Cotton woven fabrics which were previously dyed with a reactive dye were treated with a commercial cellulase preparation. Dyeing with a reactive dye for cotton apparently inhibited the weight loss activity and saccharification activity of cellulase. In addition, dyed cotton was treated with highly purified cellulases which were exo-type cellulases (Cellobiohydrolase I (CBH I) and Cellobiohydrolase II (CBH II)) and endo-type cellulase (Endoglucanase II (EG II)). Exo-type cellulases were inhibited more than endo-type cellulase by dyeing in the case of saccharification activity. CBH I was severely inhibited by dyeing as compared with CBH II or EG II from the viewpoint of morphological changes in the fiber surface. Dyes on the cellulose substrates severely influenced CBH I in spite of the rare modification, because CBH I hydrolyzed cellulose with true-processive action. The change in the activity of each cellulase component on dyed cotton can affect the synergistic action of cellulases.

Key words: action of cellulases; reactive dye; weight loss activity; saccharification activity; morphology of fiber surface

Cellulases are commonly used in textile processing to obtaining a soft feel, better surface appearance (depilling, also known as biopolishing), and stone washing effects (also known as biostoning). It is well known that commercial cellulase preparations contain several cellulase components such as endo- and exo-type cellulases and β-glucosidase. But in the textile industry we might use only a part of cellulase actions in multi components. They show different properties for adsorption and hydrolysis on cellulose. The mechanism of cellulose degradation by cellulases has not been fully established because of the high crystallinity and water insolubility of cellulose fiber. In the textile industry, cotton fabrics are usually dyed with a few types of dyes, and they are hydrolyzed by cellulases with more difficulty than the original cotton. Some researchers have reported the inhibitory effect of a few types of dyes adsorbed or reacted with cotton as substrates for the cellulase catalytic reaction. Especially, reactive dyes can form covalent bonds with the functional groups of cellulose in the amorphous regions. So dyeing with reactive dyes should be looked upon as a kind of chemical modification of cellulose. The modes of action of cellulases on cotton become more complicated by existence of dyes. Manufacture in textile processing by cellulases depends on perception and experience. We should analyze the degradation mechanism of previously dyed cotton fabrics by several cellulase components, because we must construct stable mass production at low cost in the textile industry.

In this study, cotton woven fabrics which were previously dyed with a reactive dye were treated with a commercial cellulase preparation. We investigated the inhibitory effect of reactive dyes, which reacted with cellulose by making covalent bonds, on the cellulase catalytic reaction. In addition, to investigate the modes of action of highly purified cellulases, we observed morphological changes in the fiber surface of cotton treated with cellulases by scanning electron microscopy.

Materials and Methods

Materials. Cotton woven fabric, plain woven made of cotton counts yarn of 50/1, which was desized, scoured, bleached, and mercerized by conventional methods, was used as a test material. The reactive dye, C. I. Reactive Blue 19 (Blue-19), obtained as a commercial product, was used without further purification. Cellusoft L (Novozymes, endo glucanase activity; 750 unit/g), a commercial cellulase product from Trichoderma reesei, was used without further purification. On the other hand, purified cellulases (CBH I, CBH II, and EG II) from T. reesei, used in this study, were obtained in a highly purified state through several steps.
Dyeing method. Cotton woven fabrics were dyed with Blue-19 by a continuous dyeing process (Pad→Dry→Steam→Wash). The dye liquor contained the required concentrations of Blue-19 with 20 g/l of sodium hydroxide, 50 g/l of urea, reduction inhibitor, and migration inhibitor. The cotton woven fabric was padded with dye liquor and dried at 100 °C for 2 min, steamed at 103 °C for 3 min, and then finally washed off with boiling water.

Dye uptake and degree of substitution (DS). The color depth of dyed fabrics was evaluated in K/S values from the Kubelka–Munk function measured with a Shimadzu UV-3100 spectrophotometer. The dyed fabrics were dissolved in perchloro acid and each dye uptake was determined by measuring the amounts of sulfur with a Seiko SPS1500VR Inductively Coupled Plasma (ICP) atomic emission spectrometer.

The color depth (K/S values) and dye uptake (mol/kg values) of fabrics dyed with several concentrations of reactive dye are shown in Table 1. The K/S values and mol/kg values of dyed fabrics increased with increasing dye concentration. Furthermore, we calculated the values of DS from dye uptake (mol/kg values), because the covalent bonds between reactive dye and cellulose are formed by the chemical modification of cellulose. The inhibitory effect of dyes on the cellulase reaction can be discussed from the viewpoint of the modification of cellulose. The DS values (as shown in Table 1) were from 0.5 × 10⁻³ for the pale shade (dye conc. 10 g/l) to 2.8 × 10⁻³ for the deep shade (dye conc. 80 g/l). The DS of dye reacted with cellulose was 3 or less against every 1,000 glucose units even in the deep shade.

<table>
<thead>
<tr>
<th>Dye conc. (g/l)</th>
<th>Color depth K/S</th>
<th>Dye uptake ×10⁻² (mol/kg)</th>
<th>DS ×10⁻³</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>2.96</td>
<td>0.31</td>
<td>0.5</td>
</tr>
<tr>
<td>20</td>
<td>5.37</td>
<td>0.53</td>
<td>0.9</td>
</tr>
<tr>
<td>40</td>
<td>9.80</td>
<td>1.02</td>
<td>1.7</td>
</tr>
<tr>
<td>80</td>
<td>12.5</td>
<td>1.72</td>
<td>2.8</td>
</tr>
</tbody>
</table>

Dyeing method with C. I. Reactive Blue-19 is described in “Materials and Methods”. Color depth (K/S) was calculated by Kubelka–Munk function at λmax = 600 nm. Dye uptake was determined by ICP and degree of substitution (DS) was calculated from dye uptake.

Treatment of cotton with highly purified cellulases. Each reaction mixture consisted of 20 mg of cotton yarn pulled off from each cotton fabric and 0.5 ml of purified enzyme solution (the enzyme concentration was adjusted with absorbance 1.0 at 280 nm) dissolved in 0.05 M sodium acetate buffer (pH 5.0). The reaction mixtures were incubated at 50 °C for 24, 48, and 72 h with mechanical shaking (100 strokes/min). The reducing sugar produced in the reaction mixture was measured.

Measurements of weight loss (WL) and reducing sugar (RS). The value of WL was calculated from weights of samples before and after cellulase treatment with drying at 105 °C for 120 min.

The amount of RS as glucose produced in the reaction mixture was measured by the methods of Somogyi and Nelson.

Adsorption of enzymes. The reaction mixture with highly purified cellulases was incubated at 4 °C for 180 min without shaking. The amount of enzyme in the supernatant was measured by the method of Lowry et al. 13)

Observation by scanning electron microscope. Scanning electron micrographs were obtained using a JSM-T100 scanning microscope (JEOL).

Results

Treatment of cotton with commercial cellulase preparation

WL after enzyme treatment for 200 min is plotted against DS in Fig. 1. The values of WL decreased with increasing values of DS. The inhibitory effect of dyes on the WL activity of cellulase was observed, the same as reported previously. 4–7) We also measured RS produced in the reaction mixture, as shown in Fig. 1. The amounts of RS decreased with increasing values of DS, the same tendency that the values of WL showed (Fig. 1). From these results, dyeing with a reactive dye for cotton fabrics inhibited not only WL activity but also the saccharification activity of cellulase.

The amounts of RS after treatment for 50, 100, and 200 min for undyed and dyed fabrics are plotted against WL in Fig. 2. The values of RS for undyed fabric increased linearly with increasing values of WL. On the other hand, it was clear that the values of RS for dyed fabrics were smaller than those for undyed fabrics, as compared with the same values of WL. This was
remarkable with increasing values of DS. It is thought that the role of each component on the synergistic reaction for cellulose degradation might be changed by modification of cellulose with reactive dye. Therefore, purified enzymes such as exo-type (CBH I and CBH II) and endo-type (EG II) cellulases from *T. reesei* were also used in this study, since the commercial cellulase preparation contained various components, and they catalyzed cellulose degradation synergistically.

*Treatment of cotton with highly purified cellulases*

We investigated the treatment of cotton yarns with highly purified cellulases, which were exo-type (CBH I and CBH II) and endo-type (EG II) cellulases from *T. reesei* were also used in this study, since the commercial cellulase preparation contained various components, and they catalyzed cellulose degradation synergistically.

![Graph 1: Weight Loss (%) of Undyed and Dyed Cotton Fabrics and Reducing Sugar (mg/mL) Produced in the Reaction Mixture Treated with Commercial Cellulase Preparation against Degree of Substitution. The treatment condition is described in "Materials and Methods". The reaction mixture was incubated at 50 °C for 200 min.]

![Graph 2: Reducing Sugar Produced in the Reaction Mixture against Weight Loss of Undyed Cotton Fabric (●) and Dyed Cotton Fabrics with DS = 0.5 × 10⁻³ (●), 0.9 × 10⁻³ (●), 1.7 × 10⁻³ (▲), 2.8 × 10⁻³ (▲). The treatment condition is described in "Materials and Methods". The reaction mixture was incubated at 50 °C for 50, 100, and 200 min.]

Table 2. Reducing Sugar Produced by Highly Purified Cellulases and Crude Enzyme Incubated at 50 °C for 72 h for Undyed and Dyed Cotton Fabrics

<table>
<thead>
<tr>
<th>Cellulase</th>
<th>Undyed DS = 0.5 × 10⁻³</th>
<th>DS = 2.8 × 10⁻³</th>
<th>Relative value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBH I</td>
<td>0.035</td>
<td>0.003</td>
<td>b/a = 0.91</td>
</tr>
<tr>
<td>CBH II</td>
<td>0.436</td>
<td>0.070</td>
<td>0.84</td>
</tr>
<tr>
<td>EG II</td>
<td>0.078</td>
<td>0.043</td>
<td>0.85</td>
</tr>
<tr>
<td>Crude enzyme*</td>
<td>2.791</td>
<td>1.312</td>
<td>0.70</td>
</tr>
</tbody>
</table>

The treatment condition and measurement method (the methods of Somogyi and Nelson) are described in "Materials and Methods".

*Cellusoft L was used as the control at a concentration of 3.2 g/l with absorbance 0.5 at 280 nm by the same operation as for purified cellulases.
larger than that on endo-type cellulase.

The adsorption of each purified cellulase incubated at 4 °C on undyed and dyed cotton yarns is shown in Table 3. The amounts of adsorption of exo-type cellulases (CBH I and CBH II) are larger than that of endo-type cellulase (EG II). Especially, CBH II had a high capacity for adsorption not only on undyed cotton but also on dyed cotton. But CBH I and EG II were easily influenced in the capacity for adsorption by dyeing. The behavior of adsorption of each purified cellulase is different from the behavior of RS, as shown in Table 2. It is suggested that the change in the behavior of adsorption with dyeing is not coincident with the inhibitory effect of dyes on the enzymatic reaction of cellulase. We confirmed that dyes free from cotton fabrics had little influence on the saccharification activity of cellulases.

**Table 3. Adsorption of Each Purified Cellulase Incubated at 4 °C for 180 min on Undyed and Dyed Cotton Fabrics**

<table>
<thead>
<tr>
<th>Purified cellulase</th>
<th>Adsorption of cellulase (%)</th>
<th>Undyed</th>
<th>DS = 0.5 × 10⁻³</th>
<th>DS = 2.8 × 10⁻³</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBH I</td>
<td>17.7</td>
<td>16.0</td>
<td>12.9</td>
<td></td>
</tr>
<tr>
<td>CBH II</td>
<td>33.2</td>
<td>30.6</td>
<td>31.3</td>
<td></td>
</tr>
<tr>
<td>EG II</td>
<td>4.6</td>
<td>4.0</td>
<td>2.8</td>
<td></td>
</tr>
</tbody>
</table>

The treatment condition and measurement method (the method of Lowry et al.) are described in "Materials and Methods".

**Morphology observation**

Scanning electron micrographs of the fiber surface of undyed cotton fiber, pale-shade cotton fiber (DS = 0.5 × 10⁻³), and deep-shade cotton fiber (DS = 2.8 × 10⁻³) treated with highly purified cellulases for 72 h are shown in Fig. 3.

**Fig. 3.** Scanning Electron Micrographs of the Fiber Surface of Cotton Treated with Highly Purified Cellulase for 72h.

A, Undyed cotton; B, Dyed cotton with DS = 0.5 × 10⁻³; C, Dyed cotton with DS = 2.8 × 10⁻³. Mark pitch on micrograph is 10 μm. The treatment condition is described in "Materials and Methods". The reaction mixture was incubated at 50 °C for 72 h. Scanning electron micrographs were obtained using a JSM-T100 scanning microscope at 5 kV accelerating voltage.
For undyed cotton fiber, each exo-type cellulase (CBH I and CBH II) caused the formation of a number of deep bias cracks and many fibrils on the fiber surface of cotton. On the other hand, an endo-type cellulase (EG II) caused only many fibrils and no cracks. These results are coincident with our previous study\(^{14,15}\) which reported that the degradation of cotton by exo-type and endo-type cellulases yielded quite different morphological patterns. For pale-shade cotton fiber with \(DS = 0.5 \times 10^{-3}\), CBH II caused similar but fewer cracks and similar fibrils on the fiber surface of cotton. On the other hand, CBH I caused only many fibrils and no cracks, the same as EG II. For deep-shade cotton fiber with \(DS = 2.8 \times 10^{-3}\), CBH II caused a small number of bias cracks and a few fibrils on the fiber surface of cotton. CBH II was inhibited but still had a small amount of activity because of its high potential of activity for cotton substrates. But CBH I caused no fibrils and no cracks. Perhaps CBH I was severely inhibited for enzymatic reaction by dyeing. On the other hand, EG II caused only a few fibrils and no cracks. Perhaps EG II was inhibited less than exo-type cellulases by dyeing. These results are coincident with the results for inhibition of each purified cellulase activity in Table 2.

**Discussion**

In general, crude cellulase preparation contains multi-components, and they can catalyze cellulose degradation synergistically.\(^{15}\) Endo-type cellulases catalyze cellulose degradation by a non-processive action which was accompanied by the dissociation from the cellulose chain in each catalytic action. Exo-type cellulases degrade the same cellulose chain continuously with a processive action not accompanied by the dissociation.\(^{16,17}\) Recently it has been reported that there are endo-processive enzymes like CBH II from *Humicola insolens* showing an intermediate property between the processive and non-processive types.\(^{18}\)

The endo-type cellulase EG II was little influenced by stereo-obstacles like dyes with covalent bonds on cellulose, since they can dissociate from substrates freely (Table 2 and Fig. 3). On the other hand, the exo-type cellulases CBH I and CBH II were affected by dyes more than the endo-type. Exo-type cellulase, especially CBH I, is severely influenced by stereo-obstacles in spite of the rare modification. CBH I has a tunnel structure at the binding site and attacks the same cellulose chain continuously by processive action.\(^{19}\) Therefore when CBH I proceeds to the modified glucose unit, it might stop and be stuck there. However, exo-type cellulase like CBH II is rarely influenced by stereo-obstacles like dyes. It has been reported that there are endo-processive enzymes like CBH II from *Humicola insolens* that attack the same cellulose chain at several times.\(^{18}\) If it proceeds to the modified glucose unit, it might stop but instantly dissociate from the cellulose chain because it has a short tunnel and easily opens the flexible loop which makes the tunnel structure by closing. Perhaps the properties of the enzyme originated from the tertiary structure of the enzyme are closely related to the mode of action on cellulose degradation, especially modified cellulose degradation.

**References**
