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Effects of cellulase on the modification of cellulose

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Abstract

Multicomponent cellulases, purified endoglucanases and cellobiohydrolases were assayed and shown to modify pure natural cellulose (softwood pulp). Changes in structure and properties of the cellulose caused by enzymatic treatment depend on the composition, the type of enzyme, and the treatment conditions. The reactivity of cellulose for some dissolving and derivatization processes may be improved by enzymatic hydrolysis. Endoglucanases decreased the average degrees of polymerization ($\overline{DP}$) and improved the alkaline solubility of cellulose most efficiently. The variation in the supramolecular structure estimated from the infrared spectra of the cellulose samples was found to be correlated with the reactivity and might represent wide variations in conformation caused by the breakdown of the hydrogen bonds.

Keywords: Cellulase; Cellulose; Hydrolysis; $\overline{DP}$; Alkaline solubility; Supramolecular structures

1. Introduction

Cellulases are enzymes that hydrolyze the β-(1→4) linkages in cellulose. Cellulases are produced as a multicomponent enzyme system comprised usually of three enzymes that act synergistically in the hydrolysis of cellulose: endoglucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91), and cellobiase (β-glucosidase, EC 3.2.1.21). The first two enzymes act directly on cellulose, yielding mainly cellobiose and glucose as the reaction products. The cellobiose is then hydrolyzed into glucose by cellobiase. Endoglucanases and cellobiohydrolases degrade soluble cellodextrins and amorphous cellulose. However, it is the cellobiohydrolases that degrade crystalline cellulose most efficiently.[1]

Cellulose is the most abundant and renewable biopolymer on Earth. The enzymatic modification of cellulose is a challenge in all applications using cellulose-based fibers due to the potential benefits. Currently the most widely exploited cellulase applications are in the textile industry, for example the stone washing of jeans and finishing of cotton fabrics.[2,3] In the pulp and paper industry, cellulases are used together with hemicellulases to improve the drainage and running of paper machines and to enhance the deinking of recycled fibers.[4,5]

To date, many studies on the action of cellulases or purified cellulases on cellulose have revealed the mechanism by which the enzymes degrade cellulose.[6] The substrate concentration and effect of endoglucanase and cellobiohydrolase on the properties of cellulose need further study. The aim of the present study is to investigate the treatment of dissolving pulp with different cellulases and determine whether such treatment improves its properties.

2. Results and discussion

Activity profiles of the enzymes.—Cellulase can hydrolyze carboxymethylcellulose (CMC). The structure of the endoglucanase is known, and the active-site residues in the enzymes are situated in a cleft that can accommodate the carboxymethyl groups in such a manner that the individual glucose units can be attacked.[7] Cellulases have been analyzed for pH and temperature activity using CMC, and the profiles for endoglucanase and cellulase are shown in Figs. 1 and 2. Both have pH optima in the range of pH 6.5–8 and have good activity in the temperature range of 45–60 °C, exhibiting more

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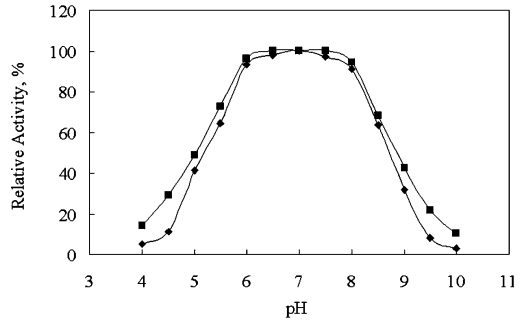

Fig. 1. Effect of pH on relative activity at 50 °C. Symbols: ■, endoglucanase; ◆, cellulase.

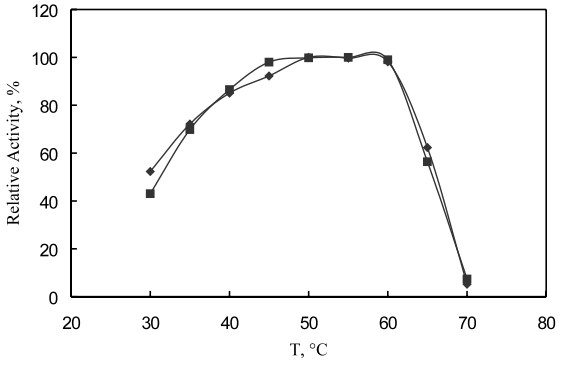

Fig. 2. Effect of temperature on relative activity at pH 7. Symbols: ■, endoglucanase; ◆, cellulase.

than 85% of the maximum activity over this range. The endoglucanases have somewhat broader pH optima.

Enzyme activities.—Before evaluating the enzymes for their potential to modify the properties of pulp, each enzyme was assessed for its ability to hydrolyze standard substrates (Table 1). Cellulase is shown to contain high levels of xylanase activity, while the endoglucanase shows lower but significant levels of xylanase activity.

Effects of cellulases on the $\overline{DP}$ of the cellulose.—Both endoglucanase and cellulase are shown to decrease the $\overline{DP}$ of cellulose, even at low enzyme dosages, as seen in Table 2. Cellobiohydrolase has a less pronounced effect on the $\overline{DP}$. The observed decreases in $\overline{DP}$ were in keeping with the specific activities of CMCase of endoglucanase and cellobiohydrolase. Table 2 shows the $\overline{DP}$ decrease relative to the increase in enzyme dosage.

Fig. 3 shows the effect of the treatment with cellulase, endoglucanase, and cellobiohydrolase on the $\overline{DP}$ of cellulose. Both curves differ notably in their characteristics. In the experiments with cellobiohydrolase, the decrease of $\overline{DP}$ is small, at most 15%. The very much smaller drop in the beginning of the curve of cellobiohydrolase is caused by the mechanism of its hydrolysis. In the case of endoglucanase, however, cellulose suffers a strong degradation at the beginning of the treatment, with a slow up to some degree in the hydrolysis as time increases. The $\overline{DP}$ moderately decreases in the beginning of the degradation of cellulose and afterwards become lower.

This behavior of cellulose under treatment of enzymes was to be expected, because the cellobiohydrolase degrades cellulose by an endwise attack, giving mainly cellobiose, causing, therefore, only a slight diminution in $\overline{DP}$. The endoglucanase, on the other hand, cleaves the cellulose chains randomly, provoking a relative strong degradation. Cellulase is a multicomponent enzyme, being comprised of the two enzymes that act synergistically.[7]

Table 1
The specific activity of enzymes

Enzymes	CMCase (IU/mg)	Mananase (IU/mg)	Xylanase (IU/mg)	Filter paper (IU/mg)
Cellulase	2.07	bdl [a]	63.2	0.21
Endoglucanase	1.57	bdl	0.12	bdl
Cellobiohydrolase	0.06	bdl	bdl	nd [b]

[a] Below detection limit.
[b] The filter paper activity of cellobiohydrolase was not measured.

Table 2
DP and viscosities of the pulp with enzymatic treatment [a]

Enzyme	Cellulase			Endoglucanase			Cellobiohydrolase			Control
Dosage (mg/g)	0.10	0.50	2.50	0.10	0.50	2.50	0.10	0.50	2.50	0.00
Viscosity (cm^3/g)	505	465	413	492	429	358	530	510	483	535
DP	706	645	566	686	590	483	745	714	672	753

[a] The treatments were carried out at 3% substrate concentration at 50 °C and pH 7 for 2 h.

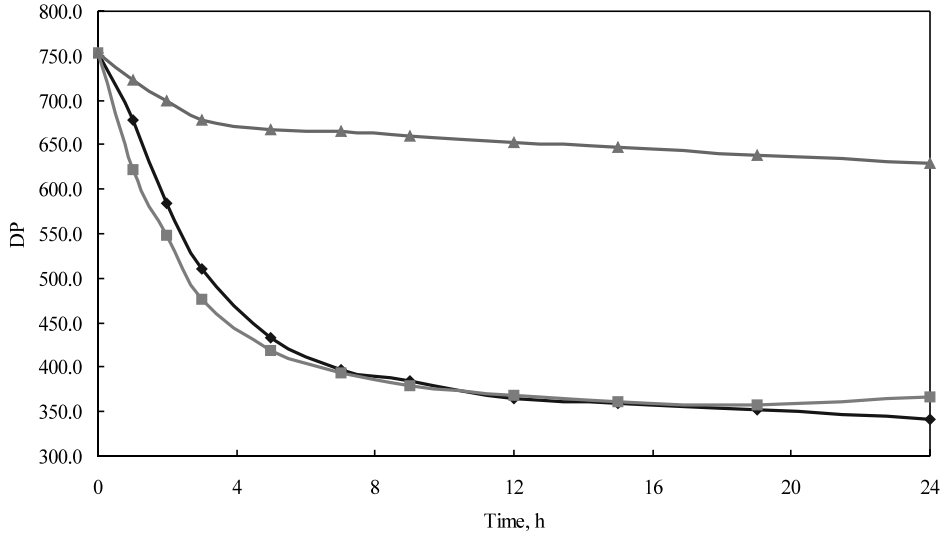

Fig. 3. Effect of enzymes on the $\overline{DP}$ of cellulose. Symbols: ■, endoglucanase; ◆, cellulase; ▲, cellobiohydrolase. Dosage of the enzymes: 2 mg/g pulp (dw). Substrate concentration: 3%.

Effects of substrate concentration on degradation of cellulose.—The normal pattern of dependence of $\overline{DP}$ on substrate concentration is that the substrate concentration does not have an effect on the decrease of $\overline{DP}$ at low concentration as shown in Fig. 4. When the concentration of substrate is over 8%, the $\overline{DP}$ drops slightly.

It is known that there are three processes that occur before onset of the enzymatic hydrolysis of cellulose: (1) the diffusion of cellulase in the liquid; (2) the transfer of cellulase from the liquid to the surface of substrate; and (3) the enzyme absorption of cellulose onto the enzyme with the formation of an enzyme–cellulose complex. High concentration of substrate hinders the transfer of cellulase so that the enzyme does not sufficiently touch the substrate, which affects the hydrolysis of cellulose in the last step.

Effect of enzymatic treatment on the alkaline solubility of the cellulose.—After enzymatic treatment, the alkaline solubility of the cellulose was investigated. As expected, endoglucanase is found to be the most efficient in hydrolyzing pulp, lowering the viscosity, and improving the alkaline solubility of the substrate. The correlation between the decrease in viscosity and the alkaline solubility is shown in Table 3. Thus, the improvement in the alkaline solubility was caused by the decrease in $\overline{DP}$ and hydrogen bonding because of hydrolysis. Cellobiohydrolase has a less pronounced effect on either the $\overline{DP}$ or the alkaline solubility.

Effect of enzymatic treatment on the structural properties of cellulose.—Enzymatic treatments have some effect on the crystallinity of cellulose. Cellulose that has undergone enzymatic treatment is crystallographically cellulose I, revealing the characteristic X-ray diffraction peaks. The crystallinity of partially crystalline cellulose increases as hydrolysis reaction proceeds. It was found that the enzymes preferentially attack the 002 crystal planes for cellulose I as shown in Figs. 5 and 6. These results indicate that the crystallite width decreases while the crystallite length remains practically constant during enzymatic hydrolysis.

The crystalline index of cellulose increased during hydrolysis as shown in Table 4. The increase in the crystalline index is good evidence that the amorphous portion of the cellulose is more readily hydrolyzed than the crystalline. The data for the apparent crystal size in Table 4 show some variation and imply that the enzyme has some effect on the crystal of cellulose during enzymatic hydrolysis.

The FTIR spectroscopic data in Figs. 7 and 8 indicate that enzymatic treatments have some effect on the structure of cellulose. The FTIR spectra show characteristic cellulose peaks in the range of 1000–1200

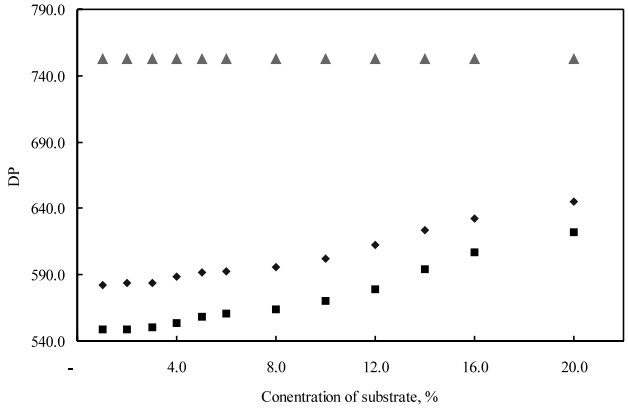

Fig. 4. Effect of substrate concentration on $\overline{DP}$ of the cellulose. Symbols: ■, endoglucanase; ◆, cellulase;▲, control. Dosage of the enzymes: 2 mg/g pulp (dw). Reaction time: 2 h.

Table 3
Influence of enzymatic treatment on the alkaline solubility of the cellulose [a]

Enzyme	Crystalline index (%)	Viscosity (cm^3/g)	Viscosity decrease (cm^3/g)	Alkaline solubility (%)
Control	71.63	535		21
Endoglucanase	79.59	402	132	59.6
Cellulase	77.04	425	112	50.1
Cellobiohydrolase	no direct	500	37	30.2

[a] The treatments were carried out at 3% substrate concentration for 2 h using an enzyme loading of 2 mg protein/g substrate.

cm^{-1}.[8] The band near 1160 cm^{-1} is representative of the antisymmetric bridge stretching of the C–O–C groups in cellulose and hemicellulose, and the band near 1318 cm^{-1} could be ascribed to CH_2 wagging vibrations in cellulose and hemicellulose. The 895 cm^{-1} band, which is characteristic for β linkages, especially in hemicelluloses,[9] was reduced after enzyme treatment. There was also a substantial reduction in the band at 1635–1640 cm^{-1}, which has been attributed to C=O groups.

The bands near 3400 cm^{-1} are representative of OH vibrations. The band of OH vibrations is wide and moves to lower wavenumber (3370 cm^{-1}) because of intermolecular and intramolecular hydrogen bonds. After the enzymatic hydrolysis, the bands near 3400 cm^{-1} became narrow and move to high wavenumber (3400 cm^{-1}). There is good evidence that a portion of the hydrogen bonds is broken in the enzymatic hydrolysis. Such effect leads to an improvement on the alkaline solubility of the pulp while the crystalline index increases. Fig. 8 also shows the effect of the reaction time on hydrogen bonding. The hydrogen bonds vary very little at the beginning of the enzymatic hydrolysis, but after reaction for some time, the hydrogen bonds became fewer in number. In short, hydrolysis with either endoglucanase or cellulase has broken, to some extent at least, the hydrogen bonds.

3. Experimental

Materials

Dissolving pulp. Softwood dissolving pulp was Alicell-Super (Western Pulp, Ltd., Vancouver, BC, Canada) with high α-cellulose content. The characteristics of the pulp are presented in Table 5. Prior to enzymatic treatment the pulp was disintegrated in an apparatus by agitating at 3000 rpm for ≈ 1 h.

Enzymes.—Multicomponent cellulase was derived from *Humicola insolens*. The monocomponent endoglucanase was prepared and purified from a genetically modified *Aspergillus* microorganism. The monocomponent cellobiohydrolase was prepared and purified from *H. insolens*. The activity and treatment conditions of the enzymes were studied.

Analysis and treatment

Enzymic assays. The activities of the enzymes are given as international unites (IU), in which, one unit of activity is defined as the amount of enzyme required to liberate 1 μmol of product per min. The endoglucanase (CMCase), xylanase, mannanase, and filter paper activities were measured on carboxymethylcellulose (1% CMC, Sigma), xylan (1% birchwood xylan, Sigma), galactomannan (1% local bean, Sigma) and filter paper (No. 1 Whatman), respectively, using methods described previously.[10] The amount of reducing sugar released was estimated by the dinitrosalicylic acid method[11] using glucose as the standard.

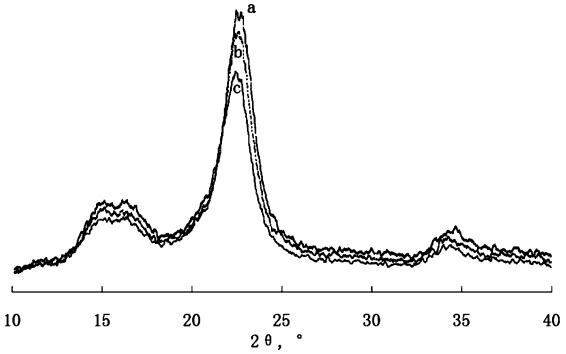

Fig. 5. Effect of endoglucanase on cellulose crystallinity. Dosage of the enzyme: 2 mg/g pulp (dw). Substrate concentration: 3%. Reaction time: (a) 15 h; (b) 2 h; (c) control.

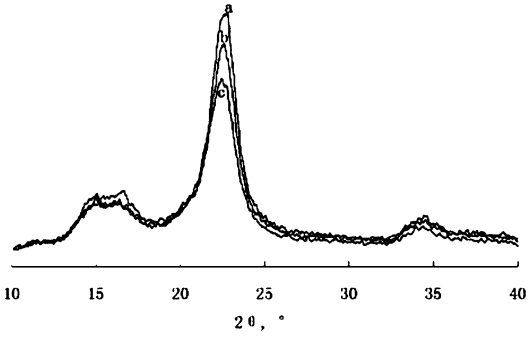

Fig. 6. Effect of cellulase on cellulose crystallinity. Dosage of the enzyme: 2 mg/g pulp (dw). Substrate concentration: 3%. Reaction time: (a) 15 h; (b) 2 h; (c) control.

Table 4
The crystalline index of cellulose with enzymatic treatment

Enzyme	Cellulase			Endoglucanase			Control
Time (h)	2	7	15	2	12	15	0
Crystalline index (%)	79.59	81.22	82.12	77.04	77.40	79.02	75.83
ACS (Å)	63.7	63.9	63.9	63.7	63.8	64.0	63.6

pH and temperature activity profiles.—Multicomponent cellulase and endoglucanase activity profiles were obtained using CMC. The buffers were prepared as either phosphate or acetic acid–sodium acetate buffers.[12]

Enzymatic treatment of pulp.—The enzyme concentration in the pulp treatments was based on the mass of oven-dry pulp. All enzyme treatments were incubated at 50 °C under continuous agitation at 175 rpm, at a respective pulp consistency in 50 mM phosphate buffer (pH 7.0). The enzyme reactions were terminated by boiling for 15 min. Control pulp treatments were run in parallel under similar conditions except that no enzyme was added.

Analyses of pulp.—The viscosity and characteristics of the pulp were measured according to the related methods of ISO/TC6 (5351/1, 1762,535).

Determination of average degree of polymerization ($\overline{DP}$).—$\overline{DP}$s were measured viscosimetrically in CuEn (copper ethylenediamine solution), and the obtained intrinsic viscosities so obtained were converted into the respective values of DP by Eq. (1):[13]

$$\overline{DP}^{0.905} = 0.75[\eta_{CuEn}\ (cm^3/g)] \quad (1)$$

Alkaline solubility of the pulp.—The alkaline solubilities of the reference and enzyme-treated pulps were determined by a modification of the method.[14] The pulps were dissolved under vigorous stirring (1500 rpm) in 9% (wt) NaOH at −10 °C at 4% pulp consistency for 1 h. Thereafter the solutions were centrifuged at 1000*g* for 10 min. The dissolved part was discarded, and the undissolved part was washed with 9% NaOH. The washed cellulose was treated with 3% HCl and then washed with deionized water in a ceramic sinter. The solubility (S) of cellulose was calculated from the following equation:

$$S = (W - W_U)/W \times 100\%$$

where W, weight (dw) of enzymatically treated or control cellulose sample; W_U, weight (dw) of undissolved cellulose.

Crystalline and apparent crystal size of cellulose.—The supramolecular structure of the cellulose (cellulose crystalline) was determined under both control and enzymatic treatment by X-ray diffraction. After drying, two slices of pulp were prepared by pressing from each sample and testing for reproducibility. Samples were scanned and recorded using the D/max RB X-ray diffractometer with a D-5000 rotating anode X-ray generator from 10° to 40° of 2θ (Bragg angle), using Cu Kα irradiation at 30 mA and 40 kV. The crystalline indices of the cellulose samples were calculated from the X-ray diffraction patterns by the following equation.[15]

$$X_c = (I_{002} - I_{am})/I_{002} \times 100\%$$

where I_{002}, the peak intensity of 002 crystal plane ($2\theta = 22.6°$); I_{am}, the peak intensity of amorphous phases ($2\theta = 19.0°$).

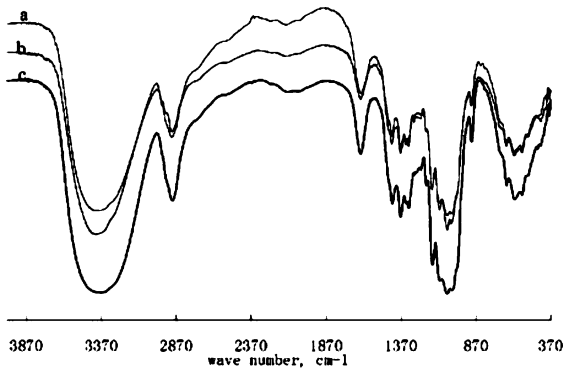

Fig. 7. FTIR of cellulose treated with endoglucanase. Dosage of the enzyme: 2 mg/g pulp (dw). Substrate concentration: 3%. Reaction time: (a) 15 h; (b) 2 h; (c) control.

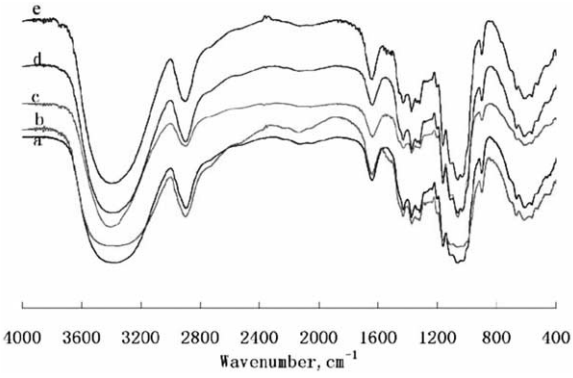

Fig. 8. FTIR of cellulose treated with cellulase. Dosage of the enzyme: 2 mg/g pulp (dw). Substrate concentration: 3%. Reaction time: (a) control; (b) 1 h; (c) 2 h; (d) 7 h; (e) 15 h.

Table 5
Characteristics of the pulp

Viscosity (cm^3/g)	α-Cellulose (%)	Ash content (%)	Fe (ppm)	Alkali absorption velocity (mm/5 min)	Swelling capacity (%)
537	92.8	0.09	7	35×33	408

Apparent crystal size (ACS) was estimated through use of the Scherrer equation:[16]

$$ACS = 0.89 \times \lambda/\beta \cos\theta$$

where λ, the wavelength of the incident X-ray (1.5418 Å); θ, the diffraction angle corresponding to 002 crystal plane; β, the half-value width of the peak angle of 002 crystal plane.

FTIR spectroscopy.—Fourier-transform infrared spectra (FTIR) were obtained with a Perkin–Elmer 2000 FTIR spectrometer. The wave number range scanned was 4000–350 cm^{-1}. After milling, the powdered samples were compacted into KBr disks and analyzed.

4. Conclusions

The three enzymes—multicomponent cellulases, purified endoglucanases and cellobiohydrolases—were assayed and allowed to modify the cellulosic material. The effects of enzymatic hydrolysis on changes in the viscosity-average degree of polymerization ($\overline{DP}$), solubility towards aqueous alkali solution, and the variety of the crystalline and hydrogen bonds as analyzed by X-ray and FTIR spectra were noted. The following conclusions can be summarized briefly from this study: (1) Both endoglucanase and cellulase decrease the $\overline{DP}$ of pulp at low enzyme dosages, but cellobiohydrolase has a less pronounced effect on the $\overline{DP}$. (2) The extent of $\overline{DP}$ drop become smaller when substrate concentration is higher than 8%. (3) Endoglucanase is the most efficient of the three enzymes in improving the alkaline solubility, and cellobiohydrolase has a less pronounced effect on $\overline{DP}$ or the alkaline solubility. (4) The crystallinity of partially crystalline cellulose increases, and the crystallite width decreases, while the crystallite length remains practically constant during enzymatic hydrolysis, as determined by X-ray analysis. Cellulose that has undergone enzymatic treatment is crystallographically cellulose I. (5) The hydrogen bonds of the OH groups were partly broken in the enzyme-hydrolyzed cellulose as estimated by FTIR spectroscopy.

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