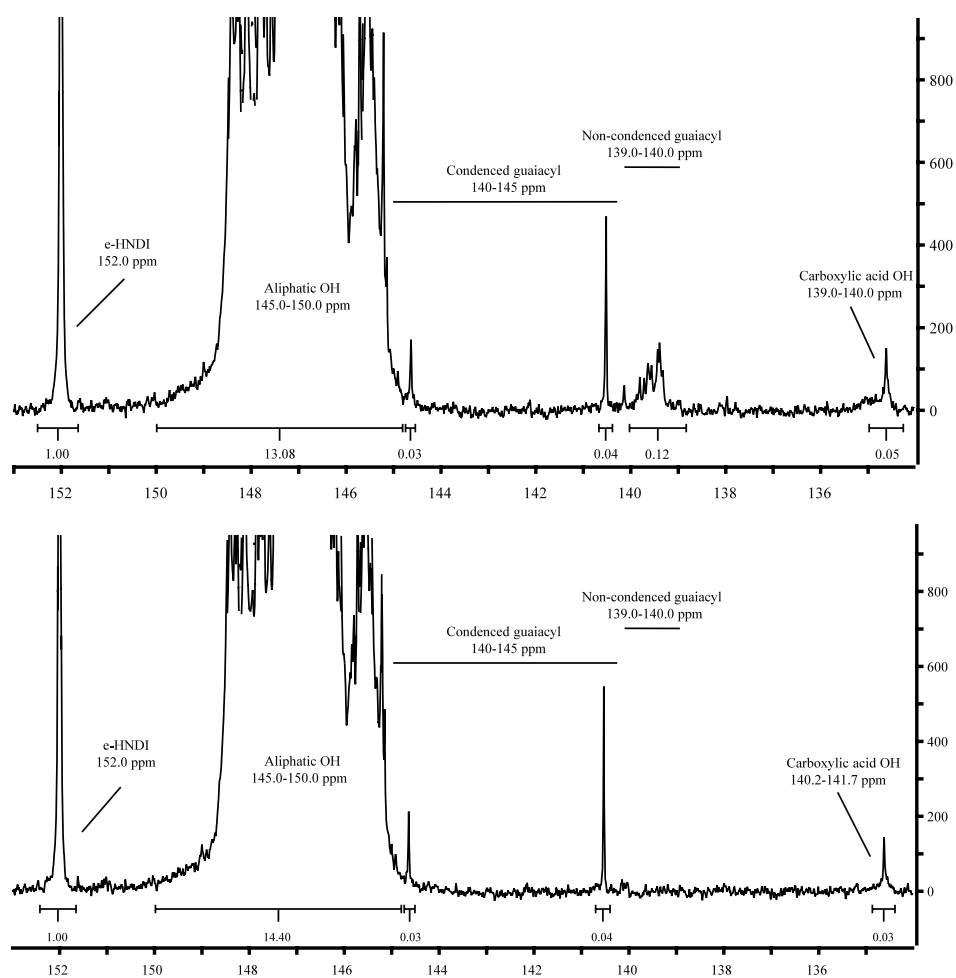
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Figure 4-4. ^{31}P-NMR of the samples LCC 5-10 kDa (top) and LCC 5-10 kDa L (bottom). The laccase treatment oxidised the unsubstituted guaiacyl unit while the C5-substituted phenols remains unreacted.

4.1.3   Infinite lignin-hemicellulose complex formation

The crosslinking phenomenon and the increased molecular weight helped us understand some structural details of Norway spruce galactoglucomannans from the amount of increased molecular weight. We used the laccase-enzyme system for the crosslinking of the hemicellulose-lignin complexes to confirm earlier research that has indicated that oxidoreductase-enzymes are believed to be involved in lignin polymerization during lignification (Berthet et al, 2011; Christensen et al, 1998; Önnerud et al, 2002). As the carbohydrate composition remains almost identical in all of the achieved samples (table 4-1). This could be an indication that:

- both the galactoglucomannans as well as arabinoxylans can undergo crosslinking via the attached guaiacyl type phenolic moieties; and
- the radical crosslinking mechanism is, as expected, not distinguishing between the different carbohydrates but the crosslinks are formed randomly creating a network of galactoglucomannans, xylans and lignin moieties

It is also likely that the technique works for all types of hemicelluloses that are bearing phenolic moieties.

The increasing of the molecular weight over two times of the polymers implies that the lignin-hemicellulose structurs are capable of forming network structures. A two-fold increase in molar mass upon laccase treatment would imply that each hemicelluloses molecule on average has only one linkage point to lignin, as depicted in figure 4-5a. Following the same reasoning the hemicellulose molecules involved in the reactions leading to a further increased molecular weight must have at least two reactive groups (phenols) covalently attached, in which case the depiction in figure 4-5b is in order. Our results thus suggest that a large fraction of hemicellulose molecules in our experiments include two or more phenolic lignin structures covalently attached, and that these by laccase treatment can form larger molecules and networks. This is in line with the work of Lawoko (Lawoko, 2013), who discussed the existence of more than one type of LCC bond present in a LCC moiety.
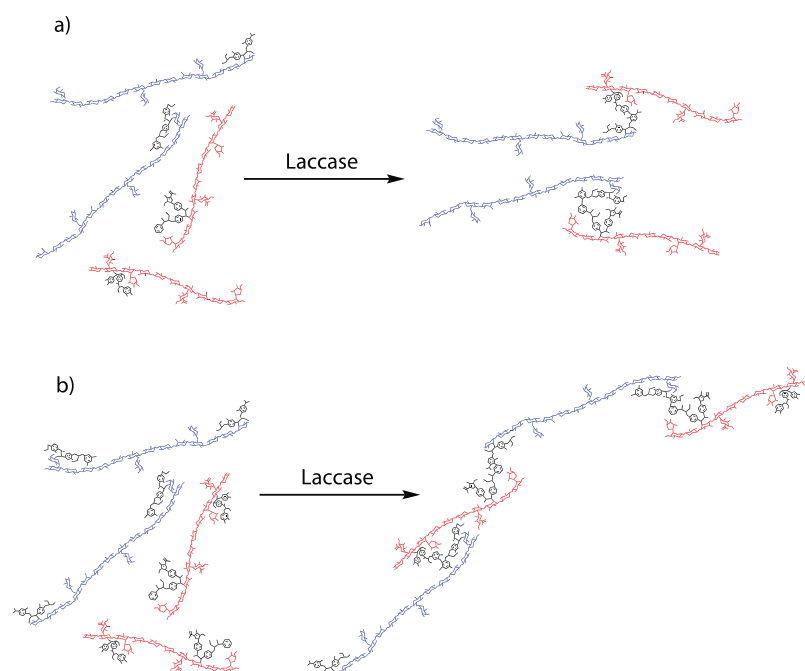
24

Figure 4-5. Schematic presentation of cross-linking of hemicellulose with covalently bound lignin structures upon oxidation with laccase. a) If the hemicellulose molecules only have one bound lignin structure with a reactive phenol, the molecular weight can maximally be doubled. b) If hemicellulose molecules include two of more lignin structures with reactive phenols, radical-radical coupling may generate molecules with far over doubled molecular weights, as is suggested by the experimental data.

Even though the results of this work was considering only hemicellulose-lignin complex formation, it is likely that also cellulose is, in a similar way linked to the infinite lignin-carbohydrate network structure in wood as is indicated by e.g. the work of Lawoko who suggested that virtually all carbohydrates in wood are linked to lignin structures (Lawoko et al, 2006).

4.1.4   The role of laccases in the early stages of lignification

It is known that hemicelluloses are synthesized inside the cells in the Golgi apparatus and then excreted out in vacuoles to be deposited to the cell wall. It is also established that phenolics play a critical role in the crosslinking of

cell wall components of grasses (Carpita, 1996). Furthermore ferulate polysaccharide esters are known to be incorporated to lignins by enzymatic oxidative coupling (Ralph et al., 1995). Paper II describes a hypothesis on how in a similar way as with grass ferulate polysaccharides, in the early stages of lignification of woody tissue, oligomer-sized hemicelluloses with attached lignin moieties could be having a similar role. Experimentally, we were able to increase the molecular weight of low molecular weight lignin hemicellulose complexes by over two fold by laccase treatment (figure 4-3) in conditions that resemble the Norway spruce wood matrix *in vivo*.

By using ^{31}P-NMR we could observe the aromatic moieties of carbohydrate rich LCC samples after first hydrolyzing the samples enzymatically. We were thus able to observe phenomenal changes in the signals assigned to phenolic hydroxyls during the enzymatic cross-linking reaction and show how hemicellulose-lignin complexes contain both condensed guaiacyl C5-substituted units and non-condensed guaiacyl type phenolic end groups in the untreated sample (figure 4-4). The disappearance of the signal assigned to the non-condensed guaiacyl type units after the enzymatic treatment indicated the radical crosslinking reaction while the condensed guaiacyl C5-substituted molecules remains unchanged. This is a similar mechanism that occurs during the crosslinking of ferulate polysaccharides (Ralph et al., 1995).

Size exclusion chromatography confirmed that the treatment resulted in cross-linking reactions and not only oxidations. The significantly increased molecular weight of the samples was a clear indication of this (figure 4-3) We were not able to find out the types of bonds formed. The amount of condensed phenolic and carbon-carbon bonds was unaltered in ^{31}P-, HSQC- and ^{13}C-NMR. However, because likely only a few units are needed for the crosslinking of the carbohydrates it is possible that these bonds were formed, but not visible in the NMR.

Laccases have been suggested to function in the early stages of lignification oxidizing monolignols to oligo-lignols and peroxidases in the later stages of xylem development so that the phytotoxic effects of H_2O_2 (that peroxidases require for the oxidoreduction) are avoided (Sterjiades et al., 1993). Further proof was found by Ranocha and co-workers who observed that the down-regulation of certain laccase-genes in Poplar,

increased the amount of total soluble phenolics in the xylem ray parenchyma cells by 2-to 3-fold (Ranocha et al., 2002). This finding suggests that the role of laccases could be the oxidation of the soluble, non-condenced phenolics, or as in case of our experiments the water-soluble carbohydrate bound non-condenced phenolics in a similar fashion as with ferulates. Furthermore, the identification of a poplar peroxidase that oxidises polymeric lignin (Sasaki et al., 2004) suggests that a direct enzymatic oxidation may be the predominant mechanism for the generation of radicals once the polymeric lignin is formed.

Cell wall cohesion has also shown to be dependent on laccase activity and concomitant oxidation of phenolic components (Ranocha et al., 2002). The mechanical strength data in the paper I, shows similarly that the tensile strength and Young´s modulus of Norway spruce galactoglucomannan-carboxymethyl cellulose composite films was significantly increased after the laccase crosslinking of the aromatic moieties (Oinonen et al., 2013). Furthermore, according to Samuels and co-workers the initiation sites of lignification is regulated through the vesicular secretion of pectin and hemicelluloses to the cell wall in gymnosperms (Samuels et al., 2002).

This made us suggest a theory where in the early stages of wood component assembly, hemicelluloses that contain non-condensed phenolic moieties could be deposited to the inter-lamellar voids as initiation sites. They could then be cross-linked by laccases, creating networks of lignin and carbohydrates and improving the cohesion of the cellulose fibrils and rigidity of the cell wall. After that, monolignols are polymerized to the hemicellulose-lignin matrix by peroxidase oxidation in the later stages of lignification increasing the hydrophobicity of the wood matrix.

A schematic suggestion for how wood might be covalently organized on molecular level is shown in figure 4-6. Our results support the existence of such complex three-dimensional network that gives a physical resistance towards separation of polysaccharides during water adsorption and could explain why wood separation into its individual components is so difficult.
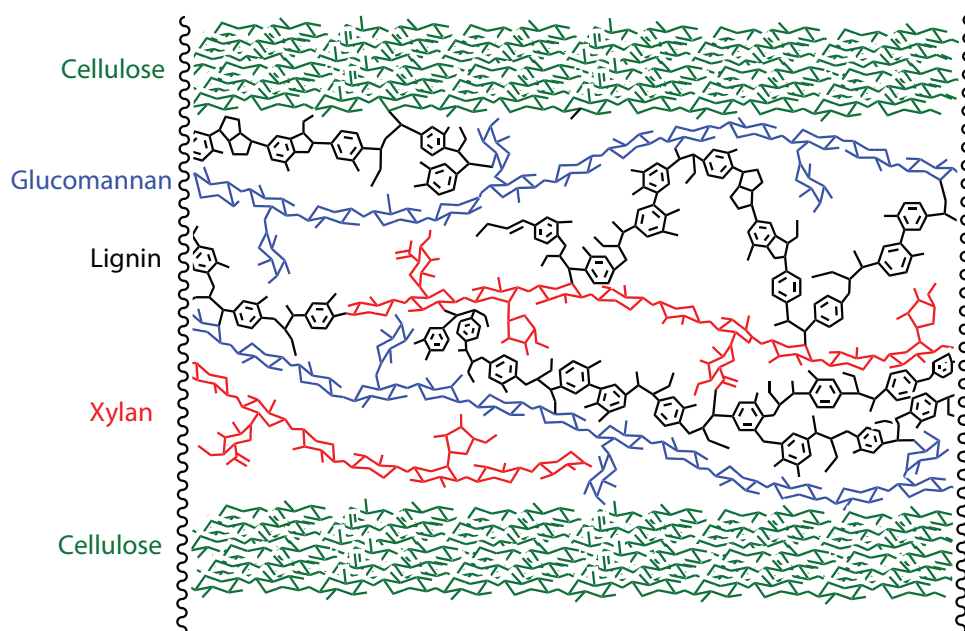
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Figure 4-6 Wood component organization

4.1.5 Cross-linking improves the material properties of the hemicelluloses

Paper I describes how after the crosslinking and fractionation of the hemicelluloses we could use them in material applications with improved properties. We made freestanding films with the produced hemicelluloses together with carboxymethyl cellulose (CMC) as a supportive polymer and glycerol as plasticiser. Stress-strain curves of the films were measured which showed us how the crosslinking improved the mechanical strength of the films while still keeping a reasonably good flexibility. Because of the crosslinking we were able to use the hemicelluloses in higher quantities (70% w/w) and still getting reasonably good film properties implicating a better cost-efficiency for the film product. To try to explain why the laccase treatment improved the film properties, we studied the mechanical and thermal properties of wood hemicelluloses before and after the crosslinking treatment in the paper V.

Sample polymer	Stress at max. load (MPa)	Strain at break (%)	E-modulus (MPa)
A. Films made with 60 wt% hemicellulose, 20 wt% CMC and 20 wt% glycerol)			
SCHw UF1	2.2 ± 0.5	23 ± 1.7	10 ± 11.7
SCHw UF1 C low Mw	1.6 ± 0.4	27 ± 3.2	6 ± 1.1
SCHw UF1 C high Mw	8.0 ± 0.7	21 ± 2.0	98 ± 16.5
TMP UF1	4.5 ± 0.7	13 ± 2.1	64 ± 20.8
TMP UF1 C low Mw	4.0 ± 0.7	24 ± 1.5	24 ± 1.0
TMP UF1 C high Mw	9.8 ± 1.5	9 ± 2.0	240 ± 7.0
B. Films made with 70 wt% hemicellulose, 20 wt% CMC and 10 wt% glycerol)			
TMP UF1	N/A	N/A	N/A
TMP UF1 C low Mw	N/A	N/A	N/A
TMP UF1 C high Mw	15.0 ± 0.1	6 ± 0.6	433 ± 35.2

Table 4-1 Stress/strain behaviour of films made with Norway spruce hemicelluloses with and without cross-linking treatment.

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4.2 NETWORK STRUCTURE OF HEMICELLULOSE-LIGNIN COMPLEXES IN NORWAY SPRUCE (PAPERS III-IV)

After finding out about the mechanism of the crosslinking phenomenon, we set out to study the molecular structure of the complexes and its meaning for the accessibility of these structures for enzymatic hydrolysis in a model system. Linkage analysis and different NMR-techniques were used for determining the structural details and hydrolytic enzymes were used both as an analytical tool but also as a means to analyze the structures by their degradation patterns. Commercial and culture filtrate-derived hydrolytic enzymes were used for the hydrolyses.

4.2.1 Detailed structure of Norway spruce lignin-hemicellulose complexes

After observing the oxidation of the non-phenolic guaiacyl units by laccase treatment in paper II, we used ^{13}C- and HSQC-NMR-techniques to analyze the aromatic structures in the samples (paper IV). The main finding was guaiacyl type phenolic structures that were bonded by β-O-4 bonds and connected to the carbohydrates by phenyl glucosidic bonds (figure 4-7).

The differences in the linkage pattern of the carbohydrates in the vicinity of the aromatic part were compared between the non-crosslinked and crosslinked samples by analysing the samples after hydrolysis by an endo-β-mannanase that hydrolyzed most of the carbohydrates in the samples to oligomer sized sugar products.

The main finding of the linkage analysis was that the polysaccharide structures in proximity to the aromatic structures are richer in branched structures and galactose side groups than the bulk of the glucomannans in spruce (table 4-2). These branched structures may take part in the formation of covalent bonds between lignin and polysaccharides, and therefore play a key role in the formation of wood properties. This can be a logical explanation also since side group monosaccharide residues are more capable of rotation, than monosaccharide residues located in a polysaccharide main chain, and thereby are expected to have higher reactivity.
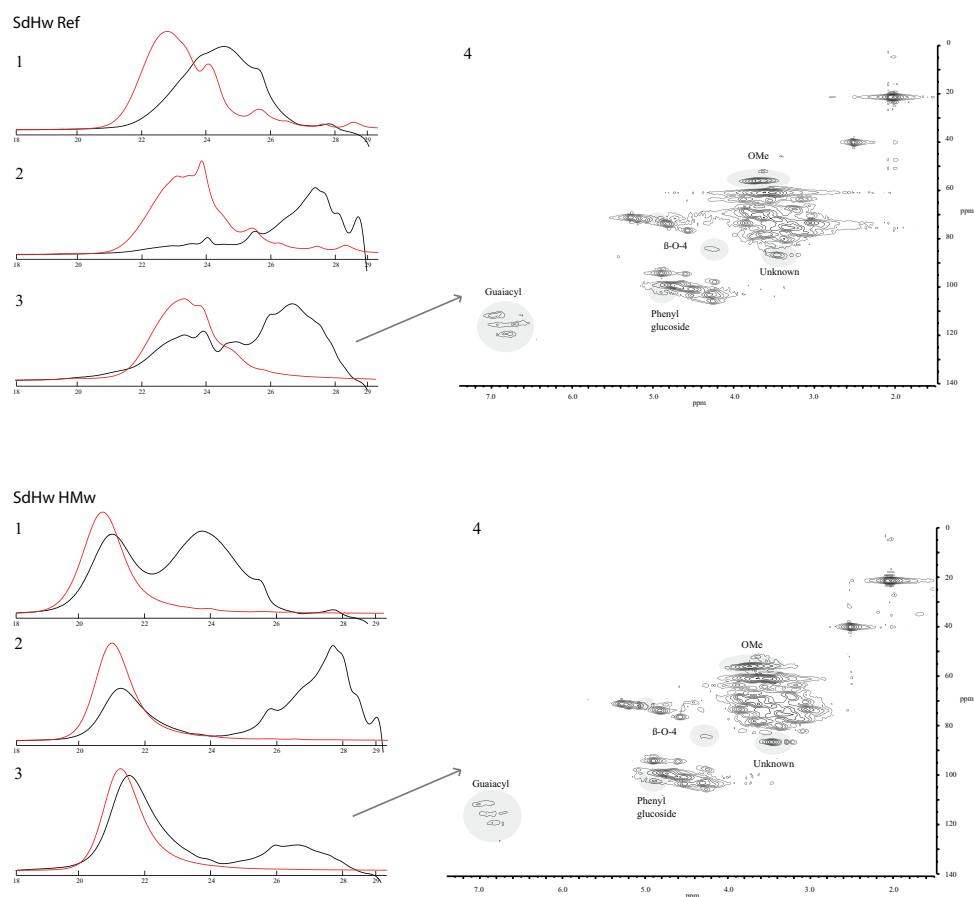
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Figure 4-7 Enzymatic hydrolysis and whole HSQC of the cross-linked and non-cross-linked GGMs. 1-3: Size exclusion chromatography graphs of the Norway spruce hotwater extract reference material (upper panel) and the cross-linked product (lower panel). 1. Starting material, 2. Hydolysis product 3. Product after hydrolysis and dialysis. Black color graph represents the RI-signal and red colour graph the UV-signal (280nm). 4. Whole HSQC-graph of the hydrolyzed and dialyzed samples.

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Residue	SdHw Ref (%)	SdHw Ref H (%)	SdHw hMw H (%)
4-Man	49,9	27,6	28,1
4-Glc	16,6	11,8	9,8
t-Man	8,1	11	10,7
t-Gal	5,4	9,6	8,8
4,6-Man	3,7	5,2	4,8
4-Xyl	2,4	6,4	3,7
3-Glc	2,3	4,5	8,8
4-Gal	2,1	3,8	2,1
t-Glc	2	2,6	3,4
4,6-Glc	1,1	2,1	1,6
3,4-Man	0,9	0,4	0,4
2,4-Man	0,9	0,6	0,7
2-Man + 3-Man	0,7	2,1	4
3,6-Gal	0,6	1,8	2,5
6-Gal	0,5	1,2	1,7
t-Xyl	0,4	0,8	0,7
6-Man	0,4	0,9	1,2
2,4-Xyl	0,3	1,2	0,5
2-Rha	0,2	0,7	0,5
3,4-Glc	0,2	0,3	0,4
3,6-Man	0,2	0,5	0,7
2,6-Man	0,2	0,6	1,3
t-Rha	0,1	0,3	0,5
6-Glc	0,1	0,3	0,3
3,4-Gal	0,1	0,2	0,2
2,4-Gal	0,1	0,1	0,1
3-Gal		2	0,6
2,3-Man		0,2	0,3
3,6-Glc		0,3	0,4
2,3,6-Man + 2,4,6-Man		0,1	0,2

Table 4-2. Glycosyl linkages of unhydrolyzed (SdHw Ref) and hydrolyzed samples with and without crosslinking (SdHw Ref H, SdHw hMw H respectively). (t=terminal residue)

4.2.2 Model system for studying the enzymatic breakage of lignin-carbohydrate complexes

As discussed in the Introduction-section of this thesis, LCC-structures are believed to be important to the pulping and biorefinery processes providing recalcitrance for the plant structures and thus making the different polymers less accessible for enzymes and chemicals. The lignin-carbohydrate complexes that were formed as described in the papers I and II, can be considered to be good model compounds for the LCC-structures in wood as they were formed by mimicking natural processes. The crosslinking products were thus subjected to different enzymatic systems to study their capabilities for degrading the complexes. We used both commercial enzyme products as well as a culture filtrate from a fungus (*Phoma herbarum*) that has showed potential towards breaking phenyl glycoside-bonds between lignin and carbohydrates (Ran et al., 2012). The enzymatically produced LCC fractions showed differences in degradation patterns when subjected to the different enzyme systems.

4.3 IMPROVING THE APPLICABILITY OF HEMICELLULOSES FOR MATERIAL PRODUCTION (PAPER V-VI)

For gas barrier film applications, it is important that the used polymer product has both flexibility and strength as well as a low oxygen transmission rate. For the product to be interesting for industrial production, it is also important that the production costs are kept low. This is usually achieved by using a simple and robust process with as few processing steps as possible. The developed process enabled us to increase the molecular weight and thermal stability of the hemicelluloses in a simple and cost-efficient two-step process (enzymatic crosslinking and ultrafiltration). Furthermore, we studied how cellulose nanoparticles could be used to further improve the properties of the films.

4.3.1 The increased molecular weight by cross-linking increases the strength and thermal stability of hemicellulose films

It is important to test the mechanical strength of a polymer product to find out about its suitability in different applications. Also the glass transition

temperature (Tg) and the thermal degradation properties are important measures of polymers as they can predict the performance of the material. Theoretically, increased molecular weight or cross-linking increases the strength and the glass transition temperature of any given polymer (Nielsen and Landel, 1969). The papers V and VI describe how the cross-linking technique can be used to increase the molecular weight and the thermal stability of the hemicelluloses in an up-scaled process that could be taken into an industrial environment.

We studied the properties of the polymers that were upgraded in the developed processing scheme (figure 4-8) for a Swedish CTMP-mill. This process was proven to be viable for isolating, cross-linking and fractionating the hemicelluloses in terms of membrane performance, process water logistics and processing tim. The performance of the runs were similar to the results given by Persson et al., (2007) indicating a very cost-efficient process.
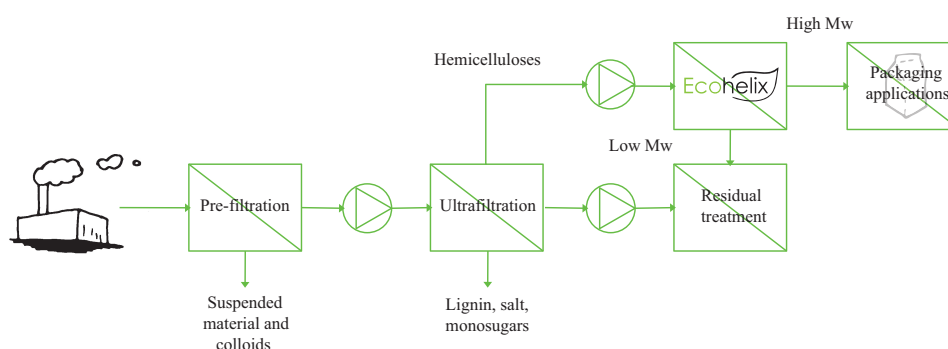

Figure 4-8. Processing scheme

To gain improved mechanical properties for polymer products, entanglement coupling phenomenon has to occur, which is dependent on the molecular weight of the polymers. The molecular weight between entanglements (Me) has been determined for cellulose and amylose with values of 3.2×10^3 g/mol and 2.5×10^4 g/mol respectively (Horinaka et al., 2011). This is the critical value of where entanglement coupling starts to occur that in turn affects the mechanical properties of polymers.

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The differences in the values for cellulose and amylose is believed to be due to that cellulose takes a linear and amylose a helical conformation due to intramolecular steric hindrance (Horinaka et al., 2011). Based on the known structure of galactoglucomannan and similarities to amylose and cellulose structures we can assume that galactoglucomannan has the Me-value between the values of amylose and cellulose.

It is therefore likely that by increasing the molecular weight by crosslinking, past the value of Me, it is possible to affect the properties of the produced material. The crosslinking in CTMP-derived hemicelluloses increased their molecular weight to about 26 kDa. This resulted in improved mechanical and thermal properties that were shown by mechanical and thermal testing.

The treatment also affected the mechanical strength of free standing films that exhibited 2-3 times increased stress at maximum load with hotwater extracted galactoglucomannans (paper I and table 4-1). The treatment also affected the thermal stability of the CTMP hemicelluloses. Similarly to previously published results for glucomannan (Moriana et al., 2014), the thermal decomposition of the produced galactoglucomannans occurred in two main decomposition stages. Figure 4-7 describes how the laccase crosslinking increases the onset temperature of second thermal decomposition process by 16 °C (from 206 to 222°C) indicating a more thermally stable polymer product. Furthermore the DSC results showed an increase in the T_g value of 15 °C for the cross-linked sample (GGM hMw, table 4-3).

Samples	T_g (°C)	ΔC_p J/(g °C)
GGM Ref	86.1 ± 1.0	0.132 ± 0.01
GGM hMw	101.3 ± 1.1	0.112 ± 0.01
GGM50/NFC50/G0	145.1 ± 1.2	0.152 ± 0.01
GGM70 NFC30/G0	145.7 ± 1.1	0.232 ± 0.01

Table 4-3. Glass transition parameters

Combined, the mechanical and thermal testing suggested greatly increased product properties. This could be due to two different reasons or their combination:

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1. The increased stability is due to the increased molecular weight beyond the assumed Me-value of the galactoglucomannans that would affect the product properties (Horinaka et al., 2011)

2. The increased stability is due to the linkages that are created between the aromatic units by the enzymatic treatment causing reduced mobility of the polymer chains (Chung et al., 2004)
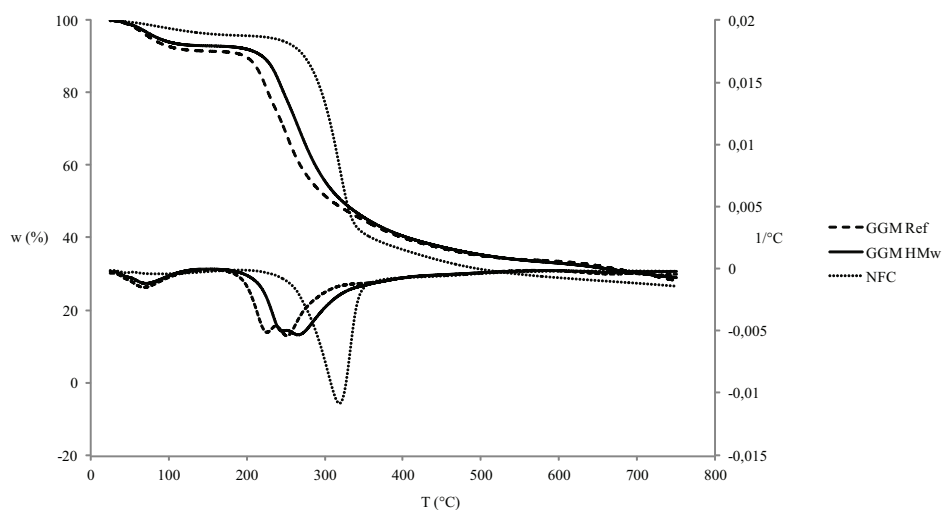

Figure 4-7. Thermal degradation pattern of the non-crosslinked and crosslinked CTMP hemicelluloses and NFC.

Although the mechanical strength and thermal stability of the polymers was significantly improved, high reinforcing polymers or nanoparticles was needed to further improve the properties of the films.

4.3.2 Nanocomposite formation with nanofibrillated cellulose to improve the film properties

The flexibility of a polymer product decreases with crosslinking and the increased Tg. On the other hand, by using plasticizers it is possible to increase the flexibility of the films. This is due to that plasticizers reduce the intermolecular interactions of the polymers. This however often lowers the oxygen transmission rate (OTR) of the polymer product. The final film product should therefore exhibit a balance of high enough molecular weight with an optimal amount of plasticizers for a high flexibility and strength and a low OTR-rate.

Eventhough we achieved an increased molecular weight and thermal stability of the hemicelluloses as described in paper V, the CTMP process derived hemicellulose product was still brittle when cast as freestanding films. We therefore used nanofibrillated cellulose (NFC) to find out if it could be used to create nanocomposites together with the hemicelluloses and get better film forming properties. Glycerol was used as a plasticiser to increase the strain behaviour of the films.

The mechanical testing showed expectedly how an increasing amount of NFC increased the strength and modulus of the films. Addition of glycerol increased the flexibility of the films, but decreased the modulus (table 4-4), which was also expected.

Sample	Stress (MPa)	Strain (%)	Modulus (GPa)
GGM50/NFC50/G0	101.8 ± 10.7	2.7 ± 0.2	5.89 ±0.37
GGM50/NFC35/G15	61.1 ± 3.4	3.4 ± 0.4	3.36 ± 0.25
GGM50/NFC25/G25	36.2 ± 10.1	11,5 ± 1.6	0.75 ± 0.12
GGM70/NFC30/G0	93.0 ± 4.3	1.9 ± 0.1	5.51 ± 0.09
GGM70/NFC15/G15	50.2 ± 2.3	2.5 ± 0.2	2.72 ± 0.02
GGM70/NFC5/G25	19.8 ± 0.9	1.9 ± 0.1	1.02 ± 0.18
NFC100	197.4 ± 14.1	16.5 ± 1.8	2.97 ± 0.52

Table 4-4. Mechanical testing of the formed composites. (GGM = the amount of cross-linked galactoglucomannan, NFC = the amount of nanofibrillated cellulose, G = the amount of glycerol in the samples
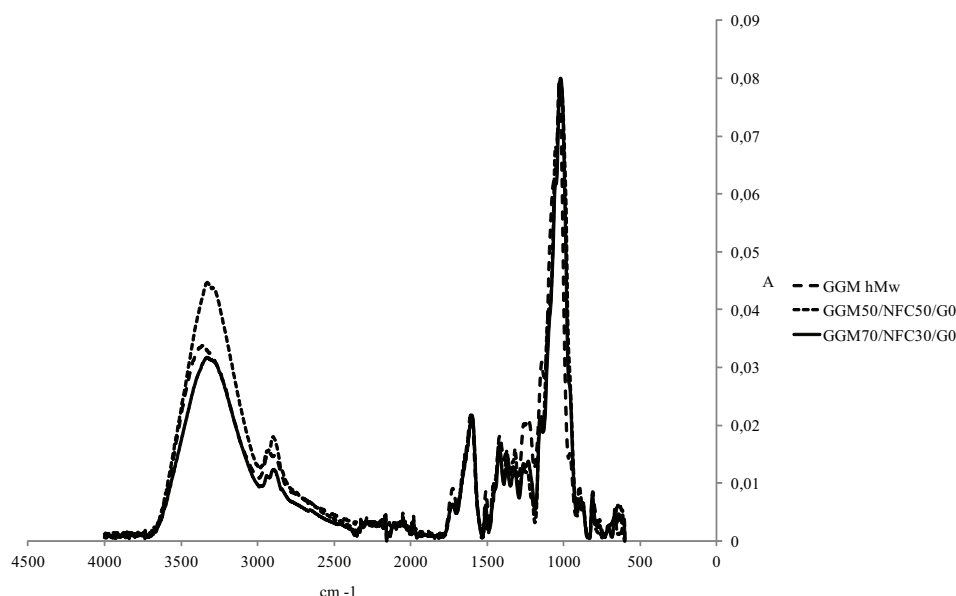
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Figure 4-8 FT-IR graphs of the cross-linked CTMP galactoglucomannan and composites formed with NFC

The thermal degradation pattern of the composites showed how composites with NFC were formed. The addition of NFC to the films had an improving effect on the mechanical strength (table 4-4) and thermal stability as can be seen on the onset temperature of the second decomposition process. The formulations with 30 and 50 % addition of the NFC resulted in a more thermal resistant material than the pure GGM Mw, indicating interaction between the pure materials (Le Normand, 2014). However, glycerol addition seemed to have a greatly deteriorating effect on the thermal stability of the films. Glycerol itself has an onset temperature of the second decomposition process at about 198°C, but the addition of glycerol to the films reduced the value to the lowest of 156.8°C with a 25% addition and a 25% NFC amount. Mikkonen experienced similar results with Norway spruce galactoglucomannans and konjac galactomannans (Mikkonen et al., 2010) and hypothesized that glycerol could have attached to the interface of the crystallites and the amorphous film matrix, lubricating the interfacial area and reducing the load transfer between these components explaining the mechanical properties phenomena. It is also known that plasticizers reduce the crystallinity of polymers that could be

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the reason for the reduced thermal stability (Zhang and Han 2010). This phenomenon was seen also in the FT-IR results where the samples with glycerol showed similar or higher OH-streching wavenumbers as the sample GGM hMw, indicating that glycerol was preventing the interaction of the hemicelluloses with the nanofibrillated cellulose and thus a composite structure was not formed.

In comparison, the galactoglucomannan together with NFC formed a nanocomposite. The interaction between the GGMhMw and the NFC was proven with the reduced wavenumber of the O-H stretching band founded for the GGM50/NFC50/G0 and the GGM70/NFC30/G0. The band related to the O-H stretching region for the pure GGMref decreased as a function of the NFC content, indicating an increase in hydrogen bond strength (figure 4-8)

As a conclusion, we found that hemicelluloses that were cross-linked by the laccase treatment had a higher thermal stability than the non-cross-linked samples. This was seen in the increased glass transition temperature and the thermal degradation profile. Furthermore, the produced high molecular weight galactoglucomannas could be used to form a nanocomposite together with NFC, which creates interest and potential to apply them in e.g. renewable packaging applications.

5 CONCLUDING REMARKS

5.1 BIOMIMETIC TECHNIQUE FOR CROSS-LINKING WOOD HEMICELLULOSES

This thesis describes the development of a new type of cross-linking technique for hemicelluloses that mimics the lignification phenomenon in wood. The results show how in the beginning of the wood component assembly oligomer sized hemicelluloses are likely cross-linked to the wood cell wall via aromatic moieties by radical chemistry catalyzed by the laccase enzyme. We believe that this is how lignin-hemicellulose complexes could be formed *in vivo* and make the wood cell wall structure more robust.

We could analyze how on a molecular level laccase catalyzes the oxidation of non-condensed guaiacyl structures attached to the hemicelluloses and as a consequence increase the molecular weight of the hemicelluloses. We saw how the carbohydrate structures stay intact during the treatment and characterized more branched structures in the proximity of the aromatics.
We up-scaled the process to small pilot scale and identified the industrial potential of the technology as a process and by analyzing the produced products. The technique enables the increasing of the molecular weight of the hemicelluloses creating improved mechanical strength in film products as well as increases the thermal stability of the polymers.

5.2 THE ECOHELIX TECHNOLOGY

The good results from this work enabled us to file a patent and start the commercialization efforts of the technology. This was started in a project funded by the Swedish Innovation Agency (VINNOVA) and made possible the up-scaling of the laboratory verified process together with an industrial partner. The technology has received a lot of interest and is currently being commercialized in a start-up company Ecohelix residing at the Greenhouse labs company incubator at KTH. For more information and updates, see: www.ecohelix.se or contact Petri at petri@ecohelix.se.

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