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Enzyme catalyzed cross-linking of spruce galactoglucomannan improves its applicability in barrier films

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ABSTRACT

Hemicelluloses are one of the main constituents of plant cell walls and thereby one of the most abundant biopolymers on earth. They can be obtained as by-products from different wood based processes, most importantly from the mechanical pulping. Hemicelluloses have interesting properties in e.g. barrier film applications. However, their relatively low molecular weight after isolation and co-extraction with lignin has limited their use. In this work, we present a novel technique for increasing the molecular weight of different wood hemicelluloses from mechanical pulping process waters as well as from pre-hydrolysis extracts. This is achieved by enzyme-catalyzed cross-linking of aromatic moieties bound to the hemicelluloses. The cross-linking treatment resulted in significantly improved mechanical properties in barrier films made with spruce galactoglucomannan. To our knowledge, this is the first time that wood hemicelluloses have been cross-linked by utilizing the bound aromatic moieties and creates new possibilities for utilizing this raw material source.

1. Introduction

Today the majority of both fuels and consumer plastics consist of petroleum-based precursors. The increased awareness of the negative environmental impacts and the unsustainable life cycle of these materials have led to an interest in new renewable raw material sources for energy and material applications. The plant cell wall, consisting of lignocellulose is likely to be the most widely distributed and abundant renewable raw material source available. 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 (Farrell et al., 2006; Shen & Patel, 2008).

Barrier films are used in packaging to increase the shelf life of the product by keeping it away from odors, oxygen gas etc. Petroleum based and aluminum films are dominating the field but films made of renewable materials have gained a lot of interest in recent years. Research has shown that each stage of the life cycle polysaccharide-based end products gives better environmental profiles than their petrochemical counterparts (Shen & Patel, 2008).

Lignocellulose is a complex material consisting of several different polymers that are linked to each other with chemical bonds (covalent and non-covalent) to form a highly resistant three-dimensional structure. The most important compounds are: *cellulose*, which consist of uniform, linear non-branched chains of anhydrous glucose with high degree of polymerization which further forms crystalline bundles, fibrils; *hemicelluloses*, a group of shorter heterogeneous polysaccharides, that form amorphous structures. The two most important classes of hemicelluloses are *xylans*, that dominate in hardwoods, but also occur in softwood, and *glucomannans* that dominates in softwood. The third main component is *lignin*, a web-shaped polymer formed from phenyl propanoid monomers. Together they form a composite type material, where the cellulose play the role of enforcing fibers, hemicellulose of flexible matrix and lignin locks the other compounds to each other by covalent and non-covalent interactions (Sjöström, 1993). Furthermore many researchers have recently reported evidence of lignin carbohydrate complexes, which can play an important role in the chemistry and processing of wood components (Laine, Tamminen, & Hortling, 2004; Lawoko, Henriksson, & Gellerstedt, 2006). The close association of the different wood components causes significant challenges in the fractionation, upgrading and the utilization of these components.

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 and 95% (Sundholm, 1999). The yield- losses of these processes

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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 process liquids from mechanical pulping of Norway spruce (*Picea abies*) can be fractionated by a combination of different filtration methods (drum filtration, microfiltration, ultra filtration and nanofiltration) yielding in a product enriched with low-molecular weight hemicelluloses. To isolate a mixture of 30 g hemicelluloses per liter with a purity of 80% aimed for barrier film production is expected to cost less than 700 €/ton (Persson, Nordin, Zacchi, & Jönsson, 2007). This price is highly competitive considering e.g. the market price of ethylene vinyl alcohol (EVOH) that is (together with aluminum) currently dominating the market of oxygen barrier applications. The low production cost thus speaks for the use of this source for e.g. material production (Persson et al., 2007).

The molecular weight of the main hemicellulose of softwoods, galactoglucomannans ranges between 30 and 60 kDa (Willför, Sundberg, Tenkanen, & Holmbom, 2008) and the main hemicelluloses of hardwoods, arabinoxylans, ranges between 0.56 and 40 kDa. The wide ranges reported likely reflect differences between tree species and methods of isolating the material (Teleman, Tenkanen, Jacobs, & Dahlman, 2002). As wood is processed, e.g., in mechanical pulping or during hydrothermal extraction, hydrolytic cleavage reactions decrease the molecular weight of the polysaccharides with increased temperature and process time (Song, Pranovich, Somerskiy, & Holmbom, 2008). Consequently, the polysaccharides that are dissolved in these processes tend to have a very low degree of polymerization ranging usually from 1 (monomers) to 100 (~20 kDa) depending on the processing conditions (Song et al., 2008). These small polysaccharides are causing discharges of oxygen demanding substances (COD), which are normally taken care of in biological purification of the outlet water. Not only is a construction and maintenance of such purification plant a significant cost for the mill, but this also results in that a major fraction from the process is wasted.

It is possible to use films made of hemicelluloses as gas barriers. Höije, Gröndahl, Tommeraas, and Gatenholm (2005) were able to create films with arabinoxylans from barley husks with reasonably good mechanical and gas barrier properties without the addition of plasticizers. To create a non-brittle film product from an industrial galactoglucomannan stream with a low average molecular weight, plasticizers needs to be used, usually in about 1:1 proportions (Edlund, Ryberg, & Albertsson, 2010; Mikkonen, Heikkilä, Helén, Hyvönen, & Tenkanen, 2010). The use of plasticizers tends to lower the strength and moisture tolerance of the films that can cause a problems in barrier film applications. This effect can be reduced by e.g. benzylation of the hemicelluloses (Hartman, Albertsson, & Sjöberg, 2006). Nanocrystalline cellulose (NCC) can be added to the hemicellulose films not only to create a nanocomposite with lowered moisture sensitivity but also greatly increased mechanical properties can be achieved (Saxena, Elder, Kenvin, & Ragauskas, 2010; Saxena, Elder, & Ragauskas, 2011). This is a highly attractive way to improve the film properties even in an industrial setup provided that the cost of the NCC is low. It is also shown that by adding high molecular weight polymers such as alginate or carboxymethyl cellulose into a physical blend with the hemicelluloses, it is possible to retain high mechanical strength together with high flexibility and low oxygen permeability of the films (Hartman, Albertsson, Söderqcist Lindblad, & Sjöberg, 2005). Higher amounts of NCC or high molecular weight polymers will however increase the price of the film product. Furthermore the high amount could lead to problems related to viscosity if a high dry matter amount in the solution needs to be used in the coating application.

Another approach to solve the problems related to the poor mechanical properties of wood hemicelluloses would be to produce a hemicellulose product with high average molecular weight, which would lead to improved mechanical properties and thus reduced need for plasticizers and supportive polymers. Cross-linking hemicelluloses through derivatization by chemical methods is possible (Voepel, Edlund, Albertsson, & Percec, 2011), but expensive and complicated in industrial production. Lignin can on the other side easily be cross-linked by oxidative treatment induced by enzymes (Hatakka, Nyyssönen-Hiekka, & Temmes, 1993; Viikari, Hase, Qvintus-Leino, Niku-Paavola, 1999; Areskogh, Li, Gellerstedt, & Henriksson, 2010). In addition to the low molecular weight, industrial wood hemicellulose products usually contain lignin contaminants. In fact, most of the researchers on this field have found it very difficult to gain a totally lignin free hemicellulose product (Willför et al., 2008).

As previously discussed, lignin–carbohydrate bonds have been shown to exist in wood. It has often been observed that these bonds are formed during the processing of the wood. This was shown e.g. by Laine and coworkers who reported residual lignin carbohydrate complexes from spruce and pine pulp (Laine et al., 2004). Lawoko and coworkers suggested that lignin is linked through covalent bonds to all the major polysaccharides in the wood cell wall through ester-, ether- or phenyl glucosidic bonds (Lawoko, Henriksson, & Gellerstedt, 2005; Lawoko et al., 2006). Evidence has also been found of the lignin polymer cross-linking various polysaccharides to each other (Lawoko et al., 2006). Grasses and cereals are known to contain hemicelluloses that contain covalently bonded phenolic moieties such as feruylated arabinoxylans. This has been used technically to crosslink the polysaccharides to aid baking processes (Schooneveld-Bergmans, Hopman, Beldman, & Voragen, 1997; Wende & Gry, 1996).

Laccase is a very useful enzyme for polymerizing and crosslinking phenolic structures. This enzyme is a multicopper oxidoreductase, which catalyzes one-electron oxidations in aromatic amines and phenols resulting in radicals ($4PhOH + O_2 \rightarrow 4PhO^{\bullet} + 2H_2O$) that may undergo polymerization. It only requires oxygen as a co-substrate and can display both ligninolytic and polymerizing abilities when acting on lignin. Laccases are found in fungi, bacteria, insects and plants. Many white-rot fungi produce laccases under ligninolytic conditions and they are believed to have a role in lignin degradation (Ten Have & Teunissen, 2001). On the other hand plants use laccases for polymerization reactions through monolignol oxidation during the formation of the secondary cell wall (Pesquet et al., 2001). Also fungal produced peroxidases can initiate phenolic radicals and lead to polymerization in similar reactions, but require hydrogen peroxide as the oxidizing agent (Hatakka, 2001).

Industrial laccase applications can be found in the textile industry applications such as denim bleacing (Tzanov, Basto, Guebitz, & Cavaco-Paolo, 2003). Also technical lignins can be cross-linked with laccase in industrial scale and applications could potentially be found in for instance resin production for fiberboard production and in cement applications. (Areskogh et al., 2010; Hatakka et al., 1993; Viikari et al., 1999). Laccase treatment has also been used in the wastewater treatment through the polymerization and precipitation of monomeric aromatic structures as well as in pitch control and in enzymatic bleaching (Widsten & Kandelbauer, 2008).

In this work, we present a method for crosslinking wood hemicelluloses obtained from pre-hydrolysis- and mechanical pulping processes based on laccase catalyzed cross-linking of aromatic moieties bound to the hemicelluloses. The produced high molecular weight spruce galactoglucomannans were used for the production of oxygen barrier films.

2. Experimental

2.1. Materials and pre-treatment

250 g batches of dried industrial chips (95% dryness), obtained from Norway spruce (*P. abies*), Eucalyptus (*Eucalyptus urograndis*) or 80 g wheat straw (*Triticum aestivum*, 94% dryness) were charged to batch autoclaves with 2 liters of de-ionized water. The Eucalyptus and wheat straw was processed in 150 °C and Norway spruce in 160 °C for 60 min after which the residue was separated from the hydrolyzate on a paper machine filter. The residue was washed with 500 ml boiling de-ionized water and the liquid fractions were combined. The sample was filtrated by microfiltration (Mini kerasep module, Novasep) with a 0.45 μm ceramic membrane (The kerasep 0.45 μm, Novasep) and upgraded either by ultrafiltration or ethanol precipitation, as described further below. After the upgrading the sample was freeze-dried.

Process waters from thermomechanical pulping (TMP) process of Norway spruce (*P. abies*) were taken from the process stream of a Swedish TMP mill. The sample was filtrated by microfiltration (Mini kerasep module, Novasep) with a 0.45 μm ceramic membrane (The kerasep 0.45 μm, Novasep) and upgraded by ultrafiltration (The kerasep, 1 or 5 kDa molecular weight cut-off membrane, Novasep) or ethanol precipitation. After the upgrading the sample was freeze-dried.

A fungal laccase (E.C. 1.10.3.2), denoted NS 51002, was kindly donated by Novozymes (Bagsvaerd, Denmark) and was used in all oxidation experiments without further purification. The enzyme originated from the basidiomycete *Trametes villosa*, and the activity was determined to be 700 U ml^{-1} as described below. The other laccase used in the studies (Sigma 53739, Sigma–Aldrich) was purchased from Sigma–Aldrich, the enzyme originates from the basidiomycete *Trametes versicolor*.

2.2. Ultra filtration, crosslinking and fractionation

10 l of the filtered hydrolyzate from the hot water extraction of Norway spruce was upgraded by fractionation using ultrafiltration (Mini kerasep module, Novasep) employing a ceramic membrane with a cut-off of 1 kDa (The kerasep 1 kDa, Novasep). The membrane filtration was performed to concentrate the retentate (the high molecular weight fraction) down to a volume of 2 l and thus giving 7 l permeate (the low molecular weight fraction). The high molecular weight fraction was further purified by diluting it with de-ionized water to a 9 l volume and then again membrane-filtering down to a volume of 2 l. The yield of high molecular weight materials thus obtained from the wood hydrolyzates by membrane filtration was 52% based on dry weight of the hydrolyzate.

Ultra filtration of the TMP sample was conducted as described in the previous section with the exception that a second sample was produced by employing a 5 kDa ceramic membrane to produce two high-molecular weight fractions with a dry content of 53.2% and 44.6% (for 1 kDa and 5 kDa membrane respectively).

Both the Eucalypt and Wheat straw hydrolyzates were further purified by ethanol precipitation to isolate the polysaccharides from organic solvent-soluble substances. The hydrolyzate was first concentrated by vacuum evaporation. The sample was then added to technical grade ethanol the volume percentage of ethanol being at least 90, and the polysaccharides were allowed to precipitate overnight in a cold room (+4 °C). The samples were centrifuged at 4000 g for 25 min with a Rotofix 32 A centrifuge (Hettich, Germany). The precipitated polysaccharides were collected and washed twice with ethanol and once with acetone. The precipitate was finally dried under vacuum.

Two different laccase-enzymes (NS51002, Novozymes Bagsværd, Denmark and Sigma 53739, Sigma–Aldrich) were used for oxidative cross-linking of the purified polysaccharides. The reaction conditions were as follows: enzyme dosage 14 U/g (~1.4 mg/g substrate) of hydrolyzate, pH 5, reaction temperature +40 °C, reaction time 3 h and hydrolyzate concentration 100 g/l. Pure oxygen gas was introduced to the samples during the reaction.

The cross-linked samples were fractionated to high and low molecular weight fractions by ultra filtration (Solvent-Resistant Stirred Cell, Millipore) employing a cellulose membrane with a molecular weight cut-off of 30 kDa (PLTK07610, Millipore). Prior to ultra-filtration, the samples were diluted ten times. Ultra-filtration was conducted under nitrogen atmosphere (3 bar) and constant stirring and continued until a retentate/permeate volume ratio of 1/10 was achieved. The retentate was further diluted 10 times and the ultrafiltration was repeated. After the repeated ultra-filtration, a total of three fractions were collected; a high-molecular weight retentate (high Mw) and two low-molecular weight permeate (low MW) fraction. The two permeate fractions were subsequently combined to a single low Mw-fraction. Control filtrations for untreated samples were committed in an identical manner.

2.3. Film preparation

Each of the samples was dissolved in water in (50 °C) under stirring. Centrifugation was performed (4000 × *g*, 15 min) to remove the small amount of non-soluble material. Carboxymethyl cellulose sodium salt (21 900, low viscosity, purity > 99.5%, Fluka Chemie A.G., Sigma Aldrich, Stockholm, Sweden) and glycerol (Ultrapure, Alfa Aesar, Ward Hill, MA, USA) were mixed into the sample as high-M_w-polymer and plasticizer respectively in the amounts of 30–40 wt. %, yielding a mixture with a concentration of 0.0013 × *g* solid/ml and a total dry mass of 400 mg.

The mixture was thereafter cast in a flat dish (9 cm × 9 cm) that was covered with a thin film of Teflon. The water was allowed to slowly evaporate at room temperature until it was completely dry, producing thin, transparent and dry films, which were manually removed from the dishes. The mechanical properties and the oxygen gas transmission rate of the films were measured.

2.4. Chemical analysis methods

Laccase activity was determined by oxidation of 2,2′-azinobis(3-ethylbenzthiazoline-6-sulphonate) (ABTS) (Bourbonnais & Paice, 1990). The reaction mixture contained 0.5 mM ABTS, 0,1 M phosphate buffer, pH 5 and a suitable amount of enzyme. Oxidation of laccase was followed by absorbance increase at 420 nm ($\varepsilon_{420\,nm} = 3.6 \times 10^4\ M^{-1}\ cm^{-1}$). Enzyme activity was expressed in units (1 U = 1 μmol ABTS oxidized per minute and ml enzyme).

The carbohydrate and lignin compositions of the samples were determined by employing the hydrolysis conditions described in the TAPPI -standard method (Tappi test methods, 2003). The mono sugar analyses were performed with a HP 6890 series gas chromatography device with a BP-70 column (60 m, 0.32 μm I.D., and 0.25 μm film thickness). Anhydro corrections of 0.9 and 0.88 were used for the hexoses and pentoses, respectively.

The ash content of the samples was measured according to the TAPPI–standard method (Tappi test methods, 1985). The ash and Klason lignin content was not measured for the wheat straw and Eucalyptus samples because of the small sample size.

2.5. Size exclusion chromatography

The samples were analyzed using a size exclusion chromatography system consisting of a Rheodyne 7725i (Rohnert Park, CA, USA) manual injector, a Waters 515 HPLC pump (Milford,

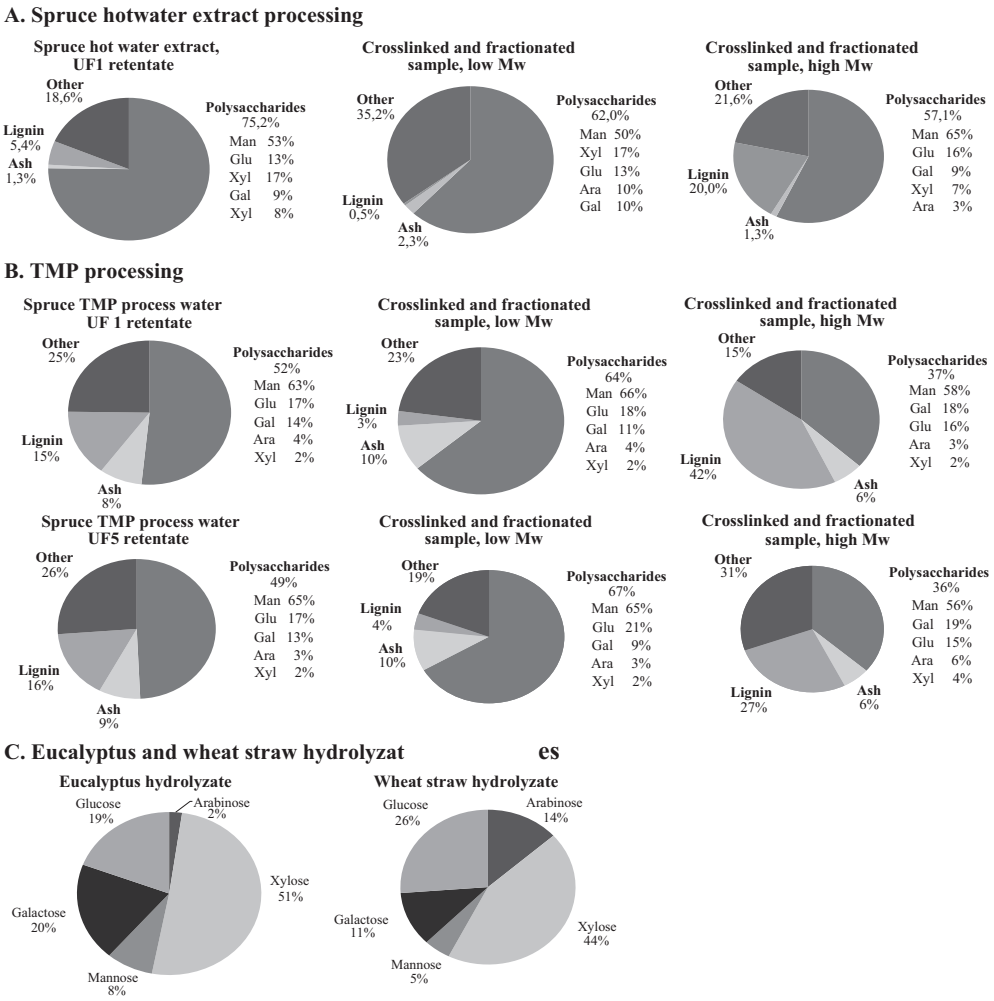

Fig. 1. (A) The composition of the spruce hotwater extract ultra filtrated with 1 kDa MWCO membrane as well as the fractions after the enzymatic cross-linking and fractionation by ultra filtration with a 30 kDa MWCO membrane. (B) The composition of the process water from the TMP process ultrafiltrated 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 hot water extracts purified by ethanol precipitation.

MA, USA) and three TSK-gel columns (Tosoh Bioscience, Tokyo, Japan) coupled in series, G3000PW (7.5×300 mm, 10 μm particle size), G4000PW (7.5×300 mm, 17 μm particle size) and another G3000PW. For detection, a Waters 2487 dual wavelength absorbance detector (Milford, MA, USA) and a Waters 410 Refractive Index (RI) detector (Milford, MA, USA) were used. The eluent system utilized was 10 mM sodium hydroxide made in ultra-pure laboratory grade Milli-Q water. A volume of 20 μl was injected, and the UV absorbance at 280 and 254 nm and RI detection was recorded. The columns were calibrated with polyethylene glycol (PEG) and polyethylene oxide (PEO) standards with specific molecular weights ranging from 1500 to 400 000. The integration and quantification of peaks were performed using the supplied Millenium 2 software.

2.6. Mechanical properties

The mechanical properties of the films were tested according to the ASTM D882-09 standard (ASTM, 2009). The stress–strain behavior of the films was determined either by using a Minimat 2000-equipment (Rheometric Scientific) equipped with a 20 N load cell, and controlled by the Minimat software (Rheometric Scientific, version 2.4.6) or with Instron 5944-machine equipped with a 50 N load cell and controlled by the Bluehill 2 software (Instron) depending on the maximum strength of the sample. Films were cut into thin rectangular strips with a width of 5 mm and a gauge length of 15 mm. The stress–strain curves of specimen samples were recorded at room temperature and 50% RH at a strain rate of 10% min^{-1}. At least three specimens were tested from each sample and results were reported for those specimens that do not show premature failure at the jaw face. Stress–strain curves were plotted, and the Young's modulus (E) was determined from the slope of the low strain region in the vicinity of 0.05% strain.

2.7. Oxygen transmission rate

The oxygen gas transmission rate of the films was measured 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).

3. Results and discussion

3.1. The samples for the crosslinking

The hydrothermal extraction liquids and thermomechanical pulping liquids produced a hydrolyzate that contained a high

Table 1
The molecular weights of the samples before and after the enzymatic treatment and fractionation. SHw = spruce hotwater extract, TMP = thermomechanical pulping water, EGHw = *eucalyptusgrandis* hotwater extract, WSHw = wheat straw hotwater extract, UF1 = sample ultrafiltrated with 1 kDa MWCO membrane, UF5 Sample ultrafiltrated with 5 kDa MWCO membrane.

Sample SHw UF1	Reference	NS 51002 treated sample		
		Unfractionated sample	Low-Mw sample	High-Mw sample
Mn (kDa)	7.0 ± 0.1	8.4 ± 0.2	7.4 ± 0.1	23.8 ± 0.2
Mw (kDa)	10.4 ± 0.2	28.1 ± 0.5	10.5 ± 0.2	62.7 ± 0.9
Mw/Mn	1.5 ± 0.05	3.3 ± 0.03	1.4 ± 0.01	2.6 ± 0.02
Sample SHw UF1, ethanol precipitate	Reference	NS 51002 treated sample		
Mn (kDa)	5.9 ± 0.0	5.5 ± 0.1		
Mw (kDa)	7.7 ± 0.1	12.9 ± 0.2		
Mw/Mn	1.3 ± 0.01	2.4 ± 0.02		
Sample TMP UF1	Reference	NS 51002 treated sample		
		Unfractionated sample	Low Mw sample	High Mw sample
Mn (kDa)	11.3 ± 0.2	15.1 ± 0.2	10.0 ± 0.1	27.1 ± 0.2
Mw (kDa)	17.6 ± 0.3	37.6 ± 0.2	14.5 ± 0.3	59.5 ± 0.2
Mw/Mn	1.6 ± 0.01	2.5 ± 0.02	1.4 ± 0.02	2.2 ± 0.01
Sample TMP UF5	Reference	NS 51002 treated sample		
		Unfractionated sample	Low Mw sample	High Mw sample
Mn (kDa)	14.8 ± 0.2	16.2 ± 0.4	9.7 ± 0.1	20.5 ± 0.1
Mw (kDa)	22.4 ± 0.1	32.9 ± 0.6	13.6 ± 0.2	40.0 ± 0.3
Mw/Mn	1.5 ± 0.01	2.0 ± 0.02	1.4 ± 0.01	1.9 ± 0.01
Sample TMP UF5	Reference	Sigma 53739 treated sample		
Mn (kDa)	14.8 ± 0.2	17.1 ± 0.1		
Mw (kDa)	22.4 ± 0.1	34.2 ± 0.2		
Mw/Mn	1.5 ± 0.01	2.0 ± 0.01		
Sample EGHw, ethanol precipitate	Reference	NS 51002 treated sample		
Mn (kDa)	7.3 ± 0.1	10.7 ± 0.2		
Mw (kDa)	12.6 ± 0.1	57.1 ± 0.1		
Mw/Mn	1.7 ± 0.01	5.3 ± 0.08		
Sample WSHw, ethanol precipitate	Reference	NS 51002 treated sample		
Mn (kDa)	17.3 ± 0.2	23.9 ± 0.2		
Mw (kDa)	37.7 ± 0.2	59.5 ± 0.2		
Mw/Mn	2.2 ± 0.01	2.5 ± 0.02		

amount of organic material consisting mostly of hemicelluloses. By using either ultrafiltration or ethanol precipitation it was possible to produce fractions with high hemicellulose content (Fig. 1). These fractions were used for the cross-linking reactions.

It is to be noted that in addition to lignin (Klason lignin), polysaccharides and ash the hydrolyzates likely contain also proteins, organic acids (incl. sugar acids), lipophilic substances and lignin degradation products. This is stated as "other" in Fig. 1.

Table 2
A. The stress at maximum load, stain at break and the elastic modulus of films made with 20 wt% CMC and 20% glycerol together with either 60 wt% of hotwater extracted Norway spruce galactoglucomannan or different processed fractions or 60 wt% TMP galactoglucomannan and processed fractions. B. The stress at maximum load, stain at break and the elastic modulus of films made with 70 wt% TMP galactoglucomannan or different processed fractions. Abbreviations: SHw = spruce hotwater exctraction; TMP = thermomechanical pulping process water; UF1 = ultrafiltration with a 1 kDa MWCO membrane; C = crosslinked sample; Low Mw = permeate after crosslinking and the following ultrafiltration with a 30 kDa MWCO membrane; High Mw = retentate after crosslinking and the following ultrafiltration with a 30 kDa MWCO membrane.

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

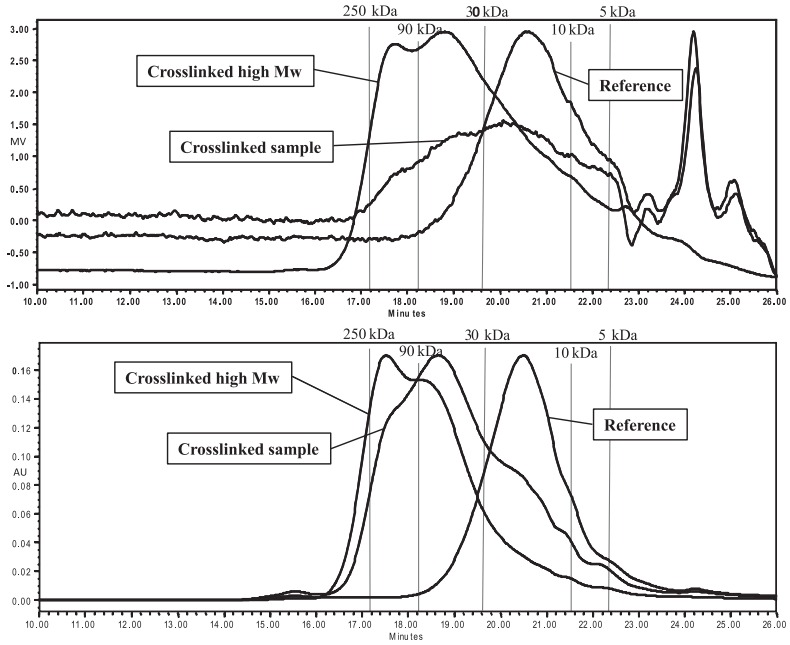

Fig. 2. Size exclusion chromatography of TMP process water upgrading experiments: a typical SEC-graph showing how the laccase treatment increases the molecular weight of the sample, as indicated by the RI-detector (upper graph) and how the absorbance of aromatic structures (UV absorption in 280 nm, lower graph) is transferred upstream, to the high molecular weight range. There are non-aromatic small molecular weight substances eluting after 24 min in the TMP samples. These were removed during the ultrafiltration after the cross-linking.

Sugar analysis revealed that the Norway spruce hot water extract and the TMP process water sample consisted mostly of galactoglucomannans and to lesser extend also arabinoxylans. The Eucalypt hot water extracts contained xylan and galactan as well as glucan. The wheat straw hydrolyzate consisted mostly of a arabino(glucurono)xylan but also of glucans and traces of other hemicelluloses.

3.2. Crosslinking of polysaccharides with bound aromatic moieties

Subjecting the hemicellulose mixtures to laccase oxidation resulted in increased average molecular weight in all of the samples (Table 1). After fractionation of the cross-linked sample by ultra filtration (30 kDa cut-off membrane) into two fractions, the retentate showed a very high average molecular weight distribution with a high UV response indicating enrichment of aromatic structures to this fraction. Furthermore the UV-detector showed how the UV signal was transferred from being evenly distributed along all molecular weights in the reference sample into the high molecular weight area after the oxidative treatment suggesting that all polysaccharides that contain lignin moieties were cross-linked and formed large molecules (Fig. 2). As the reported molecular weights were achieved by size exclusion chromatography that is giving information about the hydrodynamic volume of the molecules rather than the actual molecular weight, we are not able to report accurate molecular weight data. We are likely underestimating the actual molecular weight of the cross-linking products because the cross-linking could result in branched polymer structures that do not correspond to the three dimensional structure of the linear standard polymers used in the calibration. It is likely however that the cross-linking product is somewhat linear based on the increased tensile strength in the tested film products (Table 2).

It is well documented in the literature that the laccases catalyze the monoelectronic oxidation of phenols and aromatic or aliphatic amines in a system where laccase mediators are not present (Riva, 2006). Considering the composition of the studied samples, it is therefore likely that the oxidation that is followed by the cross-linking and increased molecular weight is due to the reactions in the phenolic structures present in the samples. It would however be of great interest to get structural data on the cross-linking positions. This could potentially be achieved by using NMR and MS-methods.

The yield of high molecular weight substances after the crosslinking and fractionation was 23% for the Spruce hotwater extracts and 52% for both TMP process water samples. Wheat straw and Eucalypt hydrolysates and ethanol precipitation samples were not fractionated. The control filtrations for untreated samples with the 30 kDa cut-off membrane yielded in only 2–5 wt% retentate yields.

Similar observations were made while processing all of the samples that were used in the study. The observed broad substrate range is due to that the oxidative cross-linking reaction is based on the aromatic moiety instead of the polysaccharide structure and suggests that all of the studied hemicelluloses contained polysaccharides with bound phenolic moieties.

The increased polydispersity after the oxidative treatment suggests that the cross-linking phenomenon occurs randomly which is to be expected considering that laccases only create phenoxy radicals and does not participate in the following coupling reactions. It also seems that some of the polysaccharides are not affected by the treatment indicated by the different shape of the UV response in comparison to the RI signal after the treatment; the UV signal is strongly moved to the high molecular weight area, while the RI signal is more evenly distributed. This suggests that the samples contain both free hemicelluloses and hemicelluloses with bound aromatic moieties.

Chemical composition analysis (Fig. 1) of the products after the oxidative cross-linking treatment and fractionation revealed that a majority of the aromatic moieties were located in the high-Mw fraction (higher than 30 kDa). On the contrary, the low-Mw fraction contained significantly lower amounts of lignin. This is a highly interesting finding as it means that the technique makes it possible to crosslink hemicelluloses that contain bound aromatic moieties into high molecular weight polymers and separate them

from non-aromatic hemicelluloses. This has not previously been reported.

3.3. The effect of the increased molecular weight to the film properties

By preparing films with the hemicelluloses from the different fractions, it was observed how the molecular weight affected the strength properties of the films. When comparing a non-treated (Norway spruce TMP derived) hemicellulose sample with a cross-linked high-Mw sample in a system with 60% hemicellulose amount, a four-time increase in the stress at maximum load was seen while the strain at break remained more or less the same (Table 2A). More interestingly the high-Mw hemicellulose fraction was the only sample that could be used for production of a film with 70% hemicellulose amount (Table 2B). This particular film gave good mechanical properties and the measured oxygen transmission rate was 0.6 ml/(m^2 24 h), indicating a very good oxygen barrier film. The ability to use a high amount of the hemicelluloses in the final film product is very important when considering the cost of a commercial product. Furthermore the small amount of plasticizer makes the product even more interesting because it potentially minimizes the plasticizer migration problem.

4. Conclusions

This manuscript describes the development of a novel enzymatic method for cross-linking and fractionating hemicellulose mixtures. We believe this to be an important technique facilitating the use of wood hemicelluloses derived from pre-hydrolysis- and mechanical pulping processes and partly solving the problems arising from the low molecular weight of the polymers giving poor material properties. By taking advantage of the bound aromatic moieties, it is possible to increase the molecular weight of the polysaccharides using oxidative cross-linking. This may facilitate the use of these hemicelluloses as barrier films industrially.

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