Effects of purified *Trichoderma reesei* cellulases on the fiber properties of kraft pulp

Jaakko Pere, Matti Siika-aho, Johanna Buchert, and Liisa Viikari

ABSTRACT: Purified cellulases from Trichoderma reesei *were applied to unbleached pine kraft pulp at various dosages. The purpose was to study the individual effects of the four main cellulases—cellobiohydrolases I and II (CBH I and CBH II) and endoglucanases I and II (EG I and II)—on pulp strength. This approach eliminates the synergy and high levels of saccharification observed for cellulase mixtures. Only 0.20–2.05% of the pulp (dry weight) was solubilized by these enzyme treatments, depending on the cellulase and the dosage applied. Both EGs dramatically decreased pulp viscosity. EG II in particular appears to attack cellulose at sites where even a low level of hydrolysis reduces pulp viscosity, resulting in a marked deterioration of strength properties.*

KEYWORDS: Biotechnology, cellulase, hydrolysis, kraft pulps, mechanical properties, trichoderma, viscosity.

Interest in the application of enzymes in the pulp and paper industry has increased considerably in recent years. Utilization of hemicellulases to improve the bleachability of kraft pulps is one of the most promising new large-scale applications of enzymes *(1, 2)*. Besides enzyme-aided bleaching, enzymes also have been used to improve deinking *(3)*, drainage of paper *(4, 5)*, and pitch removal from sulfite pulp *(6)*.

The fungi and bacteria used to produce commercial enzyme preparations for the pulp and paper industry are often capable of producing both cellulases and hemicellulases. In enzyme-aided bleaching, cellulases are considered to be detrimental to pulp strength properties, so cellulolytic activity is minimized in commercial xylanase preparations. This is achieved by using noncellulolytic strains or genetically modified production strains devoid of certain cellulase-coding genes *(7)*. However, in some pulp applications, e.g., in drainage improvement of recycled pulp *(4, 5)*, undefined cellulases together with hemicellulases constitute the major activity.

Trichoderma reesei is an industrially important fungus utilized in production of cellulolytic and hemicellulolytic enzymes. The cellulolytic system of *T. reesei* is well characterized *(8–10)* and comprises two exoglucanases (cellobiohydrolases I and II) and at least three endoglucanases.

Characterization and classification of cellulases as *endo*- or *exo*cellulases is based on experimental data obtained using pure celluloses of different origins and varying crystallinities. Endoglucanases randomly attack the amorphous regions in native cellulosic substrates, carboxymethylcellulose (CMC), or phosphoric-acid-swollen cellulose, resulting in a rapid decrease in chain length *(9)*. Cellobiohydrolases have been shown to hydrolyze crystalline cellulose without the aid of endoglucanases *(11)*. Both endoglucanases and cellobiohydrolases are composed of a cellulose-binding domain and the core protein, which contains the active site *(12, 13)*. However, the roles of

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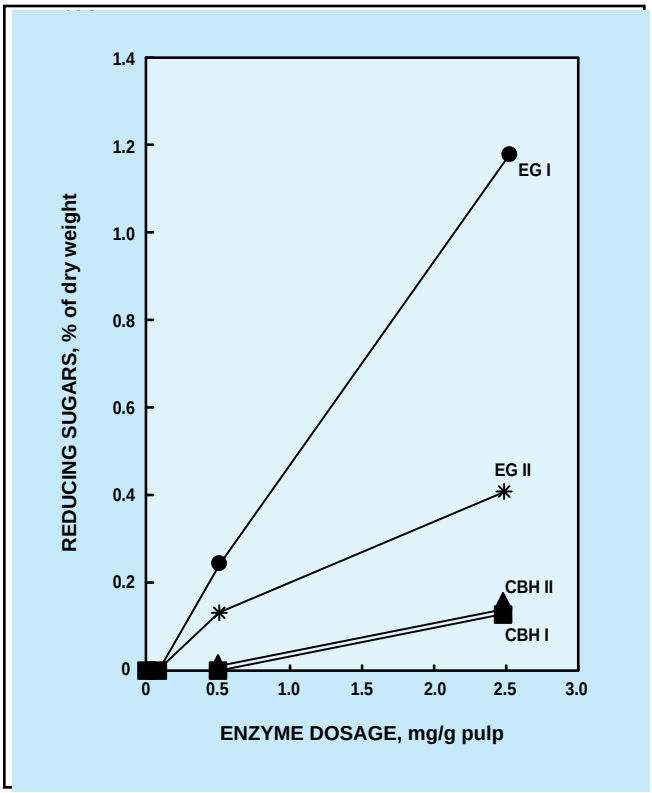
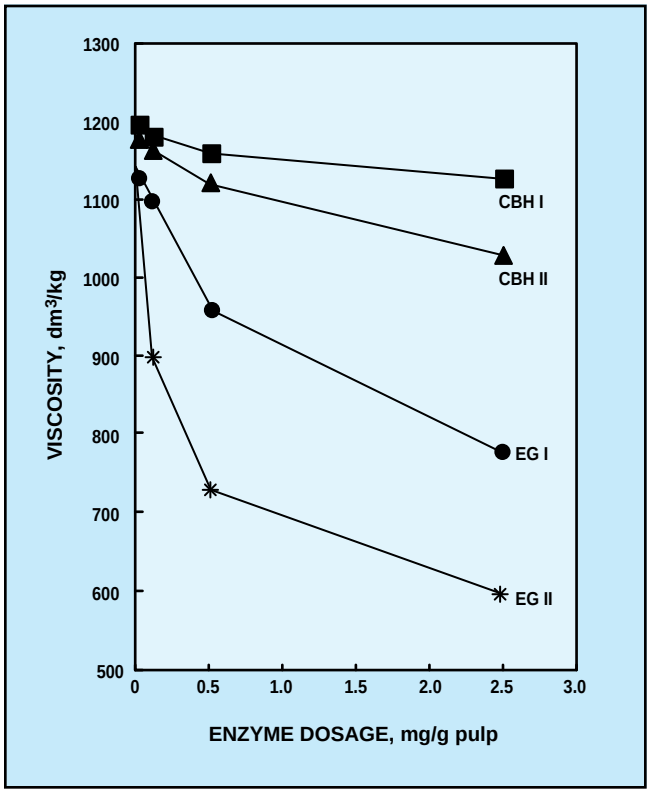
1. Liberation of reducing sugars from pine kraft pulp after treatment with purified cellulases, expressed as a function of enzyme dosage



2. Viscosity of pine kraft pulp after treatment with purified cellulases, expressed as a function of enzyme dosage



these two cellulase-molecule domains in cellulose degradation are not fully understood.

Simultaneous application of different cellulases on insoluble cellulosic substrates has a synergistic effect *(14)*. The action of individual cellulases, free of synergism, has generally been studied only on isolated cellulose substrates. However, it is also essential to understand the effects of individual cellulases on practical substrates in pulp and paper applications. This work presents the results of a study examining the effects of four individual cellulases on the strength properties of unbleached pine kraft pulp. The cellulases were obtained from *T. reesei*.

Results and discussion

Hydrolysis of kraft pulp with purified cellulases

Unbleached pine kraft pulp (kappa no. 34.4) was treated with four purified *T. reesei* cellulases—cellobiohydrolases I and II (CBH I and CBH II) and endoglucanases I and II (EG I and EG II)—over a range of enzyme dosages [0.5–2.5 mg protein/g pulp (dry weight)]. The degree of hydrolysis was measured by analyzing the solubilized carbohydrates as reducing sugars and by HPLC (high-pressure liquid chromatography) before and after a secondary hydrolysis to sugar monomers.

Both CBHs were inefficient in hydrolyzing pulp carbohydrates. The highest dosage of CBH enzyme [2.5 mg/g pulp (dry weight)] solubilized less than 0.2% of pulp (dry weight), as seen in **Fig. 1**. In contrast, similar dosages of EG I and EG II solubilized 1.2% and 0.4% of pulp (dry weight), respectively. EG I was much more efficient in hydrolyzing pulp than EG II, although the specific endoglucanase activity of EG II was almost twice that of EG I.

The solubilized carbohydrate oligomers were identified by HPLC. All of the cellulases liberated cello-oligomers as well as some unidentified oligomers derived from the hemicellulosic fraction of pulp (results not shown). In order to analyze exactly the sugar composition of solubilized carbohydrates, the supernatants were subjected to a secondary hydrolysis using an enzyme mixture, as described elsewhere *(15)*. As expected, the CBHs produced a monomeric fraction composed mainly of glucose, as seen in **Table I**. CBH I liberated some xylose-containing oligomers, which is in accordance with the measured activities. CBH II solubilized some mannose- and galactose-containing oligomers, although no mannanase activity was detected in the preparation.

EG I solubilized both xylan and cellulose from the pulp. With an EG I dosage of 0.5 mg/g, the molar ratio of liberated xylo-oligomers to cello-oligomers was about 2:1, but with a dosage of 2.5 mg/g, it decreased to 1.3:1 (calculated from the values in

I. Composition of solubilized oligomeric carbohydrates of the enzyme-treated pulps and the control pulp as analyzed by HPLC

Enzyme	Dosage, mg/g	Solubilized sugars, % dry weight	Sugar composition of solubilized carbohydrates, % dry weight				
			Glucose	Xylose	Mannose	Arabinose	Galactose
CBH I	0.5	0.20	0.15	0.05	0	0	0
	2.5	0.40	0.30	0.10	0	0	0
CBH II	0.5	0.10	0.10	0	0	0	0
	2.5	0.30	0.20	0	0.05	0	0.05
EG I	0.5	0.80	0.25	0.35	0.15	0.05	0
	2.5	2.05	0.80	0.75	0.35	0.10	0.05
EG II	0.5	0.50	0.35	0	0.15	0	0
	2.5	1.05	0.55	0.1	0.35	0	0.05
Control	. . .	0	0	0	0	0	0

Table I). Based on the original carbohydrate composition of the pulp, EG I treatment at the lowest dosage (0.5 mg/g) solubilized 0.25% of pulp cellulose and 5.0% of pulp xylan, while the higher dosage (2.5 mg/g) produced corresponding figures of 0.9% and 10.4%. Thus, at the lower dosage, EG I acted more as a xylanase. One possible explanation might be the more accessible location of xylan on the surface of the pulp, caused by the reprecipitation reactions during the cook *(16)*.

EG I also attacked pulp glucomannan, as seen in Table I. Assuming a glucose-to-mannose ratio of 1:4 in the pulp glucomannan *(17)*, 2.2% and 5.1% of the pulp glucomannan was hydrolyzed with EG I dosages of 0.5 mg/g and 2.5 mg/g, respectively.

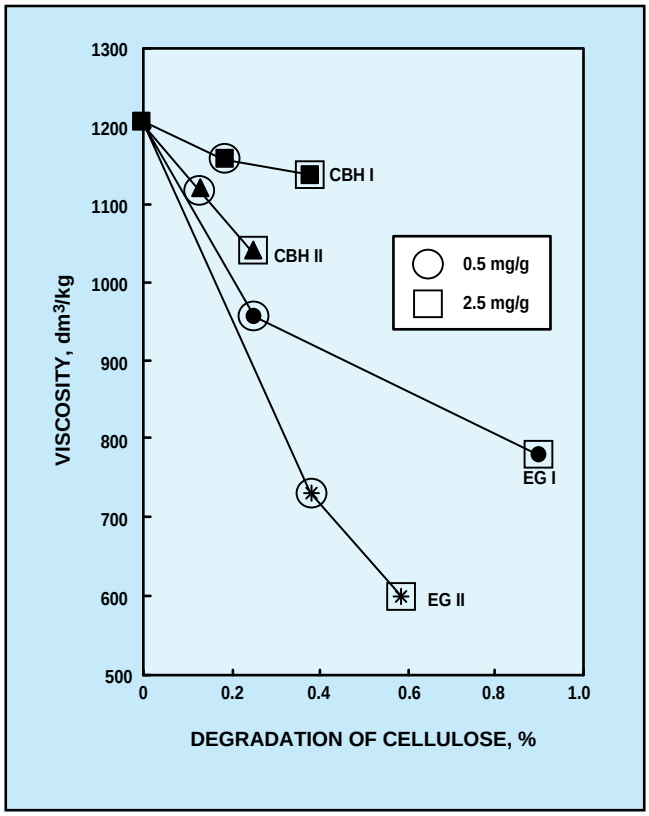
EG II was less efficient in the overall solubilization of pulp than EG I, despite its higher specific endoglucanase activity (Table I). With the EG II dosage of 2.5 mg/g, about 0.6% of the pulp cellulose was solubilized, compared with 0.9% obtained by EG I. However, using the lower enzyme dosage (0.5 mg/g), EG II hydrolyzed the cellulose component of pulp more efficiently than EG I (Table I). This was obviously due to the preference of EG I for xylan at a relatively low enzyme concentration. In addition to cello-oligomers, EG II also solubilized oligomers composed of glucose and mannose as efficiently as EG I.

The activity of CBHs and EGs against wood-derived glucomannans has not been studied in detail, but it appears that all the cellulases used in this work, with the exception of CBH I, solubilized at least some pulp glucomannan. Liberation of glucomannan oligomers could have been caused by (a) a contaminating side-activity in the preparation, (b) an unspecific hydrolysis, or (c) physical leaching. In the case of the EGs, a contaminating mannanase side-activity might partly explain the observed results. In a previous work, both CBH II and EG I from *T. reesei* expressed in yeast, i.e., without any contaminating side-activities, were shown to hydrolyze locust bean gum galactomannan, indicating the unspecificity of these enzymes *(18)*. However, it is also possible that the liberation of glucomannan-derived oligomers in the hydrolysis with the EGs was partially caused by physical solubilization (without enzymatic activity) caused by the very close association of cellulose with glucomannan in the pulp. In view of these factors, it is apparent that activity assays against soluble or modified pure substrates may not always predict the hydrolytic effects of individual cellulases on fiber-bound or solid substrates.

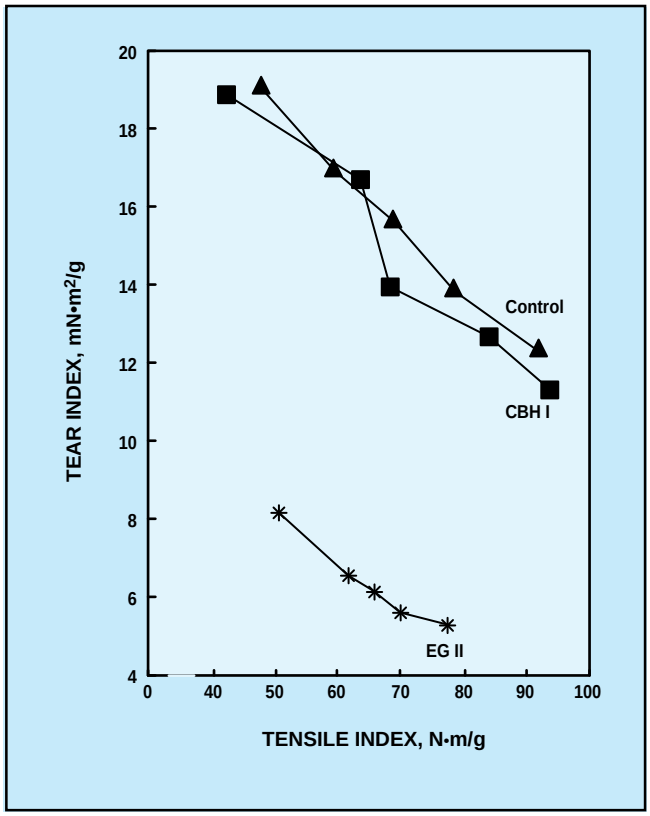
Effects of cellulases on viscosity

Both EGs decreased pulp viscosity, even at low enzyme dosages, as seen in **Fig. 2**. The observed decreases in viscosity were in keeping with the specific activities of the EGs. Sur-

3. Viscosity of enzyme-treated kraft pulps as a function of cellulose degradation. Calculation of cellulose degradation was based on the original carbohydrate composition of the pulp and on the amount of solubilized glucose. Glucomannan-derived glucose was subtracted from the values, assuming a glucose : mannose ratio of 1:4.



4. Relationship between tensile and tear indices for the CBH I- and EG II-treated pulps and for the untreated control pulp. Loading of both enzymes was 0.5 mg/g pulp (dry weight).



prisingly, however, the decrease in viscosity did not follow the solubilization of pulp cellulose, although both enzymes are known to act on amorphous parts of the cellulose *(9)*. **Figure 3** shows that EG II solubilized 0.38% of pulp cellulose at an enzyme dosage of 0.5 mg/g, while EG I solubilized 0.9% of the pulp cellulose at a dosage of 2.5 mg/g. However, as seen in Fig. 2, EG II at a dosage of 0.5 mg/g (0.38% cellulose degradation) reduced the viscosity more than EG I at a dosage of 2.5 mg/g (0.9% cellulose degradation). Hence the mode of action of EG II on the pulp cellulose results in a rapid decrease of viscosity with a lower degree of hydrolysis.

The decrease of viscosity with CBH I and CBH II was moderate. Figure 3 shows that, at the same degree of cellulose degradation, viscosity decreased more significantly with CBH II than with CBH I, indicating differences in their mode of action on the pulp substrate. For example, at a cellulose degradation level of 0.25%, the decreases of viscosity with the CBH I- and CBH II-treated samples were 4% and 13%, respectively, as compared with the untreated control. This could be explained by the partially endo-type action of CBH II, corresponding to the slight ß-glucanase activity of this enzyme, as reported by Henrissat *et al. (14)*.

Although 0.2–1% of the pulp cellulose was solubilized, depending on the enzyme used and the applied dosage, no effect on the fiber length was observed. **Table II** shows that both the arithmetic and length-weighted average fiber lengths were of the same order of magnitude in

II. Average fiber length of pine kraft pulp after treatment with the purified cellulases*

	Fiber length, mm				
Procedure	*Control*	*CBH I*	*CBH II*	*EG I*	*EG II*
Arithmetic avg.	1.07	1.10	1.06	1.06	1.06
Length-weighted avg.	1.99	2.00	1.96	1.98	1.97

* Hydrolyses were performed at a pulp concentration of 2% (w/v) and an enzyme loading of 2.5 mg/g pulp (dry weight). Incubation was at 45°C and pH 5 for 2 h.

III. Handsheet strength properties of pulps treated with CBH I (0.5 mg/g) and EG II (0.5 mg/g) and the untreated control pulp at various levels of beating

Sample	Beating, rev.	Density, g/cm³	Tensile index, N·m/g	Tear index, mN·m²/g	Zero-span tensile strength, Nm/g Dry	Zero-span tensile strength, Nm/g Wet	Air resistance (Gurley), s
Control	0	0.62	47.2	19.1	145.1	128.0	1.8
	500	0.66	68.8	15.6	153.0	131.7	2.5
	1000	0.69	78.2	13.8	169.2	142.5	3.1
	2000	0.72	91.7	12.2	164.9	143.2	4.4
CBH I	0	0.59	42.0	18.9	149.1	126.0	1.4
	500	0.65	68.3	13.9	164.1	136.1	2.6
	1000	0.71	84.0	12.6	162.3	137.9	4.2
	2000	0.73	93.7	11.2	160.3	139.3	7.3
EG II	0	0.65	50.2	8.1	108.4	54.4	1.9
	500	0.72	65.8	6.1	119.6	53.1	5.0
	1000	0.75	70.2	5.5	122.2	53.6	9.9
	2000	0.79	77.4	5.2	124.6	51.8	57.0

IV. Characteristics and specific activities of the enzymes used in the pulp treatments

Enzyme	Molecular weight, kDa	Isoelectric point (pI)	Specific activities, nkat/mg Xylanase	Endoglucanase	Mannanase
CBH I[a]	65	3.9	3	1	<d.l.[b]
CBH II[a]	53	5.9	<d.l.[b]	<d.l.[b]	<d.l.[b]
EG I	55	4.7	540	525	30
EG II	48	5.6	<d.l.[b]	950	3

[a] Activity against crystalline cellulose was not measured.
[b] Below detection limit

the enzyme-treated and control pulps, as measured prior to beating. In earlier studies *(19, 20)*, in which different pulps and paper grades were treated with cellulase mixtures, a gradual decrease of average fiber length was observed as the degree of saccharification increased from 3.5% to 15%.

A clear correlation between viscosity and cellulose degradation by cellulases was detected. Interestingly, Ramos *et al. (20)* observed no concomitant decreases in the degree of polymerization or crystallinity with the proceeding hydrolysis (up to a glucose yield of 70%) for fully bleached eucalyptus kraft pulp treated with a mixture of CBH and EG. Possibly there is a great variability in degradation modes of cellulases when acting individually or when acting synergistically in mixtures. The disparity between our results for unbleached pine kraft pulp and the observations of Ramos *et al. (20)* for bleached eucalyptus kraft pulp may also indicate differences in the accessibility of cellulose to enzymes for pulps from different wood species.

Handsheet strength properties

The strength properties of pulp handsheets were analyzed after treatments with CBH I and EG II. There were no major differences in the strength properties for the CBH I-treated and the control pulps, as seen in **Fig. 4** and **Table III**. In the sample treated with CBH I, the slight decrease of tear index was compensated by the increase in tensile index at higher levels of beating. The comparable zero-span indices in Table III indicate that CBH I did

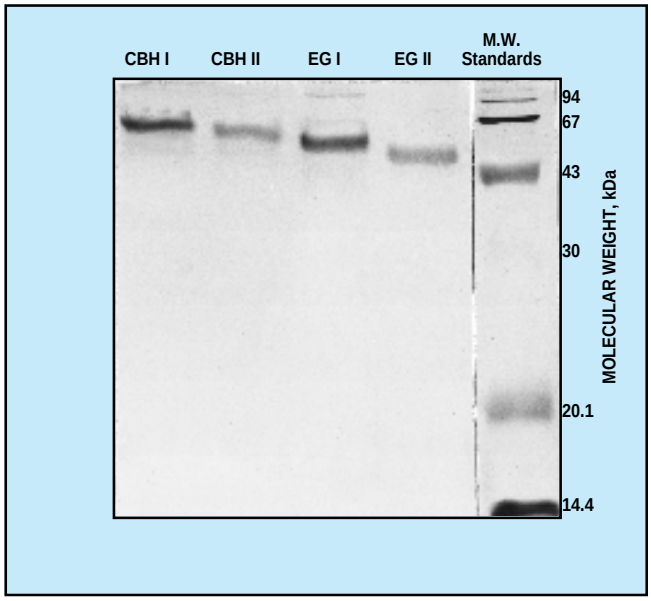
not cause structural damage to the fibers.

In contrast, the EG II treatment severely damaged the strength properties of fibers, as could be expected from the viscosity data. Twice as much beating was needed to obtain a tensile index of 70 N·m/g with the EG II pulp as compared with the control (Table III), but at the same time the strength properties of the fibers were lost. At a tensile index of 70 N·m/g, the tear index for the EG II-treated pulp was only 30% of that obtained for the untreated control, as seen in Fig. 4. The loss of the structural integrity of the fibers after treatment with EG II can also be seen by comparing the wet and dry zero-span indices of the EG II-treated sample and the untreated control (Table III). The wet and dry zero-span tensile indices for the EG-II treated sample were on average 40% and 75% of the control, respectively. The negative effects of EG II were irreversible and could not be restored by beating.

It is possible that EG II attacks certain accessible regions of the cellulose molecules within the microfibers, loosening the structure but not breaking the fibers. After handsheet manufacture and drying, the loosened cell wall might have partly collapsed, increasing fiber conformability, which can be detected as an evident change in air resistance (Gurley) and as a higher density (Table III). Hence the functional integrity of fibers was destroyed by EG II at a very low level of hydrolysis (< 0.5%). This implies a specific mode of attack on critical points of microfibers. If this is the case, it would seem that the loss of strength could not be explained by the "peeling off" type of mechanism on the surface of the fibers, as suggested by Ramos *et al. (20)*. Indeed, a specific mode of attack at critical points would suggest that the enzyme penetrates and subsequently hydrolyzes cellulose in the inner S_2 layers, at least to some extent.

Gurnagul *et al. (21)* explained the strength loss of black spruce kraft pulp after cellulase treatment by speculating that structurally irregular zones such as kinks and nodes are subjected to localized attack. These types of defects in the fiber structure, possibly amorphous and hydrated in nature, have been observed to be initiation sites for the hydrolysis of microcrystalline *Valonia* cellulose by EG II *(11)*. One important reason for the observed differences in the mode of action between different cellulases could be the varying binding properties of these enzymes at different sites along the fiber. This hypothesis is supported by the fact that there are differences in the hydrophobicities and electrostatic properties of the cellulose-binding domains of EG and CBH, as predicted by molecular modelling (A.-M. Hoffrén, personal communication).

5. Gel electrophoresis of the purified cellulases in 12% SDS polyacrylamide gel. Positions of MW standards are marked at the sides.



Conclusions

Commercial enzyme preparations used in the pulp and paper industry may contain cellulases as major or minor activities. Consequently, it is important to understand the impacts of different individual cellulases on pulp quality.

The four cellulases of *T. reesei* exhibited significant differences in their mode of action on pulp cellulose, as judged from the hydrolysis products and pulp viscosity. The cellobiohydrolases (CBH) had only a very modest effect on pulp viscosity. In contrast, the endoglucanases (EG) dramatically decreased viscosity, even at low enzyme dosages. The detrimental effect on viscosity was more pronounced with EG II, despite a low degree of hydrolysis of pulp cellulose. This suggests that EG II attacks cellulose at sites where even low levels of hydrolysis produce large decreases in viscosity and, consequently, a dramatic deterioration of tensile index.

In addition to cellulose hydrolysis, all of the cellulases expressed some activity toward hemicelluloses, despite the electrophoretic homogeneity of the enzyme preparations. The impact of these side activities, together with cellulase activities on viscosity and strength properties, remains to be clarified in further studies. However, it is clear that the four purified cellulases of *T. reesei* had profoundly different effects on pulp properties. Moreover, the results demonstrate that not all cellulases are detrimental to pulp strength properties.

Materials and methods

Pulps

Pine kraft pulp of kappa no. 34.4 was produced in the laboratory as described previously *(22)*. The carbohydrate composition of the pulp after acid hydrolysis was 81.6% glucose, 8.2% xylose, and 6.8% mannose. After acid hydrolysis, no arabinose or galactose were detected, obviously because they were destroyed under the acid hydrolysis conditions.

Enzymes

The cellulose-degrading enzymes—endoglucanases (EG I and II) and cellobiohydrolases (CBH I and II)—were purified from *Trichoderma reesei* Rut C 30 culture filtrate. The organism was cultivated in a pilot fermenter (2 m^3) on a medium containing 3% (w/v) cellulose and 3% (w/v) corn steep liquor supplemented with KH_2PO_4 and $(NH_4)_2SO_4$. The culture filtrate was clarified by treatment with bentonite and diatomaceous earth at pH 3.5 as described by Zurbriggen *et al. (23)*. Prior to the chromatographic purification, the filtrate was concentrated by ultrafiltration (ES 625 membranes, PCI, England).

The bentonite-treated and concentrated culture filtrate (100 g protein) was equilibrated with 10m*M* sodium phosphate buffer (pH 7.2) by gel filtration (Sephadex G-25 coarse, Pharmacia) and applied to an anion-exchange column of DEAE (diethylaminoethyl) Sepharose FF (Pharmacia) equilibrated with the same buffer. Elution was performed first with the starting buffer to remove unadsorbed proteins and thereafter with an increasing concentration of sodium chloride. By this method, the four major cellulases were separated. Most of EG II eluted in the breakthrough, CBH II at 25 m*M* NaCl, EG I at 120m*M* NaCl, and CBH I at 150m*M* NaCl.

Protein was assayed by the method of Lowry *et al. (24)* or estimated by absorbance at 280 nm. An absorbance at 280 nm of 1.00 corresponded to a protein concentration of 0.8 g/L.

CBH I. A pool from the first DEAE Sepharose run containing part of the CBH I (740 mg protein) was equilibrated with 10m*M* sodium acetate (pH 4.5) by gel filtration (Sephadex G-25 coarse) and applied to a column of DEAE Sepharose FF equilibrated with the same buffer. The adsorbed proteins were eluted with a gradient of sodium chloride from zero up to 150m*M* and thereafter with the buffer containing 150m*M* NaCl. Fractions containing the highest protein concentrations (37 mg protein in all) were pooled and used for the pulp treatments.

CBH II. Partially purified CBH II from the anion-exchange run was further purified using affinity chromatography as described by van Tilbeurgh *et al. (25)*. The affinity ligand was p-aminobenzyl 1-thio-ß-D-cellobioside linked to cyanogen bromide-activated Sepharose 4B (Pharmacia). Part of the pooled CBH II preparation (240 mg protein) was equilibrated with the binding buffer (50m*M* ammonium acetate, pH 5.0, containing 100m*M* glucose and 10m*M* gluconolactone) by gel filtration (Sephadex G-25 coarse) and applied to the affinity column. The adsorbed CBH II was eluted by 100m*M* lactose. The fractions containing the highest concentrations of CBH II (31 mg protein in all) were combined and dialyzed against 20 m*M* sodium acetate buffer (pH 5.0) before use.

EG I. EG I was purified further by cation exchange, hydrophobic interaction, and affinity-chromatography runs. A pool from the first DEAE Sepharose run (3.2 g protein) was buffered to 7m*M* sodium acetate buffer (pH 3.8) by gel filtration (Sephadex G-25 coarse) and applied to a cation-exchange column (CM Sepharose FF, Pharmacia) equilibrated with the same buffer. The bound proteins were eluted with a combined pH and ionic-strength gradient from 7m*M* sodium acetate (pH 3.8) to 20m*M* sodium acetate (pH 4.7). Fractions containing EG I that eluted during the gradient (2.0 g protein) were combined, and $(NH_4)_2SO_4$ was added to a concentration of 0.5 mole/L. The centrifuged pool was applied to a column of phenyl Sepharose FF equilibrated with 20m*M* sodium acetate (pH 4.5) containing 0.5 mole/L $(NH_4)_2SO_4$. The adsorbed proteins, including EG I, were eluted by a decreasing gradient of ammonium sulfate from 0.5*M* to zero in 20m*M* sodium acetate. The contaminating CBH I was removed from the EG I preparation by affinity chromatography *(25)*. A fraction (120 mg protein) of EG preparation was buffered to the binding buffer by gel filtration and applied to the affinity column. EG I was collected in the unbound protein fractions, and the adsorbed CBH was eluted by 0.1*M* cellobiose. A part of the unadsorbed material (38 mg protein) was dialyzed against 50 m*M* sodium acetate (pH 5.5) and concentrated by ultrafiltration (Amicon, PM-10 membranes) for pulp treatments.

EG II. The breakthrough of the first anion-exchange run containing EG II (5.1 g protein) was applied to a CM Sepharose FF column equilibrated with 10m*M* sodium phosphate buffer (pH 7.2). The unbound proteins including EG II were collected. The ammonium sulfate concentration of the EG II pool (2.5 g protein) was adjusted to 0.4 mole/L, and the sample was centrifuged and applied to a column of phenyl Sepharose FF equilibrated with 10m*M* sodium phosphate buffer at pH 7.2 containing 0.4 mole/L $(NH_4)_2SO_4$. The adsorbed proteins were eluted by stepwise decrease of the concentration of $(NH_4)_2SO_4$. EG II was eluted with 50m*M* ammonium sulfate. This preparation (600 mg protein) was buffered to 5m*M* sodium acetate at pH 4.6 by gel filtration and applied to a CM Sepharose FF column equilibrated with the same buffer. EG II was retarded in the column and eluted using the equilibrating buffer.

The adsorbed proteins were eluted by increasing the pH and ionic strength. Final purification and concentration was achieved by adjusting the pH of the combined EG II fractions (240 mg protein) to 4.3 and applying the preparation to a CM Sepharose FF column (50 x 100 mm) equilibrated with 5m*M* sodium acetate at pH 4.3. The bound EG II was eluted as a concentrated peak by a combined gradient of pH and ionic strength from the equilibrating buffer to 20m*M* sodium acetate at pH 5.0. The fraction with the highest activity (60 mg protein) was used for the pulp treatments.

Characterization of the cellulase preparations

The purity and specific activities of the enzyme preparations were determined before experimental work.

For purity control, conventional electrophoresis in denaturating conditions [sodium dodecyl sulfate (SDS) in polyacrylamide gel electrophoresis (PAGE)] was carried out in 12% gel slabs by the method of Laemmli *(26)* using a Midget electrophoresis unit (Pharmacia). The low-molecular-weight-calibration mixture for electrophoresis (Pharmacia) was used as standard, and proteins were stained with silver stain (Bio Rad, silver stain kit).

Endoglucanase activity was assayed by a standard method *(27)* using 1.0% (w/v) hydroxyethyl cellulose (Fluka 54290) as substrate. One nkat of endoglucanase activity liberated 1 nmole of reducing sugars per second from hyroxyethylcellulose. Activity on a crystalline cellulose substrate was not measured. Xylanase activity was assayed as described by Bailey *et al. (28)* using 1.0% (w/v) birchwood 4-*O*-meglucuronoxylan (Roth 7500) as substrate. Mannanase activity was assayed using 0.5% (w/v) locust bean gum (Sigma G-0753) as substrate *(29)*, analogously to the xylanase assay.

On the basis of the electrophoretic analysis, the enzyme preparations were pure, i.e., only a single band was obtained from each preparation in the SDS–PAGE analysis, indicating homogeneity of the samples, as seen in **Fig. 5**.

The characteristics and specific activities of the enzymes used are presented in **Table IV**. In addition to its endoglucanase activity, EG I also exhibited a high xylanase activity as well as clearly detectable mannanase activity, reflecting the unspecificity of this enzyme, as reported earlier *(18)*. The xylanase activity of EG I has also been verified on pulp substrate *(30)*.

Enzyme treatments

The enzyme treatments were carried out at 2% consistency in 50m*M* citrate buffer, pH 5. The hydrolysis temperature was 45°C and the hydrolysis time was 2 h. In the experiments, the enzymes were dosed on the basis of protein rather than activity. After the hydrolysis, the pulp was washed with distilled water.

Analytical methods

The viscosity of the pulp was measured according to the SCAN-method (SCAN C15: 1988). The solubilized carbohydrates liberated in the enzyme treatments were analyzed as reducing sugars *(31)* or by HPLC (high-pressure liquid chromatography) before and after a secondary analysis of solubilized oligosaccharides *(15)*. Handsheet strength properties were measured according to SCAN methods. The fiber length was measured using a Kajaani FS-200 analyzer.

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