

Differential Thermal and Thermogravimetric Analyses of Bound Water Content in Cellulosic Substrates and Its Significance During Cellulose Hydrolysis by Alkaline Active Fungal Cellulases

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Abstract

Various cellulosic substrates were examined for bound water content by differential thermal analysis (DTA) and thermogravimetry (TG). Samples were heated in the range of 30–100°C at a rate of 3°C/min. DTA vaporization curves for different cellulose samples indicated that the bound water (W_b) was vaporized at higher temperature than free water (W_f) at the surface. Weight loss was observed in two stages, corresponding to W_f and W_b in TG curves. The bound water content was dependent on the degree of crystallinity of cellulose. Among different cellulosic substrates, Walseth cellulose showed the highest bound water content, and it also was found to be the least crystalline. The alkaline-active, alkali-stable cellulase was obtained from the alkalotolerant *Fusarium* sp. The substrate specificity and viscometric characteristics confirmed the enzyme to be an endoglucanase. The W_b content of Walseth cellulose was lowered during the enzymatic hydrolysis. The possible application of bound water analysis in understanding the hydrolysis of cellulosic substrates of different crystallinity is discussed.

Index Entries: Cellulose; endoglucanase; differential thermal analysis; thermogravimetry; bound water; vaporization.

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Introduction

Cellulases that are active and stable in an alkaline pH range are in demand because of their potential ecofriendly applications in textile (1,2), detergent (3,4), and pulp and paper industries (5). However, alkaline-active cellulases are rare among fungi (6). Recently, we reported on an alkalo-tolerant fungus, *Fusarium* sp., that produces alkaline-active cellulases useful in deinking operations during recycling of mixed office wastepaper (MOW) (7). For successful application of cellulases in these processes, an understanding of the structural and functional relationship of enzyme to its substrate becomes important. Cellulose hydrolysis by microbial cellulases is often limited by the degree of crystallinity of the substrates (8). However, the amorphous regions of cellulose can form hydrogen bonds with water molecules owing to hydroxyl groups attached to the glucopyranose ring (9,10). The amount of bound water in biopolymers can be estimated by differential scanning calorimetry (DSC) (11–13), nuclear magnetic resonance (14), viscoelastic measurements (15), and other spectroscopic techniques (16). Various researchers have applied bound water analysis to characterize pulp samples (17–19) and other industrially important cellulose derivatives (20). The information obtained by thermal analysis of bound water content of different cellulosic substrates such as Avicel and Walseth cellulose is limited.

In the present study, various cellulosic substrates were studied for bound water content by differential thermal analysis (DTA) and thermogravimetry (TG). In spite of recent advances in X-ray crystallography (21), the amorphous structure of cellulose in both natural and regenerated cellulose is not completely understood (22). The alkaline-active fungal cellulase was produced from the alkalotolerant fungus *Fusarium* sp. The changes in the amount of bound water during enzymatic hydrolysis were monitored to determine the changes in amorphous regions. The possible application of bound water analysis in understanding the hydrolysis of cellulosic substrates of different crystallinity is discussed.

Materials and Methods

Chemicals

The following materials were purchased from the suppliers indicated: Avicel PH 101, 4-hydroxybenzoic acid hydrazide (Fluka AG, Buchs, Switzerland); Cellulose-123 (Carl Schleicher & Schuell, Germany); Solka floc BW 100 (pure fibrous spruce wood pulp) (Brown, Dassel, Germany); absorbent cotton (Bengal Chemicals and Pharmaceuticals Work, Kolkata, India); Bio-Gel P-100, Biolytes (electrolyte carrier, pH 3.0–10.0) (Bio-Rad, Richmond, CA); sodium salt of carboxymethylcellulose (CMC) (medium viscosity) (Sigma, St. Louis, MO).

Phosphoric acid-swollen cellulose (Walseth cellulose) was prepared as described by Wood (23). MOW pulp was prepared as described previ-

ously (7). All the buffer salts and microbial media components were procured from the standard commercial sources and were of the highest quality available.

Microorganism, Culture Conditions, and Enzyme Production

The alkalotolerant fungal strain was screened in our laboratory and identified as *Fusarium* sp. It is maintained by periodic transfer on potato dextrose agar (0.5% dextrose) slants at 30°C. The fungus was grown in M-1 medium (24). The pH of the medium was adjusted to 7.0 after autoclaving by using separately sterilized 1M Na₂CO₃. The enzyme production was carried out in a 250-mL Erlenmeyer flask as described previously by us (25). Samples were withdrawn at regular intervals. The mycelium was removed by centrifuging at 5000g at 4°C for 10 min to obtain a clear crude broth. This preparation was used for measurement of enzyme activities. The results given here are the mean of at least duplicate experiments.

Protein Content

Protein concentration was measured using Lowry's method (26). Bovine serum albumin was used as the standard.

Purification of Endoglucanase A

After harvesting mycelia by centrifuging at 5000g for 10 min, the supernatant was used as crude cellulase extract. This crude extract was concentrated using a freeze drier. A 5.8-mg freeze-dried sample containing 5.0 mg of protein was dissolved in 2 mL of 0.05 M sodium phosphate buffer, pH 7.0, and applied onto a Bio-Gel P100 column (160 × 1 cm) equilibrated and eluted with the same buffer. Fractions, each 2.5 mL, were collected at a flow rate of 10 mL/h. Two separate fractions containing high-specific-activity carboxymethylcellulase (CMCase), namely Endoglucanase A (Endo A) and Endoglucanase B (Endo B), were collected separately.

The Endo A fraction obtained from the gel filtration column was concentrated using an Amicon ultrafiltration membrane (PM 10) unit. Finally, a sample of the concentrated enzyme was purified using a miniscale isoelectric focusing (IEF) unit as described by Sathivel et al. (27). IEF was carried out using Biolyte carrier electrolytes in the pH range of 3.0–10.0 at 400 V for 6–8 h. After electrofocusing was completed, each fraction of 0.5 mL was removed from the column, and the samples were analyzed for CMCase activity, pH, and protein content.

Enzyme Assays and Substrate Specificity Studies

The activity of purified Endo A (1,4-β-D-glucan-4-glucanohydrolase; EC 3.2.1.4) on 1% CMC solution, filter paper (FPase), and other cellulosic substrates containing β1–4 linkages was measured in terms of the release of reducing sugars by the dinitrosalicylic acid method (28). Xylanase (1,4-β-D-xylan xylanohydrolase; EC 3.2.1.8) activity was tested using a

1% solution of oat spelt xylan. β -Glucosidase (1,4- β -D-glucan glucohydrolase; EC 3.2.1.21) and β -xylosidase (1,4-D-xylan xylohydrolase; EC 3.2.1.37) activities were determined using the synthetic substrates *P*-NPG and *P*-NPX, respectively, at pH 6.0 and 50°C.

The various buffers used for enzyme activity were 50 mM citrate buffer (pH 3.0–6.0) and sodium phosphate buffer (pH 7.0–8.0). One international unit of enzyme activity is defined as the amount of enzyme that liberates 1 μ mol of glucose/min (for cellulase) or xylose/min (for xylanases) under standard assay conditions. One international unit of β -glucosidase and β -xylosidase is defined as the amount of enzyme that liberates 1 μ mol of *p*-nitrophenol/min under standard assay conditions.

Viscometric Analysis

The relationship between the change in specific viscosity (η_{sp}) and reducing sugars released from CMC solution by Endo A was determined (29). A 13-mL reaction mixture containing 1% (w/v) CMC and enzyme in 0.05M phosphate buffer, pH 7.0, was incubated at 50°C for 30 min. The decrease in flow time was recorded at 3-min intervals for 30 min, and simultaneously aliquots were removed and analyzed using the *p*-hydroxybenzoic acid hydrazide method (30).

Sample Preparation for X-Ray Diffraction and TG/DTA Studies

All cellulose samples were purified by extracting contaminants using toluene and *n*-hexane successively. To avoid the effect of particle size, the cellulose powder samples were passed through a sieve of 200-mesh size. Cotton, filter paper, and MOW pulp samples were cut to a fine powder to eliminate the effect of fiber orientation. All cellulose samples under investigation were soaked for 4 h in deionized distilled water in order to facilitate binding of water molecules to cellulose samples. The samples were dried in vacuum desiccators for 3 d to remove excess surface water. One part of these samples was dried further and used for X-ray diffraction studies, as described in detail in the next section. The other portion of each sample was used for vaporization studies as described in Vaporization Measurements by TG/DTA.

Determination of Degree of Crystallinity of Cellulose Samples

Samples were prepared as described in the previous section. X-ray diffraction of each set of samples was recorded using a Philips Analytical X-ray diffractometer (PW 1710). The wavelength of Cu radiation source was 0.1540 (nm), and spectra were obtained at 30 mA with an accelerating voltage of 40 kV. Samples were scanned on the automated diffractometer from 9 to 40° of 2θ (Bragg angle) with data acquisition taken at intervals of 0.400 s. A peak resolution program (PC-APD diffraction software) was used to calculate crystallinity index (Crl). Crl of cellulose was also calcu-

lated by the empirical method described by Segal et al. (31) using the following equation:

$$\text{Crl (\%)} = I_{002} - I_{AM}/I_{002} \times 100$$

in which I_{002} is the maximum intensity of the 002 lattice diffraction (reflection attributed to crystalline regions of the sample), and I_{AM} is the intensity of diffraction at Bragg angle $2\theta = 18^\circ$ (reflection attributed to amorphous regions of the sample). The results obtained using both methods were in good agreement.

Vaporization Measurements by TG/DTA

A Seiko TG/DTA 32 computer-controlled unit was used for bound water analysis of different cellulose samples. A small amount of distilled water was added with a microsyringe to each sample, and then the total weight of the sample was directly recorded by a microbalance fitted in the instrument with an accuracy of 0.001 mg. The cellulose samples were heated in the range of 30–100°C at a rate of 3°C/min. The amount of bound water (W_b) was calculated from thermograms by subtracting the weight loss for free water (W_f) from the total weight loss owing to water content of the sample (W_t): $W_b = W_t - W_f$.

To evaluate the effect of endoglucanase action on the bound water content of cellulose samples, 50 mg of Walseth cellulose was incubated along with 10 µg of purified Endo A. The reaction was carried out at different time intervals. The unhydrolyzed part of Walseth cellulose was separated by centrifugation. The samples were washed to remove residual enzyme and dried and used for bound water analysis. To avoid experimental error, the samples were run in triplicate and the data are given as the mean of all three sets. The values obtained are expressed as the amount of bound water in grams per gram of the cellulosic substrates.

Results and Discussion

Enzyme Purification and Properties

The crude culture filtrate obtained from submerged fermentation of alkalotolerant *Fusarium* sp. showed different extracellular cellulolytic and xylanolytic activities (25). The enzyme preparation was active in a broad pH range of 4.0–9.0, with an optimum pH of 5.0 at 60°C. The enzyme was stable in an alkaline pH range of 8.0–10.0 at 50°C. The enzyme showed maximum activity at 60°C and retained 80% of the maximum enzyme activity at 70°C (7). Microbial cellulases are generally active in the acidic to neutral pH range, and active fungal cellulase at alkaline pH conditions is rare (6,32). The alkalotolerant *Fusarium* strain reported in our work secretes a high amount of extracellular cellulases that are alkaline active and alkali stable, indicating their potential for various industrial processes such as biodeinking of MOW (7).

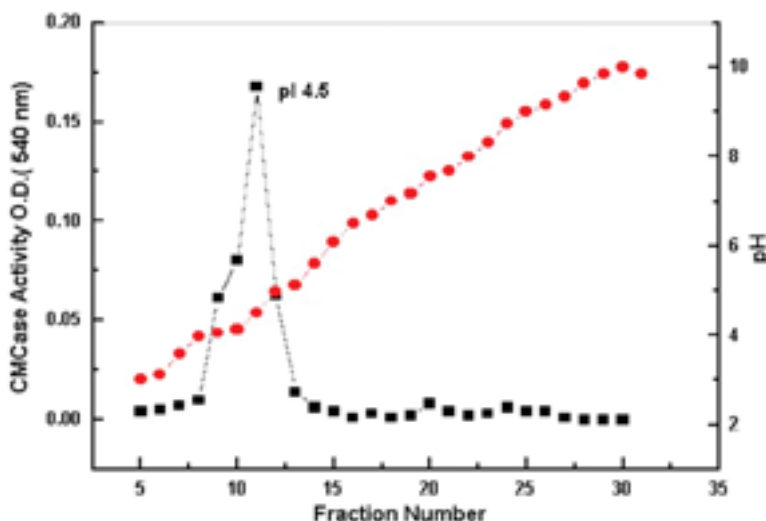


Fig. 1. Purification of Endo A using miniscale IEF unit. Electrofocusing was carried as described under Materials and Methods. The *pI* of the enzyme was found to be 4.5. (●) pH; (■) CMCase activity (OD 540).

The crude concentrated protein was fractionated on a Bio-Gel P 100 column. The elution profile demonstrated two active fractions, Endo A and Endo B, showing CMCase activities. The fractions Endo A and Endo B showed a 2.3- and 4.6-fold increase in specific activities, respectively. The Endo A fraction obtained from the gel filtration column was subjected to further purification by IEF (Fig. 1). The homogeneity of the Endo A was ascertained by observing the single protein band in polyacrylamide gel. The purified endoglucanase showed a 13-fold higher specific activity than that of crude broth.

Substrate Specificity

Purified Endo A was used to study its substrate specificity on different cellulosic substrates and D-glucans of various linkages (Table 1). Endo A did not hydrolyze laminarin consisting of β -(1 \rightarrow 3) and β -(1 \rightarrow 6) glycosidic linkages. CM pachyman with β -(1 \rightarrow 3) and sophorose with β -(1 \rightarrow 2) linkages were not hydrolyzed in the presence of the enzyme. However, CMC, Walseth cellulose, and lichenan were hydrolyzed readily by Endo A. This indicated that the substrate specificity of Endo A is restricted to only β 1-4 linkages. The enzyme did not show β -D-xylanase, β -xylosidase, and β -glucosidase activity.

Viscometric Analysis

The decrease in specific viscosity (η_{sp}) of CMC solution owing to the hydrolytic action of Endo A is shown in Fig. 2. Suitably diluted enzyme

Table 1
Substrate Specificity of Endo A

Substrate	Component	Linkage of D-glycosyl group	Reducing sugars (μg) ^a
CMC	D-Glc→D-Glc	β -(1→4)	912
Walseth cellulose	D-Glc→D-Glc	β -(1→4)	16.4
Filter paper	D-Glc→D-Glc	β -(1→4)	9.6
Lichenan	D-Glc→D-Glc	β -(1→3) and β -(1→4)	22.5
Laminaran	D-Glc→D-Glc	β -(1→3) and β -(1→6)	ND
Sophorose	D-Glc→D-Glc	β -(1→2)	ND
CM pachyman	D-Glc→D-Glc	β -(1→3)	ND
Xylan	D-Xyl→D-Xyl	β -(1→4)	ND
Dextran	D-Glc→D-Glc	α -(1→6)	ND

^aND, not determined.

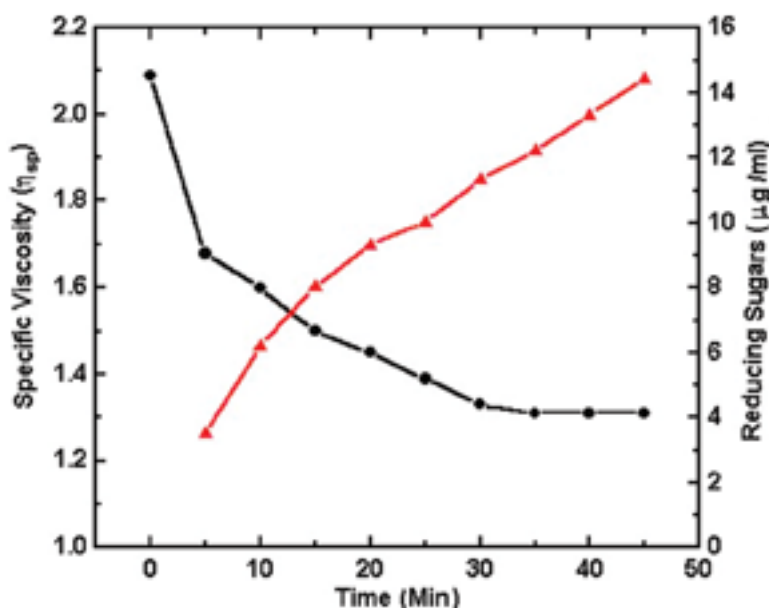


Fig. 2. Randomness of CMC hydrolysis by Endo A. The time course of CMC (1.0%) hydrolysis by 0.5 μg of purified Endo A at pH 7.0 at 50°C shows the lowering of specific viscosity (η_{sp}) of 1% CMC solution (●) with the subsequent release of reducing sugars (▲) owing to random action of Endo A.

samples were used to determine the randomness of their attack on CMC. The specific viscosity of 1% CMC solution was rapidly reduced as a function of time with the subsequent slow release of reducing sugars, indicating random cleavage of the substrate. Thus, the enzyme appeared to be typical endoglucanase, similar to some of the other endoglucanases reported from other microorganisms (33–36).

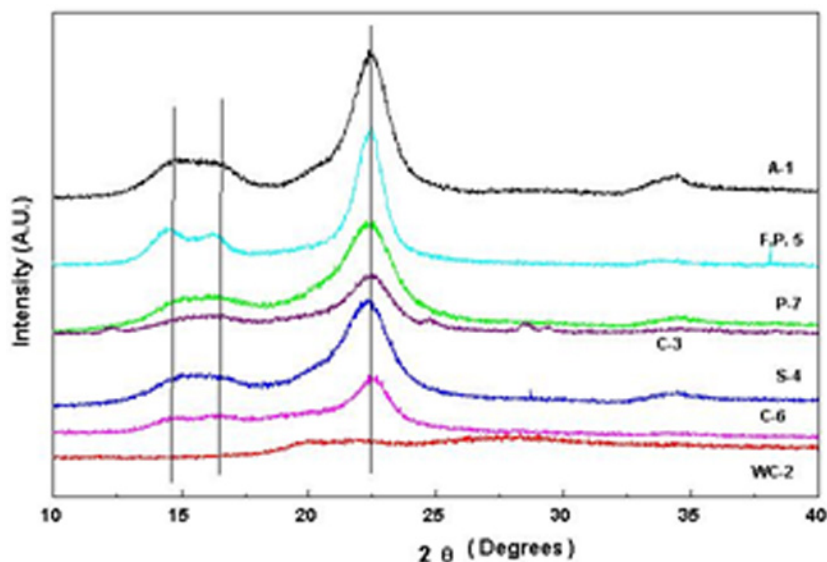


Fig. 3. X-ray diffraction patterns of different cellulose samples: A-1, Avicel PH 101; F.P.5, filter paper (Whatman no. 1); P-7, MOW pulp; C-3, Cellulose-123; S-4, Solka floc BW 100; C-6, dewaxed cotton linters; WC-2, Walseth cellulose.

Crystallinity of Cellulose Samples

X-ray diffraction patterns of different cellulose samples are presented in Fig. 3. In the case of microcrystalline substrates, filter paper, and Avicel PH 101, diffraction peaks of (101), (10 $\bar{1}$), and (002) appeared at 2θ of 14, 16, and 23°, respectively. In the case of all the cellulose samples, the highest intensity peak occurred at the (002) plane that appeared in the range of 22–24° of 2θ . However, in the case of Walseth cellulose, cotton, MOW pulp, Solka floc, and cellulose powder, there was shift of 2θ values at the (001) plane. The C_{rl} values for Avicel PH-101, Walseth cellulose, Cellulose powder 123, Solka floc BW 100, filter paper (Whatman no. 1), dewaxed cotton linters, and MOW pulp were found to be 84, 31, 80, 83, 91, 75, and 83%, respectively.

Analysis of Bound Water Content Using TG/DTA Studies

The interaction of water with the cellulose polymer markedly influences the physicochemical properties of both cellulose and water (37). It has been observed that in polymers such as cellulose, melting and crystallization temperatures of bound water are higher and lower, respectively, than those of free water (38–40). Because the heat of vaporization of water molecules is large, it can be used to determine traces of water amounts in the samples. DSC on cotton and regenerated cellulose samples suggested that the vaporization of bound water occurs at a much higher temperature than that of free water (41). In the present work, a thermogravimetric method

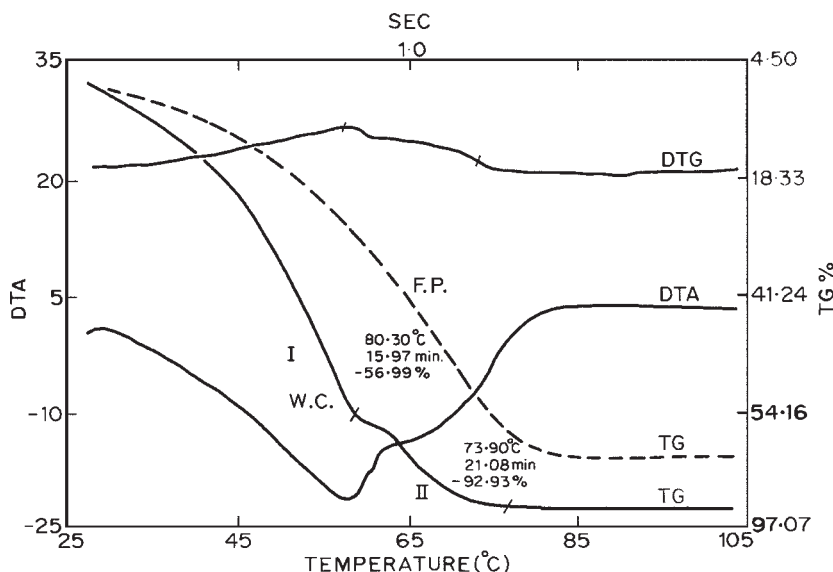


Fig. 4. TG/DTA/DTG thermograms for vaporization of water in cellulose samples. TG = (curves for thermogravimetric data, including W.C. (—) for Walseth cellulose and F.P. (---) for filter paper. DTA = the curve for DTA of the same Walseth cellulose sample. DTG = the curve for differential thermogravimetric analysis of the same sample. I and II represent the weight loss that occurred owing to vaporization of free water and bound water, respectively.

was used to evaluate the bound water content of different cellulose samples (Fig. 4). Walseth cellulose was heated at a rate of $3^{\circ}\text{C}/\text{min}$ in a range of 30 – 110°C . In this case, weight loss was observed in two stages, I and II, corresponding to W_f and W_b , respectively. The evaporation of free water at the surface occurs up to 60°C , and at about 80°C for tightly bound water. This observation was confirmed by the differential thermogravimetric (DTG) curve in Fig. 4. The values are expressed in grams per gram of cellulosic substrates. The data for other cellulosic substrates are presented in Table 2. Because bound water content depends on the molecular architecture of cellulose chains and its capacity to form hydrogen bonds with water molecules, the amount of bound water varied in different samples. DTA curves for Walseth cellulose and dewaxed cotton linters suggested that vaporization of W_b occurs at about 80°C , the highest temperature in all the samples studied. This indicated that W_b is tightly bound to cellulose chains in these substrates compared with other substrates. The highest amount of bound water (0.92 g/g of substrate) was observed in Walseth cellulose (phosphoric acid-swollen cellulose), whereas such bound water was not detected in the case of Whatman filter paper (Fig. 4, F.P. curve).

The bound water content was found to be dependent on the degree of crystallinity of cellulose; the higher the crystallinity, the lower the bound water content (Table 2). It is well known that the moisture content of cellulose depends on the molecular structure of the amorphous part of cellulose

Table 2
Effect of Crl on Bound Water Content of Different Cellulosic Substrates

Sample	Substrate	Crl (%)	Bound water (g/g of substrate) ^a
A-1	Avicel PH 101	84	0.068
W.C.-2	Walseth cellulose	31	0.920
C-3	Cellulose powder (123)	80	0.096
S-4	Solka floc	83	0.045
F.P.-5	Filter paper (no. 1)	91	ND
C-6	Cotton	75	0.276
P-7	MOW pulp	84	1.466

^aND, not determined.

(42,43). Water molecules can only diffuse through amorphous regions of cellulose samples (44). Nakamura et al. (40) described the relation between water molecules and crystallinity of various cellulose samples. According to their studies, only amorphous regions in cellulose molecules can form hydrogen bonds with water molecules and can be regarded as the site of water molecule adsorption (40). In the present work, among the different cellulosic substrates, Walseth cellulose and MOW pulp showed the highest bound water. Walseth cellulose is regenerated cellulose, and ortho-phosphoric acid treatment for its preparation resulted in a decrease in the crystallinity of cellulose. The much higher amount of bound water was seen in MOW pulp despite high crystallinity of the samples. Different fillers such as calcium carbonates and starch are used to improve brightness and strength properties of high-quality writing-grade paper. MOW pulp is produced by repulping such papers. Thus, the higher amounts of bound water content in these samples may be attributed to the capacity of these fillers to bind water molecules tightly.

The amorphous structure of natural cellulose is not completely random, but molecular chains are arranged unidirectionally along the cellulose fiber axis. It is known that the molecular rearrangement in the amorphous region of natural cellulose occurs reversibly by absorption and desorption of water (15). The molecular chains in these regions take an ordered form when a small amount of water is added. Molecular chains in the intermediate region reversibly change from a regular structure to a random molecular arrangement in the presence of a trace amount of water. Further, the higher-order structure of amorphous cellulose chains converts into a more random form by the loss of a small amount of bound water (41).

Cellulose hydrolysis by microbial cellulases is often limited by the degree of crystallinity of the substrates. Hydrogen bonds between cellulose microfibrils in crystalline regions are so strong that no further hydrogen bonds with water molecules can be formed. However, the amorphous regions of the cellulose can form hydrogen bonds with water molecules, and hence, the water molecules in these regions remain as bound water.

Table 3
Time-Dependent Hydrolysis of Walseth Cellulose
and Its Effect on Bound Water Content

Sample	Time (min)	Bound water (g/g of cellulose)	Reducing sugars formed (μg)
1	0	1.930	2.3
2	30	1.698	20
3	60	1.559	50
4	120	1.186	82
5	240	1.032	110

The Endo A reported in our work is an endo-acting cellulase that specifically acts at these amorphous regions. The changes in the amount of bound water during hydrolysis were monitored to determine the alteration in the amorphous regions. The data presented in Table 3 indicate that the W_b content of Walseth cellulose was lowered during the enzymatic hydrolysis as a function of time. Similar studies using DSC to estimate the bound water content in cotton cloths are useful in establishing new mechanisms of detergency. The bacterial endoglucanase component added to detergents acts at extensively soiled amorphous regions of cotton cloth and improvises brightness of soiled cloth (45). Further studies to evaluate the effect of enzyme dose and other conditions on bound water content of different cellulose samples are under way. Thus, DTA and thermogravimetric methods to assess bound water in cellulose can be valuable in understanding the enzymatic hydrolysis of cellulosic substrates of different crystallinity.

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