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Cellulase Hydrolysis of Rice Straw and Inactivation of Endoglucanase in Urea Solution

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ABSTRACT: In order to optimize the cellulase (from *Aspergillus glaucus*) hydrolysis of pretreated rice straw, the effects of varying enzyme concentration, temperature, and pH were studied. The best experimental conditions found to degrade the pretreated rice straws were 24 h of incubation at 55 °C and pH 5.0, with an enzyme concentration of 48 mg/L. Urea is one of the important nitrogen sources used in fungi culture, but it is also a denaturant. The model of denaturation of endoglucanase (EG) in urea solutions was established. The denaturation was a slow, reversible reaction. Determination of microscopic rate constants showed $k_{+0} > k'_{+0}$, indicating that EG was protected by the substrate to a certain extent during denaturation. Comparison with the results from fluorescence emission spectroscopy revealed that the inactivation of EG occurred before the marked conformational changes could be detected.

KEYWORDS: cellulase, hydrolysis, endoglucanase, denaturation, urea, *Aspergillus*

INTRODUCTION

Biofuels have become a hot topic among environmentalists and are gaining increased public and scientific attention in modern and growing economies, driven by factors such as oil price spikes, the need for renewable and sustainable energy, and concerns over greenhouse gas and toxin emissions from fossil fuels. Potential agricultural biomass residues in China such as sugar cane bagasse, rice straw, and wheat stalks can be exploited for bioethanol production.[1,2]

Cellulose is one of the most abundant nondegradable organic compounds on earth. About half of the agricultural solid wastes contain cellulose. Cellulosic material is insoluble and, without the presence of cellulase, is not accessible for direct bioconversion to ethanol. The hydrolysis of cellulose in agricultural residues via enzymatic action is critical in releasing monomeric sugars for fermentation to bioethanol.[3]

Fungal cellulases are inducible enzymes that are usually excreted into the environment. The production of cellulases by fungi are generally higher when organic nitrogen sources, such as urea, peptone, and yeast extract, are employed, compared to that of inorganic sources. As a convenient source of nitrogen, urea has the highest nitrogen content of all solid nitrogenous sources in common use, and it is cheaper than yeast extract or peptone. Meanwhile, urea is a chemical denaturant, which is often used to study protein conformation and unfolding, and can lead to the conformational change of the enzyme and the complete loss of enzyme activity.[4]

In our previous study,[5,6] a cellulase producing fungi *Aspergillus glaucus* XC9 was isolated, and the enzymatic properties of an endoglucanase (EG) were illustrated. In our progressing research, we have obtained the optimization of cellulase saccharification of pretreated rice straw. We also established an inactivation model and studied the conformational changes of EG during the denaturation in urea solutions monitored by fluorescence spectra.

MATERIALS AND METHODS

Materials and Reagents. Purified EG was prepared from *Aspergillus glaucus* XC9 as described previously.[6] Carboxymethylcellulose sodium (CMC-Na) was purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China); urea was purchased from Sigma (St. Louis, MO, USA). All other reagents were local products of analytical grade.

Pretreatment of Rice Straw. The rice straws were obtained locally. Washed rice straws were pretreated for 6 h at 80 °C with a 0.5 M NaOH solution using a solid−liquid ratio of 5% (w/v). After cooling down, the rice straws were filtered and neutralized. Then they were dried and chopped into small pieces by a chopper, then ground into smaller particles in a hammer mill, and finally separated by a 0.45 mm (40 mesh) sieve.[7]

Enzyme and Reducing Sugar Assay. Endoglucanase activity (CMCase) was measured by incubating 50 μL of the sample with 250 μL of 1% carboxymethylcellulose sodium (CMC-Na) in 700 μL of 50 mM NaAc buffer (pH 4.0) at 50 °C for 30 min.[8] The amount of reducing sugar produced was measured by the 3,5-dinitrosalicylic (DNS) reagent method.[9]

Enzymatic Saccharification. The *Aspergillus glaucus* XC9 strain was cultured in MS medium (0.3% w/v sugar cane bagasse as carbon source) for 3 days as described in a previous study.[6] Finally, the culture was centrifuged at 8000*g* for 20 min at 4 °C, and the supernatant was pooled as the crude enzyme. For the enzymatic saccharification study, 3% (w/v) rice straws were resuspended in solutions containing 50 mM

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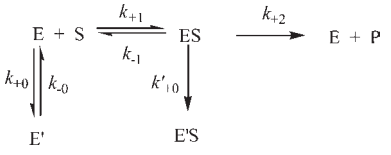
NaAc buffer (pH 5.0) and diluted crude enzyme. Hydrolysis was performed in shaken flasks in a shaking water bath (180 rpm) at the given temperature and pH for up to 24 h. The volume of the hydrolysis reaction was 100 mL. Samples were centrifuged at 8000*g* for 5 min, filtered, and stored at 4 °C awaiting sugar analysis.[10]

Enzyme Denaturation in Urea Solutions. Enzyme denaturation was carried out at 4 °C in solutions containing different concentrations of urea in 0.01 M Tris-HCl buffer (pH 7.5) for 24 h. Denatured enzyme solutions were used for activity assay and measurement of fluorescence spectra.[11] Fluorescence spectra were obtained with a Hitachi 850 spectrofluorometer. The excitation wavelength was 279 nm.

Determination of Inactivation Rate Constants. The progress-of-substrate-reaction method previously described by Tsou[12] was used for the study of the inactivation kinetics of *A. glaucus* EG. In this method, 10 μL of enzyme was added to 1.0 mL of reaction mixture with 4.5 mg/mL CMC-Na in 50 mM HAc-NaAc buffer (pH 4.0) containing different concentrations of urea. The reaction was carried out at a constant temperature of 50 °C.

The substrate reaction progress curve was analyzed to obtain the rate constants as detailed below. A reversible enzymatic reaction can be described in the following scheme:



where S, P, E, and E′ denote the substrate, product, and the native and inactivated enzyme, respectively; ES is the native enzyme−substrate complex; and E′S is the inactivated enzyme−substrate complex. k_{+0} and k_{-0} are rate constants for forward and reverse inactivation of the free enzyme, respectively; k'_{+0} is the inactivation rate constant of enzyme−substrate complex. As usual for the case of $[S] \gg [E_o]$, the product formation can be written as follows:

$$[P]_t = \frac{vk_{-0}}{A}\cdot t + \frac{v}{A^2}(A - k_{-0})(1 - e^{-At}) \quad (1)$$

where $[P]_t$ is the concentration of the product formed at time t, which is the reaction time; A is the apparent rate constant of inactivation; $[S]$ is the concentration of the substrate; and v is the initial rate of reaction in the absence of urea.

$$A = \frac{k_{+0}\cdot K_m + k_{+0}[S]}{K_m + [S]} + k_{-0} \quad (2)$$

$$v = \frac{V_m\cdot[S]}{K_m + [S]} \quad (3)$$

When t is sufficiently large, the curves become straight lines, and the product concentration is written as

$$[P]_{calc} = \frac{vk_{-0}}{A}\cdot t + \frac{v}{A^2}(A - k_{-0}) \quad (4)$$

Combining eqs 1 and 4 yields

$$[P]_{calc} - [P]_t = \frac{v}{A^2}(A - k_{-0})\cdot e^{-At} \quad (5)$$

$$\ln([P]_{calc} - [P]_t) = -A\cdot t + \text{constant} \quad (6)$$

where $[P]_{calc}$ is the product concentration to be expected from the straight-line portions of the curves as calculated from eq 4, and $[P]_t$ is the product concentration actually observed at time t. Plots of $\ln([P]_{calc} - [P]_t)$ versus t give a series of straight lines at different concentrations of denaturant with slopes of $-A$. The apparent rate constant A can be obtained from such graphs (see Figures 5B and 6B). From eq 4, a plot of $[P]_{calc}$ against time t gives a straight line with a slope of vk_{-0}/A. From the slope of the straight line, k_{-0} can be obtained.

Combining eq 2 and eq 3 gives

$$\frac{A}{v} = \frac{K_m}{V_m}(k_{+0} + k_{-0})\cdot\frac{1}{[S]} + \frac{k'_{+0} + k_{-0}}{V_m} \quad (7)$$

A plot of A/v versus $1/[S]$ gives a straight line with K_m k_{+0}/V_{max} and k'_{+0}/V_{max} as the slope and intercept, respectively. As K_m and V_{max} are known quantities from measurements of the substrate reaction in the absence of urea at different substrate concentrations, the rate constants k_{+0} and k'_{+0} can be obtained from the slope and intercept of the straight line, respectively.

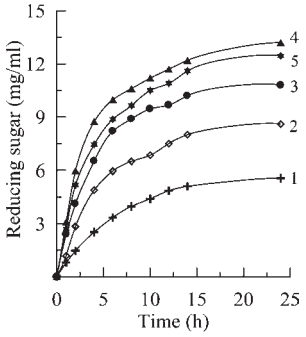


Figure 1. Enzymatic hydrolysis of treated rice straw at different concentrations of enzyme (55 °C, pH 5.0). The concentrations of the enzyme for curves 1−5 were 12, 24, 36, 48, and 60 mg/L, respectively. The values are the average of three independent experiments.

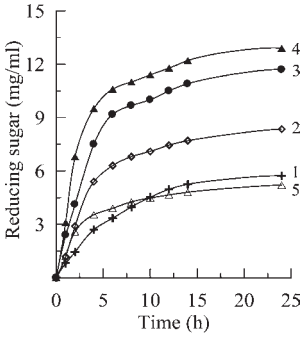


Figure 2. Enzymatic hydrolysis of treated rice straw at different temperatures (pH 5.0). The concentration of the enzymes was 48 mg/L. The temperatures for curves 1−5 were 30, 40, 50, 55, and 60 °C, respectively. The values are the average of three independent experiments.

RESULTS AND DISCUSSION

Enzymatic Hydrolysis at Different Enzyme Concentrations. Effects of enzyme concentrations on saccharification were studied at 55 °C, pH 5.0, and different concentrations of enzyme. The results showed that increasing the enzyme concentration produced a higher amount of soluble reducing sugars, although for enzyme concentrations over 48 mg/L, small differences were observed probably due to saturation of the substrate surface. For each concentration, the maximum velocity of hydrolysis occurred

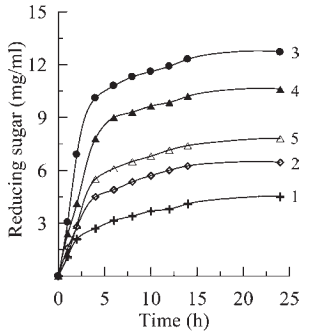


Figure 3. Enzymatic hydrolysis of treated rice straw at different pHs (55 °C). The concentration of the enzymes was 48 mg/L. The pHs for curves 1−5 were at pH 3.0, 4.0, 5.0, 6.0, and 7.0, respectively. The values are the average of three independent experiments.

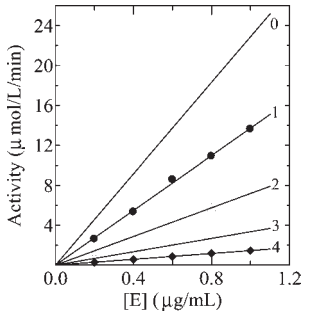


Figure 4. Effects of EG concentration on its activity for the hydrolysis of CMC-Na at different concentrations of urea. The concentrations of urea for curves 1−4 were 0, 1, 2, 3, and 4 M, respectively.

during the initial stages, becoming progressively lower as the hydrolysis reaction went up to 15 h, due to inhibition by the reaction product accumulation (Figure 1).

Enzymatic Hydrolysis at Different Temperatures. Effects of temperature on saccharification were studied at pH 5.0 and different temperatures. The concentration of enzyme was 48 mg/L. As seen from Figure 2, temperature showed a significant effect on enzymatic saccharification. The amount of reducing sugar increased as the temperature increased from 30 to 55 °C. Hydrolysis at 55 °C for 24 h yielded the highest recorded sugar release of 12 mg/mL. However, at 60 °C, the velocity of hydrolysis reached the maximum within 5 h, but increasing time did not influence sugar yields probably due to the heat inactivation of the enzymes.

Enzymatic Hydrolysis at Different pH Values. Effects of pH on saccharification were studied at 55 °C and different pHs. The concentration of enzyme was 48 mg/L. The maximum reducing sugar was observed at pH 5.0 for 24 h. When the pH was below 4.0 or above 6.0, the amount of reducing sugar decreased (Figure 3). According to the results above, the maximum sugar yield (410 mg/g pretreated rice straw) was obtained at 55 °C, pH 5.0 for 24 h by adding the enzyme to the concentration of 48 mg/L.

Urea Is a Reversible Inhibitor of *A. glaucus* EG. Figure 4 indicated the effects of EG concentration on its activity for the hydrolysis of CMC-Na at 0−4 M urea. The plots of the remaining enzyme activity versus the concentration of the enzyme in the presence of 0−4 M urea gave a family of straight lines, which all passed through the origin, indicating that urea is a reversible inhibitor of EG.

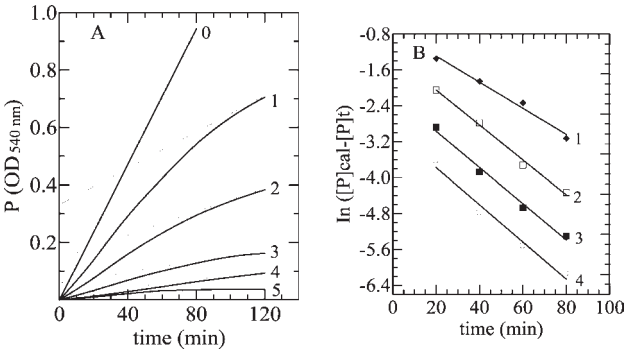


Figure 5. Course of inactivation of EG in urea solutions of different concentrations. Final CMC-Na concentrations were 4.5 mg/mL in 0.05 M NaAc buffer (pH 4.0). (A) Substrate reaction course. The concentrations of urea for curves 0−5 were 0, 1, 2, 3, 4, and 5 M, respectively. (B) Semilogarithmic plots of $\ln([P]_{calc} - [P]_t)$ against time. Data were taken from curves 1−4 in A.

Table 1. Microscopic Rate Constants for the Inactivation of EG in Urea Solutions

	rate constant ($\times 10^{-3}$ min^{-1})			
urea (M)	k_{+0}	k_{-0}	k'_{+0}	residual activity (%)
0	0	0	0	100.0
1	1.96	5.03	0.49	59.8
2	2.12	4.29	1.62	32.3
3	17.7	1.69	14.67	14.3
4	47.7	0.83	28.50	6.4
5	too fast to be determined			5.5
6	too fast to be determined			4.7

Inactivation Rate Constant of *A. glaucus* EG in Urea. The temporal variation of the product concentration during substrate hydrolysis in the presence of 0−5 M urea is shown in Figure 5A. At each concentration of urea, the rate decreased with increasing time until a straight line was approached. From the slopes of the straight line in Figure 5B, the apparent inactivation rate constants A and k_{-0} were calculated (Table 1).

The kinetic courses of the hydrolysis reaction at different substrate concentrations (4.5−9.6 mg/mL CMC-Na) in the presence of 2 M urea are shown in Figure 6A. In the presence of 2 M urea, when the time was sufficiently large, a straight line was approached at each concentration of substrate. From the slopes of the straight line in Figure 6B, the apparent forward rate constant A was calculated. Since K_m = 5.0 mg/mL and V_{max} = 44.2 μM/min,[6] the values of k_{+0} and k'_{+0} were obtained from the slope and the intercept of the straight line in Figure 6C. Similarly, the inactivation rate constants in other concentrations of urea were estimated as summarized in Table 1.

The values of k_{+0} and k'_{+0} increased with increasing urea concentration while the value of k_{-0} decreased, illuminating that the reversible intensity of the enzyme was weakened by increasing urea concentration. The value of k_{+0} was much larger than k'_{+0}, which indicated that the free enzyme molecule was more fragile than the enzyme−substrate complex in the urea solution. This may be due to the substrate protection of the enzyme during the inactivation by urea.

Fluorescence Emission Spectra of *A. glaucus* EG after Denaturation by Urea. The fluorescence emission spectra of

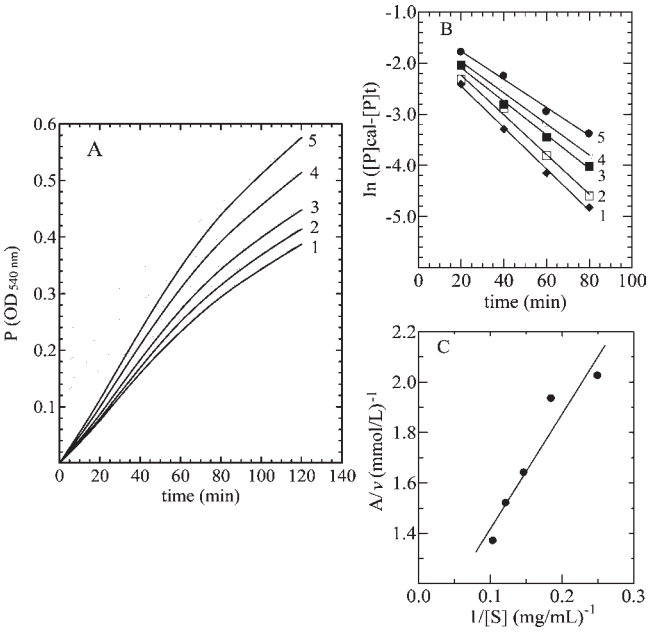


Figure 6. Determination of the inactivation rate constants of EG by 2 M urea. (A) Substrate reaction course of the enzyme in the presence of 2 M urea. Curves 1−5 are progress curves with 4.5, 5.4, 6.8, 8.2, and 9.6 mg/mL of CMC-Na, respectively. (B) Semilogrithmic plots of $\ln([P]_{calc} - [P]_t)$ against time. Data was taken from curves (1−5) in A. (C) Plot of A/v vs $1/[S]$. The values of A were obtained from the slopes of the straight lines in B.

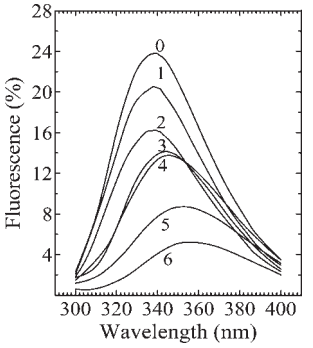


Figure 7. Fluorescence emission spectra of EG denatured in urea solutions of different concentrations. The enzyme was dissolved in 0.01 M Tris-HCl buffer (pH 7.5) containing urea at the desired concentrations. The solutions were allowed to stand at 4 °C for 24 h before fluorescence measurement. The excitation wavelength was 279 nm. Concentrations of urea for curves 0−6 were 0, 1, 2, 3, 4, 5, and 6 M, respectively.

A. glaucus EG denatured in different concentrations of urea are shown in Figure 7. The emission peak of the native enzyme was at 338 nm, which might contain contributions from both Trp residues and Tyr residues. Half inactivation of the protein was induced at 1.5 M urea, and half of the fluorescence intensity change was observed at 4 M urea (Table 1 and Figure 7). A red shift occurred when EG was denatured by 3 M urea. The red shift of the emission maximum continued to reach a final value of about 352 nm.

Comparison of Inactivation and Unfolding of *A. glaucus* EG after Denaturation by Urea. Guanidine hydrochloride (GuHCl) and urea are the most common chemical denaturants used for probing the protein conformation and unfolding studies.[13,14] These denaturants show different behavior toward different proteins, in some cases, for example, low concentrations of GuHCl activated the enzyme activity of prostaglandin-D-synthase.[15] In our research, a marked decrease of EG activity was observed with the increasing concentration of urea.

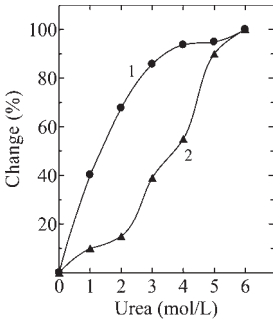


Figure 8. Comparison of inactivation and unfolding of EG during denaturation by urea. Inactivation data were from Table 1, and unfolding data were from Figure 7. Extent of inactivation (●); red shift of fluorescence emission maximum (▲).

The extent of the red shift of the fluorescence emission maximum during denaturation in urea symbolizes the unfolding of the enzyme. Comparison of the inactivation and the unfolding of EG indicated that much lower concentrations of urea were required to inactivate the enzyme than were required to produce significant conformational changes of the enzyme (Figure 8). It has been reported by many authors that during the denaturation of an enzyme by GuHCl or urea, inactivation occurred before a noticeable conformational change of the enzyme molecule as a whole can be detected.[4,16] The results implied that the active site of the *A. glaucus* EG was formed by a relatively weak molecular interaction, so it was more easily affected by urea than the whole enzyme molecule.

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Author Contributions

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ABBREVIATIONS USED

EG, endoglucanase; CMC-Na, carboxymethylcellulose sodium; CMCase, carboxylmethyl cellulase; K_m, Michaelis−Menten constant; DNS, 3,5-dinitrosalicylic; NaAc buffer, sodium acetate buffer; GuHCl, guanidine hydrochloride

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