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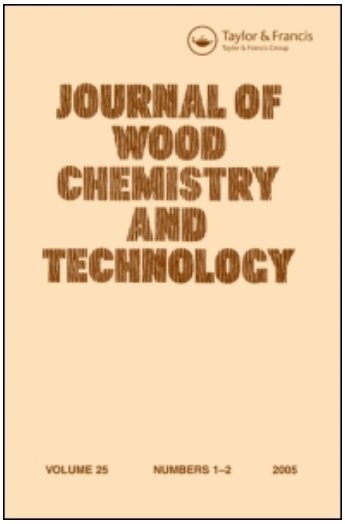

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Action of Xylanases on Chemical Pulp Fibers Part I : Investigations Gn Cell-Wall Modifications

F. Mora[a]; J. Cotrtat[a]; F. Barnoud[a]; F. Pla[b]; P. Nee[c]
[a] Centre de Recherches sir les Macromolecules Vegetales, Grenoble, France [b] Eccle Francai'st de Papeterie, Grenoble, France [c] Centre Technique de 1'Industrie des Papiers, Grenoble cedex, France

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ACTION OF XYLANASES ON CHEMICAL PULP FIBERS

PART I : INVESTIGATIONS ON CELL-WALL MODIFICATIONS.

F. Mora*, J. Comtat*, F. Barnoud*, F. Pla** and P. Noe***

* Centre de Recherches sur les Macromolécules Végétales, C.N.R.S.
B.P. 68 - 38402 Saint Martin d'Hères cedex (Grenoble, France).

** Ecole Française de Papeterie, I.N.P.G.
B.P. 65 - 38402 Saint Martin d'Hères cedex (Grenoble, France)

*** Centre Technique de l'Industrie des Papiers,
Cartons et Celluloses
B.P. 7110 - 38020 Grenoble cedex (France)

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ABSTRACT

The action of xylanases on chemical wood pulps was studied in the presence of a 1 mM $HgCl_2$ solution. In that medium, the endo-cellulases present in the crude enzyme mixture from the Basidiomycete Sporotrichum dimorphosporum were inhibited as shown by soluble sugar analysis and by molecular weight determination of the carbanilated derivatives of the enzyme-treated pulps. After enzymatic hydrolysis, although the mass loss was less than 2% of the dry material weight, important structural modifications were revealed by physical property determinations on the residual pulps. The water retention value which increases by 20%, the mean pore radius which is reduced by a factor of 10, the scanning electron microscopic photographs which show fiber flexibility and external fibrillation and the molecular weight distribution curves, allow us to come to the conclusion that xylans are actually hydrolyzed in

* Laboratoire propre du C.N.R.S., associé à l'Université Scientifique, Technologique et Médicale de GRENOBLE
** Laboratoire de génie des procédés papetiers U.A. 1100 C.N.R.S.

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the whole delignified cell walls. Therefore, because of the xylan distribution with regard to cellulose microfibrils, the selective hydrolysis by xylanases might affect the cell wall cohesion and, thus, influence the papermaking properties of pulps.

INTRODUCTION

The use of new specific markers makes it possible to gain a better understanding of the relationship of various cell wall polysaccharidic constituents with each other[1,2]. For example Ruel and Joseleau[2] making use of gold-bearing xylanases on ultrathin sections of young tissues of parenchyma cells and fibers from the reed Arundo donax, have shown that xylans are present with a high concentration in the whole cell wall. This conclusion agrees with previous results[3,4] which suggest that xylans can constitute a thin sheath around each elementary fibril or each group of fasciated fibrils[3].

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The present work is based on these conclusions. The study is, however, conducted with delignified fibers as lignin impedes enzymatic action inside fully lignified cell walls. The objective was to reveal cell-wall structural modifications following the specific hydrolysis of xylans in relation to their particular distribution and to understand why xylans seem to be "shielded from or not recognized" by the xylanases according to Paice and Jurasek[5] and Boutelje et al[6].

MATERIALS

Enzymes

The crude enzymatic complex constituting the culture filtrate of a Basidiomycete, Sporotrichum dimorphosporum, was kindly

provided by "La Rapidase" (59 - SECLIN, France) under the trade name "cellulase de Basidiomycete". During the fungus growth many enzymes are secreted into the culture medium, among which cellulases, xylanases, amylases, and β-glucosidases were identified[7].

Substrates

Xylan : This polysaccharide was the extract obtained from the bleached kraft pulp from birch wood by stirring the pulp in a N NaOH solution under nitrogen atmosphere. Its neutral sugar composition was the following : xylose 99.6 % and arabinose 0.4 % (molar ratios) as determined by trifluoroacetic acid hydrolysis[8] and analysis of the alditol acetate derivatives in a gas chromatograph fitted with a glass column containing SP 2340 (3 %) on gas chrom wax-DMCS phase.

Mannan : Mannan was obtained from ivory nut as described by Chanzy et al[9]. Its neutral sugar composition was mannose 94.8 %, glucose 4.7 % and xylose 0.5 % as determined by total acid hydrolysis according to Saeman et al[10].

Carboxymethylcellulose : This water soluble polysaccharide (CMC) is the commercial product "Blanose cellulose gum" 7H3SXF (Hercules, S.A. France).

Avicel cellulose powder : This microcrystalline cellulose was the commercial product AVICEL PH101. Glucose was the only sugar detected after total hydrolysis[10].

Chemical pulps : Industrial sulfite spruce and kraft birch pulps, fully bleached by the conventional CED bleach sequence, were employed. They were received as dry sheets.

The aspen wood pulp is a laboratory preparation from a 6 month old stem of aspen (Populus Euramericana hybride). Delignification was performed according to Marechal[11] on chips crushed in a press roll machine (Patent taken out by the Centre Technique du Papier, Grenoble, France). This employed alkaline cooking with sodium hydroxide 20 % (W/W dry wood) and anthraquinone (1 % W/W) for 8 h at 135°C as first step. It was followed by an acid

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hydrolysis for 1 h at 100° C with (.01° M H_2SO_4. The third step was a treatment with oxygen (290 P.S.I.) and sodium hydroxide (5 % W/V) at 125°C for 2h. The bleached pulp was washed with water and freeze dried.

METHODS

Enzymatic hydrolysis of the isolated substrates.

Each substrate was dissolved, or well dispersed, in water (2.5 $g.L^{-1}$), and incubated, under shaking, at 40°C with the enzymatic complex. The incubation time was 4 h for microcrystalline cellulose and mannan and 15 min only for xylan, and soluble cellulose (CMC) because of the large amounts of sugars rapidly released.The formation of reducing-end groups was measured by the Somogyi-Nelson method[12].

In the same manner, the hydrolysis of the substrates was studied in the presence of a glycanases inhibitor. Mercuric chloride in aqueous solution at the final concentration of 1mM was used and the reducing end groups produced were determined[12]. The mercuric chloride molecule has a very low dissociation in water and therefore mercuric ions do not significantly interfere with the reducing end groups determination. However, a standard curve obtained in the 1mM $HgCl_2$ medium is needed.

Enzymatic hydrolysis of xylan in pulps.

Sheet fragments of the industrial pulps and the freeze dried aspen pulp were soaked overnight in a 1 mM $HgCl_2$ solution at room temperature. Then, they were washed several times with $HgCl_2$ solution and stirred to obtain a pulp slurry.

1) Enzymatic hydrolysis of aspen pulp.

The crude enzyme complex (30 mg) was shaken for 15 min in the 1 mM $HgCl_2$ solution. It was poured into the pulp slurry contained

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in an Amicon ultrafiltration cell fitted with a non cellulosic UM10 diaflo membrane. The final volume was 1L and the pulp consistency was 3%. The incubation was performed at 40°C under gentle agitation for a period of 88 h during which the incubating medium was pressed two times through the membrane. Each time new $HgCl_2$ solution was added to continue the hydrolysis, but the enzymes were not replaced. At the end of that experiment the pulp was pressed dry and heated at 100°C for 10 min to inactivate the enzymes. The filtrates were also heated, collected and the reducing end groups[12] analyzed.

2) Enzymatic hydrolysis of industrial pulps.

The birch kraft and the spruce sulfite pulp were treated according to the same procedure as outlined above, except that the crude enzyme complex was 60 mg and 120 mg during 24 h for the birch and the spruce pulps, respectively. It should be noted however that these experimental conditions may be optimized further.

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Analyses of pulps and pulp degradation products.

Initial pulp and enzyme-treated pulp were acid hydrolyzed according to Saeman et al[10]. The neutral sugars were identified as alditol acetate derivatives by gas chromatography as described for isolated substrates. The soluble sugars carried through the membrane were separated on a Biogel P2 column eluted with distilled water at 60°C, and identified by comparison to standards.They were also analyzed after being hydrolyzed by the trifluoroacetic acid method[8].

Physical tests on pulp.

The pore formation in the aspen pulp fibers during the selective xylan hydrolysis was followed with a nitrogen porosimeter. The volume of pores (with a radius of less than 50 Å) was determined, and the specific area of the pulp fibers was measured.

The water retention value (WRV) was calculated according to Silvy et al[13] with standardized centrifugations.

Molecular weight distribution.

The weight average molecular weights $\overline{M}_w$ and the molecular weight distribution of the different pulps were determined according the procedure established by Lauriol et al[14] :

- The pulps were first submitted to carbanilation in dimethyl sulfoxide with an excess of phenyl isocyanate, for 48 h at 70°C.

- After precipitation and purification[14], the carbanilated derivatives were characterized with regard to their degree of substitution (D.S.) on the basis of their nitrogen content. This was obtained by Kjeldahl's method. In all cases the obtained values were near DS=3.

- The molecular weight distribution was obtained by size exclusion chromatography (S.E.C.) using a double detection on line : a low angle laser light scattering (L.A.L.L.S.) apparatus (Chromatix KMX-6) equipped with a 15 µl cell working at 632.8 nm, and a differential refractometer (Waters R 401). Elutions were performed with tetrahydrofuran (THF) through three columns associated in series : EHÂ, E.1000 and E.500 (Waters-µBondagel). The flow-rate was 0.5 mL.min.$^{-1}$. The refractive index gradients dn/dc, needed for calculations, were measured in THF at 20°C with a Brice Phoenix differential refractometer at 632.8 nm. The second virial coefficients, A_2, were also previously obtained, in THF, from static determination of $\overline{M}_w$ of each sample (A_2 = 5.10^{-4} mol. cm^{-3}. g^{-2}). These measurements were carried out with the Chromatix KMX-6 L.A.L.L.S. apparatus equipped with a 150 µl cell.

RESULTS

Inhibition assays and molecular weight determinations.

Figure 1 is a plot of the hydrolysis rate of the hemicellulosic substrates, xylan and mannan, in water or in the 1

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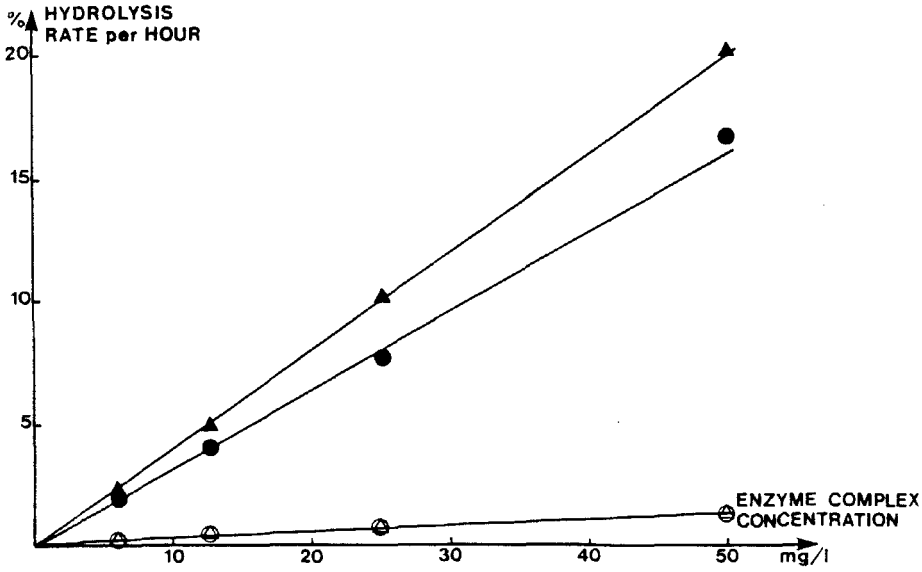

FIGURE 1. Effect of enzyme final concentration on the hydrolysis rate per hour of hemicellulosic substrates : xylan in water (▲), xylan in the presence of 1 mM $HgCl_2$ (●), mannan in water and in 1 mM $HgCl_2$ solution (○).

mM $HgCl_2$ solution versus the enzyme complex concentration. The enzyme mixture shows a strong activity on xylan in water, and this activity decreases only near 80 % of its value when the reaction medium is replaced by the 1 mM $HgCl_2$ solution.In the same way, mannan is hydrolyzed to a lesser extent in water, but the mannanase activity seems to be unaffected in the mercuric chloride medium.

Figure 2 illustrates the hydrolysis of two standard cellulosic substrates over a wider range of enzyme concentrations.

The exocellulase activity related to the enzyme activity on microcrystalline cellulose (AVICEL) can be detected at a low level in water medium. The 1 mM mercuric chloride medium does not reduce that activity. But the strong endocellulase activity which is measured in water by the hydrolysis of the carboxymethylcellulose is totally lost in the inhibiting medium. Thus, one can expect a

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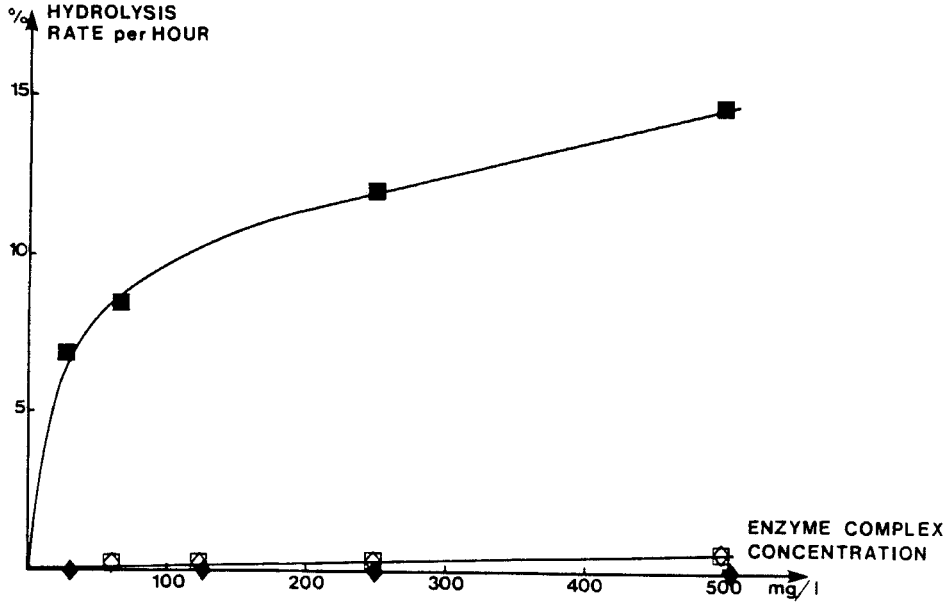

FIGURE 2. Effect of enzyme final concentration on the hydrolysis rate per hour of cellulosic substrates : carboxymethylcellulose in water (■), carboxymethylcellulose in 1 mM $HgCl_2$ solution (◆), microcrystalline cellulose in water and in 1 mM $HgCl_2$ solution (⊠).

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hydrolytic effect on the hemicellulosic part of pulp fibers without encountering a significant degradation of cellulose chains.

That conclusion was confirmed by determination of average molecular weights (Table 1) and of the molecular weight distribution curves (Figs. 3, 4).

The graphs for standard and for enzyme-treated pulps (Figs.3,4) can be superposed in the high molecular weights domain. Thus, the cellulose chains are not cleaved in spite of 88 h of attack by the enzyme solution on the aspen pulp (Figure 3) or in spite of 24 h of attack on the spruce pulp by a four times more concentrated enzymes solution (Figure 4). After enzymatic treatment, differences arise only in the low molecular weights

TABLE I

Characteristics of the Carbanilated Derivatives From the Pulps.

	dn/dc (mL.g^{-1})	$\overline{M}_w$ (a)	$\overline{M}_w$ (b)	$\overline{M}_n$ (b)	$\overline{DP}_w$ (c)	$\overline{DP}_n$ (c)	$I=\overline{DP}_w/\overline{DP}_n$
Spruce pulp standard	0.157	1,118,000	1,118,000	648,000	2,155	1,250	1.72
Enzyme-treated spruce pulp	0.160	1,150,000	1,160,000	670,000	2,235	1,290	1.73
Aspen pulp standard	0.164	840,000	843,000	245,000	1,625	470	3.46
Enzyme-treated aspen pulp	0.164	800,000	805,000	315,000	1,550	605	2.56

(a) determined by L.A.L.L.S. (Static Method)

(b) determined by Size Exclusion Chromatography (Dynamic Method using L.A.L.L.S. and differential refractometer coupled on line)

(c) molar weight of the carbanilated monomer unit = 519 g.mole^{-1}

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domain. In addition, the distribution curves (Figure 3) for enzyme-treated aspen pulp indicate a loss of low molecular weight material while it is not the case for the curves (Figure 4) obtained from enzyme-treated spruce pulp. This can be explained by differences according to wood origin and chemical pretreatment. Indeed,the yield in xylan macromolecules which can be subjected to enzymatic hydrolysis is higher in aspen pulp (Table 2) than in spruce pulp (Table 4).

Moreover, the hydrolytic action on hemicelluloses reduces the relative abundance of these macromolecules in the spruce pulp (Figure 4) so that curve 2 related to enzyme-treated pulp lays beneath curve 1 related to standard pulp. Some hydrolysis products with low molecular weights are also generated. In the same way, a marked effect was expected for experiments with aspen pulp where xylans are the only hemicelluloses (Table 2). On the contrary, the

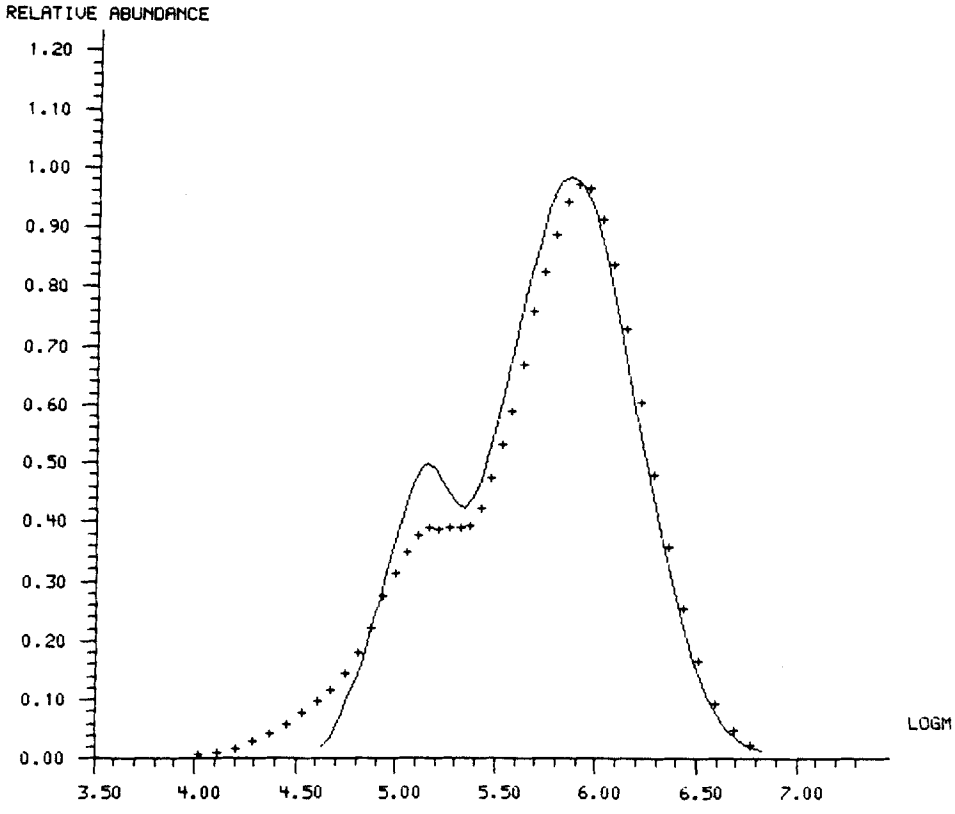

FIGURE 3. Molecular weight distribution curve of the carbanilated derivatives from the aspen pulp.Control pulp curve 1 (+++), enzyme-treated pulp curve 2 (——).

distribution curves (Figure 3) show an important increase in the relative abundance of molecules having lower molecular weights. The reason for that increase is not known but it could be explained by discrete cleavage of bonds in xylan chains with polymerization degrees higher than those usually admitted. Indeed, the average viscosimetric polymerization degree ($\overline{DP}v$) of isolated xylans from hardwoods is known to be around 150-200, but no result was ever obtained about xylan chains which cannot be extracted with alkaline medium. The use of carbanilated derivatives in this method[14] which

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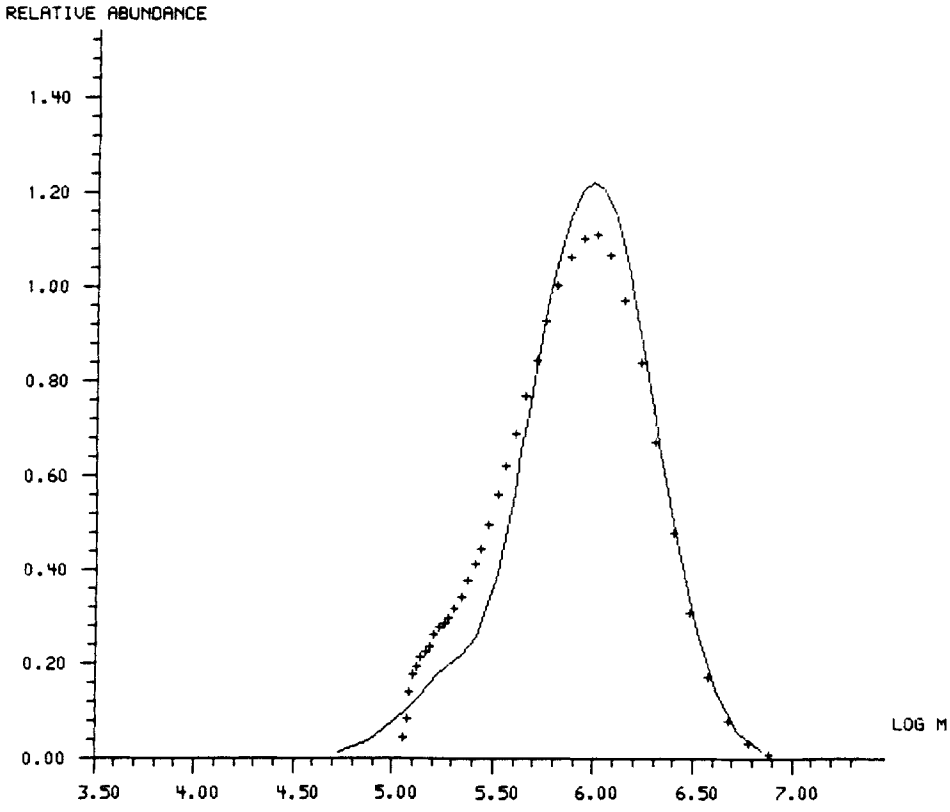

FIGURE 4. Molecular weight distribution curve of the carbanilated derivatives from the sulfite spruce pulp. Control pulp curve 1 (+++), enzyme-treated pulp curve 2 (——).

involves the solubilization of all the fiber constituents seems to be a proper way to identify the real hemicellulosic part of the plant cell-walls.

Neutral sugar composition of pulps and pulp degradation products.

Tables 2, 3 and 4 give the molar ratios of neutral sugars in the different pulps. The sugar compositions are consistent with

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TABLE 2

Neutral Sugar Composition of Initial and 88 Hours Enzyme-Treated Aspen Pulps.

	Ara	Xyl	Man	Glc
Initial aspen pulp*	0.5%	19.6%	1.9%	78%
Enzyme-treated pulp*	0.9%	19.5%	2%	77.8%

* molar ratios

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TABLE 3

Neutral Sugar Composition of Birch Kraft Pulp and Relative Proportion of Each Neutral Sugar Present in the Soluble Hydrolysis Products After Enzyme Treatment For 24 Hours.

	Ara	Xyl	Man	Glc	Per cent mass loss* (W/W)
Initial Birch Kraft pulp (molar ratios)	0.7 %	25.2 %	2 %	72.1 %	
Hydrolysis products weight** (mg)	5	374	0	72	1.5 %
Per cent of the initial content of the considered sugar (W/W)	2.8 %	5.9 %	0 %	0.3 %	

* determined from monosaccharide analysis. ** from 30 g of dry pulp.

TABLE 4

Neutral Sugar Composition of Spruce Sulfite Pulp and Relative Proportion of Each Neutral Sugar Present in the Soluble Hydrolysis Products After Enzyme Treatment For 24 Hours.

	Ara	Xyl	Man	Glc	Per cent mass loss* (W/W)
Initial spruce sulfite pulp (molar ratios)	1.4 %	7.7 %	8.9 %	82 %	
Hydrolysis products weight**(mg)	3.8	104	15.8	37.5	0.6 %
Per cent of the initial content of the considered sugar (W/W)	1.3 %	6 %	0.6 %	0.1 %	

* determined from monosaccharide analysis.** from 30 g of dry pulp.

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analysis results of industrial pulps originating from the same wood species and which have been subjected to similar cooking processes.

The analytical results on the enzyme-treated aspen pulp (Table 2) show that the enzymatic hydrolysis does not cause a significant decrease in the hemicellulose content of the pulp. The amount of soluble sugars in the incubation medium agrees with that conclusion.

The hydrolysis rate calculated as the ratio of the xylotriose-equivalent weight[12] to the dry pulp weight was limited to 0.5 %, although the incubation time was as long as 88 h with hemicellulases. The sugars released were analyzed on Biogel column as seen for birch pulp hydrolytic products (Figure 5). They belong to the xylose series from dp 1 to 8; glucose or cellobiose which are obtained in little amounts (Table 3) may be present beneath the peaks of the xylose series.

Tables 3 and 4 give the percentage of each neutral sugar initially present in the birch and spruce pulps respectively, which

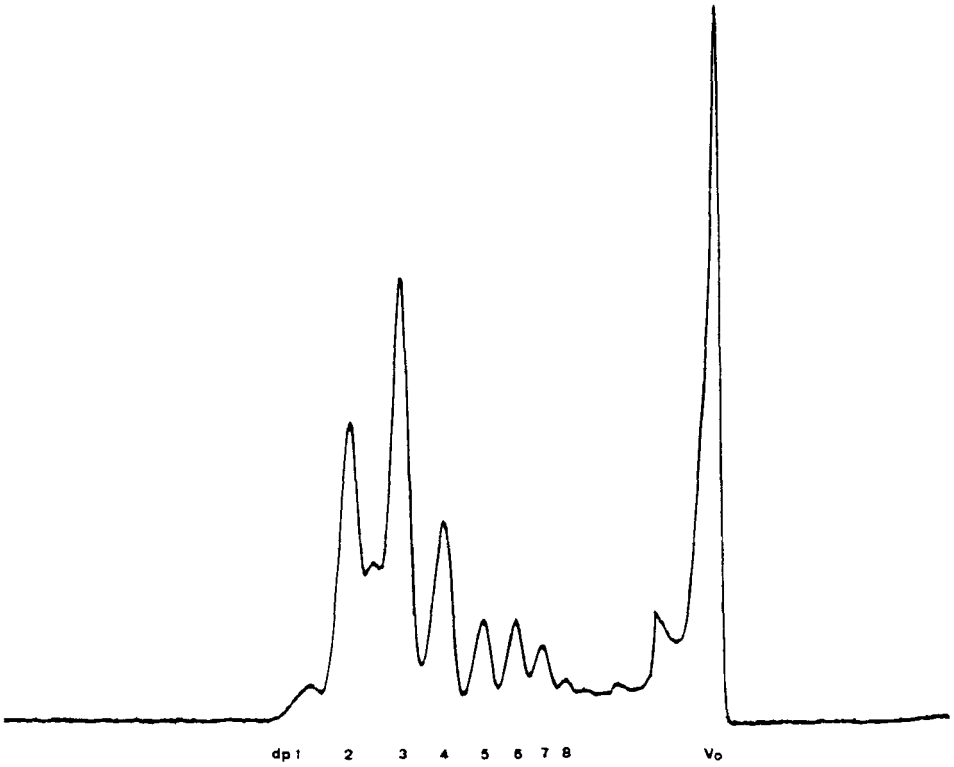

FIGURE 5. Biogel P2 analysis of the dialyzable sugars from a 24 h hydrolysis of the kraft birch pulp. Void volume: V_o.

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can be determined in the mixture of soluble sugars produced by an enzymatic degradation for 24 h.

The results illustrate again that little material is lost; they also indicate the selective hydrolysis action with regard to mannans and xylans (Table 4).

Moreover, the main enzymatic action on sulfite spruce pulp (Table 4) comes from xylanases which appear to be 10 times more efficient than mannanases with respect to recovered soluble sugars. This, of course, assumes that soluble sugar content correlates well with the number of bonds cleaved in the macromolecules inside the

TABLE 5

Aspen Pulp Characteristics

	Specific area (m2/g)	pore volume (m3/100 g)	mean radius Å	W.R.V. (g/g)
Standard aspen pulp	1.4	1.16	166	2.23
Xylanase treated pulp	2.4	0.2	17	2.70

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fiber cell wall. That selectivity could be expected for hardwood pulp, but it is more surprising with a spruce pulp where mannans are the main hemicellulose of the cell walls (Table 4). Thus, the respective localisation of the two macromolecules inside the plant cell wall are to be questioned.

Enzymes effects on pulp characteristics.

Table 5 summarises the results of the physical tests of the aspen pulp after an enzyme degradation for 88 h.

The water retention value is increased by 20 %. That is a first indication of important modifications within the cell wall of the aspen pulp fibers which was not expected on the basis of the small mass loss. The major result, however, is obtained by specific area measurements and pore radius calculations. The mean pore radius is reduced by a factor of ten following the action of xylanases, and this demonstrates the formation of micro-cracks which may be opened in the walls of pores resulting from the chemical treatments of the aspen wood.

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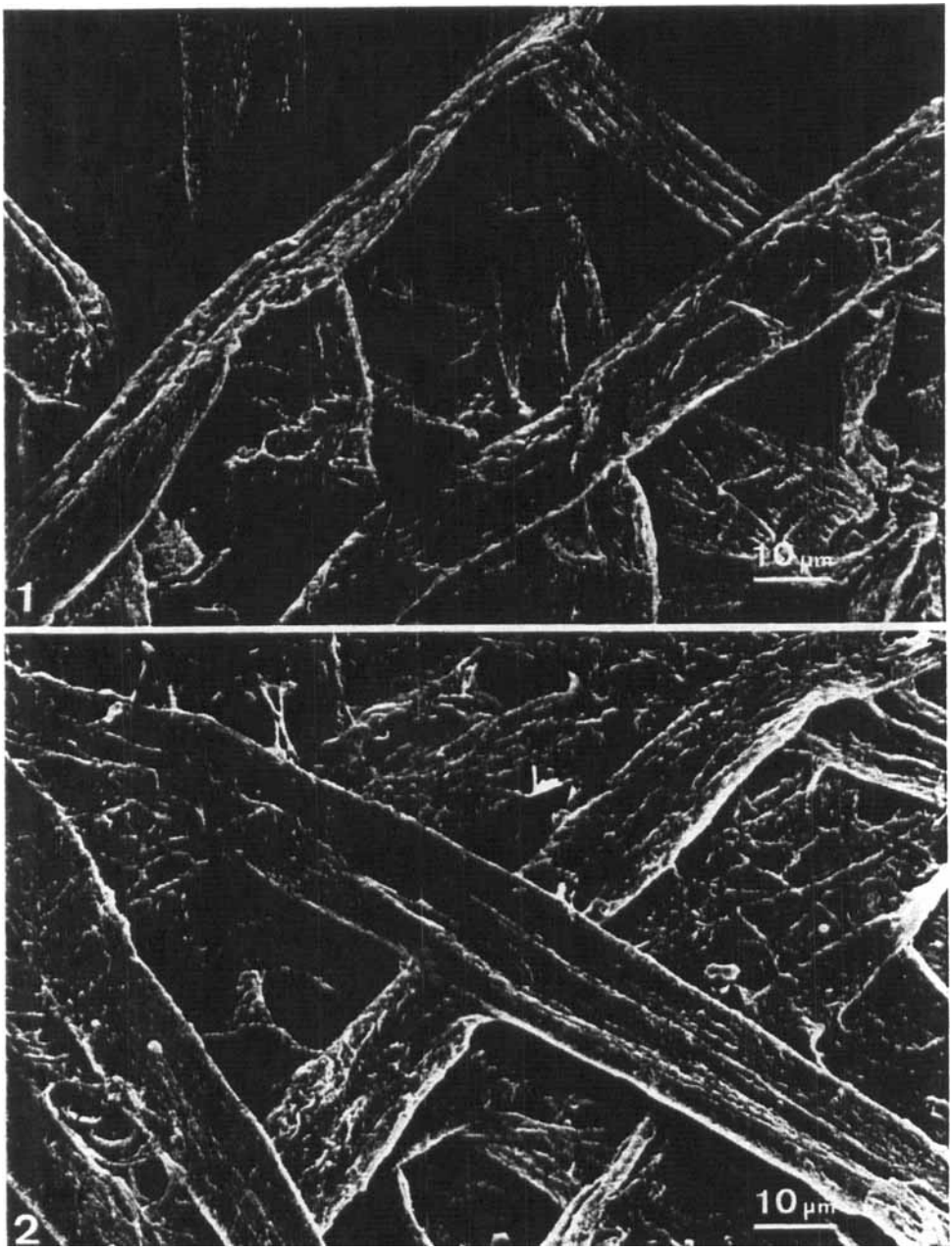

FIGURE 6. Scanning electron microscopic observations of the kraft birch pulp : control (1), enzyme-treated pulp for 24 h (2).

Electron microscopic examination of pulp.

The scanning electron microscopic observations of the bleached kraft pulp from birch (standard and 24 h enzyme-treated pulps) confirm the above conclusions. The photographs clearly show an external fibrillation and a good flexibility of fibers which also imply internal modifications (Figure 6).

Furthermore, a careful survey of the preparation did not reveal the chopped aspect characteristic of cellulase-degraded fibers,thus confirming the efficient inhibition of endocellulases.

CONCLUSION

Endo-cellulase inhibition was obtained in a mercuric chloride solution with the objective to study selective modifications inside pulp fibers. It cannot be expected that an industrial process might be built in such a medium, but we have been able to demonstrate the selective action of xylanases on a laboratory scale.

Certainly, the crude enzymatic complex includes mannanases which are not inhibited in the reaction conditions. However, since glucomannans are ten times less present than xylans in the aspen pulp fibers and since mannanases are not very active on the isolated substrate, we cannot ascribe the important changes in the physical characteristics of the aspen pulp to mannanases. Furthermore, the little exocellulase or cellobiohydrolase activity on crystalline cellulose microfibrils would not greatly affect the microfibril ultrastructural cohesion, mostly since this activity is not enhanced by synergistic effect with that of endocellulases.

All these results,the molecular weight distribution curves as well as the physical tests, indicate that xylans are more widely hydrolyzed in the fiber cell wall than it is shown by the usual mode of calculation of the hydrolysis rate based upon the release of soluble sugars. Indeed, this calculation mode does not account for xylan hydrolysis products left "in situ" in the fiber cell wall

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where they are retained via hydrogen bonding. With respect to the distribution of xylans between cellulose microfibrils[2,3,4], cell wall cohesion should be widely modified by a selective action on xylans mainly in hardwood pulps where the xylan content is important. It is the aim of part II to test pulps with regard to papermaking properties in order to confirm the role of xylans on fiber strength.

We have also pointed out that mannanases like xylanases keep their activity in the inhibiting medium. It is, then, interesting to note again that glucomannans, in contrast to xylans, are not significantly degraded in spruce wood pulp fibers. The difference must results from a lower accessibility of much of the glucomannans which are in closer association with cellulose than xylans[16,17].

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