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Distribution of methyl-esterified galacturonan in chemical and mechanical pulp fibers

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Abstract The effects of chemithermomechanical pulping (CTMP), high-temperature (HT)-CTMP pulping, and kraft pulping on the distribution of the pectin polymer, methyl-esterified polygalacturonan have been qualitatively evaluated using immunocytochemistry. Pectin was immunolocalized using an antibody (JIM7) specific for partly methyl-esterified polygalacturonan. A fluorescent antibody was linked to JIM7 and analyzed by epifluorescence microscopy. Pectin was found in both chemithermomechanical pulps in similar uneven patterns: Some fibers showed no labeling, whereas others showed extensive labeling in patches restricted to the fiber surfaces. Pectin labeling of bleached and unbleached kraft pulps did not show any presence of pectin. Labeling was correlated to the presence of compound middle lamella tissue left on the CTMP-treated surfaces. Pectin on pulp-fiber surfaces may affect the interfiber bonds and thereby the pulp properties.

Key words Chemithermomechanical pulping · High-temperature chemithermomechaical pulping · Kraft pulp · Pectin · Immunolocalization · Fiber surface

Introduction

Pectin is a heterogeneous polysaccharide, but its main constituent is polygalacturonan, a polymer of (1 → 4)-α-linked galacturonic acid units.[1] Sapwood of conifers consists of up to about 4% pectin, although at the cell-wall level the local concentration of pectin can be relatively high.[2,3] Monoclonal antibodies that recognize polygalacturonans have been developed,[4,5] and the antibody JIM7 binds specifically to galacturonan esterified to a degree of 15%–90%.[5,6] When JIM7 has been used in cell-wall studies of softwood,[7–9] pectin was shown to be mostly associated with the compound middle lamella (CML), pit membranes, and ray cell walls. However, native pectin cannot be isolated qualitatively because it is intimately entangled with lignin and hemicellulose and is susceptible to chemical degradation. Extensive deesterification occurs in alkaline conditions, as methyl-esterified galacturonic units are saponified and carboxylic groups are formed. Free carboxylic acid groups on polygalacturonan give pectin a high negative charge capable of binding cations,[10] which affect wood pulp and paper properties such as bleachability and photoyellowing.[11,12]

Because pectin is primarily concentrated in the CML, pulps derived from wood defibrated mainly through this region may have pectin remaining on the fiber surface. With high-yield pulping, where most wood components are retained in the pulp, the fiber surface is formed by the mechanical defibration process. During kraft pulping, however, lignin is removed and degraded, as are some polysaccharides including pectin, which results in a varied chemical composition and structure of the fiber surface. The nature of the fiber surface is important for fiber–fiber bond properties and thus also for paper quality. In this study, the effect of chemithermomechanical (CTMP), high-temperature (HT)-CTMP, and kraft pulping on the distribution and deesterification of methyl-esterified polygalacturonans have been examined by fluorescence microscopy.

Experimental

Sample embedding and sectioning

Industrial unbleached isothermal cooked (ITC) kraft pulp (kappa no. 25.9), bleached ITC kraft pulp [oxygen treated,

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followed by chelator (Q), oxygen (O), and hydrogen peroxide (P) sequential bleaching, Q(OP)Q(PO), kappa no. 4.3], unbleached CTMP pulp, and unbleached HT-CTMP pulp, all from Scandinavian spruce (*Picea abies* Karst.) were used.

First, they were dehydrated by solvent exchange (20%, 40%, 60%, 80%, 90%, 95%, and 100% ethanol, 20 min for each step) and then embedded in LR-white methacrylate (London Resins, Basingstoke, UK). The fibers were embedded stepwise to ensure thorough sample penetration of the resin, with resin/ethanol ratios of 1:4, 1:1, and 4:1 (4 h each) before polymerization in 100% resin in air-tight gelatin capsules at 60°C overnight. Bleached kraft pulp was also suspended in a saturated pectin solution (89% methylesterified pectin; Sigma, St. Louis, MO, USA). The suspension was frozen and then lyophilized. The freeze-dried pulp was washed in distilled water (5 min × 3), dehydrated by ethanol exchange, and embedded in LR-white methacrylate. Semithin cross sections (~1 μm) of the pulps were cut with glass knives using a Reichert-Jung Ultracut E microtome, collected on glass slides, and etched with a few drops of acetone.

Antibody labeling

Sections were preincubated for 30 min in phosphate-buffered saline (PBS) buffer containing 50 mM glycin, washed in PBS buffer (5 min × 3), and air-dried before subsequent blocking in PBS buffer containing 1.5% normal goat serum, 0.8% bovine serum albumin (BSA), and 2 mM NaN_3, for 30 min. Sections were washed in PBS buffer (5 min × 3) before labeling with the monoclonal antibody JIM7 (diluted 1:10 in PBS buffer for 38 h in 4°C) against pectin. The primary antibody-labeled sections were washed (5 min × 3) in PBS buffer prior to secondary antibody labeling. The secondary antibody was a goat anti-rat immunoglobulin G (IgG) conjugated with fluorescein isothiocyanate (FITC) (Sigma) detectable by epifluorescence microscopy. Diluted 1:20 in PBS (pH 8.0), the secondary antibody were reacted with the sections for 2 h at room temperature. Thereafter, the sections were washed in PBS pH 8.0 (5 min × 3) and distilled water (5 min × 5). After air-drying, a solution of the fluorescence anti-fading agent 1,4-diazobicyclo-(2,2,2)-octane (DABCO) 0.1 M in 9 parts glycerol and 1 part PBS buffer adjusted to pH 8.6 by 0.1 M NaOH, was dropped onto the sections before they were sealed under a coverglass.[13] Nonembedded HT-CTMP and CTMP pulps were JIM7-labeled using the same protocol as for the embedded and sectioned samples, except that the pulps were labeled and washed in microcentrifuge tubes and thereafter transferred onto glass slides for microscopic analysis.

The samples were observed using an Olympus epifluorescence microscope equipped with an excitation filter (λ 460–490 nm) and a barrier filter (λ 510–550 nm). The monoclonal antibody and its antigen specificity has been characterized previously.[9,10] As a control, the primary antibody was excluded to determine unspecific binding.

Results

Distribution of pectin on CTMP and HT-CTMP fiber surfaces

Figure 1 shows distributions of pectin present on whole CTMP and HT-CTMP pulp fibers. In Fig. 1a a CTMP fiber displays JIM7 labeling along the fiber axis, and in Fig. 1b HT-CTMP pulp also displays pectin labeling. An unlabeled CTMP fiber (i.e., negative control) shows only weak lignin autofluorescence (Fig. 1c). Labeling is located and concentrated on the fiber surface, which is also shown by labeling of cross sections of HT-CTMP and CTMP (Fig. 2). Labeled and unlabeled fibers were apparent in low-magnification cross sections of CTMP pulps (Fig. 2a). There appeared to be no distinction in labeling patterns of thin-walled early-

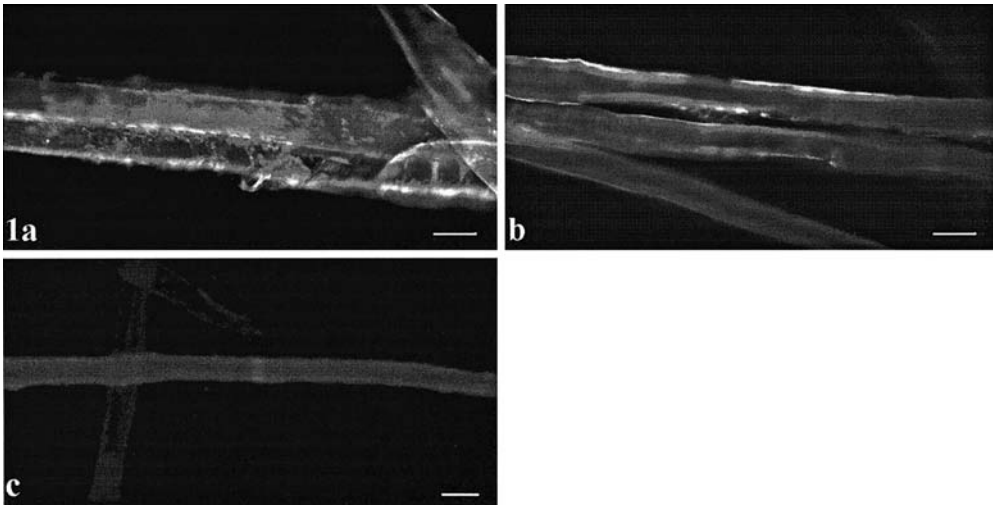

Fig. 1. Whole chemithermomechanical (CTMP) (**a**) and high-temperature (HT)-CTMP (**b**) pulp fibers immunolabeled for pectin. Labeling is localized on the fiber surfaces. **c** Unlabeled CTMP fibers (negative control). *Bars* 40 μm

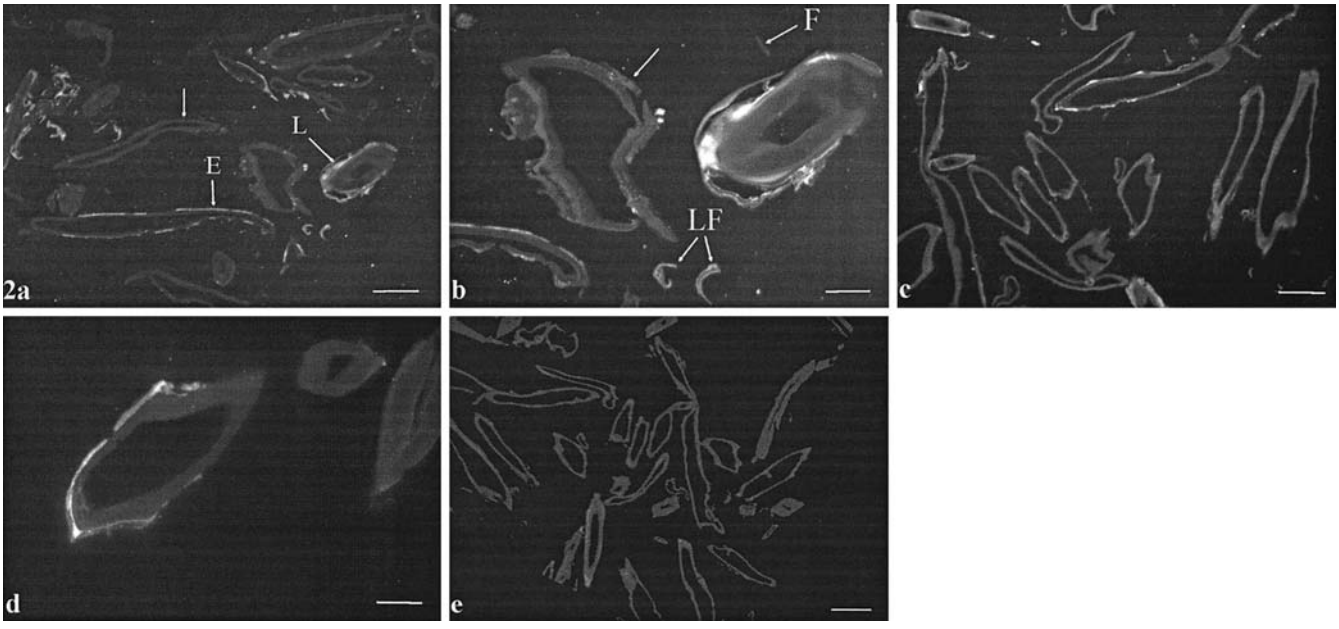

Fig. 2. Cross sections of CTMP and HT-CTMP pulp labeled with JIM7. **a** *Top arrow* indicates an unlabeled fiber; the *arrow* below it (*E*) shows the presence of pectin on an earlywood fiber. *L* labeled latewood fiber. *Bar* 40 μm. **b** Stretches of the fiber surface show strong labeling. *Unmarked arrow* shows compound middle lamella (CML) tissue without labeling for pectin. *LF*, labeled fines; *F*, unlabeled fine fragment. *Bar* 22 μm. **c** HT-CTMP fibers labeled for pectin. Only a few fibers display fluorescence. *Bar* 30 μm. **d** Pectin labeled on HT-CTMP fiber surface. *Bar* 10 μm. **e** Unlabeled HT-CTMP (negative control). *Bar* 45 μm

wood and thick-walled latewood fibers; labeled and unlabeled fibers were noted. The earlywood fiber and the latewood fiber both show strong labeling (Fig. 2a). The labeling pattern in the same CTMP sample at higher magnification (Fig. 2b) showed more clearly the fibers with areas of strong fluorescent labeling along the outer fiber surface. However, the labeling was uneven, with a few fibers having almost the entire surface completely covered by labeling; in most other fibers that show labeling, the fluorescence was present in more or less regular patches along the fiber surface. Many fibers were also unlabeled. Labeling seems to be localized exclusively to compound middle lamellae (CML) tissue, although there were areas where the CML appeared intact but did not show labeling. Pectin labeled and unlabeled fines (small wood fragments broken off during pulping) are indicated in Fig. 2b. Figure 2c shows a labeled cross section of HT-CTMP pulp at low magnification and gives an overview of the labeling. Many fibers are not labeled; and like CTMP (Figs. 1a, 2b), the labeling is unevenly distributed on the fiber surfaces. Figure 2d shows some fibers at higher magnification, with a heavily labeled fiber adjacent to another fiber that completely lacks labeling. The labeling pattern for HT-CTMP was similar to that for CTMP. Regarding the labeling intensities of the pulps, it is difficult to compare the JIM7 labeling frequencies from the micrographs, and so far no quantitative statistical analysis has been performed. Figure 2e is a negative control for HT-CTMP; no pectin is shown, although weak lignin autofluorescence can be seen.

Pectin labeling of kraft pulp

Figure 3a shows unbleached kraft pulp with no detectable pectin; only weak, uniform autofluorescence of lignin from the cell walls is apparent. No CML was detected. Micrographs of bleached kraft pulp impregnated with pectin are shown in Fig. 3b,c. Figure 3b is a low-magnification micrograph on which several fibers are apparent; intense labeling of pectin is seen along the exposed (i.e., outer fiber wall and lumen) fiber surfaces. Figure 3c shows that pectin has also penetrated all layers of the fiber wall (i.e., primary and secondary cell walls).

Discussion

Pectin distribution in the pulps

Various methods have been employed to analyze the chemical composition of pulp fiber surfaces, but most chemical methods do not give spatially resolved information (e.g., surface specific). X-ray photoelectron microscopy (XPS) can be used for chemical analysis of fiber surfaces, and it has been shown that CTMP pulp of Scandinavian spruce has about 40% carbohydrate on the fiber surface.[14] The exact polysaccharide structure or composition of surface carbohydrates is difficult to estimate; for example, ester and carboxylic groups appear in the same band in the XPS spectra, making it impossible to analyze methylated polygalacturonan specifically. Any wood component or

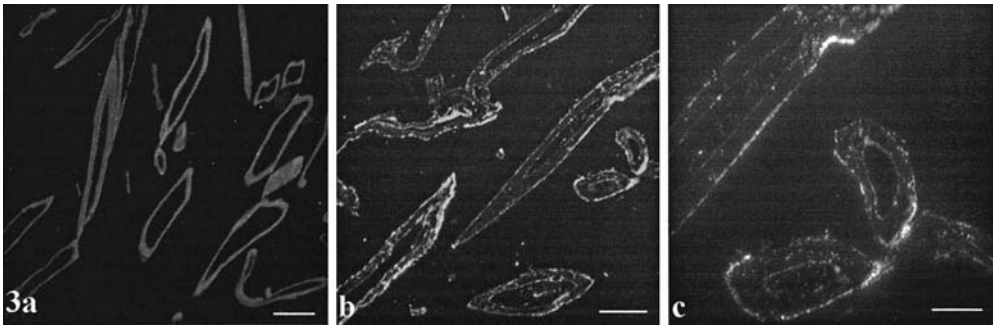

Fig. 3. Cross sections of kraft pulp fibers. **a** No pectin labeling is shown in unbleached kraft pulp. The fiber contour can be seen by weak lignin autofluorescence. *Bar* 28 μm. **b** Bleached kraft pulp impregnated by methyl-esterified pectin displays fluorescence on all fiber surfaces and in some fiber walls. *Bar* 24 μm. **c** At high magnification the pectin impregnated bleached kraft pulp-clearly displays labeling inside the fiber walls. *Bar* 8 μm

chemical supplied to pulps (to which there is a specific probe) can be localized using immunocytochemistry.

Methyl-esterified galacturonan is specifically localized on the surface of HT-CTMP and CTMP in Figs. 1 and 2. The distribution of pectin was similar in HT-CTMP and CTMP fibers, with both pulps showing heterogeneous labeling (some fibers labeled intensely and others showed no labeling for pectin). There seemed to be no difference in the labeling patterns for earlywood and latewood fibers. However, in both chemithermomechanical pulps, when pectin was present labeling was located exclusively on the fiber surface and in an irregular "patchy" pattern on the outside wall of the fibers. Labeling was found to be irregularly distributed longitudinally (Fig. 1) and transversally (Fig. 2).

Because fluorescent labeling shows the presence of antibodies, the displayed labeling pattern is completely dependent on the antibody binding to the galacturonan epitope. Changes in the chemical composition of the epitope or the nature of surrounding tissue could inhibit antibody recognition. For example, the resin used for embedding and sectioning or the lignin in the lignin-rich CML could physically cover or chemically bind the galacturonan, thereby masking the epitope. Moreover, hydrolysis or saponification of the esterified galacturonan during the defibration process would change the epitope and subsequently affect the labeling pattern of JIM7. However, based on the micrographs, the labeling of galacturonan by JIM7 indicates that the pectin-rich areas on HT-CTMP and CTMP fibers correlate with the CML remaining on the fiber surface following defibration, rather than, for example, representing reabsorbed pectic substances previously released from the wood. Previous studies have shown that pectin in *Picea abies* sapwood is uniformly distributed in the CML.[9] Therefore, the patchy distribution of pectin on HT-CTMP and CTMP fiber surfaces shown here may result from mechanical defibration.

Because the process of mechanical defibration is stochastic, the resulting pulp fiber surfaces are heterogeneous by nature. The areas lacking labeling may partly derive from randomly torn off CML or areas where fiber separation through the cell wall has exposed non-pectin containing secondary cell wall tissue on the fiber surface. Areas in the CML that lack labeling may also result from pectin having been extracted during the pulping process, and it could partly be from where the content of pectic monosaccharides in colloidal substances in the pulp liquor originate. Also, pectin-labeled and pectin-unlabeled fines were shown. Fines and dissolved material in the pulping process water have previously been shown to be enriched in pectic substances.[15–18]

Consistent with previous studies on sapwood fibers, no pectin was labeled in the cell walls of the pulp fibers.[7–9] Moreover, in contrast to the mechanical pulps, no labeling was shown in bleached or unbleached kraft pulps, as most CML would have been degraded during the pulping process. Although the unbleached kraft pulp did not show pectin labeling, some autofluorescence was present owing to residual lignin in the fiber cell walls. Bleached kraft pulp contains almost no pectin and shows no lignin autofluorescence; therefore, the sections appear black using fluorescence microscopy (data not shown). However, when the bleached kraft pulp was impregnated with a pectin solution, uniform labeling was shown around the inner and outer fiber surfaces, and some pectin had penetrated the cell wall. With high-yield pulps most of the lignin and polysaccharides are retained in the fibers, in contrast to bleached kraft pulp fibers where the main part of the lignin has been extracted; the fibers therefore are porous and open for impregnation. In the present study, however, pectin was not completely impregnated; nor did it appear in an even distribution in the bleached kraft fiber walls, which probably can be attributed to the fiber pores having a nonuniform size and distribution. Also, as pectin is a negatively charged polymer, minor pores may be blocked, further restricting penetration. In addition, to be labeled, the pectin present must be available for reacting with the antibody on the section surface. The present results allowed visualization of partial impregnation of bleach kraft pulp fibers with the relatively large negatively charged polymer polygalacturonan. Moreover, pectin impregnation served as a positive control of the primary antibody.

Acidic groups in chemithermomechanical and kraft pulps

In the wood fiber cell wall most carboxylic groups are derived from methylglucuronic acid subunits in xylan, and in the wood CML they are from the polygalacturonan in pectin. In studies of coniferous sapwood, most pectin seemed to be partly methyl-esterified.[8,9] Thus, the steam and sodium sulfite pretreatment of the wood chips prior to HT-CTMP and CTMP defibration did not seem to affect the esterification pattern of the residual pectin completely, as JIM7 does not recognize pectin with less than 15% esterification;[5,6] moreover, the labeling clearly indicates methylated pectin in the sulfonated and defibrated HT-CTMP and CTMP pulps. Even though the defibration process does not seem to cause complete saponification of the pectin during CTMP or elevated temperatures during HT-CTMP, subsequent alkaline bleaching would probably reduce the degree of methyl esterification of pectin. Alkaline peroxide bleaching releases some galacturonic acid from the fibers[17] but also increases the fiber charge by introducing new carboxylic end groups on the lignin.[19] It has also been shown that the increased negative charge was evenly localized throughout the fiber, with no selective additional charging of the surface, and that increased fiber charge could be correlated with the increasing strength properties of the pulp. In this respect, a defibration process specifically demethylating yet retaining the pectin on the pulp surfaces could be beneficial for some pulp qualities. In contrast to high-yield pulps, alkali bleaching does not increase the negative fiber charge in bleached kraft pulp. Uronosidic bonds are susceptible to alkaline conditions; and during kraft pulping the number of acidic groups decreases as pectin and xylan are released from the pulp. The lack of labeling in kraft pulp would corroborate this, however, if any residual pectin consisted only of completely de-esterified polygalacturonan, it would not be recognized as an antigen by JIM7.

Conclusions

CTMP and HT-CTMP pulps have esterified pectin on the fiber surface when compound middle lamellae are present. The pectin is unevenly distributed on the fibers, with some fibers showing intense labeling for pectin whereas others are negative. Neither sulfonation nor elevated temperatures induce complete deesterification of the pectin in chemithermomechanical pulps. Kraft pulp did not show any labeling for pectin, indicating complete removal, degradation, or total saponification of methyl-esterified galacturonan.

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