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NOTE

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Antibody-based assay for galacturonan deesterification on wood pulp fibers during bleaching

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Abstract Antibodies recognizing galacturonan were used in immunoassays for industrial unbleached and hydrogen peroxide-bleached chemithermomechanical pulp (CTMP). The assays were based on a colorimetric method using specific monoclonal antibody recognition of acidic and methylated homogalacturonan, respectively, on the pulp fiber surfaces. Alkaline phosphatase-conjugated antibodies were linked to the galacturonan specific antibodies, and an alkaline phosphatase substrate (*p*-nitrophenylphosphate) was used to develop a yellow reaction product that can be followed spectrophotometrically. Together the newly developed immunoassays were able to describe the deesterification, specifically, of surface-localized galacturonan on CTMP, induced by alkaline hydrogen peroxide bleaching. Unbleached CTMP showed relatively more labeling by methylated galacturonan recognizing antibodies, whereas bleached CTMP was relatively more labeled for acidic galacturonan. The increase in negative charge of the bleached pulp was also shown by polyelectrolyte titration; the negative surface charge was $9.3\,\mu eq\,g^{-1}$ in unbleached CTMP and $21.7\,\mu eq\,g^{-1}$ in bleached pulp.

Key words Charge · Chemithermomechanical pulping · Immunoassay · Pectin

Introduction

The properties of chemithermomechanical pulp (CTMP) depend on, among other things, the wood used and the defibration conditions. The quality of the finished paper product is also influenced by fiber-to-fiber bond strength, which in turn is affected by the structure and chemistry of the pulp fiber surface. Therefore, the pulp surface is an important aspect for the paper.[1] Negative fiber charge is one property that has been shown to affect the pulp, and in mechanical pulp, pectin is a source of negative charge. The main pectic substructure, galacturonan, has a high negative charge density because the polysaccharide is mainly composed of (1→4)-linked α-D-galacturonic acid residues that are partly esterified by methanol, with single units of rhamnose interspersed within the galacturonic backbone.[2] In solid wood, pectin containing galacturonan is localized between the fibers.[3] Therefore, after fiber separation by mechanical pulping, some pectic galacturonic acid is exposed at the fiber surface.[4] Acidic groups have previously been suggested to relate to strength properties in mechanical pulp; in pulps with different surface and total fiber charges, a positive correlation between some paper strength properties and negative charge was indicated.[5] Industrially bleached mechanical pulp has higher negative charge than unbleached pulp when bleached with alkaline hydrogen peroxide. This is due to extensive de-esterification of polysaccharides and introduction of new carboxylic groups on lignin. To better understand, predict, and develop the pulping and paper-formation processes, detailed information of the pulp surface chemistry of bleached and unbleached pulp is desirable. However, because wood fibers are structurally and chemically heterogeneous, detailed and spatially resolved chemical information of specific components with restricted localization within the wood pulp are difficult to obtain, even with advanced spectrometric and spectroscopic methods. Therefore, biomarker-based analyses are being developed, and recently a bioassay based on a colorimetric method using specific monoclonal antibody recognition of methyl-esterified homogalacturonan was developed for analysis of wood pulp surfaces.[6] In this study, a bioassay for nonesterified galacturonan is presented, and the combined immunoassays for acidic and methylated galacturonan have been applied to unbleached and alkaline hydrogen peroxide-bleached CTMP to study the de-esterification of the pulp fiber surface galacturonan during the bleaching process. The increase of negative charge on the pulp fiber surface, due to bleaching treat-

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ment, was also analyzed by polyelectrolyte titration, and correlated to the presence of carboxylic groups on the pulp fiber surface.

Experimental

Pulps and antibodies

Unbleached and hydrogen peroxide-bleached CTMPs, predominantly derived from Norway spruce (*Picea abies* Karst.), were used. The pulps were industrially produced at the Östrand mill in Timrå, Sweden, by SCA, and delivered to the laboratory and used fresh without prior pretreatments. The antibodies PAM1 (antihomogalacturonan phage display monoclonal antibody) and JIM7 (rat antihomogalacturonan IgA) were gifts from W.G.T. Willats and J.P. Knox at University of Leeds, and K. Roberts at John Innes Institute, UK, respectively. Monoclonal antipolyhistidine clone HIS-1 (H-1029) and antimouse IgG alkaline phosphatase conjugate (A3562) were from Sigma-Aldrich. Goat antirat IgG conjugated to alkaline phosphatase was from Amersham Pharmacia Biotech.

Immunoassays for galacturonan

The general labeling procedure for the pulps was performed as described earlier,[6] using PAM1 against acidic galacturonan and JIM7 for partially methyl-esterified galacturonan. Pulps samples (10 mg dry weight) were placed in sodium phosphate-buffered saline (PBS buffer, pH 7.2) containing 3% (w/v) bovine serum albumin (BSA) for 30 min. Then 100 μl of PAM1 (diluted 1:100 in PBS with 3% BSA) replaced the blocking buffer, and after overnight incubation at 4°C the samples were washed in PBS (5 min × 3) and then labeled with 100 μl of antipolyhistidine clone HIS-1 (diluted 1:100 in PBS with 3% BSA) for 4 h, at room temperature. Thereafter, samples were washed again in PBS (5 min × 3) and labeled overnight at 4°C with 100 μl of antimouse IgG alkaline phosphatase conjugate (diluted 1:1000). Afterward, the pulps were washed in PBS (containing 0.1% Tween 20, 5 min × 3) and distilled water (5 min × 3). Alkaline phosphate liquid substrate (*p*-nitrophenylphosphate, pNPP) was added (160 μl/sample) and the color development of 150-μl samples was monitored at 405 nm. All spectrophotometric assays were performed using a BioTek EL311 microplate autoreader. By omitting PAM1 in the labeling procedure and by using galacturonan-free pulps, labeling-specificity controls were performed. Negative controls showed no significant labeling. The procedure for labeling pulps with JIM7 was the same as outlined above for PAM1, with the exception that no intermediate linker antibody was needed. For the colorimetric analysis, a secondary antibody with alkaline phosphatase conjugated with goat antirat IgG was used.

Fiber composition and surface charge

Neutral sugar compositions of the pulps were analyzed after the pulp samples were hydrolyzed by sulfuric acid treatment. The dissolved sugars were derivatized and analyzed as alditol acetates by gas chromatography,[7] while the lignin-containing residue was filtered off and determined gravimetrically (Klason lignin).[8] The fiber surface charge was determined by polyelectrolyte titration,[9] and the Canadian Standard Freeness (CSF) of the pulps were analyzed using a standard ISO test method.[10]

Results and discussion

Alkaline hydrogen peroxide treatments induce complex chemical reactions and alterations to the pulp fiber surface properties during bleaching. However, chemical analysis of large surface-specific areas are not readily performed on insoluble, heterogeneous, and natural biopolymer composite materials such as wood fibers. Therefore, alternative methods based on biomarkers have been developed for fiber surface studies. Recently, an antibody-based bioassay for partially methyl-esterified galacturonan on wood pulp fibers has been developed.[6] To a galacturonan-specific antibody, a secondary antibody conjugated with alkaline phosphatase was bound; thereafter, when adding *p*-nitrophenylphosphate (an alkaline phosphatase substrate), a yellow product forms at a rate correlating to the amount enzyme present in the sample, and the increase in optical density is monitored using a spectrophotometer. The linear regression-fitted slopes of the absorbance increase over time correlate to the specific amount of labeled galacturonan present on the pulp surface. When the colorimetric assay for methylated galacturonan were used for surface characterization of two wood pulps, it was able to successfully recognize different qualities.[6]

Methylated galacturonan on the pulp fiber surface

When CTMP is bleached in alkaline conditions, the carboxylic group at the C-6 position of the galacturonic acid residue is deesterified, which results in the liberation of carboxylic acid (Fig. 1). The base-catalyzed deesterification produces randomly distributed free carboxylic acids along the galacturonan polymer backbone; thus, CTMP contain-

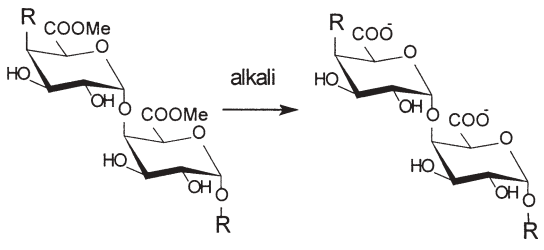

Fig. 1. Alkali deesterification of homogalacturonan (R = galacturonan backbone)

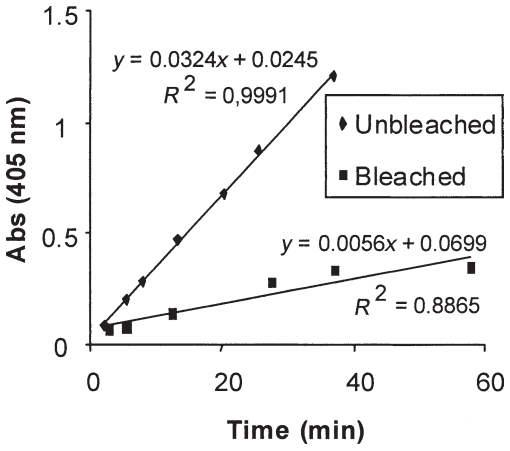

Fig. 2. Chemithermomechanical pulp (CTMP) fibers labeled for methylated galacturonan. Unbleached CTMP shows a higher rate of change in absorbance than bleached CTMP. The data are mean values of duplicates

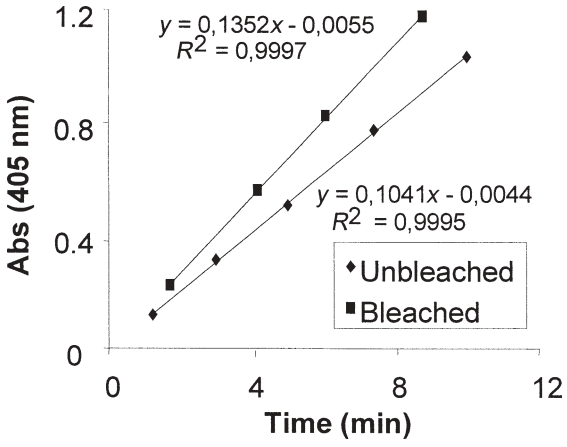

Fig. 3. CTMP fibers labeled for acidic galacturonan. Bleached CTMP shows a higher rate of change in absorbance than unbleached CTMP. The data are mean values of duplicates

ing methyl-esterified galacturonan will become more acidic during alkaline bleaching as demethylation generates new carboxylic groups in the fibers. When bleached and unbleached CTMP samples were subjected to colorimetric measurements using the JIM7 monoclonal antibody, specific to partially methyl-esterified homogalacturonan,[11,12] the rate of color development was significantly reduced for the bleached CTMP sample, which indicate a reduction in the degree of methylation of the galacturonan exposed on the CTMP surface (Fig. 2).

Assay of acidic galacturonan

Because bleaching is known to dissolve some pectic substances,[13] a colorimetric assay giving a positive response with increased absorbance, as a result of the demethylation, was developed in order to corroborate the results from Fig. 2. The bioassay was adapted for analysis of acidic homogalacturonan using an antibody called PAM1, which specifically recognizes regions of about 30 nonesterified galacturonic acid residues. PAM1 comes from a naive phage display library and is a recombinant single chain antibody fragment that has a N-terminal polyhistidine (HIS).[14,15] The HIS tag was used for linking the alkaline phosphatase-conjugated antibody to the epitope. The alkaline-treated pulp (i.e., bleached) generated faster optical density development than unbleached pulp (Fig. 3), indicating an apparent increase in the amount of accessible nonesterified galacturonic acid residues on the surface of the bleached pulps, which is in agreement with the results in Fig. 2. The loss of some galacturonan during bleaching probably reduced the total amount of galacturonan on the fiber surface; however, the deesterification of the galacturonan still present, seems to add more negative charge to the fiber surface than what is lost to galacturonan dissolved from the pulp fiber surface.

Table 1. Characterization of chemithermomechanical pulp before and after hydrogen peroxide bleaching

Analysis monosaccharide[a]	Unbleached	Bleached
Arabinose	1.1	1.2
Xylose	5.0	5.1
Galactose	1.7	1.6
Glucose	43.3	44.1
Mannose	12.1	11.7
Lignin[a]	27.1	27.1
CSF[b](ml)	443	401
Surface charge[c] (μeq g^{-1})	9.3	21.7

[a] Dry weight percentage, mean values of duplicates
[b] Canadian standard freeness, according to standard test method
[c] According to standard test method

Fiber composition and charge

The overall chemical composition, freeness, and surface charge were analyzed for the two different pulp qualities (Table 1), and the neutral monosaccharide composition and lignin content indicated that the bleaching had not produced any major differences in chemical composition between the pulps. The Canadian standard freeness (CSF) showed the bleached pulps to have somewhat lower values but the pulps were similar enough to expect it to not significantly affect the colorimetric assays. The surface charges of the pulps were analyzed by polyelectrolyte titration, and there was an evident increase in overall surface charge as a result of bleaching of the pulp. In hydrogen peroxide-bleached high-yield pulps, most of the polysaccharides and lignin are retained in the fibers, but some process-induced chemical alterations of the wood components are known to occur. The increased surface charge would presumably be the result of the combined effect of the bleaching process on all carboxylic acid esters and lactones in the fibers (i.e., also hemicelluloses). Hydrogen peroxide treatment also increases negative charge in CTMP through hydroperoxide anion reactions with enone structures in lignin to form carboxylic end groups. In addition, other reactive species (hydroxyl and superoxide anion radicals), formed during metal

ion-catalyzed hydrogen peroxide decomposition, can also lead to the formation of carboxylic acid groups in the lignin structure.[16] Thus, the higher negative charge of hydrogen peroxide-bleached CTMP compared with unbleached CTMP results from both polysaccharide and lignin modifications. Titrations for fiber-charge analysis determine the carboxylic acid content unspecifically, whereas the immunoassay method presented in this study provides a useful tool for surface analysis of specific structures; any structures that can function as epitopes for antibody recognition. A physically masked or changed epitope would leave it unrecognized by an antibody. If JIM7 labeling of bleached CTMP were reduced (compared with unbleached) due to something other than deesterification, the PAM1 antibody labeling would also have been negatively affected. Therefore, the decreased amount of labeling of bleached CTMP, by JIM7, and the subsequent increased amount of labeling of PAM1 of the same pulp, together indicate that the immunoassays can successfully be used to show the galacturonan deesterification on wood pulp fibers during bleaching.

Conclusions

Changes in the methylation of galacturonan on CTMP during bleaching can be detected using immunoassays based on specific antibody recognition of methylated and acidic galacturonic acid residues, respectively. The decrease in methyl-esterified galacturonan labeling corresponded with an increase in labeling of acidic galacturonan in bleached CTMP when compared with unbleached CTMP. The successful application of the developed bioassays for analyzing changes in esterification on wood pulps due to industrial processing were corroborated by surface charge analysis by polyelectrolyte titration. The approach of combining two immunoassays to analyze the decrease and increase in antibody-labeling intensity, has in this study been shown to be a useful tool for analyzing process-related surface-localized changes in specific chemical structures. A similar approach should also be generally applicable to other modifications of surface-localized biopolymers.

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