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Co-immobilization Mechanism of Cellulase and Xylanase on a Reversibly Soluble Polymer

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Abstract Cellulase and xylanase from *Trichoderma reesei* were immobilized simultaneously on Eudragit L-100, a reversibly soluble polymer. The effects of polymer concentration and polymer precipitation pH on enzyme activity recovery were investigated at an enzyme complex concentration of 1%. The immobilization mechanism of cellulase and xylanase on the polymer was discussed. An activity recovery of 75% and 59% was obtained for the cellulase and the xylanase, respectively, under the condition of a polymer concentration at 2% and a polymer precipitation pH at 4.0. Most zymoproteins might be connected to the polymer by electrostatic attraction in a medium of pH 4.8. In addition, the covalent coupling between the zymoproteins and the polymer was demonstrated by the infrared spectrograms. It was suggested that dehydration–condensation reaction occurred between the zymoproteins and the polymer during the immobilization.

Keywords Immobilization · Cellulase · Xylanase · Covalent coupling · Ionic adsorption

Introduction

Lignocellulose is the most abundant natural renewable resource. Cellulose and hemicellulose contained in lignocellulose can be converted to glucose-based six-carbon sugars and xylose-based five-carbon sugar by enzymatic hydrolysis using cellulase and xylanase [1, 2]. When an integrated wet-milling and alkali pretreatment is applied to lignocellulose prior to enzymatic hydrolysis, its crystalline structure is disrupted and lignin is removed, which significantly enhances the reducing-sugar yield in enzymatic hydrolysis [3]. On the other hand, some problems in cellulase and xylanase, such as (1) the poor stability, (2) the high

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production cost, and (3) the difficulty in recovery, should be overcome for industrial application of the enzymes in lignocellulose saccharification [4].

Immobilization is a technology for increasing the stability and realizing the reuse of enzymes [5]. In case of the immobilization of cellulase or xylanase, reversibly soluble carriers must be used since the lignocellulosic materials are insoluble. Eudragit L-100, a copolymer of methacrylic acid and methyl methacrylate with a mass ratio of 1:1, is soluble at a pH higher than 5.7 and insoluble at a pH lower than 4.4. It has been used to immobilize cellulase and xylanase separately. By changing the pH, the solubility of the immobilized cellulase or xylanase is switched, thus making recycling of the enzymes possible. The immobilization of cellulase or xylanase on the polymer allowed for improved stability, without major loss of activities [6, 7]. On the other hand, both cellulase and xylanase are simultaneously produced by microorganisms such as *Trichoderma* and *Aspergillus* [8]. The two enzymes are required for the effective hydrolysis of lignocellulosic materials, but only few reports on the immobilization of individual cellulase or xylanase could be found.

The co-immobilization, i.e., simultaneous immobilization, of cellulase and xylanase using Eudragit L-100 as the reversibly soluble carrier was proposed by the authors. It was found that the highest pH for the precipitation of Eudragit L-100 was increased from 4.4 to 4.7 by adding 1‰ of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) to the medium, so that the immobilized enzymes with EDC had more tight structures in comparison with that without EDC [9]. In the present research work, the effects of polymer concentration and polymer precipitation pH on enzyme activity recovery were investigated. The covalent coupling between zymoproteins and polymer was investigated by an infrared spectroscopic analysis, and the ion adsorption of zymoproteins on the polymer was discussed according to the isoelectric points (PI) of zymoproteins.

Materials and Methods

Enzymes and Carrier

A liquid enzyme complex (NS50013, Novozymes Investment) produced from *Trichoderma reesei* was used as the test enzymes. One milliliter of the enzyme complex had a cellulase activity of 100 FPU and a xylanase activity of 800 U under the condition of a temperature at 50°C and a pH at 4.8.

Eudragit L-100 (Evonik Degussa Investment) was used as the carrier. Its solubility was determined by measuring the absorbance at 470 nm with a spectrophotometer (752 S, Shanghai Lingguang Technology). The absorbance of 0% and 100% indicated the dissolved and the undissolved status of polymer, respectively [10].

Immobilization

Eudragit L-100 (0.05–2 g) was mixed with 40 mL of distilled water. The suspension was stirred by a magnetic stirrer (HJ-3, Changzhou Guohua Electric Appliance), and its pH was measured by a pH meter (PHS-3C, Shanghai Precision Scientific Instrument) in real time. A few drops of 2.5 M NaOH solution were added to raise the pH to 9.0. After the polymer was completely dissolved, the pH was adjusted to 6.0 with 10% acetic acid. EDC (0.05 g, 99%, Shanghai Medpep) was added as the condensing reagent for zymoproteins and polymer. Finally, the pH was reduced to 4.8 with 10% acetic acid, and the volume was

brought to 50 mL with 0.01 M acetate buffer (pH 4.8). The concentration of polymer solution ranged from 0.1% to 4%.

The commercial enzyme (0.5 mL) was added to 50 mL of the polymer solution at room temperature. After stirring for 10 min, the polymer was precipitated by lowering the pH to 3.0–4.6 with 10% acetic acid. The supernatant and the precipitate were separated by 30-min centrifugation (TDI-5, Shanghai Anting Scientific Instrument Factory) at 5,000 rpm. The precipitate was collected as the immobilized enzyme.

Enzyme activity recovery, protein recovery, and enzyme activity yield were calculated for cellulase and xylanase, respectively, as follows:

$$\text{Enzyme activity recovery}(\%) = \text{activity of immobilized enzyme}/\text{activity of commercial enzyme} \times 100\% \quad (1)$$

$$\begin{aligned}\text{Protein recovery}(\%) &= \text{mass of protein in immobilized enzyme(g)}/\text{mass of protein in commercial enzyme(g)} \times 100\% \\ &= [\text{mass of protein in commercial enzyme(g)} - \text{mass of protein in supernatant(g)}]/\text{mass of protein in commercial enzyme(g)} \times 100\%\end{aligned} \quad (2)$$

$$\begin{aligned}\text{Enzyme activity yield}(\%) &= \text{experimental activity of immobilized enzyme}/\text{theoretical activity of immobilized enzyme} \times 100\% \\ &= \text{activity of immobilized enzyme}/(\text{activity of commercial enzyme} - \text{activity of enzyme in supernatant}) \times 100\%\end{aligned} \quad (3)$$

Measurement of Enzyme Activity

Cellulase and xylanase activities of the commercial enzyme, immobilized enzyme, and supernatant of centrifugation were measured as follows. The immobilized enzyme was dissolved at pH 4.8 before the measurement.

1. Cellulase activity

The cellulase activity was measured using the method reported by Mandels et al. [11]. A unit of FPU was defined as the amount of cellulase that produces 1 μmol of reducing sugar (as glucose) from 50 mg filter paper in 1 min under the optimum condition of temperature at 50°C and pH at 4.8.

2. Xylanase activity

The xylanase activity was measured using the method reported by Bailey et al. [12]. A unit of U was defined as the amount of xylanase that produces 1 μmol of reducing sugar (as xylose) from 1% birchwood xylan in 1 min under the optimum condition of temperature at 50°C and pH at 4.8.

In the measurement of cellulase and xylanase activities, the reducing sugar was determined by the dinitrosalicylic acid (DNS) method using glucose and xylose as the standard, respectively [13].

Measurement of Protein

The protein contained in the commercial enzyme and the supernatant was determined by the Bradford method [14].

Infrared Spectroscopic Analysis

Infrared spectroscopic analysis was performed for the polymer, the commercial enzyme and the immobilized enzymes, using a Fourier transform infrared spectrometer (Avatar-360, Nicolet, USA). The polymer was mixed with KBr and then compressed into chips, while the commercial and the immobilized enzymes were coated on KBr chips. During the analysis, the resolution was set at 2 cm^{-1}, and the scan range was set at 500 – 2,000 cm^{-1} [15].

Results and Discussion

Effect of Polymer Concentration on Co-immobilization

Figure 1 shows the change of enzyme activity recovery with the polymer concentration. The polymer was precipitated at pH 4.0. For both cellulase and xylanase, the enzyme activity recovery increased with increasing polymer concentration in a polymer concentration range of 0.1% to 2%, and then it decreased when the polymer concentration was higher than 2%. The activity recovery of cellulase and xylanase reached a maximum of 75% and 59%, respectively, at the polymer concentration of 2%.

The change of protein recovery with the polymer concentration is shown in Fig.2. The protein recovery was only 48% at the polymer concentration of 0.1%. It increased with increasing polymer concentration in the polymer concentration range of 0.1% to 2%, and then was almost constant at 93% when the polymer concentration was higher than 2%. The polymer did not have enough capability to carry zymoproteins at a low concentration.

The change of enzyme activity yield with the polymer concentration is shown in Fig. 3. The enzyme activity yield of cellulase and xylanase was almost constant at 78% and 69%, respectively, in the polymer concentration range of 0.1% to 2%. It decreased with increasing polymer concentration when the polymer concentration was higher than 2%. The

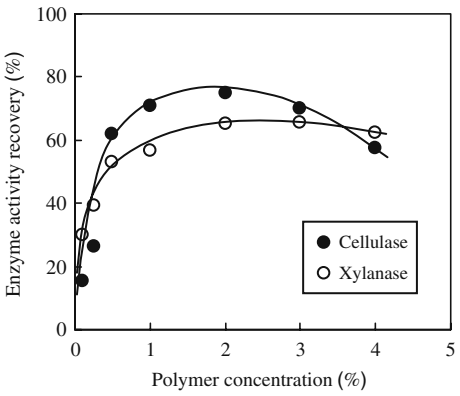

Fig. 1 Change of enzyme activity recovery with polymer concentration (polymer precipitation pH at 4.0)

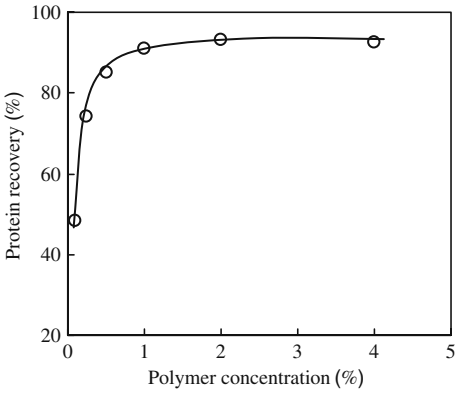

Fig. 2 Change of protein recovery with polymer concentration

decrease of enzyme activity yield at a high polymer concentration related to enzyme inactivation, probably due to the covering up of enzymes' active center by the polymer.

The protein recovery and enzyme activity yield were high at the polymer concentration of 2%, which resulted in a high enzyme activity recovery. It can be seen from Eqs. (1) to (3) that the enzyme activity recovery depended on the protein recovery and the enzyme activity yield, assuming that the proportion of "theoretical activity of immobilized enzyme"/"experimental activity of commercial enzyme" is equal to that of "protein mass of immobilized enzyme"/"protein mass of commercial enzyme".

Effect of Polymer Precipitation pH on Co-immobilization

The effect of polymer precipitation pH on co-immobilization was investigated at a polymer concentration of 2%. As shown in Fig. 4, the polymer precipitation pH affected significantly the enzyme activity recovery. The enzyme activity recovery had a maximum of 71% for cellulase and 58% for xylanase at pH 4.0.

When the polymer solution was mixed with the commercial enzyme complex and the EDC, it could only be precipitated at a pH lower than 4.6. At a lower pH, the precipitation of polymer became easier, which would result in a higher protein recovery. A lower pH, however, might cause the denaturation of cellulase and xylanase since the enzymes are sensitive to acidic pH conditions. For example, the activity of cellulase and xylanase

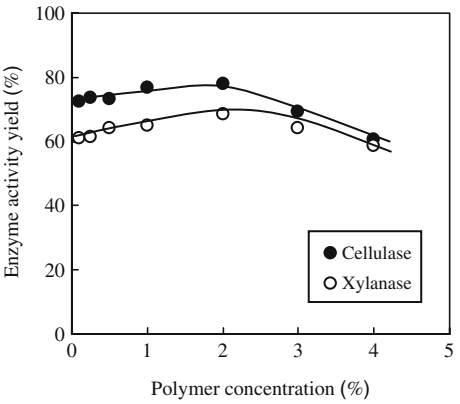

Fig. 3 Change of enzyme activity yield with polymer concentration

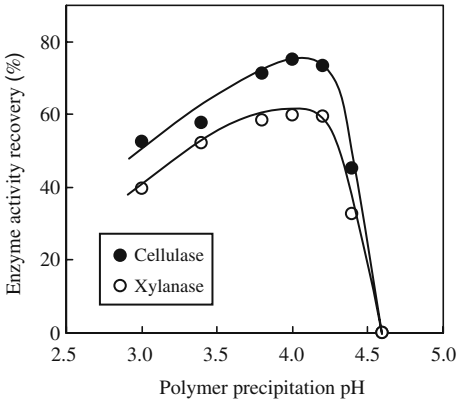

Fig. 4 Change of enzyme activity recovery with polymer precipitation pH (polymer concentration 2%)

decreased in 2 h from 100 to 86 FPU and from 800 to 634 U, respectively, in 0.01 M acetate buffer (pH 3.0) [9].

Interaction Between Enzymes and Polymer

1. Electrostatic attraction

Eudragit L-100 is a copolymer of methacrylic acid and methyl methacrylate with a proportion of 1:1. It had negative charges when mixed with distilled water, as shown in Fig. 5.

On the other hand, *endo*-1,4-β-D-glucanase (EG), *exo*-1,4-β-D-glucanase (CBH), and 1,4-β-D-glucosidase (BG) were the main active components of cellulase, and 1,4-β-D-xylanohydrolase and 1,4-β-D-xylan xylohydrolase were the main active components of xylanase [16, 17]. A two-way focusing gel electrophoresis showed that the PI was 4.0 for EGI and 4.6 for EGII, respectively, 4.5–5.2 for the most of CBHI(lowest at 3.5), 4.6–5.6 for CBHII, 5.8–6.6 for CBH III, and 6.0–6.5 for the most of BG [18]. The PI of 1,4-β-D-xylanohydrolase and 1,4-β-D-xylan xylohydrolase ranged from 5.1 to 5.5 [19].

In general, proteins carry positive charges in a solution with a pH lower than their PI [20]. It is suggested that at the pH condition (pH 4.8) for determining the activities of cellulase and xylanase, the BG and the most of CBH in cellulase, and the 1,4-β-D-xylanohydrolase and the 1,4-β-D-xylan xylohydrolase in xylanase might be connected with the polymer by an electrostatic attraction in the immobilized enzyme.

2. Covalent coupling

The infrared spectrograms for the commercial enzyme, the immobilized enzyme, and the polymer are shown in Fig. 6. Most absorption peaks of the immobilized enzyme

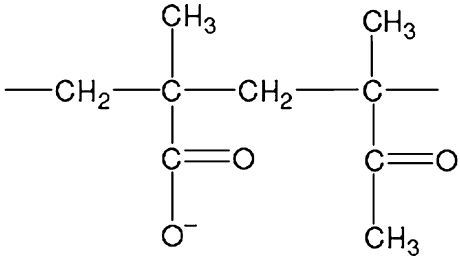

Fig. 5 Eudragit L-100 with negative charges in water

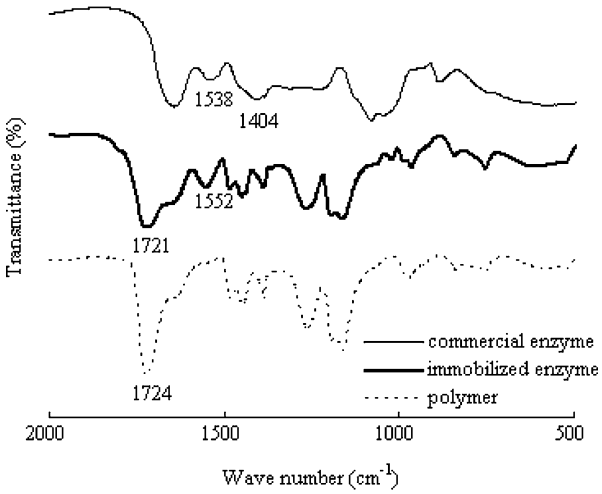

Fig. 6 Infrared spectrograms of the commercial enzyme, the immobilized enzyme, and the polymer

corresponded to those of the polymer, indicating that the basic structure of the polymer was retained after the immobilization. The absorption peak of the immobilized enzyme at 1,552 cm^{-1} was derived from the characteristic peak of the commercial enzyme at 1,538 cm^{-1}, which expressed the enzymes' α-NH specific conformation. This indicates that the zymoproteins were retained after the immobilization.

In addition, the absorption peak of the immobilized enzyme at 1,721 cm^{-1} was derived from the carbonyl's absorption peak of the polymer at 1,724 cm^{-1}. The absorption peak of the immobilized enzyme, however, had a lower and wider peak in comparison with that of the carbonyl. Meanwhile, the absorption peak of the commercial enzyme at 1,404 cm^{-1}, which expresses the β-NH conformation of enzymes, disappeared after the immobilization. It means that the dehydration–condensation reaction occurred between the zymoproteins and the polymer during the immobilization as shown in Fig. 7. Before the reaction, the polymer did not have nitrogen atoms, while the zymoproteins had primary amine groups. In the reaction, one oxygen atom of carboxyl in the polymer was replaced by a nitrogen atom in the zymoproteins. Therefore, the immobilized enzyme had a secondary amine. The nitrogen atom contained a lone-pair electron and provided an electronic track, which affected the absorption peak of the immobilized enzyme at 1,721 cm^{-1}.

Covalent coupling of enzymes, such as cellulase, α-amylase, and β-glucosidase, to Eudragit (L-100 and S-100) was also reported in earlier studies [21-25]. However, ultraviolet (UV) and fluorescence emission spectra indicated only slight structural changes in the protein conformation of immobilized trypsin, suggesting that the immobilization depended mainly on non-covalent methods, i.e., adsorption and entrapment [26]. Size exclusion chromatography and differential scanning calorimetry analysis suggested that the

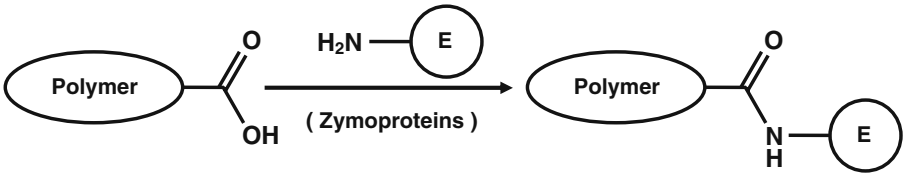

Fig. 7 Dehydration–condensation reaction between the zymoproteins and the polymer

amount of cellulase bound covalently to Eudragit was very small, and the intermolecular forces that bind the protein to the polymer were weak [7]. It is suggested that enzymes are immobilized on Eudragit by both covalent and non-covalent methods.

Covalent coupling of enzymes to Eudragit may result in a more stable preparation, in comparison with the adsorption of enzymes on the polymer by non-covalent forces. Further research work is necessary for increasing the covalent coupling in co-immobilization and the application of co-immobilized cellulase and xylanase in lignocellulose saccharification.

Conclusions

Cellulase and xylanase from *T. reesei* were immobilized simultaneously on Eudragit L-100, a reversibly soluble polymer. The experimental results are concluded as follows:

(1) An activity recovery of 75% and 59% was obtained for the cellulase and the xylanase, respectively, under the condition of an enzyme complex concentration at 1%, a polymer concentration at 2%, and a polymer precipitation pH at 4.0.
(2) The protein recovery decreased when the polymer concentration was lower than 1%. On the other hand, the enzyme activity yields of cellulase and xylanase decreased when the polymer concentration was higher than 2%. The activity recovery decreased at a low polymer precipitation pH due to the denaturation of enzymes.
(3) BG and most of CBH in the cellulase and 1,4-β-D-xylanohydrolase and 1,4-β-D-xylan xylohydrolase in the xylanase might be connected to the polymer by electrostatic attraction in a medium of pH 4.8.
(4) The covalent coupling between the zymoproteins and the polymer was demonstrated by the infrared spectrograms. It was suggested that dehydration–condensation reaction occurred between the zymoproteins and the polymer during the immobilization.

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