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Interaction of cellulase with three phenolic acids

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ABSTRACT

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.

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 8000*g* 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).

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.

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 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 ($n = 3$).

Kingdom) using silica quartz spectrophotometer cells with 0.1 cm path length. Full-scale spectra were recorded at 0.5 nm/s.

3. Results and discussion

3.1. Effects of the different phenolic acids on cellulase activity

The enzyme activity increased as the concentration of the phenolic acids increased (Table 1). A maximum increase of 28.32% and 15.17% for ferulic and *p*-coumaric acids, respectively, were attained when the concentration of the phenolic acids reached 0.83 mg/ml, and by 10.15% for salicylic acid at a concentration of 0.67 mg/ml. The release of reducing sugars did not increase or even slightly decrease when the concentration of the phenolic acids further increased (Table 1).

3.2. Kinetics study

The k_m value of cellulase decreased after the addition of ferulic acid, *p*-coumaric acid, and salicylic acid (Table 2), which indicates that the phenolic acids enhanced the affinity of cellulase for its substrate (Horton et al., 2002). Moreover, the increase in the V_{max} value of cellulase suggests that the phenolic acids also promoted enzyme–substrate complex formation to release the final products (Horton et al., 2002), namely, the reducing sugars.

3.3. Effects of the phenolic acids on the UV spectra

The absorbance peak at around 280 nm, which is attributed to the absorption of tryptophan and tyrosine (Kroll, Rawel, & Rohn, 2003), is characteristic of most protein molecules. Changes in the UV spectrum of the enzyme were observed when the aromatic amino acid residues reacted with the phenolic compounds (Kroll et al., 2003).

The absorbance peak of cellulase was 280 nm (Fig. 1). The absorbance intensity of cellulase increased with increasing phenolic acid concentration (Fig. 1). Moreover, an absorbance peak shift was observed: redshifts of 4, 5, and 4 nm were observed after the interaction of cellulase with ferulic acid, *p*-coumaric acid, and salicylic acid, respectively (Fig. 1). Given that the UV absorbance of the phenolic acids was excluded in the current research, the increase in the UV absorbance of cellulase after the addition of the phenolic acids is mainly attributed to the interaction between the phenolic acids and the enzyme.

3.4. Effects of the phenolic acids on the fluorescence spectra

Tryptophan and tyrosine, which have aromatic rings and conjugated double bonds, are the primary amino acids that exhibit fluorescence excitation and emission in proteins (Wang, Xu, Liu, & Xie, 2001). Tryptophan has activation wavelengths at 278 and 295 nm, whereas tyrosine is only excited at 278 nm. The emission wavelengths of these amino acids are at around 340 nm. Quenching of the fluorescence intensity is an indicator of microenvironmental changes in tryptophan, tyrosine, and the enzyme (Eftink & Chiron, 1981; Wang et al., 2001).

In this study, the fluorescence results showed that the emission wavelengths of cellulase were 340 and 337 nm after excitation at 278 and 295 nm, respectively. As the phenolic acid concentrations increased, the fluorescence intensity was attenuated successively. In addition, fluorescence quenching occurred after the addition of 1 mg/ml each of ferulic and *p*-coumaric acids (Fig. 2). After the interaction of cellulase with ferulic acid, *p*-coumaric acid, and salicylic acid, redshifts of 20, 16, and 3 nm were observed at 278 nm excitation, respectively, whereas redshifts of 21, 16, and 5 nm were found at 295 nm excitation, respectively. Thus, the addition of the phenolic acids changed the structure and hydrophobic environment of cellulase, which may have affected its activity.

Fluorescence quenching can be classified into dynamic and static quenching. In dynamic quenching, the quenching agent diffuses into fluorescent protein chromophores during excitation, after which the chromophores return to the ground state. In static quenching, the quenching agent reacts with the chromophores and forms a new stable compound that has no fluorescence (Eftink & Chiron,1981; Wang et al., 2001).

If the phenolic acid-induced change in enzyme fluorescence is due to dynamic quenching, then the reaction would satisfy the Stern–Volmer equation (Eftink & Chiron, 1981; Wang et al., 2001).

$$F_0/F = 1 + K_q \times \tau_0 \times [Q] = 1 + K_{sv} \times [Q]$$

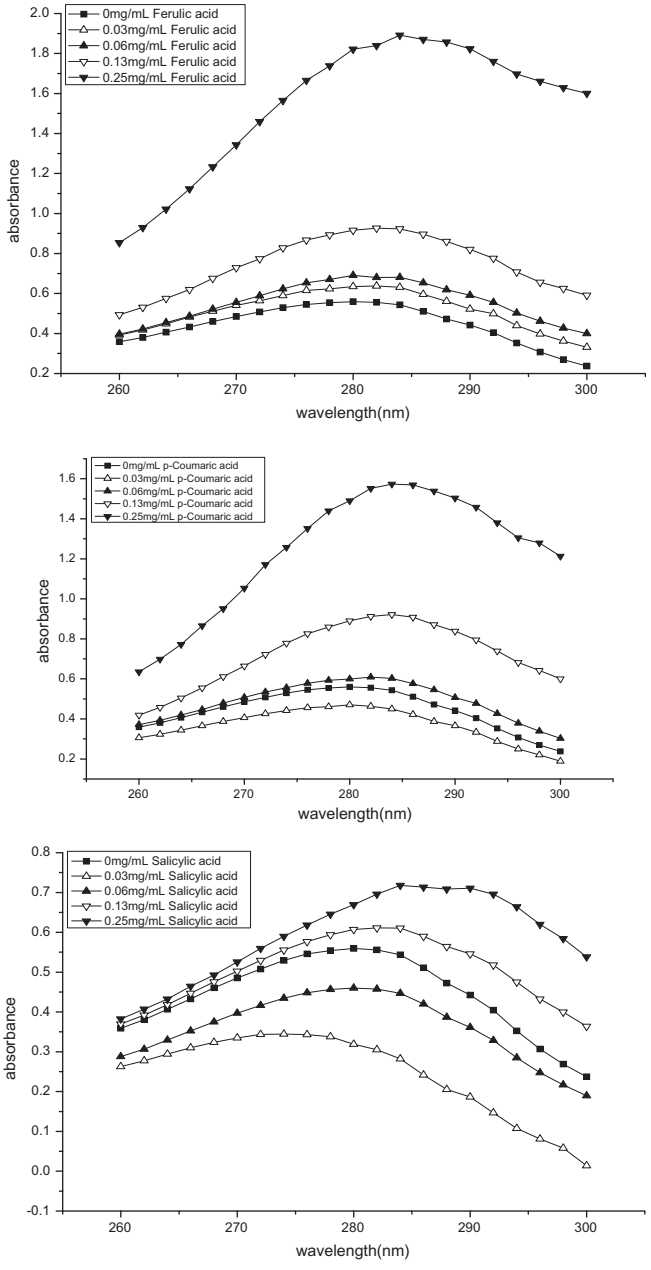

Fig. 1. Ultraviolet spectra of cellulase after its reaction with different concentrations of ferulic acid (top), *p*-coumaric acid (middle), and salicylic acid (bottom). The absorbance in the figure is the absorbance of the enzyme after its reaction with the phenolic acids minus the absorbance of the phenolic acids at the same concentrations in the buffer.

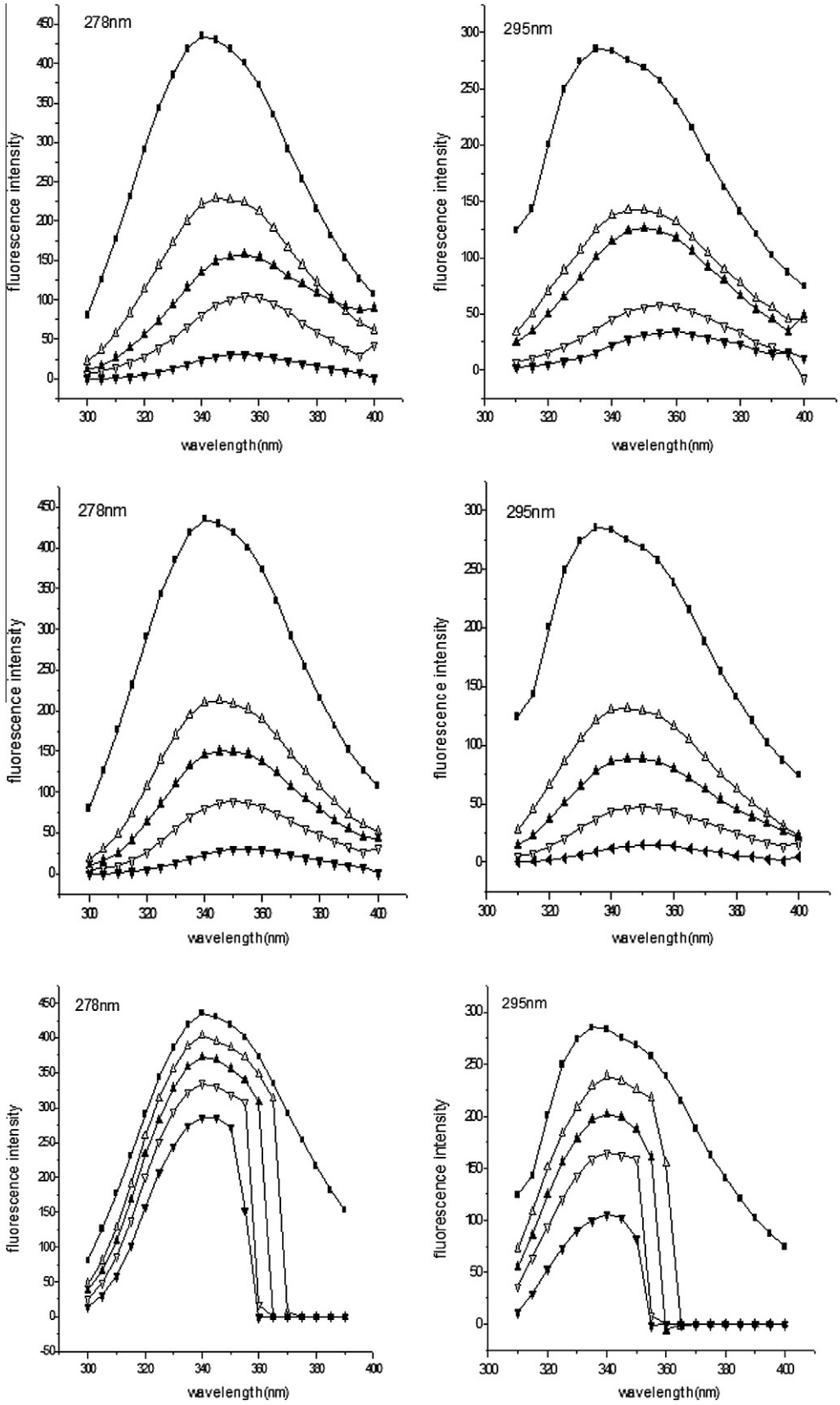

Fig. 2. Fluorescence spectra of cellulase treated with different concentrations of ferulic acid (top), *p*-coumaric acid (middle), and salicylic acid (bottom). The phenolic concentrations in each figure are 0, 0.125, 0.25, 0.5, and 1.0 mg/ml, from top to bottom; the fluorescence intensities of the phenolic acids were excluded.

where F_0 and F are the fluorescence intensities in the absence and presence of the quenching agent Q, respectively; K_q is the bimolecular rate constant for the quenching process; τ_0 is the duration of fluorescence in the absence of the quenching agent Q and is 10^{-8} s for a biomacromolecule (Xu & Wang, 2006); K_{sv} is the Stern–Volmer constant for the collisional quenching process, the

Table 3
Parameters of the Stern–Volmer equation after cellulase reaction with different phenolic acids.

	$Q \times 10^3$ (mol/L)	F_0/F	Stern–Volmer equation	K_q ($mol^{-1}\ s^1$)
Ferulic acid	0.32	1.58	$F_0/F = 1.15 \times [Q] + 1.21$	1.15×10^{11}
	0.64	1.90	$R^2 = 0.998$	
	1.29	2.76		
	2.57	4.16		
Coumaric acid	0.38	1.96	$F_0/F = 1.24 \times [Q] + 1.08$	1.24×10^{11}
	0.76	2.89	$R^2 = 0.999$	
	1.52	5.78		
	3.05	9.59		
Salicyclic acid	0.91	1.08	$F_0/F = 0.07 \times [Q] + 1.04$	6.75×10^9
	1.81	1.17	$R^2 = 0.992$	
	3.62	1.30		
	7.24	1.52		

Table 4
Static quenching parameters of cellulase after reaction with different concentrations of ferulic acid and coumaric acid.

	log[Q]	log $[(F_0 - F)/F]$	Static quenching equation	K_A	Binding sites (n)
Ferulic acid	−3.49	−0.23	$\log[(F_0 - F)/F] = 0.83 \times \log[Q] + 2.63$ $R^2 = 0.994$	428	0.8
	−3.19	−0.04			
	−2.89	0.25			
	−2.59	0.50			
Coumaric acid	−3.42	−0.23	$\log[(F_0 - F)/F] = 0.90 \times \log[Q] + 2.84$ $R^2 = 0.997$	702	0.9
	−3.12	0.01			
	−2.82	0.28			
	−2.52	0.59			

value of which can be calculated from the slope of the $F_0/F - [Q]$ curves; and [Q] is the concentration of the quenching agent (phenolic acids).

The maximum value of the biomacromolecule diffusion-controlled quenching in water is approximately 1.0×10^{10} L/(mol s), which is an index that differentiates dynamic from static quenching (Lakowicz, 2006; Xu & Wang, 2006). Dynamic quenching is considered when K_q is below this value, whereas static quenching is considered when K_q is higher than this value. Table 3 showed that salicylic acid acts as a dynamic quenching agent during its interaction with cellulase. By contrast, ferulic acid and *p*-coumaric acid act as static quenching agents, suggesting that these phenolic acids covalently reacted with the chromophore amino acids in the enzyme (Kroll et al., 2003).

According to the static quenching equation, the binding constant and the number of binding sites can be calculated using the following formula (Lakowicz, 2006; Xu & Wang, 2006).

$$\log[(F_0 - F)/F] = \log K_A + n \times \log[Q]$$

where K_A is the binding constant, n is the number of binding sites, and [Q] is the concentration of the quenching agent.

Table 4 showed that only one binding site is present in the enzyme for ferulic acid and for coumaric acid. However, the binding capacities of the enzyme significantly differ among the phenolic acids because the binding constant (K_A) of the enzyme for coumaric acid is considerably higher than that for ferulic acid (Table 4).

As previously reported (Boukari, O'Donohuec, Rémond, & Chabbert, 2011; Ximenes, Kim, Mosier, Dien, & Ladisch, 2010, 2011), higher phenolic concentrations (10–50 mM) inhibited cellulase and xylanase activities, whereas lower plant phenolic concentrations (0.05% or below 1.0 mg/ml) promote these activities. Decrease in fluorescent intensity was observed both for xylanase

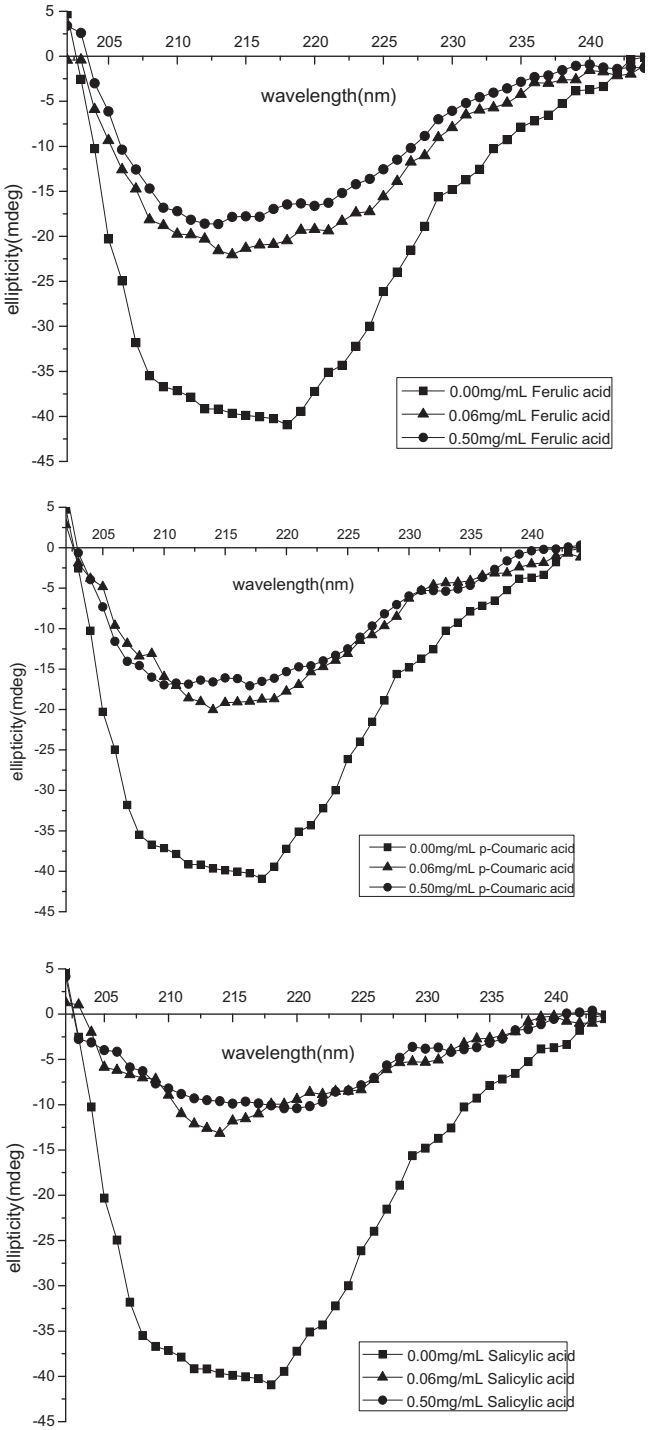

Fig. 3. Circular dichroism spectra of cellulase before and after the addition of different phenolic acid concentrations.

after addition of higher concentration of phenolic acids, and for cellulase after addition of lower concentration of phenolic acids (Boukari, O'Donohuec, Rémond, & Chabbert, 2011). However, the addition of different concentrations of phenolic acid resulted in different wavelength shifts. A substantial blueshift (12–14 nm) was observed for xylanase after the addition of higher phenolic acid concentrations in research conducted by Boukari, O'Donohuec, Rémond, and Chabbert (2011), whereas a redshift occurred for cellulase after the addition of lower phenolic acid concentrations in our research. These results seems to suggest that different concen-

Table 5
Effects of different phenolic acids on the change of the secondary structure of cellulase.

	Phenolic acid con. (mg/ml)	Content of secondary structure (%)*			
		α-Helix	β-Sheet	β-Turn	Random coil
Control	0	77.3 ± 3.5^{d}	3.6 ± 0.4^{a}	9.8 ± 0.2^{a}	10.1 ± 0.2^{a}
Ferulic acid	0.06	41.6 ± 2.7^{c}	13.3 ± 1.1^{b}	15.5 ± 0.7^{b}	29.6 ± 0.4^{b}
	0.50	35.0 ± 1.8^{b}	16.2 ± 1.3^{c}	16.5 ± 0.7^{b}	32.3 ± 0.9^{c}
Coumaric acid	0.06	35.2 ± 2.3^{b}	16.0 ± 0.7^{c}	16.4 ± 0.6^{b}	32.4 ± 0.7^{c}
	0.50	33.7 ± 2.6^{b}	16.7 ± 0.8^{c}	16.7 ± 0.8^{b}	32.9 ± 1.0^{c}
Salicyclic acid	0.06	23.3 ± 1.7^{a}	19.1 ± 1.1^{d}	17.4 ± 0.3^{c}	40.2 ± 1.3^{d}
	0.50	22.9 ± 2.1^{a}	19.9 ± 0.9^{d}	17.0 ± 0.5^{c}	39.8 ± 1.2^{d}

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

trations of phenolic acid showed different effects on the activity of cellulase or xalanase.

3.5. Effects of the phenolic acids on the CD spectra

Cellulase showed two negative peaks at 212 and 218 nm, with ellipticities of −37.15 and −40.94 mdeg, respectively (Fig. 3). The addition of the phenolic acids changed the CD spectrum of cellulase. After the addition of 0.06 mg/ml ferulic acid, the peaks blue-shifted to 210 and 214 nm, respectively, and the ellipticities decreased to −19.76 and −22.04 mdeg, respectively (Fig. 3). The addition of 0.5 mg/ml ferulic acid led to similar changes in the CD spectrum. Increased extents of blue-shifting, as well as greater decreases in the ellipticity, were observed after the addition of coumaric acid and salicylic acid respectively (Fig. 3).

The changes in the secondary structure of cellulase after the addition of the phenolic acids were analysed using a deconvolution software; the results are listed in Table 3. α-Helix, β-sheet, β-turn, and random coil accounted for 77.25%, 3.63%, 9.85%, and 10.08% of cellulase, respectively. The addition of the phenolic acids significantly decreased the content of α-helix but significantly increased the contents of β-sheet and random coil (Table 5).

The mechanism of action of the phenolic acids can be determined by the specific structure of cellulase. Cellulase has a cellulose-binding domain that contains several aromatic amino acids, including three tyrosine molecules and two tryptophan molecules, which form a hydrophobic surface (Macarron, Henrissat, Claeyssens, & Family, 1995). This domain plays a key role in the degradation of water-insoluble cellulose. Thus, the phenolic acids increase the activity of cellulase through the following mechanisms. First, the binding of the phenolic acids to the enzyme slightly increases the hydrophilic capacity of the cellulose-binding domain. This phenomenon is beneficial for maintaining the adjacency of the enzyme domain to its substrate (cellulose) on the water-cellulose surface. The higher hydrophilicity of the phenolic acids compared with other amino acids may be attributed to their hydroxyl and carboxyl groups. Second, the tryptophan and tyrosine residues in the enzyme are readily oxidised (Macarron et al., 1995). Thus, the binding of these antioxidant phenolic acids may protect them from oxidation.

The CD results also partly explain why lower phenolic acid concentrations increased the activity of cellulase. Proteins vary in their α-helix contents, with the average α-helix content of the examined proteins is 26% (Horton et al., 2002). A high α-helix content produces a compact and rigid enzyme (Chen, Yang, & Martinez, 1972). This rigidity can reduce the affinity of the enzyme for the substrate. The addition of moderate phenolic acid concentration decreased the content of α-helix but increased the content of flexible structure, the random coil. As a result, the enzyme structure became more flexible and its affinity for the substrate increased.

4. Conclusion

Addition of 0.83 mg/ml of ferulic acid, *p*-coumaric acid, and the addition of 0.67 mg/ml of salicylic acid increased the activity of cellulase against filter paper by 28.32%, 15.17%, and 10.15% respectively. Ferulic acid and *p*-coumaric acid covalently reacted with the aromatic amino acid residues in cellulase, while salicylic acid interacted non-covalently with cellulase. These phenolic acids may increase the activity of cellulase due to decreased the content of rigid structure of the enzyme, α-helix, but moderately increased the content of flexible structure, the β-sheet and random coil. It can be concluded that the activity of cellulase could be increased by addition of these three phenolic acids at proper concentrations.

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