ADSORPTION CONTROL OF CELLULASE ONTO CELLULOSE BY MODIFICATION WITH AMPHIPHILIC COPOLYMER

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The effects of the hydrophilic and hydrophobic properties of cellulase on adsorption onto and desorption from two substrates were studied. Cellulase was modified with amphiphilic copolymers made of polyoxyalkylene glycol alkylallylether and maleic acid anhydride. The polyoxyalkylene glycol (PAG) consists of ethylene oxide (EO) and propylene oxide (PO), with an EO to PAG ratio ranging from 0 to 100%. A copolymer with a high concentration of EO is more hydrophilic. The hydrophilic or hydrophobic properties of modified cellulase were varied the degree of modification and the type of copolymer. As the hydrophilic property of a modified cellulase increases, the conversion of substrate increases while the quantity of adsorbed enzyme decreases. Cellulase modification with amphiphilic copolymer is very useful for controlling cellulase adsorption onto and desorption from a substrate and can improve the saccharification reaction.

Introduction

Enzymatic decomposition of cellulosic materials into useful chemicals is a very promising process because cellulose is the most abundant renewable resource. The major obstacle to the development of large-scale processes for enzymatic hydrolysis of cellulose is the high cost and consumption of cellulolytic enzymes. Adsorption of cellulase onto a substrate is a prerequisite for the initiation of hydrolysis. Enzyme adsorption onto the substrate is very rapid at the start of the reaction. The adsorbed enzyme then gradually desorbs from the substrate as the hydrolysis progresses, although the desorption is incomplete. From the viewpoint of adsorption, reversible and irreversible adsorption occur at the same time. With respect to the hydrolysis reaction, productive and nonproductive adsorption are also involved in the adsorption process. The enzyme, once strongly adsorbed, remained immobile on the substrate even after extensive hydrolysis and strong adsorption might decrease its ability to form new active cellulose-cellulase complexes. Therefore, cellulase loses its apparent activity.

For this reason, several methods to control enzyme adsorption onto a substrate have been proposed. One of these is to use a nonionic surfactant during enzymatic hydrolysis. In this case, the surfactant, which makes a hydrophilic environment, played an important role in the desorption of cellulase from cellulose functional groups and enhanced saccharification.

In our previous work, cellulase was modified by synthetic polymers, such as polyethylene glycol (PEG) derivative, to obtain the additional properties of a nonionic surfactant and/or a synthetic polymer. The PEG derivative copolymer with a maleic acid anhydride functional group could modify cellulase without great deactivation. The modified cellulase displayed a high stability for activity against temperature and pH.

According to the above results, it may be expected that combining cellulase with amphiphilic polymer will change its hydrophilic properties due to the properties of the polymer. This is because the hydrophilic properties of synthetic copolymers can be controlled by changing the concentration of ethylene oxide (EO) and propylene oxide (PO) in the polyoxyalkylene glycol (PAG) chain.

In this study the effects of hydrophilic or hydrophobic modified cellulase on adsorption onto and desorption from two substrates during enzymatic hydrolysis were studied. The hydrophilicity of modified cellulase was controlled by varying the EO concentration in the PAG chain of amphiphilic copolymers and by changing the degree of modification.

1. Experimental

1.1 Enzyme source and activity
Cellulase from fungus, Acremonium cellulolyticus mutant strain, was kindly provided by MEIJI Ltd., Japan. The cellulase showed both C1 and C4 activities and had an average molecular weight of 48,000, which was measured by the GFC method. In this study, activity of the cellulase was represented by FPase activity, which was assayed as reported by Mandels et al. with FP-5C (Toyo Roshi Ltd., Japan) as a substrate and was represented by a filter paper unit (FPU) which produced 1.0 μmole of reducing sugar from the substrate per minute. Other chemicals used were of analytical grade.
Table 1. Characters of amphiphilic copolymers

<table>
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<th>EO/AGP (wt%)</th>
<th>n</th>
<th>k</th>
<th>MW</th>
<th>CP (°C)b</th>
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<tr>
<td>AKM-1511</td>
<td>100</td>
<td>33</td>
<td>10</td>
<td>16,000</td>
<td>100&gt;</td>
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<tr>
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<td>30</td>
<td>22</td>
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<td>27</td>
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<td>11</td>
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<tr>
<td>ACM-1611</td>
<td>20</td>
<td>29</td>
<td>10</td>
<td>18,000</td>
<td>16</td>
</tr>
</tbody>
</table>

\[
\begin{align*}
\text{CH}_3 & \quad \text{CH}_2 \quad \text{CH} \quad \text{CH} \quad CH \\
\text{O} & \quad \text{C} \quad \text{O} \quad \text{C} \quad \text{O}
\end{align*}
\]

\(\text{CH}_n\)

\(\text{(AO)}_n\)

\(k\)

a) (AO)_n is the total of EO and PO in the PAG chain. Abbreviations: AO, alkylene oxide; EO, ethylene oxide; PO, propylene oxide.
b) Cloud point (CP) is measured in aqueous solution of 10 mg/mL copolymer.

1.2 Synthetic copolymer as modifier

The synthetic copolymers used are listed in Table 1. The alternating copolymers of PAG alkylallylether and maleic acid anhydride were kindly supplied by Nippon Oil & Fats Co. (Japan). These copolymers are characterized by k, which indicates the degree of copolymerization, and by n, which indicates the number of alkylene oxide (AO). The AO chain consists of EO and PO. The weight ratio of the total EO to the total AO, denoted by EO/AG, is variable from 0 to 100% as shown in Table 1. As the value of EO/AG increases, the hydrophilic properties of the copolymer increase. Similarly, as EO/AG decreases, the hydrophobicity increases. Therefore, the relative hydrophobicity of modified celluloses may be changed by varying the EO/AG ratio of the copolymer as a modifier.

1.3 Cellulase modification

Maleylation is a chemical modification of protein with maleic acid anhydride\(^{11}\). Amino groups of the cellulase molecule were covalently coupled with the maleic acid anhydride function of the copolymer at 4°C and at a pH level higher than 8.0. The weight ratio of copolymer to cellulase varied over a range of 0.25 to 2 (w/w) to change the degree of modification (DM) of cellulase. DM is defined as the ratio of modified amino groups of modified cellulase to total amino groups of native cellulase\(^{12}\).

1.4 Substrate

Filter paper FP-5C (Toyo Roshi Ltd., Japan) and Avicel (E. Merck, Darmstadt) were used as standard substrates. In the case of filter paper, mechanical pretreatment was performed for 2 minutes with an electric blender in 0.1 M sodium acetate buffer\(^{13}\), and Avicel was incubated for 1h in the above buffer with same swelling condition. The mixture of pretreated filter paper and buffer solution showed a state of slurry.

1.5 Saccharification

The reaction condition for saccharification is pH 5.0 and 50°C. The experimental condition is characterized by the weight ratio of enzyme to substrate, namely the E/S (mg enzyme/mg substrate) ratio. A batch reactor of 200 mL capacity containing 100 mL reactant and enzyme was stirred by a magnetic stirrer. After a suitable reaction time, samples were centrifuged for 3 minutes at 2,000 rpm (1000 g) and the reducing sugar and free enzyme concentrations in the supernatant were measured.

1.6 Assay procedures

Reducing sugar, which was produced by the hydrolysis of cellulose, was determined by the dinitrosalicylic acid (DNS) method\(^{10}\) with glucose as a standard. The degree of conversion was defined by the ratio of reducing sugar to the initial substrate.

To determine the DM of modified cellulase, amino groups of the cellulase were determined with trinitrobenzene sulfonic acid (TNBS)\(^{9}\). Remaining native and modified cellulase quantities in the supernatant were measured by the GFC method\(^{15}\). The quantity of cellulase adsorbed onto the substrate was calculated by subtracting the free enzyme quantity in the supernatant from the initial enzyme quantity with consideration of deactivation by temperature.

2. Results and Discussion

2.1 Adsorption behavior of native cellulase

Adsorption of native cellulase onto substrate was studied to explain the adsorption behavior during hydrolysis. Ten mg/mL of pretreated filter paper was placed in native enzyme solutions having concentrations of 0.26, 0.52, 0.78 mg/mL respectively. The conversion of filter paper and relative free cellulase concentration during the reaction at 50°C are shown in Fig. 1-a, b.

The relative free enzyme concentration is defined as the ratio of free enzyme concentration (E) in the supernatant to initial enzyme concentration (E_i). In the case of an E/S ratio of 0.026 (mg enzyme/mg substrate), a linear relation of conversion to reaction time is shown in the initial period (0-100 minutes) of the reaction (Fig. 1-a). Conversion gradually increases up to a time of 30 hours, reaching a conversion maximum of 58% (Fig. 1-b). In contrast, cellulase adsorbs onto the substrate very rapidly within 20 minutes and the concentration of free cellulase decreases slightly through the reaction. In the cases of higher E/S ratios, 0.052 and 0.078, it is found that conversion shows a similar pattern. Namely, conversion increases rapidly at the start of reaction, and there is scarcely any change after 30 hours. In this case, the conversion ratios at 30 hours are 82% and 95% respectively. The values of E_i/E decrease very rapidly within 20 minutes and increase slightly with reaction time, reaching 0.4 and 0.51 respectively after 30 hours. From these results it was found that cellulase adsorbed rapidly onto substrate and slowly desorbed from the substrate as the
conversion proceeded. However, as the \( E/S \) ratio decreased, the value of \( E_f/E_o \) decreased.

For Avicel, another standard substrate, a similar pattern of adsorption was found. The enzyme was adsorbed rapidly on the Avicel at the start of reaction, and the adsorbed enzyme desorbed slightly with reaction. When the \( E/S \) ratio was 0.05, conversion and \( E_f/E_o \) ratio changed only slightly after 50 hours. Their values at 50 hours were 73% and 35% respectively.

**Figure 2** shows the relation of adsorbed enzyme concentration per unit substrate, Avicel, to free enzyme concentration at 20 minutes at different temperatures. The initial adsorption equilibrium of Avicel is almost achieved at 20 minutes, as in the case of filter paper. \( S_o \) and \( P \) are respectively the initial substrate and produced reduced sugar concentration. \( E_{ad}/S_o-P \) is the adsorbed enzyme concentration per unit unsaccharified substrate. As free enzyme concentration increases, \( E_{ad}/S_o-P \) increases. The maximum adsorption concentration was calculated by extrapolation of \( 1/E_f \) vs. \( (S_o-P)/E_{ad} \) at 20 minutes. The relation between \( 1/E_f \) and \( (S_o-P)/E_{ad} \) showed a straight line, and it was indicated that the experimental data fit the Langmuir-type adsorption isotherm. The maximum values for each temperature 4°C, 20°C and 50°C were 0.061, 0.096 and 0.170 (mg enzyme/mg substrate) respectively. At 4°C there is no saccharification reaction, and it is expected that the quantity of input substrate does not change. Despite the lack of saccharification, cellulase rapidly adsorbed onto the substrate surface. Therefore, a strong affinity between cellulose and cellulase was found despite the absence of hydrolysis reaction. On the other hand, with increasing temperature the value of \( E_{ad}/S_o-P \) increased. For the initial period of the reaction it was thought that as hydrolysis proceeded the number of desorption sites on the substrate increased with the change of cellulose conformation by the enzyme. Also, the enzyme, which adsorbed strongly to the cellulose surface, did not desorb completely.

**2.2 Adsorption control of cellulase by modification with amphiphilic copolymer**

Enzymatic hydrolysis using polyoxyethylene glycol (PEG), which makes a hydrophilic environment,
played an important role in the desorption of cellulase from cellulose. On the basis of this information, cellulase was modified by using a hydrophilic copolymer, AKM-1511, which was synthesized from pure EO in PAG chain. Figure 3 shows the relation of $E_{ad}/S_0$ to the initial enzyme concentration against $DM$ at 60 minutes and 4°C. The enzyme concentrations ($E_{ad}$, $E_{ad}$) are calculated from the weight of cellulase included in the modified cellulase. Native and modified cellulase show a similar adsorption pattern. Specifically, as the initial enzyme concentration increases, $E_{ad}/S_0$ increases. At the same $E/S_0$ ratio the value of $E_{ad}/S_0$ decreases incrementally with increase of $DM$. These results suggest that as the hydrophilic property of the cellulase surface increases, the quantity of adsorption onto substrate decreases. Therefore, it is expected that enzyme adsorption can be controlled by varying $DM$.

Adsorption of modified cellulase ($DM$, 50%) with AKM-1511 was considered at a practical reaction temperature, 50°C. Figure 4 shows adsorbed enzyme quantity per unit substrate and conversion during the reaction at 50°C. The same initial enzyme concentration ($E_{ad}$) (0.25 mg/mL) of native and modified cellulase was added to each reaction system. There was rapid adsorption of both within 20 minutes, and the $E_{ad}/S_0$ of native cellulase increased gradually with reaction time. In contrast, modified cellulase maintained a constant quantity of adsorbed enzyme with reaction time and there was scarcely any change of $E_{ad}/S_0$ against reaction time after 2 hours. From these results it was thought that modified cellulase with hydrophilic copolymer played an important role in the buffering action against strong enzyme adsorption onto the substrate. In addition, the native cellulase shows greater conversion than the modified cellulase in the initial period of the reaction. But after 120 hours the modified cellulase shows 20% greater conversion than the native cellulase. It is expected that the hydrophilic copolymer of modified cellulase, which desorbed enzyme from substrate surface, enhanced saccharification. In contrast, mixed copolymer containing both EO and PO may be very useful for varying the relative hydrophobicity of modified cellulase. Adsorption of modified cellulase with hydrophobic copolymer was considered. $E_{ad}/S_0$ values against $DM$ at 20 minutes and 4°C are shown in Fig. 5. As the $DM$ increases, the values of $E_{ad}/S_0$ decrease slightly in all modified cellulases. It is believed that this is because the modified cellulase, which had a larger molecular weight than native cellulase, was less likely than native cellulase to adsorb onto substrate surface by the steric hindrance of PAG chains. Also, at the
same $DM$ value, $E_{ad}/S_0$ increases with the order of hydrophobicity of modifier, specifically ACM > AEM > AKM. From these results it was thought that the PO of the copolymer, which makes a hydrophobic environment, increased the adsorbed enzyme concentration.

The relation of the $DM$ to relative FPase activity with respect to species of amphiphilic copolymer is shown in Fig. 6. Three species of copolymer, AKM-1511, AEM-1511 and ACM-1511, which have PO concentrations of 0%, 60% and 80% respectively, in PAG chain were used to modify cellulose. The FPase activity was assayed at pH 5.2 and 50°C. The relative FPase activity is defined as the ratio of the FPase activity of modified cellulose to that of native cellulose. A $DM$ value of zero corresponds to native cellulose. As the $DM$ increases, all the relative FPase activities decrease until a maximum $DM$ is achieved. In the case of modification with AKM-1511, the FPase activity retains more than 80% of the native cellulase activity at the maximum $DM$. In contrast, as PO concentration in the PAG chain of copolymer increases, the relative FPase activities decrease. It was thought that the hydrophobic property decreased cellulase activity.

Figure 7 shows the relative conversion of Avicel and relative free cellulase concentration against $EO/ AO$ at 50°C, 100 hours. In this case the modification degrees of cellulose were 50%. The relative conversion was defined as the ratio of conversion of modified cellulase to that of native cellulase. As the concentration of EO in AO increases, the relative conversion increases. For $EO/ AO$ above 75%, the modified cellulase shows higher conversion than does native cellulase. Also, it is thought that at the higher value of free cellulase concentration it is easy to recover cellulase for reuse. Most modified cellulases show higher values than native cellulase. However, for $EO/ AO$ values under 30% the values of $E_f/E_o$ and relative conversion of modified cellulase become lower than those of native cellulase. The decreases in $E_f/E_o$ may be explained by the hydrophobic property, while the decrease in relative conversion of the modified cellulase with polymers having higher PO concentration may be explained by the cloud point of the polymer. A polymer of a higher PO concentration shows a lower cloud point, as shown in Table 1. Also, the cloud points of ADM and ACM are much lower than the reaction temperature of 50°C. From these results it was found that a modified cellulase with hydrophilic copolymer showed greater conversion and higher free-concentration cellulase than did native cellulase. In this case, it was thought that the modifier created a hydrophilic environment and played an important role in the desorption of enzyme from substrate surface, also enhancing saccharification. Further, it is expected that adsorption of cellulase onto cellulose can be controlled by modification using an amphiphilic copolymer, which has different hydrophilic properties.

**Conclusions**

Effects of the hydrophilic and hydrophobic properties of cellulase on adsorption onto and desorption from two substrates by using modified cellulase with amphiphilic copolymer were studied. Amino groups of the cellulase molecule were covalently coupled with the maleic acid anhydride functional group of the polymer. Moreover, the degree of modification and the function of polyoxalkylene glycol changed the hydrophilic and hydrophobic properties of modified cellulase. Native cellulase adsorbed tightly on the cellulose surface and hindered desorption from the binding site after saccharification. In this study, cellulase adsorption could be softened by using cellulase modification with hydrophilic copolymer while retaining high conversion of substrate. As the hydrophilicity of modified cellulase increases, conversion of substrate increases, while adsorbed enzyme quantity decreases. In addition, adsorption of cellulase onto cellulose can be controlled by modification using an amphiphilic copolymer, which has different hydrophilic properties, and can improve the saccharification by utilization of a hydrophilic copolymer.

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**Nomenclature**

- $E_{ad}$ = adsorbed enzyme concentration [mg/mL]
- $E_f$ = free enzyme concentration [mg/mL]
- $E_i$ = initial input enzyme concentration [mg/mL]
- $P$ = reducing sugar concentration [mg/mL]
- $S_i$ = initial input substrate concentration [mg/mL]
- $EO/AO$ = ethylene oxide concentration in PAG chain [%]
Literature Cited
