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Ultraviolet Difference Spectroscopic Study on the Interactions of Cellulase from *Trichoderma reesei* with Cellodextrins

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ABSTRACT

The formation of cellodextrin–cellobiohydrolase complex was studied by ultraviolet difference spectroscopy. Upon the binding of cellodextrins (G_7-G_3), cellobiohydrolase (EC 3.2.1.91) purified from *Trichoderma reesei* produced difference spectra having maxima at 289–293 nm and 283–286 nm. These spectra are consistent with prior observations reported for lysozyme and amylase. In this case, water soluble cellulose oligomers (i.e., cellodextrins) are shown to interact with tryptophan residue(s) on cellobiohydrolase. The difference spectral maxima observed at acidic or alkaline pH were shifted. This was accompanied by a marked decrease of binding ability of cellobiohydrolase for cellodextrins. The standard free energy change for the association of cellodextrins to the cellobiohydrolase was an order of 4 kcal/gmol. The association constant of enzyme for substrate decreases by 15–20% as temperature increases from 20 to 48°C. At 25°C, the dissociation constants for the enzyme with respect to cellohexose and cellotriose were estimated to be 1.19 and 1.37 m*M*, respectively. A decrease in dissociation constants was observed with an increase in the number of glucosyl units from 3 to 6. This suggests that there may be six or more subsites in the active center of cellobiohydrolase.

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Index Entries: Cellulose; cellulase; cellobiohydrolase; cellodextrin(s); difference spectroscopy; ultrafiltration; subsite; degree of polymerization.

INTRODUCTION

Cellulose is an important renewable carbohydrate for the generation of fermentable sugars. Many researchers have been undertaken to hydrolyze cellulose to glucose for the production of liquid fuels and chemicals currently derived from petroleum (*1–3*). The economics of cellulose conversion are a strong function of substrate cost and enzyme cost (*4*). Hence, studies of cellulase action are important to reduce substrate and enzyme costs.

Cellobiohydrolase (C_1), the major component of the *Trichoderma reesei* cellulase system, has been intensively studied. It can attack the nonreducing end of cellulose and exhibit synergism with endoglucanase (C_x) on cellulose (*5–7*). Although many efforts have been put on the enzymatic hydrolysis of cellulose, the exact mechanism is still unsolved. It is believed that information on the residue or residues participating in the substrate binding might help to elucidate the basic features of catalytic process of cellulase.

Difference spectroscopy is a useful technique for studying enzyme–substrate interaction. Binding of a substrate to an enzyme may cause a change in the environment of any chromophores that are part of the active site or are close to the active site. For example, Hayashi et al. (*8*) added glycol chitin, which does not absorb in the ultraviolet range, to lysozyme solution and produced a red shift in the adsorption of an indole chromophore. This technique is applied to study the interaction(s) between cellobiohydrolase and cellodextrins. Through this study it might shed some light on the binding property of cellobiohydrolase with its substrates.

MATERIALS AND METHODS

Preparation of Cellulase

Trichoderma reesei (QM9414, ATCC 26921) was used for cellulase production. The growth medium for cellulase production was similar to that developed by Mandels and Reese (*9*). Batch cultivation was carried out in a 7-L fermentor for 6 d. Ammonium hydroxide addition supplied sufficient nitrogen source while maintaining the pH at 4.5, and the temperature was controlled at 30°C. After fermentation, Avicel® (10 g/L) was suspended in the culture filtrate and the filtrate was stirred at room temperature for 1

h. Then, Avicel® was settled by gravity. The treated Avicel® was washed with 20 m*M* Tris-hydrochloride buffer (pH 7.7). The enzyme was eluted from the Avicel® with 1% solution of triethylamine, and the eluent was immediately neutralized with 10% acetic acid. Protease inhibitor, phenylmethylsulfonylfluoride (PMSF) 23 m*M* (Sigma Co.), was added to enzyme solution. The enzyme solution was concentrated by ultrafiltration (PM-10 membrane, Amicon Co.) before freeze-drying.

Determination of CMCase Activity

Carboxymethyl cellulose hydrolysis activity (CMCase) of cellulase solution was determined by the generation of reducing sugar from low viscosity CMC (Sigma Co.). Sodium acetate buffer 0.49 mL (pH 4.8) containing 0.5% CMC was incubated with 10 μL of enzyme solution at 50°C for 20 min. This was followed by adding 1.5 mL dinitrosalicylic acid reagent (DNS) to stop the reaction. The solution was heated in boiling water for 10 min and cooled to room temperature. The absorbance was measured at 540 nm to determine the reducing sugar content (*10*).

Determination of FP Activity

Filter paper activity was determined by placing 0.5 mL enzyme solution and 0.5 mL of 0.05 *M* sodium acetate buffer (pH 4.8) in a test tube. Fifty milligrams of Whatman #1 filter paper were added and incubated at 50°C for 1 h. Three mL of DNS reagent was added to stop the reaction. The tube was heated in boiling water for 10 min. After cooling to room temperature, the absorbance at 540 nm was measured to determine the reducing sugar content (*10*).

Determination of Protein Concentration

Protein concentration was determined by the method of Lowry et al. (*11*) using bovine albumin (Sigma Co.) as the standard.

Sodium Dodecyl Sulfate (SDS) Polyacrylamide Gel Electrophoresis

Electrophoresis was performed on 12% polyacrylamide slab gel using the buffer system of Laemmli (*12*) with 4.5% stacking gel. Sample was diluted five times with sample buffer, consisting of 62.5 m*M* Tris-HCl pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) 2-β-mercaptoethanol and 0.125% (w/v) Bromophenol blue and subsequently heated at 95°C for 4 min. Gels were stained in 0.1% Coomassie blue R-250 for ½ h for protein detection. Phosphorylase b (130,000), bovine serum albumin (75,000), ovalbumin (50,000), carbonic anhydrase (39,000), soybean trypsin inhibitor (27,000) and lysozyme (17,000) were used for calibration (Bio-Rad Lab.).

Purification of Cellulase

A high-performance anion-exchange column (AX-300, Synchrom Inc.) was employed for cellulase purification. One mL (10 mg/mL) of cellulase was injected. The column was eluted by 20 m*M* phosphate buffer (pH 6.2) for 10 min, followed by a gradient (0 to 1 *M* NaCl) in 20 m*M* phosphate buffer. Protein concentration was determined at 280 nm. All chromatography experiments were carried out at room temperature and all buffers contained 0.01% NaN_3 as the preserving agent. Fractions were collected and desalted for future use. Flowrate was 1 mL/min. The result is shown in Fig. 1.

The CMCase activity and filter paper (FP) activity of these fractions were determined and shown in Table 1. Fractions I, II, and III show high CMCase activity but low FP activity. These fractions consisted of mainly the endoglucanase. Fractions IV and V, containing predominantly FP activity, but low CMCase activity, were cellobiohydrolase.

Avicel® column (2.5×10 cm) was used for further purification of fraction V obtained from the anion-exchange column. The Avicel® column was washed with 500 mL of 0.1 *M* sodium phosphate buffer pH 6.0 and then eluted with a pH gradient in 0.1 *M* sodium phosphate buffer from pH 6.0 to pH 12.0. Flowrate was 2 mL/min. The resulting chromatogram is shown in Fig. 2. Fraction V-B, showing single band on SDS-gel electrophoresis (Fig. 3) and releasing cellobiose as the only product during incubation with Avicel® cellulose, was confirmed to be cellobiohydrolase. The molecular weight of fraction V-B is estimated to be 64,000 by the SDS-electrophoresis method.

Chemical Modification of Enzyme by *N*-Bromosuccinimide

The procedure used for the modification of cellobiohydrolase by *N*-bromosuccinimide (Aldrich Co.) was from Patchornik et al. (*13*). Aliquots of *N*-bromosuccinimide (5.5 m*M*, 10 μL) were mixed with cellobiohydrolase (7.5 μ*M*, 0.5 mL) in 10 m*M* sodium acetate buffer (pH 5.0) and maintained at room temperature for 2 min. After incubation, 50 μL enzyme solution was withdrawn and quenched by adding 15 m*M* L-tryptophan in 50 m*M* sodium acetate (pH 6.0, 200 μL) to destroy any residual *N*-bromosuccinimide. Modified enzyme activity was determined by adding 250 μL of modified enzyme to 0.5 mL of 3 m*M* cellotetraose in 10 m*M* sodium acetate (pH 5.0) and incubated at 50°C for 55 min.

Preparation of Cellodextrin

Fifty g of Whatman CF-11 cellulose powder were dissolved in 500 mL of ice-cold fuming HCl. The solution was kept in an ice bath for 2–2½ h. After reaction, the solution was mixed with 600 mL of precooled distilled water. The resulting precipitate was filtered and the filtrate was subjected

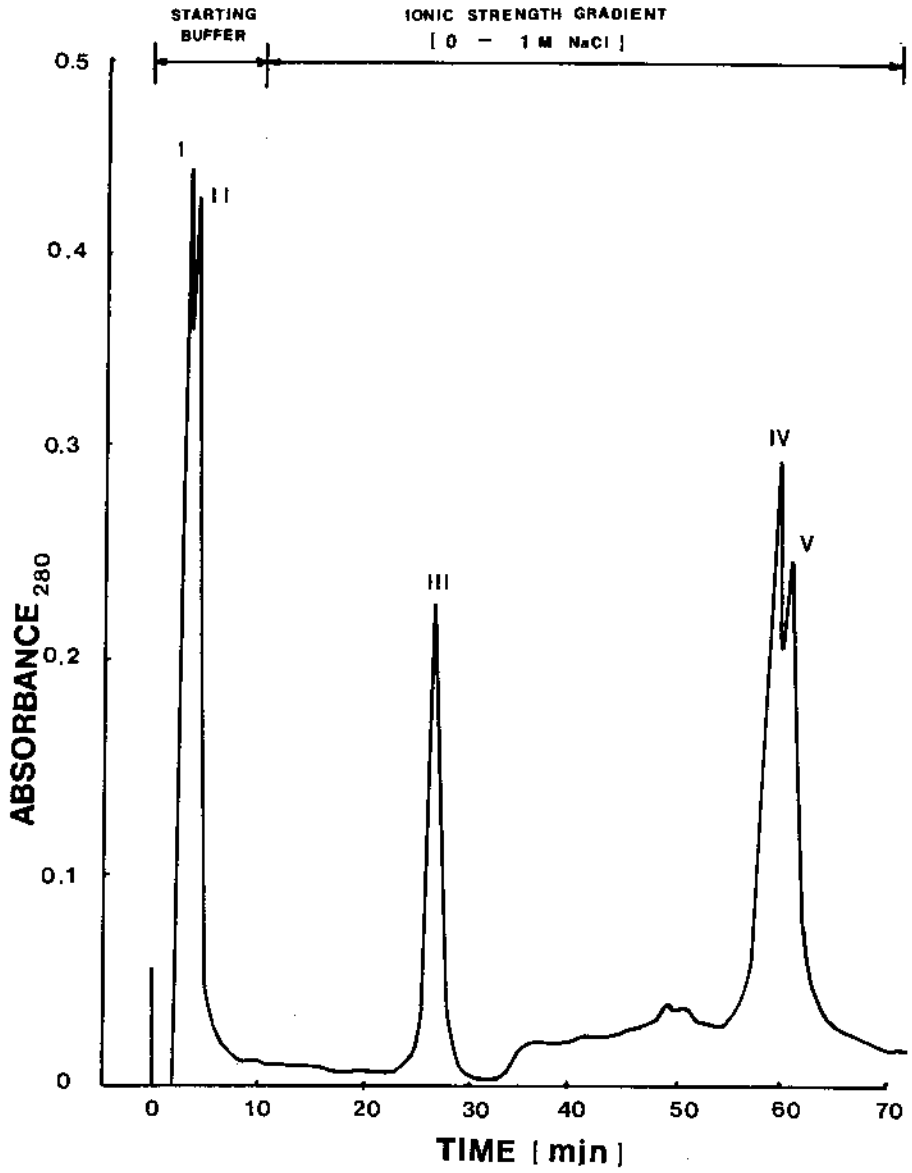

Fig. 1. Fractionation of cellulase complex from *T. reesei* (QM 9414) on AX-300 ion exchange column.

to neutralization. Sodium bicarbonate was added to neutralize the solution to pH 4.0–5.0, followed by evaporation at 45°C in a rotary evaporator to crystalize out excess NaCl. Desalting of the remaining solution was done by using YM-2 ultrafiltration membrane (Amicon Co.). Salt-free solution was freeze-dried for further purification (*14,15*). Before purification, assays were conducted by reacting cellodextrins with both 1% HCl

Table 1
Cellulase Activity of Various Fractions

Fraction	CMCase activity	Filter paper activity
	mg glucose/mg protein/min	
I	25.4	0.128
II	23.1	0.122
III	15.6	0.099
IV	11.2	0.198
V	8.5	0.164

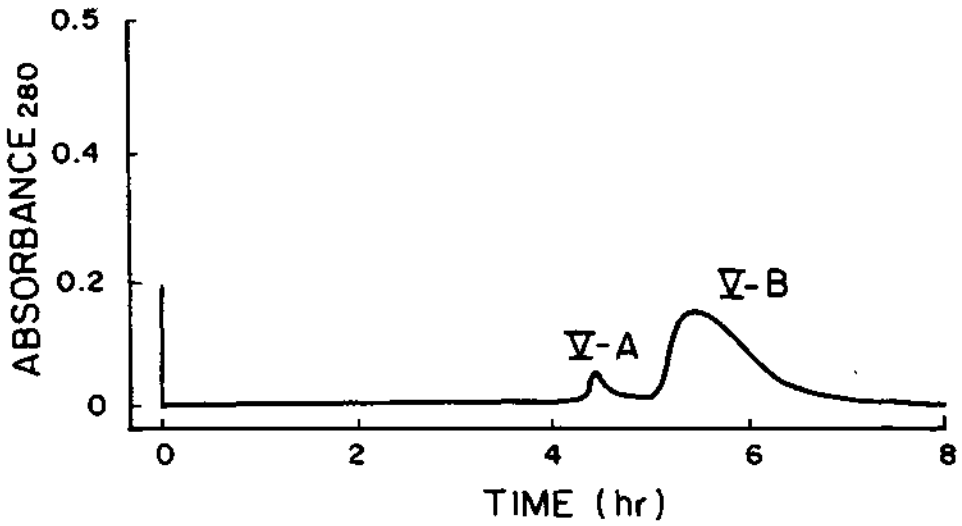

Fig. 2. Avicel®chromatography of fraction V.

at 80°C for 16 h and cellulase in 50 m*M* sodium acetate (pH 4.85) at 50°C for 12 h. Glucose measured by Aminex HPX-42A column was the only product. This is an indication of homogeneous cellodextrins.

Purification of Cellodextrin

Two water-jacketed glass columns (2.5×60 cm) were connected and packed with Bio-gel P-2 (−400 mesh) (Bio-Rad Lab.). The column was kept at constant temperature (65°C). An ISMATEC pump (Cole-Parmer Instrument Co.) was used to provide constant flow rate (0.6 mL/min) and a differential refractometer (Waters, R401) was used for detection.

Analytical determination of cellodextrin was performed by using Aminex HPX-42A column (Bio-Rad Lab.). The column, eluted by distilled water, was kept at 80°C. Flowrate was 0.5 mL/min.

The chromatogram of cellodextrins purification is shown in Fig. 4. Each fraction was collected and redetermined the purity by HPLC method.

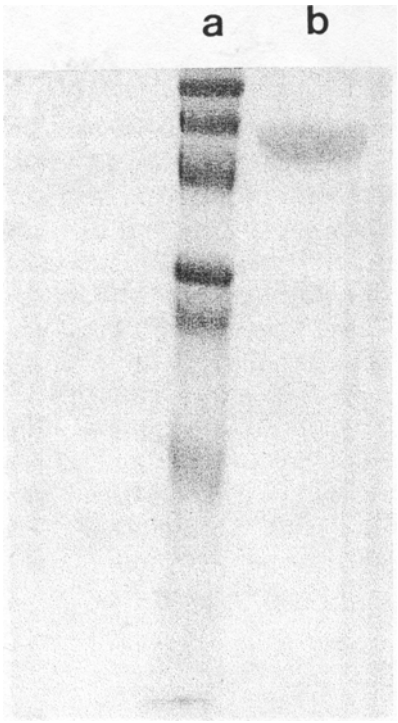

Fig. 3. SDS polyacrylamide gel electrophoresis of cellobiohydrolase. (a) Standards: phosphorylase b (130,000), bovine serum albumin (75,000), ovalbumin (50,000), carbonic anhydrase (39,000), soybean trypsin inhibitor (27,000), and lysozyme (17,000) (b) purified cellobiohydrolase.

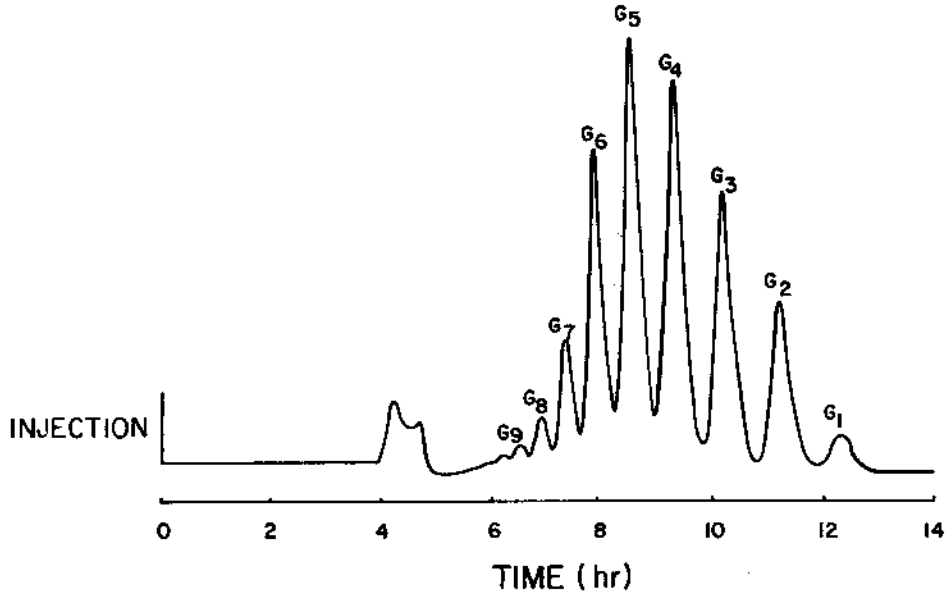

Fig. 4. Chromatogram of cellodextrin mixtures separated by Bio-gel P-2 (−400 mesh) column.

Measurement of Ultraviolet Difference Spectroscopy

The difference spectra were measured by using Cary 210 (Varian Instrument Co.) spectrophotometer equipped with a wavelength scanner and an expended scale recorder (0.01–0.1 absorbance). The spectral bandwidth and the period were set at 1 nm and 10 s, respectively. Under these conditions, the instrument can provide an acceptable noise level in the recorded spectrum. All solutions were filtered through ultraclarification filter (0.45 μm) before use. The measuring cuvets were kept at constant temperature ($\pm$0.1°C) by circulating water from a water bath. All spectral data were obtained at pH 7.0, rather than pH 5.0 where the enzyme is most active, because hydrolysis rate can be minimized at this pH level. Aliquots (0.8 mL) of either enzyme solution (7.5 μM) or substrate (1–5 m*M*) in 10 m*M* sodium phosphate buffer pH 7.0 were placed into separated compartments of 8.0 mm pathlength cuvette. After the background had been scanned and stored in the instrument, the contents in the sample cell were gently mixed and spectra were recorded at 0.5 nm/s following 2 min incubation.

RESULTS AND DISCUSSION

Difference Spectra of Cellobiohydrolase Induced by Cellodextrins

The λ_{max} at pH 7.0 of *N*-acetyl-DL-tyrosine amide is 274.5 nm and that of *N*-acetyl-DL-tryptophan amide is approximately 279.5 nm, but the adsorption bands of these chromophore are usually shifted to the red when they are incorporated into proteins (*16*). In the presence of substrates that produce red shifts, the difference spectra of tyrosine and tyrosine-rich proteins typically show a maximum at 278–281 nm and a larger maximum at 286–288 nm, whereas the difference spectra of tryptophan and tryptophan-rich proteins typically show a maximum at 281–284 nm and a larger maximum at 291–293 nm (*17*).

The binding of substrates (G_3–G_7) to native cellobiohydrolase produces difference spectra such as those shown in Fig. 5. The spectra produced by substrates (G_3–G_7) are characterized by two peaks at 289–292 and 283–286 nm, owing to the red shift in the adsorption of both tryptophan and tyrosine residues.

The positive peak at 289–293 nm, which is specifically ascribed to tryptophan, indicates that the environment of a tryptophan residue or residues becomes more hydrophobic on the binding of these substrates. From the characteristic spectra, one can speculate that these substrates interact with a specific tryptophan residue or residues located at or near the active site of cellobiohydrolase. Hayashi et al. (*8*) has shown that the difference spectrum at 293.5 nm of muramidase with glycol chitin is caused by a specific tryptophan residue located at the active center of enzyme.

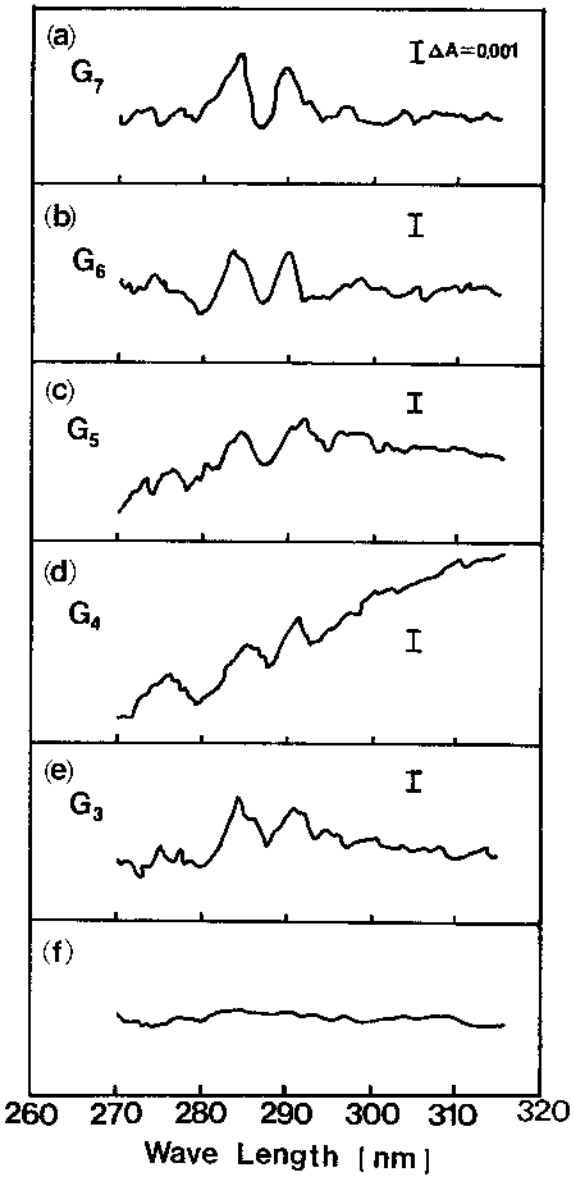

Fig. 5. Ultraviolet difference spectra of cellobiohydrolase induced by the binding of cellodextrins. (a) 5 m*M* G_7, (b) 5 m*M* G_6, (c) 5 m*M* G_5, (d) 5 m*M* G_4, (e) 5 m*M* G_3 and (f) baseline. The vertical bars represent 0.001 absorbance unit.

Collins and Stark (*18*) found the difference spectrum at 289.1 nm of aspartate transcarbamylase is a result of the tryptophyl residue perturbed by the binding of carbamyl phosphate. Ohnishi (*19*) added maltose to α-amylase and caused difference spectrum with two peaks at 286 nm and 292–293 nm. The difference spectrum at 292–293 nm was identified that one tryptophyl residue located in the maltose-binding site of the enzyme

was perturbed. Difference spectra produced by adding cellodextrins and cellobiose to endoglucanase of *Schizophyllum commune* were observed by Clarke and Yaguchi (*20*). They concluded that the difference spectrum at 291–292 nm was a result of two tryptophan residues specifically perturbed by cellodextrins.

The result of hydrolysis of cellotetraose by native and chemically modified cellobiohydrolase is shown in Fig. 6. After 55 min reaction, only 4% of the cellotetraose was hydrolyzed by modified enzyme. This result suggests that tryptophan residue(s) is/are important to the activity of cellobiohydrolase. The catalytic mechanism of lysozyme, which is functionally similar to cellobiohydrolase (both hydrolyze β-1,4-glucosidic bonds), is well known (*21*). Tryptophan 62 and tryptophan 63, which form hydrogen bonds with sugar residue C, play important roles in the formation of enzyme–substrate complex. Although generalizations are difficult to make, the data presented here suggest some similarities between lysozyme and cellobiohydrolase. Tryptophan residue(s) is/are necessary for the activity of cellobiohydrolase.

The shape of the spectra induced by different degree of polymerization (DP) of cellodextrins is slightly different. These differences might reflect the changes in the microenvironment of the tryptophan residue(s) at active site on the binding of cellodextrins with increasing DP.

The mechanisms of amylases and lysozyme are fairly well defined, with their catalytic activity attributed to their extended binding sites. Large active centers, containing several subsites, appear to be a common feature among the amylases from different sources (*22*). Similarly, cellulase from different species have been reported to have a large active center with several subsites (*23–27*), although proof of this model is still incomplete.

Determination of Association Constants for Binding of Cellodextrins to Cellobiohydrolase

In enzyme reaction, the binding of substrates to a specific site on the enzyme plays a vital role in determining the specificity of the catalytic reaction. In Michaelis-Menten's enzyme kinetics, the Michaelis constant K_m is a measure of the binding process (*28*). If the rate of product formation is small, a large K_m value indicates weak binding and a small K_m value indicates strong bindings. Thus, a study of this phenomenon should provide some insight into the binding process.

The difference molar absorptivity ($\Delta\epsilon$), which is owing to the specific binding of substrate to the enzyme, is proportional to the concentration of the enzyme-substrate complex. Thus, Michaelis-Menten equation can be modified to the following equation:

$$1 / \Delta\epsilon = (K_d / \Delta\epsilon_{max})(1 / [S]) + 1 / \Delta\epsilon_{max} \quad (1)$$

and K_a is obtained by

$$K_a = 1 / K_d \quad (2)$$

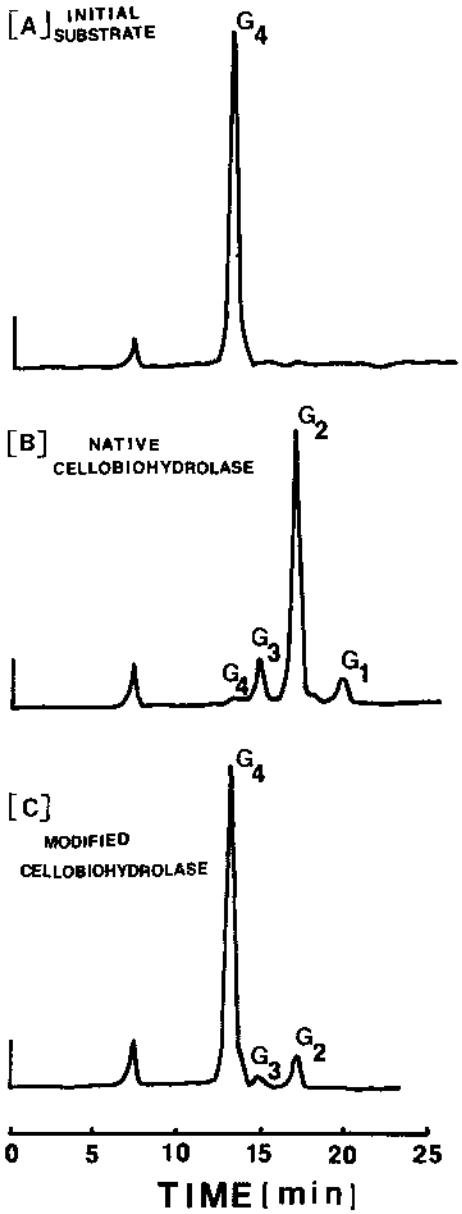

Fig. 6. Hydrolysis of cellotetraose by cellobiohydrolase and chemically modified enzyme. (a) Initial substrate. (b) After 55 min of hydrolysis by native enzyme. (c) After 55 min of hydrolysis by modified enzyme.

Table 2
Dissociation and Association Constants
for the Binding of Various Cellodextrins to Cellobiohydrolase at pH 7.0

Substrate	Temperature, °C	K_d, mM	K_a, M^{-1}
G_7	30	1.28	784.3
	40	1.37	728.9
	48	1.44	694.4
G_6	20	1.12	888.9
	30	1.27	786.2
	40	1.36	737.5
G_5	20	1.19	843.2
	30	1.34	747.9
	40	1.43	699.3
G_4	20	1.25	801.3
	30	1.39	719.4
	40	1.46	686.8
G_3	20	1.31	765.9
	30	1.44	694.4
	40	1.54	649.4

in which K_d is the dissociation constant of enzyme-substrate complex; K_a is the association constant for binding of cellodextrin to cellobiohydrolase; $[S]$ is the concentration of cellodextrin; $\Delta\epsilon$ is the difference molar absorptivity; $\Delta\epsilon_{max}$ is the maximal difference molar absorptivity where cellobiohydrolase was saturated with cellodextrin. The value of K_d and $\Delta\epsilon_{max}$ can be obtained from the linear double-reciprocal plot of $\Delta\epsilon_{289-293}$ vs cellodextrin concentration.

The dissociation and association constants of cellobiohydrolase-cellodextrins are determined by Eqs. (1) and (2) and summarized in Table 2. The dissociation constants of cellodextrins to cellobiohydrolase decreases with increasing DP. This finding is similar to the other cellulases in which the kinetics of the splitting β-1,4-oligoglucosides are chain length dependent (*29,30*). Li et al. (*31*) observed that cellulase from *T. viride* showed a decrease in Michaelis constants with increasing chain length of oligoglucosides and reported that the optimum substrate chain was at least 6 glucosyl units long.

Effect of Temperature

There are no noticeable changes in the overall shape of difference spectra taken at various temperatures. As shown in Table 2, it is clear that the affinity of cellodextrins to cellobiohydrolase decreased with increasing temperature. From the association constants taken at different temperatures, thermodynamic parameters can be obtained from van't Hoff equa-

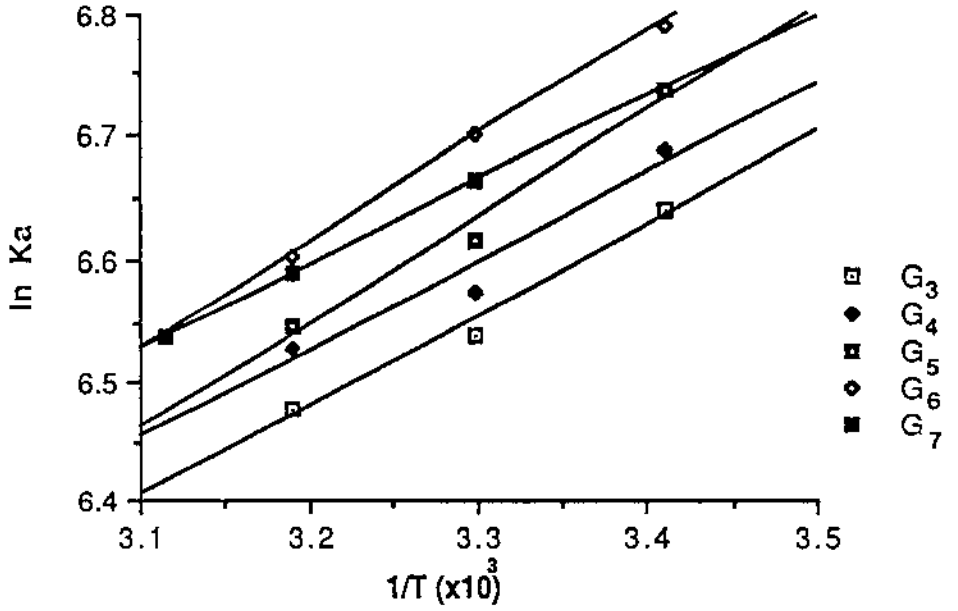

Fig. 7. van't Hoff plots for the interactions of cellobiohydrolase with G_7 (■), G_6 (◇), G_5 (□), G_4 (◆), and G_3 (⊡).

Table 3
Standard Free Energy Change for the Association of Cellobiohydrolase with Cellodextrins

Substrate	$-\Delta G°$, kcal/mol
G_7	3.97
G_6	3.99
G_5	3.96
G_4	3.93
G_3	3.91

tion. The interactions between cellodextrins and cellobiohydrolase have linear relationship over the temperature range from 20 to 48°C, as shown in Fig. 7. The standard free energy changes for the association of cellodextrin-cellobiohydrolase complex, $\Delta G°$, which is given by

$$\Delta G° = -RT \ln K_a \quad (3)$$

are summarized in Table 3. The interactions between the subsites and the substrate residues give rise to a decrease in free energy, or an increase in affinity, contributing to the binding of the substrate molecules to the active site of the enzyme. The decrease of $\Delta G°$ values from G_6 to G_3 could be

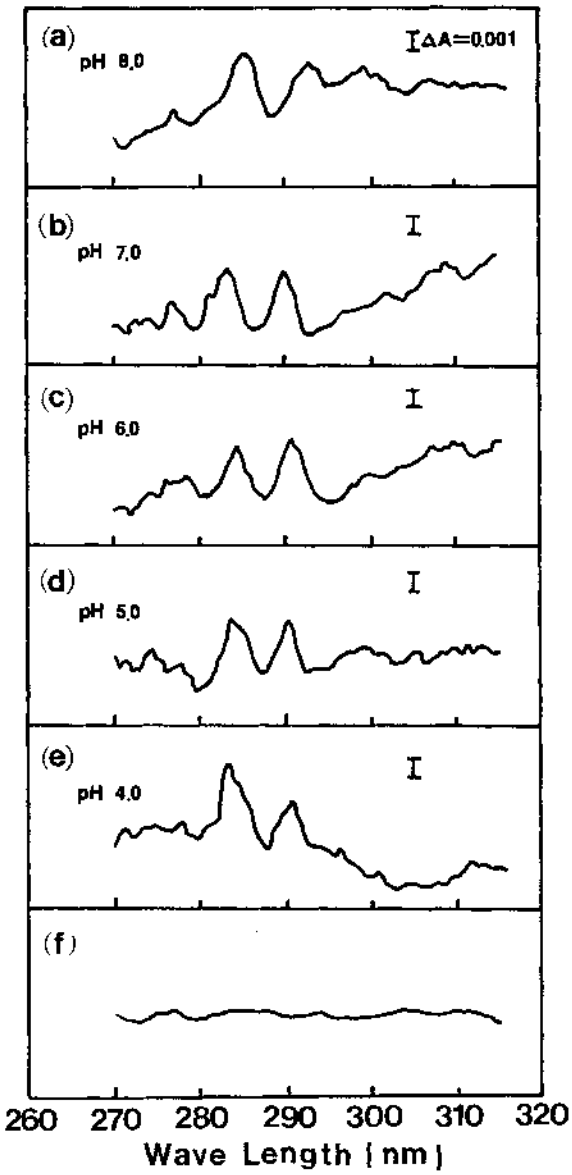

Fig. 8. Ultraviolet difference spectra of cellobiohydrolase induced by the binding of cellohexose at different pH levels. (a) pH 8.0, (b) pH 7.0, (c) pH 6.0, (d) pH 5.0, (e) pH 4.0, and (f) baseline.

taken to be the contribution of the extra glucopyranosyl group to the binding of this ligand to the proposed subsite structure of cellobiohydrolase.

Effect of pH

Figure 8 shows the difference spectra of G_6-cellobiohydrolase at various pH levels. The shapes and maxima shift slightly at various pH levels.

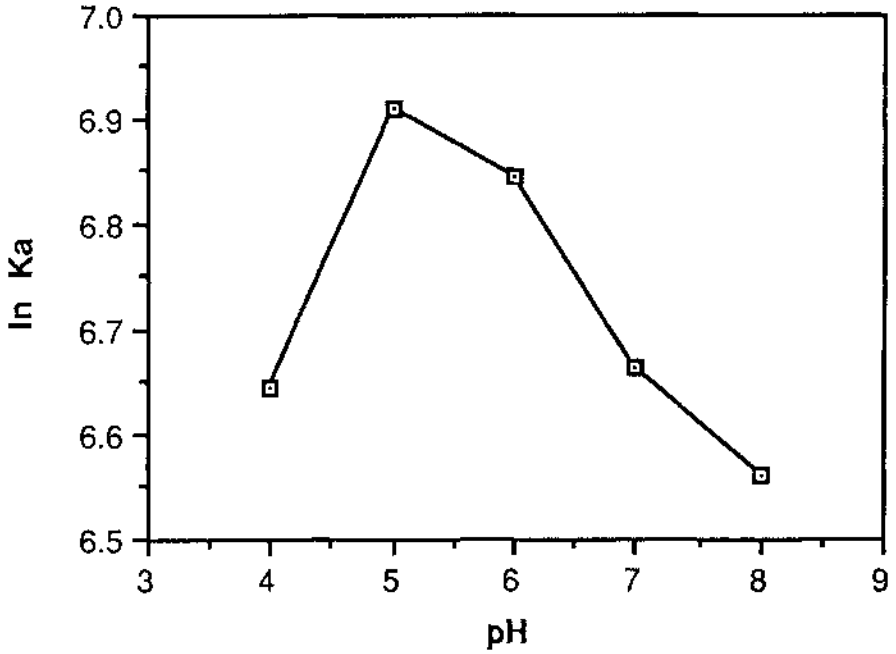

Fig. 9. pH-Dependence curve for the binding ability of cellobiohydrolase.

The shift of maximum in the acidic or alkaline region suggests that the microenvironment of tryptophan residue(s) near the active site was altered because of the local conformation change in the complex. This conformation change caused a marked decrease in the binding ability of cellobiohydrolase. The association constants for the binding of cellodextrins were calculated and the logarithms were plotted vs pH in Fig. 9. Cellobiohydrolase shows maximal affinity at pH 5.0. This might be because the microenvironment of active center is suitable for enzyme–substrate formation. The catalytic rate of lysozyme is strongly pH dependent. The enzyme activity drops sharply on either side of its optimal pH. The decrease on the alkaline pH is a result of the ionization of glutamic 35, whereas the decrease in rate on the acid pH reflects the protonation of aspartate 52 (*21*).

CONCLUSION

Difference spectral method is a useful technique to get information concerning the involvement and location of chromophoric groups in enzyme–substrate interaction. The specific role of tryptophan residue(s) located at or near the substrate-binding site of enzyme is shown to be crucial to the activity of cellobiohydrolase. The association constant of cellobiohydrolase–cellohexose complex is temperature and pH dependent.

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