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Proteins in Norway spruce thermomechanical pulp

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Abstract Two methods of cytochemical staining using Coomassie dye and Cu^{+}-bicinchoninic acid, respectively, showed that there are proteins in thermomechanical pulp (TMP) of Norway spruce. Protein isolated from TMP was analyzed for amino acid composition. There was about twice the amount of acidic amino acid material compared with basic amino acids, and the presence of glucosamine indicated that the isolated polypeptides also contained glycoproteins. The presence of proteins in ray cells and fiber tracheids in TMP adds to the chemical heterogeneity of the structurally complex high-yield pulp.

Key words Amino acid · Cytochemical staining · Norway spruce · Protein · Thermomechanical pulp

Introduction

Plant fibers consist of different groups of natural polymers. Lignin and polysaccharides are the major polymeric constituents of mature tracheid cell walls of wood. Polynucleotides and polypeptides are commonly associated with living reactions inside the cell plasma membrane, but peptide-containing polymers (proteins, enzymes, and glycoproteins) are also a vital part of the plant apoplast. The walls of developing tracheids contain cell wall-bound enzymes, and structural proteins constitute an integrated part of the cell wall structure.[1–3] After the fibers have matured, and the cell wall has lignified, some proteins once involved in the biogenesis of the wood tissue remain within the sealed lignified cell wall and compound middle lamella matrix. During wood pulping, when the fibers are separated, proteins are dissolved in the wastewater or cooking liquor. However, some of the proteins are retained in the pulp. Several lignifying and polysaccharide-modifying enzymes have been identified,[2] but the proteins in softwood fibers and pulps have been less studied compared with the major wood polymers. Because wood is a heterogeneous material, detailed and spatially resolved chemical information of components with restricted localization within the pulp may sometimes be difficult to obtain. Even though protein is a minor component in lignified wood tissue in total, there are areas of protein concentration. Primary cell walls and fractions of mechanically defibrated spruce wood contain relatively more proteins than other areas of the wood.[1,4] The wood of Norway spruce contains about 0.2% proteins (dry weight). Proteins are vital for the fiber development and constitute an essential part of the mature plant cell wall that needs to be chartered in order to obtain a truly comprehensive idea of the structure and chemistry of the wood fiber. In this study the distribution of protein in thermomechanical pulp (TMP) made from Norway spruce has been analyzed using cytochemical staining, and the amino acid composition of polypeptides isolated from TMP has been analyzed. In addition, the effects of proteins on pulping and fiber properties are discussed.

Materials and methods

Wood and pulp samples

All wood and TMP samples were from Norway spruce (*Picea abies* Karst.). Unbleached TMP was produced in the pilot plant of Metso in Sundsvall, Sweden. Hydrogen peroxide-bleached TMP and bleached Kraft pulp (softwood) were provided by the forestry company SCA in Sundsvall, Sweden. The wood and pulps were used as delivered and were stored frozen in darkness until use. Cotton, which was used as a control sample, was commercially available and was defatted prior to use.

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Cytochemical staining of proteins

TMP (bleached and unbleached), bleached Kraft pulp, and cotton-cellulose samples were stained for proteins using Bio-Rad (Bradford method) protein staining method, and the Biuret reaction with bicinchoninic acid.[5] Transverse sections of Norway spruce were cut on a slide microtome (100 μm thickness) and stained for proteins using Coomassie brilliant blue (Bradford). The cytochemical staining by Coomassie blue was performed according to Bio-Rad Protein Assay (Dye Reagent Concentrate), and a Biotrace protein staining kit was used for Cu^{+}-bicinchoninic acid staining. The samples were analyzed using a Leica light microscope. The cotton sample was used as a negative control. For spectrophotometric assay of Coomassie dye binding to the pulp, 10.0 mg of sample (dry weight) was stained with 1.25 ml Coomassie dye solution (Bio-Rad Reagent Concentrate/deionized water, 1 : 4) for 10 min. After brief centrifugation, the absorbance (465 nm) of the supernatant was analyzed using a Shimadzu UV-160A spectrophotometer.

Amino acid analysis of protein extracted from TMP

Protein-containing material was extracted from TMP using phenol/acetic acid/water (PAW).[6] About 10 g (dry weight) of unbleached TMP was dispersed in 140 ml PAW solution, containing 40 ml glacial acetic acid and 100 ml 80% aqueous phenol (w/w). The mixture was placed in a fume cupboard and was thoroughly stirred while heated at 70°C for 40 min. After cooling, the suspension was filtered. The pulp residue was washed twice with PAW diluted by 20% with distilled water. The filtrate and washings were pooled and 5 ml 10% (w/v) ammonium formate was added, followed by 500 ml absolute ethanol. The mixture was stored overnight at 4°C, and thereafter the precipitate was collected by centrifugation (5 min at 2500 g). The pellet was washed twice in 80% ethanol before it was resuspended in water and lyophilized. For determination of the amino acid content in the extracted material, a known amount of the freeze-dried sample (about 1 mg dry weight) was hydrolyzed with 2 ml 6 M HCl containing 1 mg/ml phenol and 49 nmol norleucine as internal standard. The samples were hydrolyzed for 24 h at 110°C in thoroughly evacuated and sealed Pyrex tubes. Thereafter, the hydrolyzates were evaporated to dryness and the residues were dissolved in 200 μl of pH 2.2 sample application buffer and aliquots (100 μl) were analyzed with a Biochrom 20 amino acid analyzer using a sodium citrate buffer and ninhydrin detection. The results were normalized on the basis of the recovery of the internal standard and the weight of sample taken for analysis.

Wood and pulp composition

Neutral sugar composition of the wood and pulp was analyzed after the samples were hydrolyzed by sulfuric acid treatment. The dissolved sugars were determined after hydrolysis by high-performance liquid chromatography (HPLC) equipped with an electrochemical detector,[7] while the lignin-containing residue was filtered off and determined gravimetrically (Klason lignin).[8] The Canadian standard freeness (CSF) of the pulp was analyzed using the standard ISO test method.[9]

Results and discussion

Proteins in wood and TMP

In order to show the presence and distribution of protein in wood and pulp, proteins were cytochemically stained and visualized using light microscopy. In Fig. 1a, unstained reference samples and samples stained with Coomassie brilliant blue are shown, while Fig. 1b shows the samples stained with bicinchoninic acid. Positive protein staining by Coomassie dye is blue and the protein-Cu^{+}-bicinchoninic acid complex is purple. For both stains, an increasing protein concentration results in an increased color intensity, and TMP showed the most intense staining for both cytochemical stains. Bleached and unbleached TMP showed some coloring, whereas bleached Kraft pulp and cotton showed little positive staining. However, small differences in color intensities can be difficult to analyze visually; therefore, the Coomassie blue staining was also used for spectrophotometric assays. The color change from brown (465 nm) to blue (595 nm), when Coomassie dye binds to protein in acidic medium, can be used for measuring removal of unbound dye in the presence of immobilized protein (e.g., on wood fibers).

In Fig. 2, TMP, Kraft pulp, and cotton were stained using Coomassie dye, and the reduction of absorbance at 465 nm was measured. The larger change in absorbance at 465 nm for TMP correlates with the color changes in Fig. 1, and

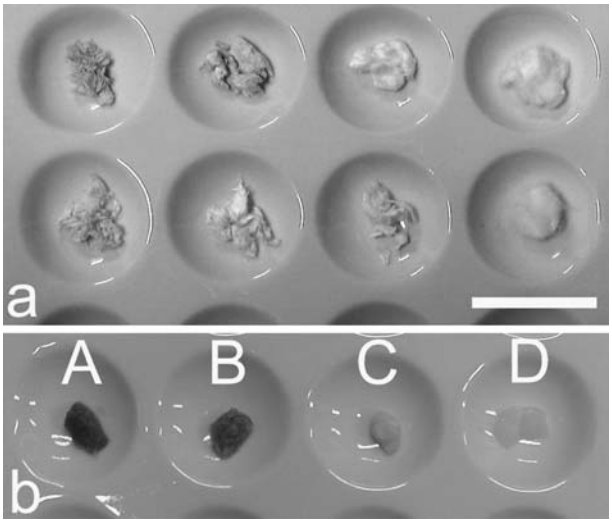

Fig. 1a,b. Cytochemical staining of thermomechanical pulp (TMP) of Norway spruce. **a** *Top row*, Coomassie blue staining; *bottom row*, unstained reference samples. **b** Cu^{+}-bicinchoninic acid stained samples: *A*, unbleached TMP; *B*, bleached TMP; *C*, bleached Kraft pulp; *D*, cotton. *Bar* 2.5 cm

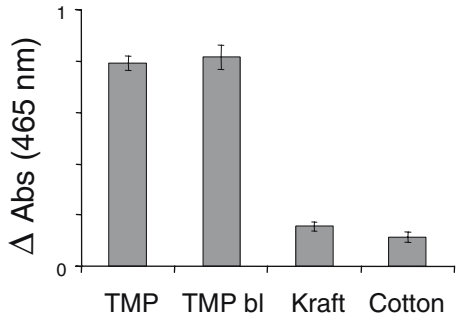

Fig. 2. Coomassie dye bound onto fibers reduced absorbance at 465 nm in solution. *TMP*, unbleached TMP; *TMP bl*, bleached TMP; *Kraft*, bleached Kraft pulp. Data represent mean values and standard deviations of triplicates

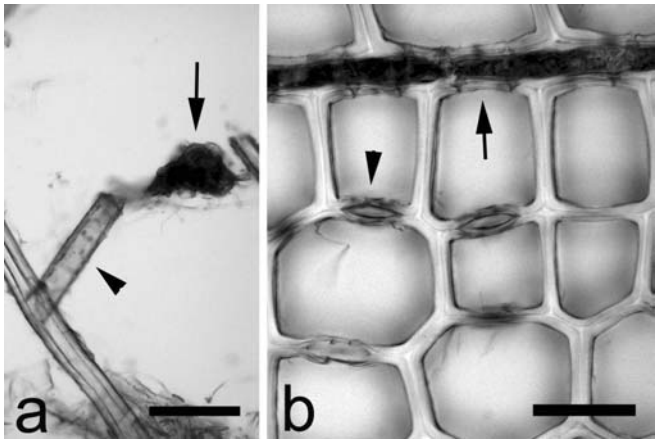

Fig. 3a,b. Coomassie blue staining of protein. **a** Unbleached TMP, unstained fiber below, medium stained fiber fragment (*arrowhead*) and heavily stained fiber material (*arrow*). *Bar* 60 μm. **b** Transverse section of Norway spruce sapwood, stained pit membrane (*arrowhead*) and intensively stained ray cell (*arrow*). *Bar* 30 μm

indicates the presence of more protein in TMP than in Kraft pulp or cotton fibers. Figure 3 shows the staining of TMP fibers and wood using Coomassie dye. In Fig. 3a, protein staining of whole unbleached TMP fibers is shown. The staining was unevenly distributed; some fines and parts of fibers showed staining whereas some did not show any staining. Figure 3b shows a stained cross section of mature sapwood of Norway spruce. As expected there was intense staining in ray cells, but pit membranes also showed staining. In the cell walls and compound middle lamellae, no obvious staining could be detected by the two cytochemical stains. Apparently, in the cross section of intact mature lignified xylem tracheids of Norway spruce, the protein concentration in cell walls is too low for visual detection using the cytochemical staining methods used in this study. The uneven distribution of proteins in TMP might possibly indicate that different fibers were subject to different mechanical forces, or there was an uneven dissolution of cell wall material during processing. Previously, several proteins were identified in plant material,[2] and were utilized for various applications.[10,11] Also, it should be noted that any proteins present on the surface of, for example, TMP could influence fiber-to-fiber bond strength and subsequently the paper strength.[12]

Table 1. Relative amount amino acids in proteins isolated from thermomechanical pulp (TMP)

Amino acid	Relative amount (%)
Aspartic acid	11
Threonine	6
Serine	8
Glutamic acid	12
Proline	7
Glycine	7
Alanine	6
Valine	7
Methionine	1
Isoleucine	6
Leucine	8
Tyrosine	5
Phenylalanine	5
Histidine	2
Lysine	4
Arginine	5
Total	100

Tryptophan and cystine were not determined

Composition of proteins isolated from TMP

Unbleached TMP was subjected to PAW extractions to extract and isolate protein-containing material. PAW extracts noncovalently bound proteins,[6] and extracts protein-containing material mainly from inside ray cells and the fiber outer layers, due to the tight lignified structure (on polymer level) of unbleached mechanical pulp fibers. The total amount of material extracted from the TMP was about 0.8 mg/g pulp (dry weight). The extracted matter was analyzed for amino acid composition (Table 1), and the relative amounts of amino acids showed that there are both hydrophilic and hydrophobic amino acids present in the extracted material, and about twice as much acidic amino acid material (Asp + Glu = 23%) as basic amino acid material (Lys + Arg + His = 12%). Also, not listed in Table 1, the amount of glucosamine present in the samples was about the same as, for example, glycine. The presence of glucosamine is an indication that the extracted polypeptide-containing material also included glycoproteins, which are typical of cell walls. The glycoproteins can form physical structures, such as beta-pleated sheets or rods, and cross-link or associate with other cell wall polymers and thereby affect the cell wall structures and properties. Compared with the amino acid content of whole wood,[13] the material isolated from Norway spruce TMP fiber surface seems to contain relatively more aromatic amino acids (Phe + Tyr). Neutral monosaccharide and lignin content analyses confirmed that the samples used were representative for Norway spruce TMP (Table 2).

TMP fiber surface

In TMP, pectin, xylan, and lignin are known sources of carboxylic groups, and negative charge is a property that

Table 2. Characterization of unbleached TMP of Norway spruce

Monosaccharide	% Dry weight
Arabinose	1.1
Xylose	5.3
Galactose	2.0
Glucose	42.7
Mannose	13.0
Rhamnose	0.1
Lignin	28.4
CSF (ml)	410

CSF, Canadian standard freeness

has been shown to affect pulp. Especially pectin has a high negative charge density because it is mainly composed of (1→4)-linked α-D-galacturonic acid residues partly esterified by methanol, with single units of rhamnose interspersed within the galacturonic backbone.[14] Pectin and lignin are localized in higher concentrations in between the fibers.[15–17] Therefore, there are polysaccharides, including pectin, and lignin exposed at the surface on high-yield pulp fibers.[18–21] As can bee seen in Figs. 1-3 and in Table 1, there was protein present also in TMP.[22] These findings are of interest for the understanding of the nature of mechanical-pulp fiber. Moreover, there have been studies on protein and biotechnological modifications of wood or wood pulp; for example, proteinase pretreatment of radiata pine chips has been shown to decrease energy consumption during primary refining by up to 10%.[23]

Conclusions

To better understand the nature of wood and to further develop the pulping and paper-formation processes, detailed information of the wood and pulp is desirable. In this study, it was shown that the heterogeneous TMP also contains proteins and glycoproteins, which is of importance for the understanding of the general chemistry of TMP. Furthermore, the TMP proteins could potentially be of importance for biotechnological applications.

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