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# Effect of a Nonionic Surfactant on the Adsorption and Kinetic Mechanism for the Hydrolysis of Microcrystalline Cellulose by Endoglucanase I and Exoglucanase II

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Effect of a nonionic surfactant, Tween 20 on the adsorption and kinetic mechanism for the hydrolysis of a microcrystalline cellulose, Avicel PH 101, by endoglucanase I (Endo I) and exoglucanase II (Exo II) isolated from *Trichoderma viride* were studied. The Langmuir isotherm parameters, amount of maximum adsorption ($A_{max}$) and adsorption equilibrium constant ($K_{ad}$) for the adsorption, were obtained in the presence and the absence of nonionic surfactant. On the addition of Tween 20, the $K_{ad}$ and $A_{max}$ values of Exo II were decreased, while those of Endo I were not affected. These indicate that the adsorption affinity of Exo II on the cellulose is weakened by nonionic surfactant, and the surfactant enhanced desorption of Exo II from insoluble substrate. The enzymatic hydrolysis of the cellulose can be described by two parallel pseudo-first order reactions using the percentages of easily ($C_a$) and hardly ($C_b$) hydrolyzable cellulose in Avicel PH 101 and associated rate constants ($k_a$ and $k_b$). The $C_a$ value was increased by adding Tween 20 for all enzyme samples (Exo II, Endo I and their 1 : 1 mixture) implying that the low-ordered crystalline fraction in the cellulose may be partly dispersed by surfactant. The $k_a$ value was not affect by adding Tween 20 for all enzyme samples (Exo II, Endo I and their 1 : 1 mixture). The $k_b$ value of Exo II was increased by adding Tween 20, while that of Endo I was not affected. This suggests that the surfactant helps the Exo II desorb from microcrystalline cellulose, and increase the hydrolysis rate. These results were show that the increase of hydrolysis of cellulose by the nonionic surfactant is due to both the activation of Exo II and partial defibrillation of the cellulose.

## Introduction

The enzymatic degradation of cellulose is catalyzed by three classes of enzymes: endoglucanases, exoglucanases, and β-glucosidase working in a synergistic way.[1,2] The multiplicity of the cellulases, the structural complexity of cellulosic materials, and product inhibition cause the difficulty in the elucidation of the hydrolysis mechanism.

The adsorption of cellulase on the cellulose is very important because adsorption is a prerequisite step in the en-

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zymatic hydrolysis of cellulose. The investigation on the adsorption phenomenon has provide very useful information on the action of cellulase.[3-5] These include the effects of various pretreatments of cellulose and sample preparations on the adsorption of cellulase. Nevertheless, the adsorption mechanism of cellulases on the cellulosic material are not fully understood yet.

We have previously described the adsorption behaviors on microcrystalline cellulose of endo-and exo-type cellulases partially purified from *Trichoderma viride*. It was shown that the maximal synergistic degradation occurs at a specific weight ratio of cellulase components at which the maximal affinity of cellulase components was obtained.[6] Our another work have described the adsorption kinetics of exoglucanase in combination with endoglucanase from *Trichoderma viride* on microcrystalline cellulose and its influence on synergistic degradation. It was shown that synergistic degradation of microcrystalline cellulose is dependent on the randomness of the endoglucanase and the tightness and affinity of adsorption.[7]

Various kinetic models have been developed to describe the enzymatic hydrolysis of cellulosic and lignocellulosic materials. Some of them are based on the assumption that the reaction rate is proportional to the amount of adsorbed enzyme on the cellulose surface,[3] some of the models are based on the structural features of the substrate, like pore size distribution, index of crystallinity, and specific surface area,[8] others are based on the properties of the cellulase enzyme and mass transfer in the reaction system.[9] Some researchers[10-14] have developed semiempirical models based on the assumption that the reaction between cellulose and cellulase can be described by a two parallel pseudo-first order reactions.

Castanon and Wilke[15] and Ooshima *et al.*[16] have reported that the addition of nonionic surfactants to the enzyme solution prior to hydrolysis accelerate both the cellulose hydrolysis and enzyme recovery. Ooshima *et al.*[16] also suggested that nonionic surfactants play an important role in the hydrolysis of crystalline cellulose: Tween 20 disturbs the adsorption of endoglucanase, *i.e.*, varies the adsorption balance of endoglucanase and exoglucanase, enhancing the hydrolysis rate. This result, however, could not explain whether the surfactant changes the structure of cellulose.

Recently we have reported the adsorption behaviors of cellulase on cellulose with different crystallilities in nonionic surfactant solution.[17] It was shown that the surfactant disturbs the enzyme from cellulose, and enhance the hydrolysis of the cellulose.

In this work, we investigated the adsorption behaviors and kinetics on hydrolysis of a microcrystalline cellulose, Avicel PH 101, in the nonionic surfactant solution by cellulase components (Endo I, Exo II and their 1 : 1 mixture) purified from *Trichoderma viride*.

## Experimental

**Materials.** Major cellulase components, such as endoglucanase I (Endo I) and exoglucanase II (Exo II), were isolated from a commercial cellulase (Meicelase TP 60) derived from the fungus, *Trichoderma viride*, by a series of chromatography procedures such as Bio-Gel P 10, Bio-Gel P 100, DEAE-Sephadex A-50, SP-Sephadex C 50, and Avicel PH 101.[18] The purified Endo I and Exo II showed a single band on a SDS-polyacrylamide gel electrophoresis with the molecular weights of 52,000 and 62,000, respectively.[18] Endo I was identified as a typical endoglucanase, showing the highest activity towards carboxmethylcellulose. Exo II was identified as typical exoglucanase, having high specific activity toward Avicel and very low activity towards carboxmethylcellulose compared to endoglucanase.[18] Exo II was the major exoglucanase component, and occupied more than 90% of the total exoglucanase from *Trichoderma viride*. The substrate for the hydrolysis was Avicel PH 101 (FMC Co., USA). A nonionic surfactant, Tween 20, was obtained from Sigma Chemical Co.,(USA). All other reagents were of analytical grade.

**Adsorption of Cellulases on the Cellulose.** Avicel PH 101 was used as the cellulase adsorbent. A 50 mg of the cellulose was suspended in 5.0 mL of 0.1 M sodium acetate buffer, pH 4.8 containing 0, or 0.05 wt% Tween 20, and preincubated for 30 min at 15 °C. After preincubation, 4.0 mL of enzyme preparation (0.1-2.5 mg/mL) was added. The reaction mixture was subjected to reciprocal shaking at 120 strokes/min for 30 min, to attain the adsorption equilibrium, then centrifuged for 5 min at 5000 rev·min$^{-1}$. The amount of enzyme in a supernatant was determined by the Lowry method[19] using bovine serum albumin as a standard.

**Enzymatic Hydrolysis.** Avicel PH 101 was used as the substrate for the hydrolysis. A 25 mg of Avicel PH 101 was accurately weighed, immersed in 2 mL enzyme buffer solution at pH 5.0, then incubated at 50 °C with shaking at 120 strokes/min. The final enzyme concentration was 2 mg/mL. The reaction was stopped at a desired time by boiling for 10 min. The amount of reducing sugar release was estimated by the dinitrosalicylic acid (DNS) method using glucose as a standard.[20]

## Results and Discussion

**Adsorption Behaviors of Cellulase on Microcrystalline Celluloses.** From the Langmuir adsorption isotherm, the adsorption of cellulase can be described as follows:

$$[A]=\frac{[A_{\max}]\cdot K_{ad}[E]}{1+K_{ad}[E]} \quad (1)$$

where $A_{max}$ and $K_{ad}$ are the maximum amount of enzyme adsorbed per unit weight of cellulose and the adsorption equilibrium constant, respectively. [$E$] is the concentration of enzyme in liquid phase at the adsorption equilibrium. Figure 1 shows adsorption as a function of varying enzyme concentration at 15 °C. The adsorption isotherm, Eq. (1), can be rearranged as follows:

$$\frac{[E]}{[A]}=\frac{1}{K_{ad}\cdot[A_{\max}]}+\frac{1}{[A_{\max}]}\cdot[E] \quad (2)$$

Adsorption equilibrium constant ($K_{ad}$) and amount of maximum adsorption ($A_{max}$) were determined from the slope of plots of [$E$]/[$A$] *vs.* [$E$] using a least-square analysis. Then the plot of [$E$]/[$A$] *vs.* [$E$] gave fairly good straight lines as shown in Figure 2. These results imply that the adsorption of Endo I, Exo II and Endo I-Exo II mixture (1 : 1, mass ra-

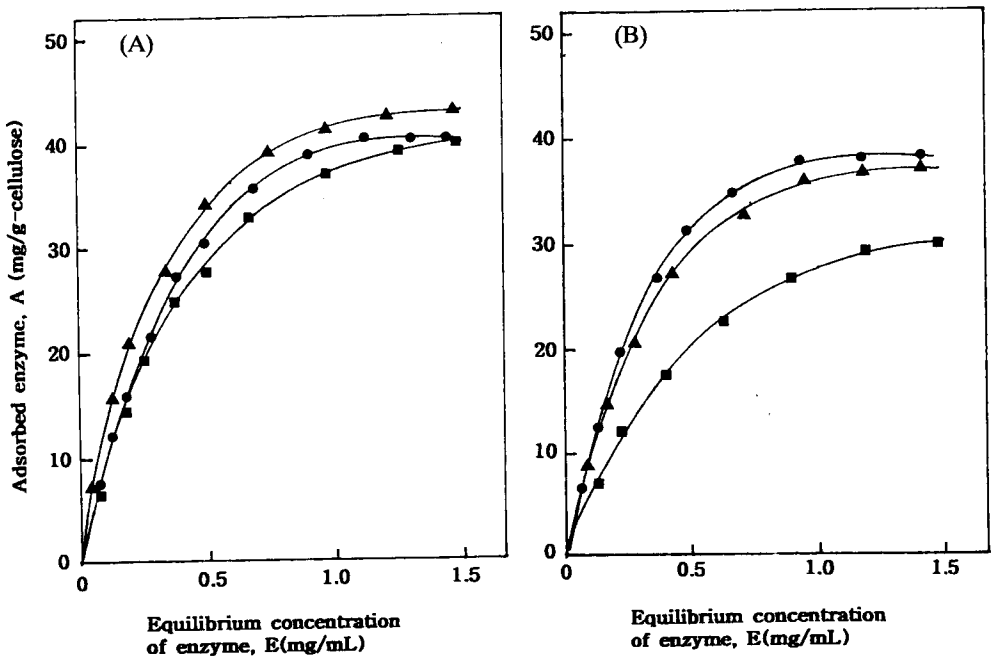


**Figure 1.** Adsorption isotherms of Endo I, Exo II, and Endo I-Exo II mixture on Avicel PH 101 in the presence and the absence of nonionic surfactant at 15 °C. Endo I (●), Exo II (■), Endo I-Exo II mixture (▲); (A), No surfactant; (B), Surfactant concentration (wt%) 0.05.

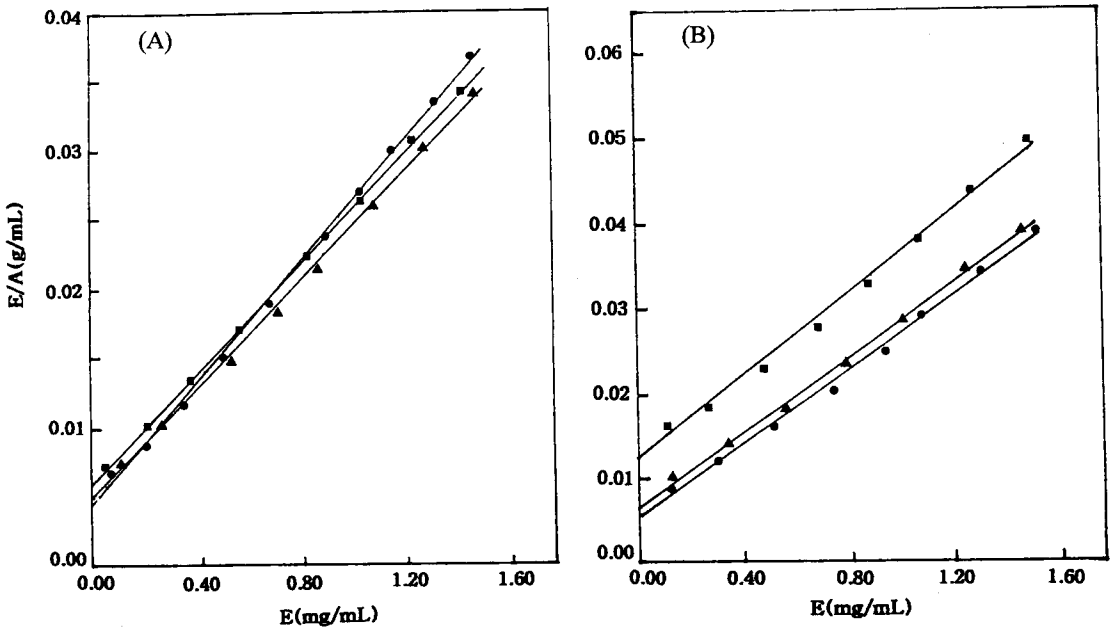


**Figure 2.** Langmuir plot for the adsorption isotherms of Endo I, Exo II, and Endo I-Exo II mixture on Avicel PH 101 in the presence and the absence of nonionic surfactant at 15 °C. Endo I (●), Exo II (■), Endo I-Exo II mixture (▲); (A), No surfactant; (B), Surfactant concentration (wt%) 0.05.

tio) on cellulose in the presence and absence of nonionic surfactant follow the Langmuir isotherm, as also observed by other investigations.[21,22] Eq. (2) is valid only if the adsorption site and adsorbate molecules are dependent and equivalent, and is not applicable to multi-equilibrium systems comprising two different adsorbates in general. Nevertheless, adsorption data for Endo I, Exo II, and their mixture are known to obey the Langmuir adsorption isotherm. Table 1 shows the Langmuir parameters for cellulase which were estimated from the data in Figure 2. The $K_{ad}$ value is an intensive property of adsorption and is a measurement for the adsorption affinity. The $K_{ad}$ value of Exo II was decreased by adding Tween 20. This indicates that the adsorption affinity of Exo II was decreased by the addition of the surfactant. On the contrary, $K_{ad}$ of Endo I was not affected by the nonionic surfactant. Therefore, there seems to be a difference in the driving force of adsorption between Endo I and Exo II. The $A_{max}$ value of Exo II and their mixture was decreased by adding Tween 20, while the $A_{max}$ of Endo I was not affected. These results indicate that the sur-

factant enhanced desorption of Exo II from insoluble substrate, while the surfactant has no effect on the desorption of Endo I. From these results may suggest that the surfactant have effect on the binding domain of Exo II.

**The Kinetics on the Hydrolysis of Cellulose.** Figure 3 shows the kinetics on the hydrolysis of cellulose with different cellulase components, such as Endo I, Exo II and Endo I-Exo II mixture (1 : 1, mass ratio) for 80 h in the presence or absence of Tween 20. For all enzyme-substrate mixtures an initial fast reaction phase during the initial 10 h is followed by a slow reaction phase. In addition, Exo II in combination with Endo I produced larger amount of reducing sugar than that of Endo I or Exo II alone in both the presence and absence of the surfactant. These phenomena are due to the synergism of Exo II in combination with Endo I. Exo II in the initial reaction produced lesser amount of reducing sugar than Endo I. But, the hydrolysis in later reaction time produced larger than Endo I. These phenomena indicated that the extent of hydrolysis highly depended upon the structural properties of the cellulose, mode of action of the cellulase, and the multiplicity of the cellulase complex. Also, all hydrolysis reactions by respective components in the presence of nonionic surfactant produced larger amount of reducing sugar than that in its absence. This indicates that the surfactant enhances the saccharification. The semilogarithmic plot of ln $C(100C_t/C_0)$ versus reaction time of the hydrolysis in Figure 4 also support that the kinetic has a two-phasic behavior which can be described by two parallel first order reactions as confirmed by others.[11~13,23] Two parallel first order reactions can be described by Eq. (3):

$$C_t = C_a \exp(-k_a \cdot t) + C_b \exp(-k_b \cdot t) \quad (3)$$

where $C_t$ is the residual concentration of cellulose in g/L;

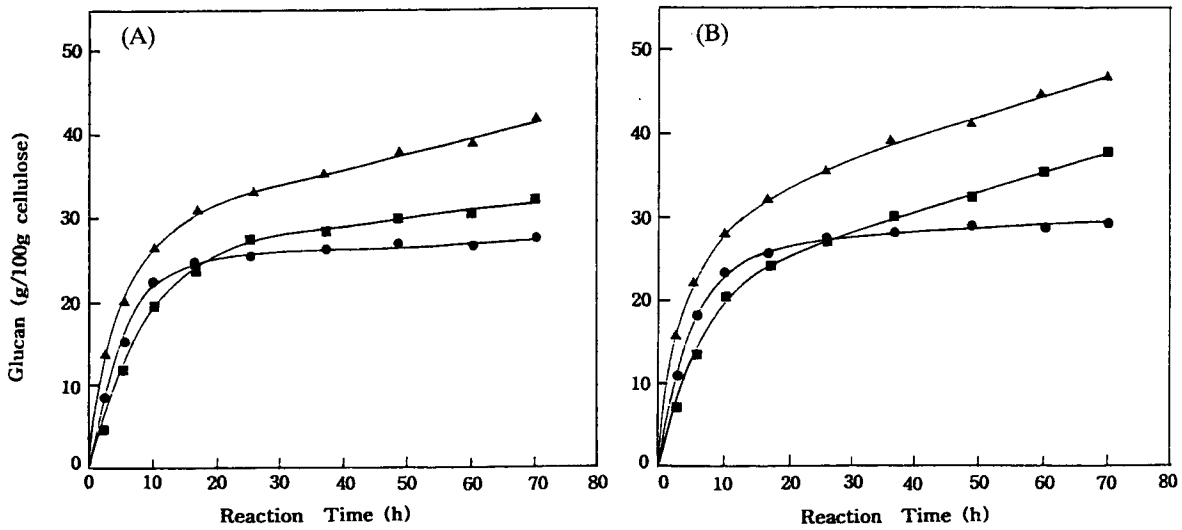


**Figure 3.** Kinetics on hydrolysis of Avicel PH 101 with Endo I, Exo II, and Endo I-Exo II mixture in the presence and the absence of 0. 05% tween 20. Endo I (●), Exo II (■), Endo I-Exo II mixture (▲); (A), No surfactant ; (B), Surfactant concentration (wt%) 0.05.

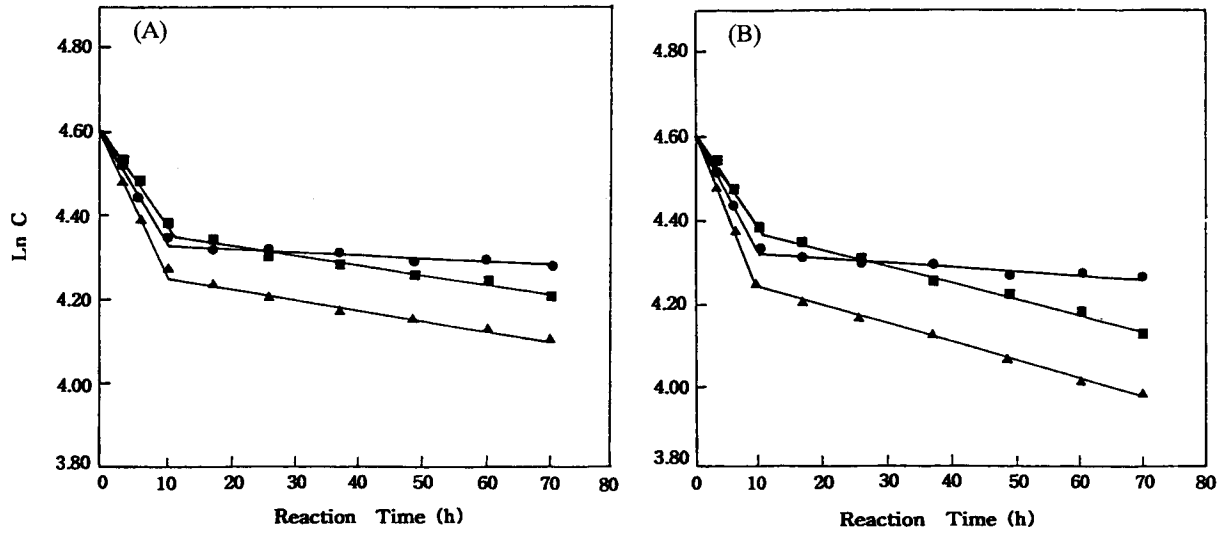


**Figure 4.** Semilogarithmic plot of residual unhydrolyzed cellulose *vs.* reaction time. Endo I (●), Exo II (■), Endo I-Exo II mixture (▲); (A), No surfactant; (B), Surfactant concentration (wt%) 0.05.

and $C_a$ and $C_b$ are the initial concentrations of the easily ($C_a$) and the hardly ($C_b$) hydrolyzable portions of cellulose in g/L. Rate constants (h $^{-1}$) of $k_a$ and $k_b$ are associated with $C_a$ and $C_b$, respectively. The symbol of $t$ respresents hydrolysis time. In the experiment, one measures the concentration of the solubilized reducing sugars at different times from which the concentration of cellulose hydrolyzed (Y) can be calculated. The following formula gives the relationship between hydrolyzed cellulose and residual cellulose:

$$Y=(C_a+C_b)-C_t \tag{4}$$

Inserting Eq. (3) in Eq. (4) gives:

$$Y=(C_a+C_b)-C_a\exp(-k_a\cdot t)-C_b\exp(-k_b\cdot t) \tag{5}$$

Taking into account the initial substrate concentration $C_0$, the fraction of substrate hydrolyzed is given by Eq. (6):

$$\frac{Y}{C_0}=\frac{C_a+C_b}{C_0}-\frac{C_a}{C_0}\exp(-k_a\cdot t)-\frac{C_b}{C_0}\exp(-k_b\cdot t) \tag{6}$$

Only for cellulosic substrates consisting of 100% glucan, the sum of $C_a$+$C_b$ is identical to the initial substrate concentration ($C_0$). For the Avicel PH 101 used in our experiment, we have estimated the glucose content and found on average 98% glucan and traces of mannans. Therefore, under this experimental condition, the ratio $(C_a+C_b)/C_o$=1. The amount of $Y/C_o$ is given by

$$\frac{Y}{C_o}=1-\frac{C_a}{C_o}\exp(-k_a\cdot t)-\frac{C_b}{C_o}\exp(-k_b\cdot t) \tag{7}$$

where the ratio $Y/C_o$ is the fraction of hydrolyzed cellulose in $g/g$ initial substrate at time $t$, and $C_a/C_o$ and $C_b/C_o$ give the fractions of the two types of the cellulose in the substrate used. This Equation should be valid for all cases in which the substrate used for the enzymatic hydrolysis consists entirely of cellulose. We have applied the nonlinear least square (NLS) method[11,13] using Eq. (7) to determine $k^a$, $k_b$, $C_a/C_o$, and $C_b/C_o$. Figure 3 are those calculated by NLS method and the experimental data are in a very good agreement with the calculated curve.

**Table 1.** Langmuir parameters for the adsorption of cellulases on Avicel PH 101 at 15 °C

| Surfactant | Enzyme | $A_{max}$ | $K_{ad}\times10^{-4}$ |
|---|---|---|---|
| No surfactant | Endo I | 45.80 | 4.60 |
| | Exo II | 50.30 | 3.40 |
| | Endo I-Exo II mixture | 51.02 | 3.95 |
| Surfactant | Endo I | 44.96 | 4.40 |
| conc.(wt%) 0.05 | Exo II | 40.05 | 1.90 |
| | Endo I-Exo II mixture | 40.78 | 3.20 |

The percentages of easily ($C_a$) and hardly ($C_b$) hydrolyzable cellulose in Avicel PH 101 and associated rate constants $k_a$ and $k_b$ are given in Table 2.

As shown in Table 2, the $C_b$ values for Exo II are very large. This means that the enzyme action of Exo II towards cellulose is not effective by alone. The $C_a$ values were increased by adding Tween 20 in all cases. This result suggests that low-ordered crystalline structure in the cellulose fiber exists and surfactant may decrease $C_b$ part and increase $C_a$ part of the cellulose. That is, the low-ordered crystalline or crystalline part in the cellulose may be partly dispersed by the surfactant. The $k_a$ values are in general about 100 fold greater than those of the $k_b$ value. The $k_a$ value was not affected by adding Tween 20 for Endo I, Exo II and their mixture. Also, the high $k_b$ value for Endo I-Exo II mixture is due to the synergistic effect. The $k_b$ value for Exo II was increased by adding Tween 20, while $k_b$ for Endo I was not affected. This may suggest that the surfactant play an important role in action of Exo II. Perhaps this may suggest that the surfactant help the Exo II desorb from the crystalline cellulose, and increase hydrolysis rate. This indicates that the enhancement of saccharification of the cellulose by adding Tween 20 is caused by increment of Exo II action.

The heat of adsorption of Exo II is much larger than that of Endo I.[17] Kim *et al.*[7] described the tightness of adsorption as change of adsorption enthalpy. Therefore, the tightness of adsorption for Exo II are much larger than that of Endo I. The $K_{ad}$, and $A_{max}$ value for Exo II decreased by adding Tween 20. This indicated that the tightness of the adsorption of Exo II was reduced by the addition of the surfactant. This may be related to surfactant binding on a cellulase. Exoglucanase was known to have hydrophobic region.[24] It is thought that the hydrophobic interaction between Exo II and cellulose by surfactant help the Exo II to desorb from the binding site on the cellulose. Exo II was the major exoglucanase component occupying more than 90% of the total exoglucanase from *Trichoderma viride*. Also, Exo II was the major cellulase component in Meicelase TP 60 occupying more than 90% of the total cellulase content.[18] The improved hydrolysis rate by adding a nonionic surfactant was postulated to the result from a reduced Exo II loss by irreversible binding to the substrate. That is, by using surfactant the Exo II deactivation can be prevented, and the saccharification can be enhanced. From these results, it was found that the increased sacchrification by surfactant is due to the activation of Exo II and partial defibrillation of cellulose.

**Table 2.** Precentages of easily ($C_a$) and hardly ($C_b$) hydrolyzable cellulose in Avicel PH 101 and associated hydrolysic rate constants, $k_a$ and $k_b$. The values for $100(C_a/C_0)$, $100(C_b/C_o)$, $k_a$, and $k_b$ were obtained by a nonlinear least-square analysis of the experimental results as shown in Figire 3

| | No surfactant | | | | Surfactant conc. (wt%) 0.05 | | | |
|---|---|---|---|---|---|---|---|---|
| | $C_a$ (%) | $C_b$ (%) | $k_a$ (h$^{-1}$) | $k_b\times10^2$ (h$^{-1}$) | $C_a$ (%) | $C_b$ (%) | $k_a$ (h$^{-1}$) | $k_b\times10^2$ (h$^{-1}$) |
| Endo I | 28 | 72 | 0.22 | 0.43 | 39 | 61 | 0.23 | 0.45 |
| Exo II | 15 | 85 | 0.13 | 0.58 | 23 | 77 | 0.14 | 0.90 |
| Endo I-Exo II mixture | 34 | 66 | 0.25 | 0.67 | 45 | 55 | 0.24 | 0.98 |

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# Cycloplatinated Complexes of Thiosemicarbazones. Synthesis and Crystal Structure of $[Ph_2PC_6H_4CHNNC(S)NHCH_3PtCl]$

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The synthesis and characterization of the platinum heterocyclic carboxaldehyde thiosemicarbazone complexes $[NC_5H_4CRNNC(S)NHR'PtCl]$ (R=H, R'=$CH_3$(**1**); R=$CH_3$, R'=$CH_3$(**2**); R=$CH_3$, R=H(**3**)) and diphenylphosphinophenyl carboxaldehyde thiosemicarbazone complexes $[Ph_2PC_6H_4CHNNC(S)NHRPtCl]$ (R=$CH_3$(**5**); R=$^iC_3H_7$ (**6**); R=Ph(**7**)) are described. Compounds **1-3** were prepared by reaction of $Pt(SEt_2)_2Cl_2$ with 2-acetylpyridine-4-alkylthiosemicarbazone in the presence of $NEt_3$. Compounds **5-7** were prepared using $Pt(SEt_2)_2Cl_2$ in toluene with diphenylphosphinophenyl carboxaldehyde alkylthiosemicarbazone. The compounds have been characterized by microanalysis, NMR ($^1H$, $^{13}C$, $^{31}P$) spectroscopy, and single-crystal X-ray diffraction. X-ray single crystal diffraction analysis reveals that compound **5** is a mononuclear platinum compound with P,N,S-coordination mode.

## Introduction

Cyclometalated compounds have been extensively studied because of their potential utility in organic synthesis, and a number of their synthethic approaches have been investigated.[1] Recent reports of platinum(II) cyclometalated compounds concern their photochemical and electrochemical properties.[2] Moreover, there is an increasing interest in platinum(II) with either bidentate (C,N; P,N)[3] or terdentate (N, C,N'; C,N,N'; P,C,P'; P,N,C; P,N,P'; S,N,C)[4] ligands. However, platinum(II) complexes of the types [PtX(N,N',S)] or [PtX(P,N,S)] have not been reported. Recently, we have extensively studied the terdentate heterocyclic carboxaldehyde thiosemicarbazone ligands $[NC_5H_4CRNNHC(S)NHR']$ which is a potentially terdentate N,N',S-chelating system.[5] The terdentate heterocyclic carboxaldehyde thiosemicarbazones have been shown to from complexes with various metal ions including Cu(II),[6] Ni(II),[7] Co(II),[8] Fe(II),[9] Hg(I),[10]