

Determining the Role of Corn Wall Peroxidases in the Formation of Dehydrodiferulates That Cross-Link Arabinoxylans

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Introduction

Grasses contain a significant amount of ferulic acid within their cell walls. Recently we have shown that grass walls contain most of the dehydrodimers of ferulic acid predicted from free radical coupling mechanisms (Ralph et al. 1994). The most predominate diferulates were coupled 8-8, 8-5, 8-O-4, with the 5-5 dimer being relatively minor in all samples. We are interested in knowing more about cross-linking mechanisms within wall matrices and have undertaken a project to investigate the role of wall peroxidases in forming ferulate dehydrodimers.

Materials and Methods

Peroxidases were isolated from walls or media of 14-18 day old cultures (*Zea mays*, cv. Black Mexican). Initial work revealed that the peroxidases secreted into the culture medium were the same as those extracted from isolated walls of corn cell cultures using 200mM CaCl₂. The crude culture media was fractionated into major subgroups of peroxidases based on their isoelectric points (pI) using ammonium sulfate fractionation, DEAE anion exchange chromatography, and chromatofocusing chromatography. Fractions containing high peroxidase activity were pooled and concentrated for subsequent chromatography steps. Specific activity against methyl ferulate, methyl-*p*-coumarate, methyl sinapate, sinapyl alcohol, and coniferyl alcohol was determined spectrophotometrically (see Table 1 for conditions). Product formation was determined using methyl ferulate as the peroxidase substrate and analyzing for dehydrodimers.

Results

The crude peroxidase mixture was separated into several subfractions using combinations of anion exchange and chromatofocusing chromatography. This fractionation scheme did not produce single protein bands but rather 2-4 closely related peroxidases in each subfraction. Four subfractions were selected for further study—P1, P2, P3 and PNa. The isoelectric points of the subgroups based on IEF PAGE 3-10 gels using diaminobenzidine to stain for peroxidase activity were: P1 pH 8-9 (2 protein bands), P2 pH 6.5-8 (4

protein bands), P3 pH 6 (1 protein band) and PNa pH 3-4 (4 protein bands). The pH optimum for each of the subgroups was nearly the same—5 to 5.5.

Activity of the peroxidases using different substrates varied with the substitution upon the aromatic ring (Table 1). For all peroxidase groups, the highest activity was against methyl ferulate, followed by coniferyl alcohol. Both of these have a methoxyl group at C3 on the aromatic ring. Methyl-*p*-coumarate (no methoxyl substitution) was also actively utilized by the peroxidases but at roughly half the rate of the methyl ferulate and coniferyl alcohol (Table 1). Methyl sinapate and sinapyl alcohol (methoxyl substitution at both C3 and C5 of the aromatic ring) were poorly utilized by all of the peroxidases tested (Table 1).

Methyl ferulate was used as a substrate to determine if the peroxidase subgroups produced different dehydrodimers. For each subgroup of peroxidases, the dehydrodiferulates detected arose from radical coupling reactions involving C8 and producing dehydrodiferulates coupled at 8-8, 8-O-4 and 8-5. No detectable 5-5 or 4-O-5 dimers were produced by the peroxidases. Although 5-5 dimers have been identified in plant extracts, no 4-O-5 dehydrodiferulate has been detected.

Conclusions

All peroxidase groups readily utilized the methyl ferulate to produce the same dehydrodimers. For methyl ferulate, 8-coupled products are the preferred radical coupling mechanism for forming dehydrodimers. Within the wall matrix, positioning of xylan chains may aid in 5-5 dehydrodiferulate formation. Alternatively, the 5-5 product may readily undergo additional radical coupling to form trimers in the free solution state, therefore being lost to detection. All of the dimers could potentially undergo additional radical reactions forming higher order products accounting for the decrease in total recovery of methyl ferulate (unreacted + dimers) with increased reaction time.

Reference

Ralph, J., S. Quideau, J.H. Grabber and R.D. Hatfield.
1994. Identification and synthesis of new ferulic
dehydrodimers present in grass cell walls. J. Chem.
Soc., Perkin Trans. 1. 23:3485-3498.

Table 1. Substrates utilized by the peroxidase subgroups. All values are the amount of substrate used in mmol/min/mg of protein. (Reaction contained 0.15 mM substrate, 0.25 mM H₂O₂, in 1 mL of 50 mM Tris -acetate pH 5)

Peroxidase subgroup	Methyl ferulate	Methyl <i>p</i> -coumarate	Methyl sinapate	Coniferyl alcohol	Sinapyl alcohol
Pna	564	328	2	564	12
P1	148	51	0.2	116	2.7
P2	75	21	0.1	54	0.1
P3	14	11	0	10	0.6

Table 2. Reaction products produced from peroxidase subgroups utilizing methyl ferulate as substrate (0.15mM Me-FA, 0.25 mM H₂O₂ in 5 mL of 50 mM Tris-acetate pH 5). Values are the fractional amounts produced of each of the dehydrodimers. No 5-5 or 4-*O*-5 dimers were detected.

Peroxidase subgroup	Dehydrodimer linkage		
	8-5	8- <i>O</i> -4	8-8
Pna	0.36	0.27	0.38
P1	0.25	0.38	0.37
P2	0.29	0.36	0.36
P3	0.31	0.08	0.61