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Interaction of cellulase with three phenolic acids

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The activity of cellulase against filter paper was enhanced by 28.32% and 15.17% after the addition of 0.83 mg/ml of ferulic acid and *p*-coumaric acid, respectively, and by 10.15% after the addition of salicylic acid at 0.67 mg/ml. The effects of three phenolic acids on the structure of cellulase were investigated via ultraviolet spectrophotometry, fluorescence spectroscopy, and circular dichroism (CD) spectroscopy. Ultraviolet spectroscopic results indicated that the peak absorbance of cellulase significantly increased and exhibited a 4–5 nm redshift after the addition of the three phenolic acids, suggesting that the phenolic acids strongly interacted with the enzyme. Fluorescence investigation of the interaction between the enzyme and the phenolic acids showed that ferulic acid and *p*-coumaric acid covalently reacted with the aromatic amino acid residues in cellulase, whereas salicylic acid interacted non-covalently with cellulase. CD analysis revealed that the addition of the phenolic acids significantly decreased α-helix content but increased β-sheet and random coil contents. The possible mechanism underlying the effects of these phenolic acids on cellulase activity was also discussed.

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1. Introduction

Lignocellulosic materials, such as wheat straw, rice straw, sugarcane bagasse, and corn stover, are renewable sources of energy, feeds, or even food. Approximately 90% of the dry weight of most plant materials is stored in the form of cellulose, hemicellulose, lignin, and pectin. Cellulose and hemicellulose must be broken down into their corresponding monomers for conversion of lignocellulose (Kumar, Barrett, Delwiche, & Stroev, 2009). Lignin and the cross-linking agents ferulic and coumaric acids in lignocelluloses form a protective barrier that prevents enzymatic hydrolysis. Thus, lignocelluloses require pretreatment to release lignin and phenolic acids (Kumar et al., 2009), such as alkali treatment or using ferulic acid esterase (Kumar et al., 2009; Yu et al., 2003).

Ferulic and *p*-coumaric acids are abundant in lignocellulose. The content of ferulic acid varies from 5 g/kg in wheat bran to 9 g/kg in sugar beet pulp and 50 g/kg in maize bran (Ou & Kwok, 2004). By contrast, the content of coumaric acid in sugarcane and maize straws nearly reaches 2% (Eylen, Dongen, Kabel, & de Bont, 2011; Xu et al., 2005).

Ferulic acid and other phenolic acids, such as caffeic, chlorogenic, and gallic acids, were reported to react with proteins through non-covalent interactions (aromatic interaction), hydrogen bonding, hydrophobic or ionic interactions, and covalent bonding (Kroll, Rawel, & Rohn, 2003). Whether these released (by ferulic acid esterase) or residual (by alkali treatment) phenolic acids would influence the activity of cellulase or xylanase and their action mechanism need to be investigated.

In the current research, effects of three phenolic acids on the activity of cellulase and the mechanisms of interaction of three phenolic acids (ferulic acid, *p*-coumaric acid, and salicylic acid) with cellulase were investigated using ultraviolet (UV) spectrophotometry, fluorescence spectroscopy, and circular dichroism (CD) spectroscopy.

2. Materials and methods

2.1. Reagents

Cellulase with an activity of 140 FPU/mg was purchased from Guangzhou Qiyun Biotechnology Company (Guangzhou, China). Ferulic acid, *p*-coumaric acid, salicylic acid, and 3,5-dinitrosalicylic acid were purchased from Sigma–Aldrich Company (St. Louis, MO, USA). All other reagents were of analytical grade.

2.2. Influence of phenolic acids on cellulase activity

Thirty millilitres of acid–sodium acetate buffer (0.1 mol/L, pH = 5.0) in a 100 ml flask containing cellulase (4.0 mg/ml), and 0.17, 0.33, 0.50, 0.67, 0.83, and 1.0 mg/ml of ferulic, *p*-coumaric, and salicylic acids were respectively prepared and pre-incubated in a water bath at 50 °C for 5 min.

One gram of the filter paper strips (1 × 1 cm) was added into the flask and reacted at 50 °C for 3 h in a SHA-BA model shaking water bath (Jiangsu Jintan Shenke Instrumetal Company, Jiangsu,

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China) at 100 rpm. The flasks were kept in a boiling water bath for 15 min to inactivate the enzyme, and then cooled in an ice water bath. The slurries were centrifuged at 8000g for 20 min. The reducing sugars in the supernatant were determined using the 3,5-dinitrosalicylic acid method (Eveleigh, Mandels, Andreotti, & Roche, 2009).

2.3. Kinetics investigation of phenolic acids on cellulase

Twenty millilitres of acid–sodium acetate buffer (0.1 mol/L, pH = 5.0) containing cellulase (8.0 mg/ml) and 2.5 mg/ml each of phenolic acids; and 20 ml of acid–sodium acetate buffer (0.1 mol/L, pH = 5.0) containing sodium carboxymethyl cellulose (CMC) solution at concentrations of 2, 4, 6, 8, and 10 mg/ml, were incubated in a water bath at 50 °C for 10 min.

Two millilitres solution of cellulase–phenolic acids and 2 ml of CMC solution were mixed in a 15 ml test tube and reacted in a SHA-BA model shaking water bath (100 rpm) incubated in a water bath at 50 °C for 5 min. The reaction was stopped by placing the test tubes in a boiling water bath for 15 min. The mixture was cooled in an ice water bath and then centrifuged as above. The reducing sugars in the supernatant were determined using the 3,5-dinitrosalicylic acid method. The k_m and V_{max} values were calculated from the enzyme kinetics curve using the Lineweaver–Burk double-reciprocal method (Horton, Moran, Ochs, Rawn, & Scrimgeour, 2002).

2.4. Analysis of cellulase–phenolic acid interactions using UV spectrophotometry

Cellulase (4.7 mg/ml) and phenolic acids (0.22, 0.44, 0.88, and 1.75 mg/ml) were separately prepared using 0.1 mol/L acetic acid-sodium acetate buffer (pH 5.0). A 30 ml cellulase solution and a 5 ml solution of the phenolic acids at different concentrations were mixed in flasks and kept at room temperature for 15 min (final cellulase concentration: 4.0 mg/ml; final phenolic acid concentrations: 0.03, 0.06, 0.13, and 0.25 mg/ml). The mixture was placed in a 1 cm quartz cuvette and then scanned using a Lambda 35 UV–visible spectrophotometer (Perkin Elmer Instruments, MA, USA) in wavelength range of 220–320 nm. Acetic acid–sodium acetate buffer was used as the blank, whereas acetate buffer containing the phenolic acids but without the enzyme was used as the control.

2.5. Analysis of cellulase–phenolic acid interactions using fluorescence spectroscopy

A reaction system similar to that used for UV spectrophotometry was designed, with the final concentrations of the phenolic acids changed to 0.125, 0.25, 0.5, and 1.0 mg/ml. The fluorescence intensity of the mixtures was determined using an LS50B fluorescence spectrophotometer (Perkin Elmer Instruments, MA, USA) at excitation wavelengths of 278 and 295 nm. Acetic acid-sodium acetate buffer was used as the blank, whereas acetate buffer containing the phenolic acids but without the enzyme was used as the control.

2.6. Analysis of cellulase–phenolic acid interactions using CD spectroscopy

A reaction system similar to that used for UV spectrophotometry was designed. The CD spectra (200–250 nm) were obtained on a Chirascan spectrophotometer (Applied Photophysics Ltd., United

Table 1
Effects of phenolic acids on the release of reducing sugars from filter paper by cellulase.

Concentration of phenolic acids (mg/ml)	Concentration of reducing sugars (mg/ml)[a]		
	Ferulic acid	*p*-Coumaric acid	Salicylic acid
0.00	8.51 ± 0.17^{A}	8.04 ± 0.18^{A}	8.67 ± 0.16^{A}
0.17	9.19 ± 0.45^{AB}	8.30 ± 0.13^{B}	8.90 ± 0.01^{B}
0.33	9.61 ± 0.13^{BC}	8.46 ± 0.06^{B}	9.19 ± 0.14^{C}
0.50	10.00 ± 0.10^{BC}	8.74 ± 0.39^{C}	9.31 ± 0.20^{CD}
0.67	10.38 ± 0.25^{CD}	9.13 ± 0.27^{D}	9.55 ± 0.38^{E}
0.83	10.92 ± 0.46^{D}	9.26 ± 0.16^{D}	9.54 ± 0.61^{E}
1.00	10.80 ± 0.06^{D}	9.18 ± 0.01^{D}	9.32 ± 0.15^{D}

[a] Values (means ± SD, n = 3) with different letters within a column are significantly different at the 5% level.

Table 2
Kinetic effects of phenolic acids on cellulase.

	CMC (mg/ml)	Rate (mg/ml min^{-1})	Kinetics results	k_m (mg/ml)	V_{max} (mg/ml min^{-1})
Blank	1.00	0.19 ± 0.01[b]	$1/v = 1.68 \times 1/S + 3.72$	0.45 ± 0.03	0.27 ± 0.00
	2.00	0.22 ± 0.00	$R^2 = 0.9928$		
	3.00	0.23 ± 0.01			
	4.00	0.24 ± 0.00			
	5.00	0.25 ± 0.00			
FA[a]	1.00	0.25 ± 0.01	$1/v = 0.77 \times 1/S + 3.25$	0.24 ± 0.04	0.31 ± 0.00
	2.00	0.28 ± 0.01	$R^2 = 0.9962$		
	3.00	0.28 ± 0.01			
	4.00	0.29 ± 0.00			
	5.00	0.29 ± 0.01			
CA	1.00	0.22 ± 0.00	$1/v = 1.26 \times 1/S + 3.26$	0.39 ± 0.03	0.31 ± 0.01
	2.00	0.25 ± 0.01	$R^2 = 0.9901$		
	3.00	0.27 ± 0.00			
	4.00	0.28 ± 0.00			
	5.00	0.29 ± 0.00			
SA	1.00	0.19 ± 0.00	$1/v = 1.45 \times 1/S + 3.74$	0.39 ± 0.04	0.27 ± 0.01
	2.00	0.22 ± 0.00	$R^2 = 0.9913$		
	3.00	0.24 ± 0.01			
	4.00	0.25 ± 0.01			
	5.00	0.25 ± 0.01			

[a] FA, ferulic acid; CA, *p*-coumaric acid; SA, salicylic acid.
[b] Means ± SD ($n = 3$).

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