Effect of Carboxylated and Quaternized Lignin and Epoxidized Lignin on Enzymatic Hydrolysis of Corn Stalk

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Lignin structure is known to have significant effects on the enzymatic hydrolysis efficiency (EHE) of lignocellulose. In this study, the lignin produced from corn stalk pretreated with p-toluenesulfonic acid (PCS) was used to prepare carboxylated and quaternized lignin (CQCL) and epoxidized lignin (ECL), and the effect of the two modified lignin forms on the EHE of PCS was investigated. The results showed that EHE after adding CQCL (83.7%) was higher than adding ECL (60.8%). To explore the reasons, the unproductive adsorption, cellulase-lignin interaction, and molecular dynamics experiments were conducted. The results showed that the smaller hydrophobic interaction and electrostatic attraction between CQCL and cellulase caused less unproductive adsorption between CQCL and cellulase than ECL. Additionally, CQCL and ECL changed differently the conformations of key amino acid residues of cellobiohydrolase I and endoglucanase II, which was also responsible for the higher EHE after adding CQCL.

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INTRODUCTION

As an abundant and renewable energy source, lignocellulose is the best candidate to replace fossil fuels (Silveira and Skaf 2015). When using lignocellulose to produce energy, enzymatic hydrolysis is a key step in the conversion of cellulose to glucose (Huang *et al*. 2023). However, the lignin in lignocellulose inhibits the enzymatic hydrolysis efficiency (EHE) of cellulose (Silveira and Skaf 2015). Effective removal of lignin by pretreatment is an effective method to improve the hydrolysis efficiency of lignocellulose (Tang *et al.* 2023; Wu *et al.* 2021). However, most methods can't completely remove lignin, so the studies of lignin are still important. The chemical structures of lignin are related to EHE (Li *et al*. 2018; Huang *et al*. 2023). The phenolic hydroxyl group (PhOH) is an important functional group affecting the EHE, and its content is positively correlated with the adsorption amount of lignin on cellulase (Mou *et al*. 2020). In general, greater adsorption between lignin and cellulase results in less adsorption between cellulase and cellulose, resulting in a lower EHE (Huang *et al*. 2023). Therefore, it is necessary to reduce the content of PhOH in lignin to increase the EHE. Epoxidation can reduce the content of PhOH through the reaction between epichlorohydrin and PhOH (Pan *et al*. 2013).

Carboxymethyl and quaternary ammonium groups can also be grafted to PhOH of lignin, reducing the content of PhOH. (Zhan *et al*. 2019).

Additionally, Xiong *et al*. (2021) found that modified lignin changed the conformation of cellulase, but the phenomenon was not explained clearly. Molecular dynamics (MD) has been used to analyze the dynamic structure information of cellulase (Zhao *et al*. 2022). Silveira and Skaf (2015) studied how the mutation in the binding site affected the structure and enzymatic hydrolysis through MD. Zong *et al*. (2016) also found through MD that the hydrophobic protein HFBII enhanced the flexibility of the tunnel loops of cellulase CBHI. Therefore, MD was used for further investigation in this study.

In this study, the lignin isolated from the corn stalk was pretreated with p-toluenesulfonic acid (p-TsOH). The treated lignin was used to prepare carboxylated and quaternized lignin (CQCL) and epoxidized lignin (ECL) *in situ*. The different effects of the two types of modified lignin on the enzymatic hydrolysis and cellulase adsorption were analyzed through FT-IR, hydrophobicity, and zeta potential analyses. To clarify the interactions between the two lignins and cellulase, MD was conducted for the simulation and analysis of enzyme-lignin complexes. This study elucidates the effect of modified lignin on the enzymatic hydrolysis of cellulose.

EXPERIMENTAL

Materials

The untreated corn stalk sample was purchased from Xinping Nanen Sugar and Paper Co., Ltd (Yunnan, China). The cellulase (CTec2) was purchased from Novozymes (Tianjin, China). Epichlorohydrin (AR) and sodium chloroacetate (AR, 98.00%) were purchased from Shanghai Aladdin Biochemical Technology Co., Ltd (Shanghai, China). The (3-chloro-2-hydroxypropyl) trimethylammonium chloride (65 wt.%) was purchased from Shanghai Macklin Biochemical Co. Ltd (Shanghai, China). All other reagents used in the study are of analytical grade.

Preparation of Lignin

The corn stalk was pretreated with p-toluenesulfonic acid (PCS). As described in the literature (Xiong *et al.* 2021), p-TsOH pretreatment reduced the lignin content of corn stalk from 23.3% to 9.2%. The corn stalk lignin obtained during p-toluenesulfonic acid pretreatment (CL) and CQCL were prepared according to Xiong *et al*. (2021). ECL was prepared according to Pan *et al*. (2013).

Enzymatic Hydrolysis

The enzymatic hydrolysis was conducted according to Lan *et al*. (2020). EHE was calculated by Eq. 1,

$$EHE = W_1 * 0.9 / W_2 * 100\% \quad (1)$$

where W_1 is the weight of the reducing sugar in the hydrolysate (g), 0.9 is the conversion coefficient for the stoichiometry of cellulose conversion to reducing sugar, and W_2 is the weight of the carbohydrate in PCS (g).

Enzymatic Adsorption

The greater the adsorption capacity of lignin to cellulase, the less the enzyme protein content in the supernatant of the reaction solution, and the color of Coomassie Brilliant Blue turns cyan from dark red when combined with protein; accordingly, the enzymatic adsorption was tested by Bradford method according to Xiong *et al*. (2021). The percentage of cellulase adsorbed on lignin (PCAL, %) was calculated using Eq. 2,

$$PCAL = (m_1\text{-}m_2)/m_1 *100\% \quad (2)$$

where m_1 is the total amount of cellulase protein added to the reaction solution (mg) and m_2 is the amount of cellulase protein in the supernatant (mg).

Characterization of Lignin

FTIR

The dried ECL and CQCL were respectively mixed with KBr (1:50, w/w), pressed into thin sheets, and analyzed by FTIR spectrometer (TENSOR27, Bruker, Karlsruhe, Germany). The scanning range was 4000 to 400 cm^{-1}, and the resolution was 4 cm^{-1} (Xiong *et al*. 2021).

Hydrophobicity

The Rose Bengal dye method (commonly used to determine the hydrophobicity of samples including lignin) was used to determine the hydrophobicity of lignin according to Lan *et al*. (2020). The partitioning quotient (PQ) was calculated with Eq. 3,

$$PQ=M_1/M_2 \quad (3)$$

where M_1 is the amount of Rose Bengal dye adsorbed on the lignin (mg), and M_2 is the amount of dye not adsorbed on the lignin (mg). The slope of the fitted straight line plotted with PQ value as abscissa and lignin concentration as ordinate represented the hydrophobicity (L/g) of the lignin sample.

Zeta potential

The zeta potentials of ECL and CQCL were measured according to Xiong *et al*. (2021) using a dynamic light scattering analyzer (Zetasizer Nano ZS90, Malvern, UK). All experiments were performed at room temperature and were performed in triplicate.

MD

Construction of lignin models

The trimers of CQCL and ECL were designed as Fig. 1-(a), containing syringyl (S), guaiacyl (G), and *para*-hydroxyphenyl (H) units. According to Boltzmann distribution, the conformational isomer with the lowest energy represents the largest proportion of the distribution (Achinivu *et al*. 2021). In order to make the lignin structure more reasonable, the conformational isomer with the lowest energy was used to represent CL.

In the preparation of CQCL and ECL, both modifications were reacted at the position of PhOH (Zhan *et al*. 2019), so the β-O-4 bond which is widely distributed in lignin (Sheng *et al*. 2021). The β-1 bond, which is often used to construct lignin dimers (Zhan *et al*. 2019; Xiong *et al*. 2021), was designed as the linking bond of the trimer. All lignin models were constructed and optimized using the software Chem3D (Huang *et al*. 2022).

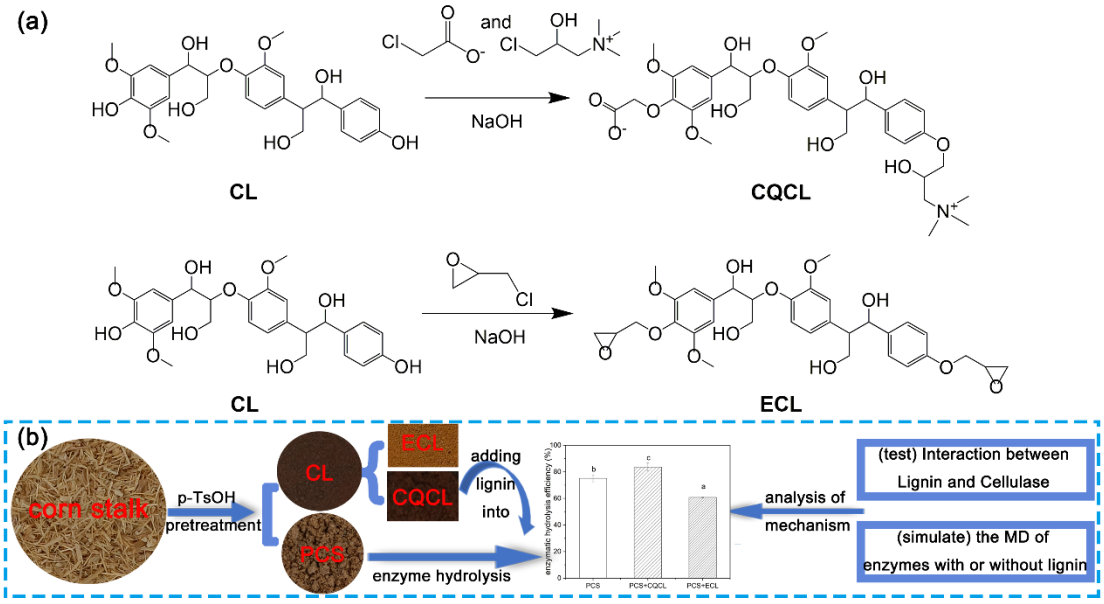

Fig. 1. (a) The schematic diagram of formation and structure of modified lignin, (b) the scheme about related mechanism in the study

Construction of cellulase models

CTec2, a commercial mixed cellulase, is produced by *Trichoderma reesei* (Jung *et al*. 2020). CBHI (a kind of cellobiohydrolase), EGII (a kind of endo-glucanase), and β-GL (β-glucosidase) are the key components in cellulase produced by fungi (Davison *et al*. 2019). Therefore, in this study the catalytic domains of CBHI, EGII, and β-GL were used to explore the effect of lignin on cellulase conformation. According to the literature (Xiong *et al*. 2021), 5CEL (CBHI), 3QR3 (EGII) and 3ZZ1 (β-GL) were obtained from the protein database (https://www.rcsb.org) and used in subsequent simulation and analysis.

MD of lignin and cellulase

The initial relative positions of lignin and cellulases were determined by Vina docking of Auto Dock (Huang *et al*. 2022). All the simulation systems were built using CHARMM-GUI (http://www.charmm-gui.org) (Jo *et al*. 2008). The cellulase-lignin complexes were placed in cube boxes with periodic boundary 1.2 nm, and the boxes were filled with water molecular models of TIP3P (Bharadwaj *et al*. 2020). In addition, to maintain the electric neutrality of the systems, Na^+ and Cl^- were added to the boxes. CHARMM General Force Field (CGenFF) and CHARMM36m were selected as the system force fields (Bharadwaj *et al*. 2020), and the temperature was set to 323.15K. All MD simulations and analysis were performed using Gromacs 2021.3.

Statistical Analysis

The software IBM SPSS Statistics 21.0 (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. The data were analyzed using independent sample t-test and one-way analysis of variance (ANOVA). The statistical significance was set at a level of $p < 0.05$. All experiments were performed in triplicate.

RESULTS AND DISCUSSION

Enzymatic Hydrolysis of PCS and Cellulase Adsorption of Lignin

To explore the effects of CQCL and ECL on the EHE of PCS, the two modified lignins were added to the enzymatic hydrolysis system of PCS, and the data of EHE are shown in Table 1. The EHE of PCS was 75.11%. After adding CQCL, the EHE of PCS was increased to 83.69%. These results were similar to previous reports (Zhan *et al*. 2019; Xiong *et al*. 2021). However, the EHE of PCS after adding ECL (60.77%) was lower than the EHE of PCS without modified lignin (75.11%). To learn the reason why CQCL improved but ECL inhibited the EHE of PCS, further exploration was conducted.

Table 1. Cellulase Adsorption and the Characteristics of Lignin

Lignin Samples	PCS	Adding CQCL	Adding ECL
EHE (%)	75.11% ±2.64^{b}	83.69±2.98^{c}	60.77±0.32^{a}
PCAL (%)	---	13.53±0.17^{a}	25.18±2.92^{b}

Because the unproductive adsorption of lignin on cellulase plays an important role in the enzymatic hydrolysis of cellulose, the cellulase adsorption amount of lignin was determined. The results associated with the percentage of cellulase adsorbed on lignin (PCAL) are shown in Table 1. The PCAL of CQCL was 13.53%, much lower than that of ECL (25.18%). The smaller cellulase adsorption amount of lignin is usually more beneficial to the EHE of lignocellulose (Huang *et al*. 2023). Therefore, the smaller cellulase adsorption amount of CQCL was responsible for the promotion of the EHE of PCS. To understand the reason for the less cellulase adsorption of CQCL, the interactions between cellulase and lignin were investigated.

Interaction between Cellulase and Lignin

The adsorption between lignin and cellulase is affected by three interactions: hydrogen bonding, hydrophobic interaction, and electrostatic interaction (Huang *et al*. 2023). Therefore, these three interactions were determined and analyzed.

To study the hydrogen bonding interaction, the FT-IR spectrum is the most commonly used method (Lan *et al.* 2020; Ou *et al*. 2023). FT-IR is also the most commonly used method to test the changes of lignin structure and functional groups (Lan *et al.* 2020; Wu *et al.* 2023). The FT-IR spectra of two kinds of lignin are shown in Fig. 2. In the spectrum of ECL, a new absorption peak appeared at 754 cm^{-1}, which means the success of ECL's modification (Pan *et al.* 2013). Because both carboxymethyl and quaternary ammonium were introduced into CQCL, carboxymethyl (1640 cm^{-1}) was not obvious in the FT-IR spectrum, but the peak area of CQCL at 1480 cm^{-1} was smaller than that of CL, indicating the success of CQCL's modification (Zhan *et al*. 2019). These results indicated that the modification of lignin was successful in this study.

The peak intensity of CQCL at 3410 cm^{-1} was larger than that of ECL. The peak at 3410 cm^{-1} corresponds to the total hydroxyl group of lignin (Jablonskis *et al*. 2018), suggesting that the hydroxyl content of CQCL was higher than that of ECL. The hydroxyl content is positively correlated to the number of hydrogen bonds (Ou *et al*. 2023). Therefore, the hydrogen bonding interaction between CQCL and cellulase was stronger than that of ECL. In general, stronger hydrogen bonding interaction means more cellulase adsorption on lignin (Xiong *et al*. 2021). In this study, CQCL adsorbed less cellulase,

indicating that hydrogen bonding interaction was not the main interaction between cellulase and lignin.

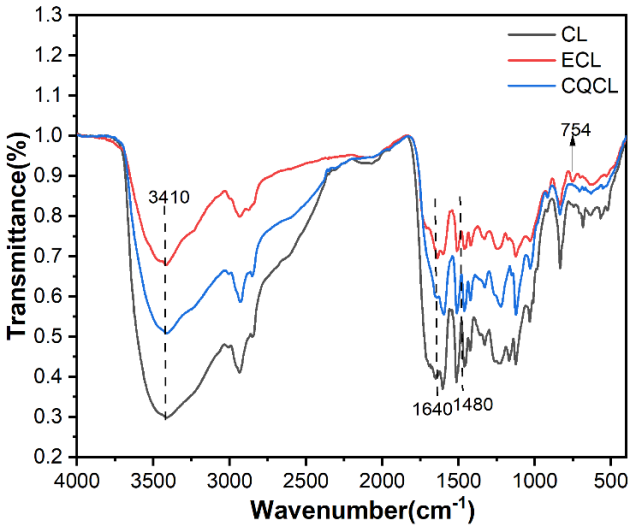

Fig. 2. The FT-IR spectrum of CQCL and ECL

The hydrophobicity and zeta potential of CQCL and ECL are shown in Table 2. The hydrophobicity of CQCL (0.04 L/g) was less than ECL (6.84 L/g). The hydrophobicity of ECL was stronger because the epoxy group induced in the epoxidation reaction is hydrophobic (Jablonskis *et al*. 2018). The hydrophobicity of CQCL was less because of the introduction of hydrophilic carboxymethyl groups (Zhan *et al*. 2019). Therefore, the hydrophobic interaction between CQCL and cellulase was weaker than that between ECL and cellulase, causing less cellulase adsorption on CQCL compared with ECL. In this study, the hydrophobicity interaction was a main force between cellulase and lignin.

Table 2. Hydrophobicity and Zeta Potential of Lignin

Lignin samples	CQCL	ECL
Hydrophobicity (L/g)	0.04±0.00[a]	6.84±0.31[b]
Zeta potential (mV)	-8.72±0.47[a]	-3.79±0.11[b]

The zeta potentials of CQCL and ECL are listed in Table 2. Both CQCL and ECL carried negative charges, and CQCL carried more negative charges (-8.72 mV) than ECL (-3.79 mV). According to the previous literature, the zeta potential of CTec2 in the enzymatic hydrolysis was also negative (Lan *et al*. 2020). Therefore, the electrostatic repulsion between CQCL and cellulase was greater than ECL, meaning that CQCL adsorbed less cellulase via electrostatic interaction. As a result, the electrostatic force was also the main interaction between cellulase and lignin in this study.

In sum, CQCL absorbed less cellulase because of smaller hydrophobic interaction and greater electrostatic repulsion. The hydrogen bonding interaction was not the key force between cellulase and lignin in this study.

MD Simulation

Root mean square deviation (RMSD)

RMSD results can be used to analyze the equilibrium state of the systems and the overall structural stability of enzymes (Huang *et al*. 2022). The RMSD values of six systems (5CEL-CQCL, 3QR3-CQCL, 3ZZ1-CQCL, 5CEL -ECL, 3QR3-ECL, and 3ZZ1-ECL) are presented in Fig.3. According to the study of Achinivu *et al*. (2021), when the fluctuation of RMSD was less than 0.1 nm, the system was stable. As can be seen in Fig.3, the systems of 5CEL, 3QR3, and 3ZZ1 adding CQCL and ECL were stable at 200, 280, and 100 ns, respectively. Therefore, the equilibrium states of systems at these time points were used to analyze the conformation of three kinds of cellulase.

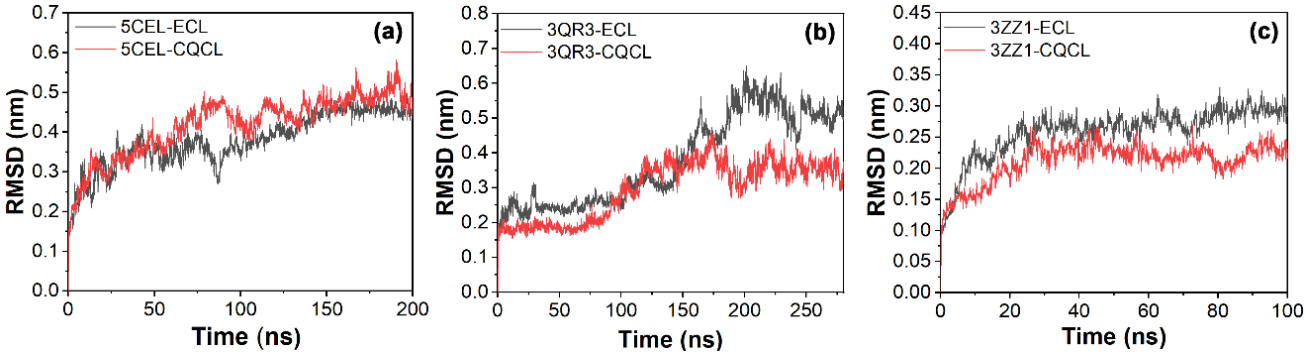

Fig. 3. The RMSD of cellulase in the cellulase-lignin complexes

Conformation analysis

The root mean square fluctuation (RMSF) can reflect the fluctuations of ammino acid residues in enzymes (Zong *et al*. 2016). The flexibility of the ammino acid residue loops plays a key role in the enzymatic hydrolysis (Zong *et al*. 2016). Therefore, RMSF values were determined in this study (Fig. 4). In Fig. 4, the key ammino acid residue loops in enzymes owning an absolute value of ΔRMSF more than 0.1 nm were marked, because these loops had significant differences in the conformation changes of ammino acid residues (Silveira and Skaf 2015). The loop conformations of cellulase-lignin systems in the equilibrium state are presented in Fig. 5.

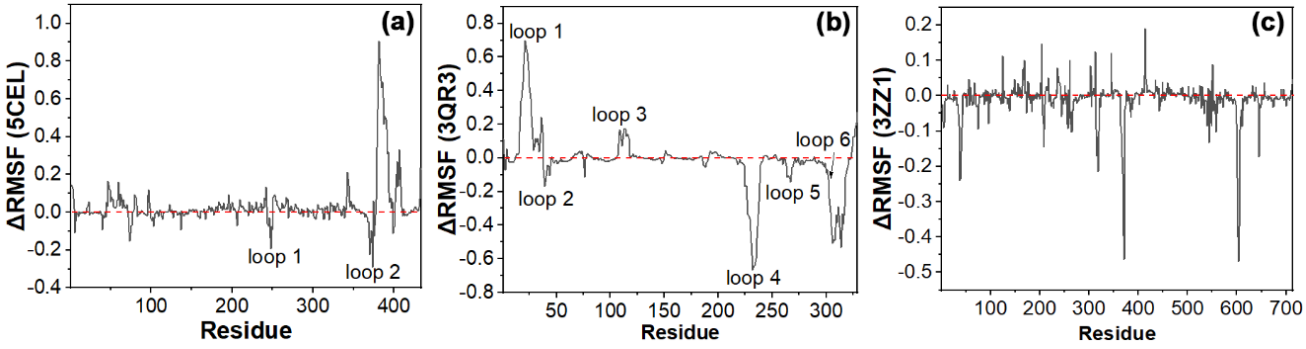

Fig. 4. The ΔRMSF of (a) 5CEL, (b) 3QR3 and (c) 3ZZ1 in the systems of enzyme-lignin complex. As for 5CEL, loops1 and 2 consist of 245-250 and 370-375 residues. As for 3QR3, loops 1 to 6 consist of 14−28, 34−40, 108−112, 222−234, 264−267 and 297-302 residues, respectively. *ΔRMSF (enzyme) = RMSF (enzyme-CQCL) - (enzyme-ECL) (Silveira and Skaf 2015)

From Fig. 4 and 5, CQCL and ECL had different effects on the conformations of three kinds of cellulase. As for 5CEL, after adding CQCL and ECL, there were two loops with a different RMSF in its three key loops (Zong *et al*. 2016). 3QR3 had eight key loops (Shu *et al*. 2014), and its six loops were significantly different in RMSF. 3ZZ1 had one key loop (Karkehabadi *et al*. 2014), but it did not have any loops with a significantly different RMSF. This suggested that the effect difference of CQCL and ECL on 5CEL and 3QR3 conformations were more significant than 3ZZ1, and these results helped to reveal the effect of CQCL and ECL on the enzymatic hydrolysis of PCS and cellulase adsorption on modified lignin.

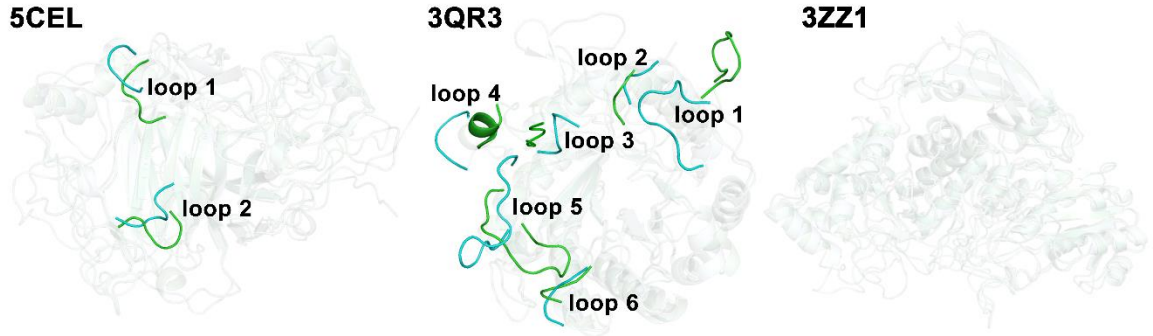

Fig. 5. The loop conformations of cellulase-lignin systems in the equilibrium state.
The loops in cellulase-ECL were shown in green, and those in cellulase-CQCL were in blue.

This study has provided theoretical support for further exploring the influence mechanism of modified lignin on enzymatic hydrolysis and the pretreatment scheme of lignocellulose. However, before the practical application of bio-refining, some other work not involved in this study needs to be completed, such as whether the reagent ratio is the best in the modification method used in this study.

CONCLUSIONS

1. Enzymatic hydrolysis efficiency (EHE) when adding carboxylated and quaternized lignin (CQCL) was significantly higher than with epoxidized lignin (ECL) because the cellulase adsorption of CQCL was less than ECL.
2. Weaker hydrophobic interaction and larger electrostatic repulsion between cellulase and CQCL caused less cellulase adsorption on CQCL compared to ECL.
3. Differences in conformation changes of the key amino acid residue loops of the cellulase enzymes 5CEL and 3QR3 might be responsible for PCS' EHE and cellulase adsorption of ECL and CQCL.

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