# Diferulate Cross-Links Limit the Degradation Rate of Nonlignified Maize Walls by Fungal Hydrolases

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## Introduction

Ferulic acid is esterified to the C5-hydroxyl of a-L-arabinose moieties of grass xylans. Xylans are cross-linked by oxidative coupling of ferulate monomers into dehydrodimers. The substitution and cross-linking of xylans by ferulates is thought to limit the enzymatic degradation of grass walls, but unambiguous evidence for such a role is lacking. We specifically manipulated ferulate substitution and diferulate cross-linking in nonlignified walls to elucidate how ferulates restrict fiber degradation by fungal hydrolases.

## Methods

Maize cell suspensions (*Zea mays* cv. Black Mexican) were grown with 0 to 50 µM 2-aminoindan-2-phosphonic acid (AIP) to manipulate the deposition of ferulate esters into nonlignified walls. In a separate study, nonlignified walls from cell suspensions grown with 0 or 40 µM AIP were incubated with mercaptoethanol to inhibit diferulate formation or with dilute hydrogen peroxide to stimulate diferulate formation by wall-bound peroxidases. Cell walls were analyzed for neutral sugars, uronic acids, hydroxycinnamic acids, and lignin. Cell walls were degraded with hydrolases from *Trichoderma reesei* (Celluclast, NOVO) and *Aspergillus niger* (Viscozyme L, NOVO). Periodically during enzymatic hydrolysis, wall residues were pelleted by centrifugation and an aliquot of the supernatant was analyzed for total carbohydrate, uronic acids, and for neutral sugars after hydrolysis with 2 N trifluroacetic acid. The kinetics of sugar release were described using a first order model.

## Results and Discussion

Cell walls from maize cell suspensions contained 197 mg $g^{-1}$ of arabinose, 180 mg $g^{-1}$ of xylose, 75 mg $g^{-1}$ of galactose, 320 mg $g^{-1}$ of glucose, 110 mg $g^{-1}$ of uronic acids, 17 mg $g^{-1}$ of ferulate esters, trace amounts of ferulate ethers, 0.5 mg $g^{-1}$ of *p*-coumaric acid, and 3 mg $g^{-1}$ of guaiacyl lignin. Overall, the chemical composition of cell walls was representative of nonlignified primary walls of grasses. Ferulate ester deposition into cell walls was reduced up to 75% by growing cell suspensions in the presence of AIP, a specific inhibitor of phenylalanine ammonia lyase. Hydrogen peroxide treatment of walls with normal or low feruloylation increased the proportion of diferulates to total ferulates from *ca* 18% to 44% (Table 1). Other wall components were not modified significantly by AIP and $H_2O_2$ treatments.

Concurrent reductions in ferulate substitution and cross-linking caused by AIP treatment increased the release of carbohydrate from walls by fungal enzymes. When walls with normal or low feruloylation were treated with hydrogen peroxide, peroxidase-mediated coupling of ferulate monomers into dehydrodimers reduced carbohydrate release by 122 mg $g^{-1}$ after 3 h and by 48 mg $g^{-1}$ after 54 h of enzymatic hydrolysis (Table 1). These results provide compelling evidence that the enzymatic hydrolysis of walls was controlled by diferulate cross-linking and not by ferulate substitution of xylans. Averaged over all treatments, release rate was greatest for galactose and uronic acid (*ca* 0.265 $h^{-1}$), intermediate for arabinose and glucose (*ca* 0.160 $h^{-1}$), and lowest for xylose (0.093 $h^{-1}$). Hydrogen peroxide treatment reduced the rate of sugar release by an average of 42% (Table 2), indicating that diferulate cross-linking of xylans restricted the rate at which all polysaccharides were released from walls. Averaged over all treatments, the extent of sugar release from walls was slightly greater for glucose and galactose (*ca* 0.884) than arabinose, xylose and uronic acids (*ca* 0.858). Alteration of ferulate substitution and cross-linking generally had little effect on the extent of sugar release from walls. However, hydrogen peroxide treatment of walls with normal feruloylation reduced arabinose release by 8% and xylose release by 21%, suggesting that the degradation of xylans was reduced by high levels of diferulate cross-linking.

**Conclusions**

Diferulate cross-linking of arabinoxylans impedes the release of all polysaccharides from nonlignified walls by fungal enzymes. Except for xylans, the extent of polysaccharide degradation was not affected by diferulate cross-linking. Simple substitution of xylans with ferulates did not affect wall hydrolysis.

**Impact Statement**

Research with this cell-wall model system provides a unique means of elucidating factors which limit efficient utilization of cell walls for nutritional and industrial purposes. Ultimately, these studies should allow rational approaches to maximizing plant utilization and farm sustainability while minimizing adverse impacts on the environment.

Table 1. Ferulate composition and fungal hydrolase degradability of nonlignified maize walls (n = 2). Feruloylation of cell walls was manipulated by growing maize cell suspensions with and without AIP, a specific inhibitor of phenylalanine ammonia lyase. Peroxidase-mediated coupling of ferulate monomers into dimers was limited by isolating and incubating cell walls with mercapoethanol or stimulated by incubating cell walls with hydrogen peroxide.

| | | Ferulate esters | | | Total carbohydrate released | |
|---|---|---|---|---|---|---|
| AIP | $H_2O_2$ | monomers | dimers | total | 3 h | 54 h |
| µM | mmol | - - - - - - - - - mg $g^{-1}$ cell wall - - - - - - - - - | | | -mg $g^{-1}$ wall carbohydrate- | |
| Normal feruloylation | | | | | | |
| 0 | 0 | 14.53 | 2.62 | 17.15 | 357 | 856 |
| 0 | 0.4 | 8.96 | 6.65 | 15.61 | 243 | 794 |
| Low feruloylation | | | | | | |
| 40 | 0 | 3.75 | 1.31 | 5.06 | 460 | 898 |
| 40 | 0.4 | 2.27 | 2.25 | 4.52 | 329 | 865 |
| Analysis of Variance | | | | | | |
| AIP | | * | * | * | * | * |
| $H_2O_2$ | | * | * | NS | * | * |
| AIP X $H_2O_2$ | | * | * | NS | NS | NS |

* = Significant at the 0.05 level of probability.
NS = Not significant.

Table 2. Rate of sugar release during fungal hydrolase degradation of nonlignified maize walls as affected by hydrogen peroxide treatment of nonlignified walls (averaged over AIP treatments).

| $H_20_2$ | Diferulate | Total ferulate | Arabinose | Xylose | Galactose | Glucose | Uronic acids |
|---|---|---|---|---|---|---|---|
| mmol | - - mg $g^{-1}$ of cell wall - - | | - - - - - - - - - - -Rate constant ($h^{-1}$, LSD† = 0.025) - - - - - - - - - | | | | |
| 0 | 1.97 | 11.11 | 0.186 | 0.127 | 0.323 | 0.229 | 0.337 |
| 0.4 | 4.45 | 10.07 | 0.099 | 0.059 | 0.175 | 0.132 | 0.230 |

†LSD to compare means within columns ($P$ = 0.05).