Adsorption of *Trichoderma reesei* Cellulase on Softwood Lignin-based Lignophenol

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Lignophenol is a lignin-based polymer which highly conserves the native lignin structure. Through simple adsorption experiments between *Trichoderma reesei* cellulase and softwood lignophenol synthesized from Hinoki cypress and p-cresol, several ideas were obtained on non-productive cellulase binding to lignin expected during enzymatic saccharification of cellulosic substrates. Cellulase is promptly bound to lignin within 10 min. The adsorption reaches a saturated value, indicating that cellulase adsorption on lignin is not multi-layered. Maximum adsorption is observed at around the isoelectric point of cellulase and, at the same time, phenolic hydroxyl groups in lignin seem to enhance cellulase binding. Cellulase bound to lignin maintains its enzymatic activity and probably functions as an immobilized cellulase in saccharification liquor.

**Key Words**
Enzymatic saccharification, Enzymatic hydrolysis, Cellulosic biomass, Cellulase, Lignin, Lignophenol, Non-productive binding, Adsorption

1. Introduction

The cost of cellulase still has a big impact on the price of the chemicals derived from cellulosic biomass. It has been noticed that a part of cellulase is non-productive due to cellulase binding to lignin in cellulosic substrates during enzymatic saccharification 1–5. The addition of a surfactant 6 or another protein like bovine serum albumin 5–7 has been extensively studied to suppress cellulase adsorption on lignin and to enhance saccharification efficiency. It is also important to develop a process to recover and reuse cellulase after enzymatic saccharification, when cellulase remains in the saccharification liquor with resultant glucose or is bound to unhydrolyzed residue mainly composed of lignin 8–10. Adding fresh cellulosic substrates is one easy method to recover free cellulase from the liquor. After several times of dosing fresh substrates, most of the cellulase is expected to be bound to lignin-rich residue accumulated in saccharification liquor. For the purpose of understanding the influence of lignin on enzymatic saccharification, many studies have been recently coming up about adsorption of cellulase on various pretreated cellulosic substrates 11,12 and isolated lignins, e.g. Bjorkman lignin 13, alkali lignin 14, and cellulolytic enzyme lignin 15,16. However, we still have lacked information on cellulase binding to lignin because of impossibility to isolate lignin in a high yield as it is in the substrates without changing its molecular structure.

Lignophenol is a lignin-based functional phenolic polymer synthesized from lignocellulosic material and a given phenol derivative at room temperature through "phase separation system" 17. In this system, lignin is considered not to suffer from structural change except grafting of the phenol derivatives to its benzyl position (Fig. 1). Namely, the resulting lignin (lignophenol) highly conserves native lignin structure. In this study, we examined binding of *Trichoderma reesei* cellulase to lignin by simple adsorption experiments using lignophenol as a model lignin, and tried to evaluate the nature of lignin-cellulase complex.

2. Experimental

2.1 Synthesis of ligno-p-cresol

Ligno-p-cresol was isolated from extractive-free wood meals of Hinoki cypress (*Chamaecyparis obtusa*) through the two step process II of the phase separation system 17 as a representative of softwood lignophenol. The procedure was shown in Fig. 2. Seventy two percent of sulfuric acid...
was added to wood meals sorbing \( p \)-cresol. The mixture was stirred vigorously for 1 hour at room temperature, and then poured into excess water with vigorous stirring. The precipitate was washed and extracted with acetone. The acetone solution was added dropwise to an excess amount of cold diethyl ether with vigorous stirring. Ligno-\( p \)-cresol is collected by centrifugation and dried.

The molecular weight distribution of the ligno-\( p \)-cresol was measured by GPC (Shimadzu Class LC-10 system) equipped with four columns (Shodex KF804, KF803, KF802 and KF801 in series) using THF as an eluent. The obtained \( M_w \) and \( M_n \) were 5500 and 3200, respectively. The FT-IR spectrum was recorded between 4000 and 400 cm\(^{-1}\) by KBr method. The KBr disk was prepared with a press kit (Shimadzu SSP-10A) under vacuum.

2.2 Adsorption of \( \textit{Trichoderma reesei} \) cellulase on ligno-\( p \)-cresol

Cellulase derived from \( \textit{Trichoderma reesei} \) was selected as a representative cellulase. Cellulase powder was purchased from SIGMA for adsorption tests. The adsorption procedure was shown in Fig. 3. The cellulase powder was dissolved in acetate buffer (pH=5) to be 1.4, 5, 8, 10 mg/mL. Ten mg of ligno-\( p \)-cresol powder is thoroughly dispersed in 0.5 mL of acetate buffer in a 2 mL vial using ultrasonic wave, to which 0.5 mL of cellulase solution was added. In order to study the effect of pH on the adsorption of cellulase, 0.01 M hydrochloric acid, phosphate buffers and 0.01 M sodium hydroxide were used instead of acetate buffer. The mixture was stirred by a magnetic stirrer for 10 min at 20°C. After centrifugation and filtration of the supernatant, the 0.1 mL of supernatant solution was taken for the quantification of the cellulase remaining in the supernatant based on Bradford method. The amount of the adsorbed (immobilized) cellulase on lignophenol was calculated by subtracting the remaining cellulase from the amount of the cellulase initially added to the solution.

2.3 Acetylation of ligno-\( p \)-cresol

Eighty milligrams of ligno-\( p \)-cresol was completely dissolved in 0.8 mL of pyridine. Then, 0.8 mL of anhydrous
acetic acid was added and stirred for 48 hours. The mixture was poured into 32 mL of cold water. The insoluble material was recovered by centrifugation (5 min, 3500 rpm), washed thoroughly and dried over P₂O₅. Acetylation was confirmed by FT-IR.

2.4 Enzymatic activity of lignophenol-cellulose complex
The 0.1 mL cellulase solution of 2, 5, 10 mg/mL was added to 10, 13, 25 mL of acetate buffer and mixed with 6.8, 13, 25 mg of ligno-p-cresol for 10 minutes, respectively. The resultant lignophenol-cellulose complex was obtained by centrifugation and was added into 5 mL of acetate buffer. Fibreform α-cellulose (0.2 g, Nacalai Tesque) was provided as a substrate. The mixture was stirred at 37°C for 2 hours and then filtrated. The produced sugar in the filtrate was quantitatively determined by absorbance measurement at 490 nm based on phenol-sulfuric acid method.

3. Results and discussion

3.1 Adsorption of cellulase on ligno-p-cresol
Fig. 4 shows the amount of adsorbed cellulase at the corresponding dosing time. The maximum adsorption around 75 mg/g ligno-p-cresol was achieved within 10 min. It was found that cellulase promptly constructs a complex with lignin only by simple stirring.

Fig. 5 shows the effect of the initial cellulase concentration on the adsorption of cellulase. Even with an excess amount of cellulase, the adsorption seemed to be limited to around 75 mg/g ligno-p-cresol. Therefore, this value is likely to be the maximum adsorption capacity, suggesting that the cellulase binding on lignin is not multi-layered. On the other hands, in the case of dilute cellulase concentration, the cellulase seemed to be totally adsorbed on ligno-p-cresol because the absorbance of the supernatant is nearly zero based on Bradford method. The result indicates that the adsorption of cellulase is favored rather than the detachment from lignin. All cellulase must be recovered from the liquor by the corresponding amount of lignin-rich residue during enzymatic saccharification of cellulose substrates.

Adsorption capacity has reached above 100 mg/g ligno-p-cresol in the latest adsorption assay with a lower concentration of ligno-p-cresol using a microtube and a shaking rotator. Dispersibility of ligno-p-cresol in aqueous solutions seems to be an important factor to increase the immobilization capacity.

3.2 Effect of pH on adsorption of cellulase
The maximum adsorption was observed around pH=2 ~ 5 including its optimum pH (Fig. 6). The adsorbed cellulase decreases with increase of pH, which is consistent with the previous studies using bovine serum albumin (BSA) and other proteins. Protein adsorption on lignophenol generally reaches maximum at around the isoelectric point of the protein. This phenomenon has been explained by the consideration that the affinity between protein and lignophenol is attributed to their hydrophobic interactions. Under alkaline conditions, proteins are negatively charged and the phenolic hydroxyl groups in lignophenol tend to dissociate. Consequently, protein and lignophenol probably repel each other at higher pH. Since the optimum pH of *Tricoderma reesei* cellulase is close to the isoelectric point, cellulase
would be bound to lignin as well as cellulose during enzymatic saccharification. The development of cellulase whose optimum pH is far from the isoelectric point might be a good option to overcome non-productive binding to lignin.

3.3 Acetylation of ligno-p-cresol
Since acetylated ligno-p-cresol has little hydroxyl groups (Fig. 7), the molecule becomes much more hydrophobic. However, adsorption was hardly observed on acetylated ligno-p-cresol. This data apparently contradicts the above-mentioned consideration that the cellulase adsorption on lignin is attributed to their hydrophobic interactions. In reality, the hydrophobicity caused the decrease of the surface area due to the self-aggregation in aqueous solutions rather than promoting adsorption. Hydroxyl groups, probably phenolic hydroxyl groups seem helpful to promote cellulase adsorption. This is consistent with the fact that lignophenols with higher frequency of phenolic hydroxyl groups show much higher protein capacity than ligno-p-cresol. Some pretreatment methods for cellulosic materials increase the phenolic hydroxyl groups of the lignin in the substrate through the cleavage of aryl ether linkages, which would enhance cellulase binding to lignin.

3.4 Enzymatic activity of lignophenol-cellulase complex
Lignophenol-cellulase complex maintains relatively high activity: about half of the enzymatic activity of free cellulase (Fig. 8). This data suggests that cellulase bound to lignin does not lose its enzymatic activity and functions as an immobilized cellulase in saccharification liquor. The decrease of the activity is larger than that for immobilized β-glucosidase reported in a previous study. Solid substrates like cellulose are more likely to suffer from the decrease of contact frequency than water-soluble substrates due to immobilization.

Once the lignophenol-protein complex is constructed, the protein did not tend to leach into the solution due to the strong hydrophobic interaction. In our latest study, the release of cellulase from ligno-p-cresol was confirmed to be small (~ 7%) when mixing the complex in fresh acetate buffer. The enzymatic activity is estimated to mostly come from lignophenol-cellulase complex rather than the cellulase detached from ligno-p-cresol to the solution. Since cellulase should be bound to cellulose more strongly than lignin, it is possible that lignin-binding cellulase “migrates” to fresh substrates by simple mutual contact, which would be evaluated in our future work.

4. Conclusion
Through simple adsorption experiments between Trichoderma reesei cellulase and Hinoki cypress ligno-p-cresol, we obtained several ideas on cellulase binding to lignin expected during enzymatic saccharification of cellulosic substrates as follows.

[1] Trichoderma reesei cellulase is promptly adsorbed on lignin within 10 min. The adsorption reached a saturated value, indicating that cellulase adsorption is not multi-layered on lignin.

[2] Maximum adsorption is observed at around the isoelectric point of cellulase, suggesting that hydrophobic interaction is dominant between cellulase and lignin. At the same time, phenolic hydroxyl groups in lignin seem to enhance cellulase binding.
Cellulase bound to lignin maintains enzymatic activity and is probably working as an immobilized cellulase in saccharification liquor.

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