

# Effect of Diferulate Cross-linking on the Hydrolysis of Xylans and Nonlignified Walls by a Fungal Enzyme Mixture Containing Feruloyl Esterase and Potent Xylanase Activities

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## Introduction

Ferulates are esterified to  $\alpha$ -L-arabinose sidechains on xylans in grasses. During wall biosynthesis and lignification, xylans are cross-linked by oxidative coupling of ferulate monomers into dehydrodimers. In previous work (1996 Research Summaries), we found that diferulate cross-links reduced the rate and, to a lesser degree, the extent of cell wall hydrolysis by Celluclast and Viscozyme, a fungal enzyme mixture free of feruloyl esterase activity. Preliminary studies revealed that another fungal enzyme preparation, Biofeed Beta, contained 15 mU of feruloyl esterase activity per mg of solid, using feruloylated arabinose as a substrate. It was also observed that a mixture of Biofeed and Celluclast degraded xylans at a two-fold greater rate than Viscozyme and Celluclast. The degradation of cellulose was similar for both enzyme mixtures. Therefore, the Biofeed and Celluclast mixture was used to determine whether the inhibitory effects of diferulate cross-linking on wall hydrolysis could be mitigated by a fungal enzyme mixture containing feruloyl esterase and high xylanase activities.

## Methods

Nonlignified cell suspensions of maize (*Zea mays*) were grown with 0 or 40 mM 2-aminoindan-2-phosphonic acid (AIP) to produce walls with normal (17.2 mg g<sup>-1</sup>) or reduced (5.1 mg g<sup>-1</sup>) ferulate concentrations. Walls were then incubated with mercaptoethanol to inhibit diferulate formation or with hydrogen peroxide to stimulate diferulate formation by wall bound peroxidases. A xylan-rich indigestible residue fraction and an 80% EtOH insoluble xylo-oligosaccharide fraction were isolated from the hydrolysate of hydrogen peroxide treated cell walls degraded for 72 h with Viscozyme and Celluclast (each added at 0.04 mL mg<sup>-1</sup> of cell wall). Cell walls and wall fractions were then degraded with Biofeed Beta (CT form, 0.04 mg mg<sup>-1</sup> of cell wall) and

Celluclast 1.5 L (0.04 mL mg<sup>-1</sup> of cell wall). Periodically, hydrolysates were clarified by centrifugation and an aliquot was analyzed for total sugars or reducing sugars by colorimetric procedures. Ferulates released from walls or wall fractions by saponification or esterases were quantified by GC-FID.

## Results

Xylo-oligosaccharides and xylan-rich indigestible residue fractions (Table 1) were hydrolyzed with a Biofeed and Celluclast mixture containing 0.6 mU of feruloyl esterase activity per mg of substrate, theoretically enough activity to completely release all ferulates within 5 to 9 h of incubation. A 24 h incubation released 168 mg g<sup>-1</sup> of reducing sugars from the oligosaccharide fraction and 737 mg g<sup>-1</sup> of total carbohydrate from the indigestible residue fraction. Substantial quantities of ferulate monomers but only small amounts of 5-5 and 8-5 coupled diferulates were released from the fractions. As noted in earlier work with Viscozyme and Celluclast, hydrogen peroxide/peroxidase-mediated coupling of ferulate monomers into dehydrodimers reduced carbohydrate solubilization from walls after 3 h of hydrolysis with Biofeed and Celluclast. Differences were not significant after 54 h of hydrolysis (Table 2). Walls with similar cross-linking (2.62 vs 2.25 mg g<sup>-1</sup> of diferulates) but substantially different ferulate substitution (17.15 vs 4.52 mg g<sup>-1</sup> of total ferulates) had roughly the same degradability. Although diferulate cross-linking reduced the initial hydrolysis of walls by both enzyme mixtures, degradation was more rapid and extensive with Biofeed and Celluclast, particularly for walls with high levels of diferulate cross-linking.

## Discussion and Conclusions

This study provides additional evidence that the rate of wall degradation is restricted by diferulate cross-linking of xylans. In contrast, simple feruloylation of

xylans does not appear to influence cell wall hydrolysis. The activity of feruloyl esterases on diferulates was extremely low, even on soluble substrates. Therefore, the effects of feruloyl esterases on cell wall degradation are probably nil. However, our results indicate that the inhibitory effects of diferulate cross-linking on wall hydrolysis may be partially overcome if enzyme mixtures contain high xylanase activity.

### Impact Statement

Research of this kind 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 and diferulate composition of nonlignified walls and wall fractions recovered after a 72 h incubation with Viscozyme and Celluclast. Values in parentheses indicate the percentage of each constituent released as free acids from wall fractions after a 24 h incubation with Biofeed and Celluclast. Data represent the means of duplicate analyses.

	(Z)-Ferulate	(E)-Ferulate	(E)-Diferulates			
			8-8	8-5	8-O-4	5-5
----- mg g <sup>-1</sup> -----						
Cell wall	2.51	6.64	1.05	5.17	1.53	1.24
Xylo-oligosaccharides	1.69 (0)	9.74 (83)	3.08 (0)	13.83 (12)	3.65 (0)	4.27 (24)
Xylan-rich indigestible residues	7.21 (0)	21.90 (52)	3.22 (0)	17.92 (8)	6.00 (0)	5.66 (13)

Table 2. Ferulate concentration and degradability of structural carbohydrates (SC) in nonlignified cells (n = 2). Feruloylation of walls was manipulated by growing 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 walls with mercaptoethanol or stimulated by incubating walls with H<sub>2</sub>O<sub>2</sub>. Walls were hydrolyzed with a mixture of Biofeed and Celluclast.

AIP	H <sub>2</sub> O <sub>2</sub>	Ferulates			Carbohydrate released	
		monomers	dimers	total	3 h	54 h
mM	mmol	----- mg g <sup>-1</sup> cell wall -----			----- mg g <sup>-1</sup> SC -----	
Normal feruloylation						
0	0	14.53	2.62	17.15	546	871
0	0.4	8.96	6.65	15.61	416	856
Low feruloylation						
40	0	3.75	1.31	5.06	570	898
40	0.4	2.27	2.25	4.52	511	916
Analysis of Variance						
AIP		*	*	*	*	*
H <sub>2</sub> O <sub>2</sub>		*	*	*	*	NS
AIP X H <sub>2</sub> O <sub>2</sub>		*	*	NS	NS	NS

\*, NS Significant at the 0.05 level of probability and not significant, respectively.