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Incorporation of Proline and Aromatic Amino Acids into Cell Walls of Maize Coleoptiles[1]

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ABSTRACT

Sections excised from maize coleoptiles incorporated radioactivity from proline, tyrosine, and phenylalanine into structural components of the cell wall. Only about 2% of radioactivity from proline taken up by sections was incorporated into cell wall; about 24% of that incorporated was in hydroxyproline and the rest remained in proline. In contrast, as much as 40% of the radioactivity from phenylalanine and 30% from tyrosine was incorporated into cell wall material. Most of this radioactivity was in saponifiable ferulic acid. Small amounts of *p*-coumaric and diferulic acid were found, but only a small fraction of the hemicellulose can possibly be immobilized directly through cross-linking of diferulic esters. Substantial amounts of radioactivity from aromatic amino acids remained insoluble after strong alkali extractions of wall material, and a large fraction of polysaccharide was solubilized by dilute alkali following oxidation of phenolics by acidic $NaClO_2$. Hence, hemicellulosic material in the cell walls of maize coleoptiles may be organized and cross-linked primarily through alkali-resistant etherified aromatics.

The chemical structure and proportion of hemicellulosic and pectic constituents of the primary cell walls of monocotyledonous species differ markedly from those of dicotyledonous species (30). Although accurate three-dimensional models of cell wall structure are yet to come, the organization of the polysaccharide components into a rigid matrix must involve participation of glycoproteins and phenolic substances as cross-linking agents (10, 14, 15, 22, 23, 26). Just as there are differences in the kinds of polysaccharides that comprise the walls of monocots and dicots, so too are there differences in the kinds of cross-linking substances. Hydroxyproline-rich glycoproteins are abundant in walls of dicots (10, 22), but are minor constituents of the walls of cereals and other grasses. On the other hand, phenolic substances have been observed by fluorescence microscopy throughout the primary walls of grass tissues (17, 19); ferulic acid has been identified unequivocally as a major phenolic compound (12, 13, 19, 21, 27, 29, 31), and its esterification to hemicellulosic arabinoxylans has been documented (21, 27). Moreover, Markwalder and Neukom (23) have also identified diferulic acid in wheat flour and implicated this ferulate biphenyl dimer in cross-linking the water-soluble pentosans into an insoluble matrix. Others have identified diferulate in other tissues and have demonstrated its peroxidative synthesis (28).

In studies of polysaccharide structure and organization in maize coleoptiles, hemicellulosic polymers were extracted by increasing concentrations of alkali and were grouped into three major fractions: GAX[2] I comprising highly-substituted glucuronoarabinoxylans (GAX) released by dilute alkali; GAX II comprising xylans with much less side-chain substitution and some (1-3),(1-4)-β-D-glucan(β-glucan) released by up to 1.0 N KOH; and MG-GAX composed of additional GAX, β-glucan, and xyloglucan released by 2.0 N KOH and greater and representing about half of the total hemicellulosic material (5). The proportion and composition of these fractions varied markedly during coleoptile development (7). In related studies, treatment of walls of maize coleoptiles with acidic sodium chlorite to oxidize phenolic substances rendered much of the hemicellulosic material soluble in as little as 0.02 N KOH (6). Hence, a significant proportion of the hemicellulosic constituents may be cross-bridged. Ferulic acid is indisputably a component of the walls of coleoptiles (29, 31), and a large proportion of [^{14}C]cinnamate or [^{14}C]phenylalanine is incorporated into readily saponifiable components (29). Much more concentrated alkali is required to solubilize most of the hemicellulosic polysaccharide before sodium chlorite treatment, so many of these polymers may be cross-linked by phenolic linkages other than esterified diferulic acid.

Maize coleoptiles are classic tissues for study of cell elongation; while auxin induces obvious changes in the rheological properties of the cell walls (9), the chemical bases for these changes are not understood. Circumstantial evidence has implicated hydrolysis of load-bearing polysaccharides as the primary action to initiate wall loosening during growth; less emphasis has been placed on the role that cross-linking agents may play in hormone-induced wall expansion, although Fry (15) has recently reintroduced this concept. To provide a framework to study the role that phenolic cross-bridging plays in the organization of maize hemicelluloses, this current study focuses on the uptake of specific amino acids and the incorporation of their carbon skeletons into the cell walls of developing maize coleoptiles.

MATERIALS AND METHODS

Plant Material and Incubation of Seedlings. Seeds of maize (*Zea mays* L. cv WF9 × Bear 38) were obtained from Custom Farm Seed (Momence, IL) and were stored and incubated as described previously (8). Upon incubation of seeds at 28°C in darkness, the coleoptile ruptures the pericarp on the 2nd d and elongates to about 4.5 cm by the 5th d before senescence begins (Fig. 1, inset).

Pulse-Labeling of Coleoptile Sections. Five-mm sections were excised from the coleoptiles 1 to 2 mm below the tip with a double-bladed cutting device and were floated on deionized H_2O until enough had been collected for each experiment (about 30 min). Seventeen to 20 sections were blotted dry and floated on 3 ml of 5 mM K-phosphate, 5 mM K-citrate (pH 6.0), supplemented with 10 mM D-glucose, 5 mM KCl, 0.01% tetracycline (HCl), ± 2 × 10^{-5} M IAA, and 100 μCi of [U-^{14}C]proline (255 mCi/mmol), [U-^{14}C]phenylalanine (415 mCi/mmol), or [U-^{14}C]

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[2] Abbreviations: GAX, glucuronoarabinoxylan; β-glucan, (1-3),(1-4)-β-D-glucan; Freon, 1,1,2-trichlorofluoroethane; TMSi, trimethysilyl.

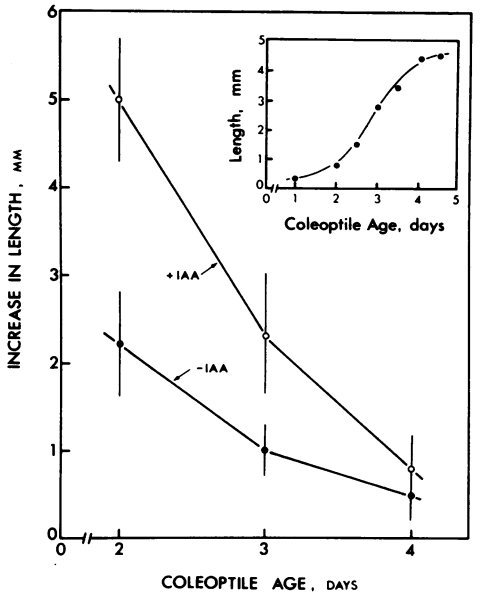

FIG. 1. Elongation of coleoptile sections excised from coleoptiles of varying stages of development. Maize seeds were soaked overnight in running tap water and incubated in darkness for 1 to 4 d at 28°C. One-half-cm sections were excised 1 to 2 mm below the tip from 2 d- through 4 d-old coleoptiles and floated on a K-phosphate, K-citrate buffer ± 2 × 10^{-5} M IAA for 12 h in darkness at 28°C. Elongation was measured with the aid of a photographic enlarger (8). Inset: Changes in total length of the coleoptile during development.

tyrosine (415 mCi/mmol). The sections were incubated for 12 h at 28°C in 4.5-cm diameter Petri dishes 1.5-cm deep sealed with Parafilm. Final lengths of the sections were recorded using a photographic enlarger (8). The sections were rinsed briefly with deionized H_2O, and a squirt bottle was used to rinse excess medium from the coleoptile cylinders. The coleoptile sections were frozen in liquid N_2.

Fractionation of Coleoptile Radioactive Pools. Frozen sections were homogenized as described previously (5) in about 5 ml of 0.05 M K-Tes (pH 7.2), supplemented with 10 mM ascorbate, and cell walls and other debris were pelleted by centrifugation at 1200*g*. Cell walls were purified by sequential washes in 0.5 M K-phosphate (pH 7.0), water, chloroform: methanol (1:1, v/v), acetone, methanol, and water as described previously (8). Samples of each fraction after K-phosphate washes were assayed for radioactivity by liquid scintillation spectroscopy; these combined measurements constituted the fraction labelled "other" in Table I. The K-Tes and K-phosphate soluble materials were brought to 80% ethanol (v/v) and stored at −20°C overnight. Material precipitated by the ethanol was collected by centrifugation, and samples of both the 80% ethanol-soluble and ethanol-precipitable materials were assayed for radioactivity. The cell wall material was suspended in water, frozen, and lyophilized, and wall weights were used to standardize the relative incorporation of radioactivity.

Fractionation of the Cell Wall Material. Wall material was extracted with anhydrous DMSO to remove starch and was extracted in hot 0.5% ammonium oxalate to remove pectic substances (5). Hemicelluloses were extracted under N_2 with increasing concentrations of KOH from 0.01 to 4 N as described before (5). Samples of each extract were neutralized with glacial acetic acid, and sugars were determined by the phenol-H_2SO_4 method (11). Esterified hydroxycinnamic acids were completely liberated from any solubilized polymers by bringing each supernatant to 5 N KOH and incubating the mixture at 30°C for 1 h. Solutions were bubbled with N_2 during addition of KOH and sealed under N_2 during incubations to prevent oxidation. In preliminary experiments, $NaBH_4$ was added to the alkali solutions to prevent end elimination of polysaccharide (1) but was excluded in experiments described here because it reduces the α,β-unsaturated carbonyl side-chain of hydroxycinnamic acids. The mixture was chilled after saponification, and an equal volume of 6 N HCl was added quickly. Hydroxycinnamic acids and other phenolic materials were partitioned into 3 × 0.5 volumes of 1-butanol. The butanol extracts were combined, and the material was repartitioned into 3 × 0.5 volumes of 5% $NaHCO_3$. The combined $NaHCO_3$ fractions were acidified to pH 0.5 with 6 N HCl, and the hydroxycinnamic acids were repartitioned into 3 × 0.5 volumes of butanol. Samples of each fraction were assayed for radioactivity, and the butanol extracts were dried under a stream of N_2.

After extraction overnight in 4 N KOH, the α-cellulose slurry in KOH was neutralized with glacial acetic acid, washed with water, and assayed directly for radioactivity.

Synthesis of Diferulic Acid. Ferulic acid (Sigma) was oxidized by horseradish peroxidase and H_2O_2 as reported by Baumgartner and Neukom (3). One hundred μmol of ferulic acid were dissolved in 10 ml of 0.1 M K-phosphate (pH 6.0) and 100 units of horseradish hydrogen peroxide oxidoreductase (EC 1.11.1.7; Sigma) and 75 μmol of H_2O_2 were added to the solution. The mixture was stirred in darkness for 24 h, then acidified to pH 0.5 with HCl, and the phenolics were partitioned into 1-butanol and repartitioned as described above. The final butanol phase was evaporated under reduced pressure. The residue was dissolved in methanol, streaked on a plate of silica gel (Kieselgel; Scientific Products), and developed in benzene:methanol:acetic acid (45:8:4, v/v/v). The band of diferulic acid (R_F = 0.21) located by UV fluorescence was scraped from the plate, extracted with methanol, and dried under a stream of N_2.

Separation and Identification of Hydroxycinnamic Acids. Residues after evaporation of butanol were dissolved in methanol, and aliquots were diluted to 15% methanol and 85% 0.05 M Na-acetate (pH 4.7). Hydroxycinnamic acids were separated by HPLC on a 0.5-cm × 25.0-cm C_8 column (Applied Science) equilibrated in 15% methanol and 85% 0.05 M Na-acetate (pH 4.7). HPLC was with a Spectra Physics 8000 with A_{254} monitored by Spectra Physics 8310 detector, and peak areas were quantified with a Spectra Physics 4100 computing integrator. The phenolics were eluted at 1.0 ml/min with a program of 15% methanol and 85% 0.05 M Na-acetate (pH 4.7), for 15 min, increased to 30% methanol in the next 5 min, held at 30% methanol for 10 min, increased to 100% methanol in the next 10 min, and held at 100% methanol for 10 min (18). One ml fractions were collected, and samples were assayed for radioactivity. Hydroxycinnamic acids identified by retention time were *p*-coumaric acid, caffeic acid, ferulic acid, and diferulic acid; *o*-coumaric acid was used as internal standard for quantitation.

After separation, fractions containing phenolics were acidified to pH 0.5, and hydroxycinnamic acids were partitioned into diethyl ether or ethyl acetate. The organic phases were concentrated under a stream of N_2 and samples of the concentrate were subjected to solid probe MS. Spectra were obtained at a source temperature of 300°C at 70 eV on a Finnigan/MAT 4021 quadrupole MS interfaced to a Finnigan/MAT 2100C INCOS data system.

Other samples were dried completely over P_2O_5 in a vacuum desiccator, and 100 μl of BSTFA in pyridine (Pierce) was added to the residue under N_2. The mixture was incubated at 45°C for 18 h with occasional vortex mixing. The resultant TMSi derivatives were separated by GLC on a 0.2-mm × 25-m vitreous silica WCOT capillary column OV-1 (Supelco) temperature programmed from 200 to 240°C at 2°C/min in a Finnigan/MAT 9610 GC coupled to the quadrupole MS system described above.

Table I. *Uptake of ^{14}C-Amino Acids and Incorporation of Radioactivity into Cellular Fractions*

One-half-cm sections were excised from 2-d through 4-d-old coleoptiles 1 to 2 mm below the tip and floated on media containing the appropriate amino acid ± 2 × 10^{-5} M IAA. Sections were pulsed for 12 h; uptake was assayed from disappearance of radioactivity from the medium. Sections were rinsed with water, frozen in liquid N_2, and homogenized in 0.05 M Tes (pH 7.2). The wall material was washed with 0.5 M K-phosphate (pH 7.0) and combined supernatants were brought to 80% ethanol. Ethanol soluble material represented mostly the endogenous pool of amino acid, and the ethanol-insoluble pool contained mostly protein. The wall material was washed further with water, chloroform:methanol, acetone, and water; samples of each extract were assayed for radioactivity and represented as "other." The cell wall fraction represents the sum of all further fractionations. Values represent the mean ± SD of three experimental samples of 17 to 20 coleoptile sections each; values in parentheses are the percent of radioactivity partitioned into each fraction.

Amino Acid: Coleoptile Age	IAA	Uptake	Cytoplasmic		Other	Cell Wall
			Ethanol soluble	Ethanol insoluble		
			cpm/mg cell wall (× 10^{-3})			
[U^{14}C]phenylalanine						
2 d	−	1413.2 ± 31.1	709.1 ± 20.7 (50.1)	418.0 ± 16.4 (29.6)	14.5 ± 0.2 (1.0)	272.7 ± 1.4 (19.3)
	+	1413.8 ± 41.1	700.1 ± 18.0 (50.8)	389.3 ± 18.9 (28.2)	17.5 ± 1.2 (1.3)	272.1 ± 7.0 (19.7)
3 d	−	1862.9 ± 8.4	642.9 ± 34.9 (38.4)	476.9 ± 18.2 (28.5)	21.4 ± 0.5 (1.3)	531.7 ± 13.9 (31.8)
	+	1971.3 ± 20.1	665.7 ± 29.0 (38.5)	494.8 ± 16.6 (28.6)	27.7 ± 1.7 (1.6)	542.3 ± 26.8 (31.3)
4 d	−	2131.5 ± 108.6	647.4 ± 16.3 (30.5)	546.5 ± 34.1 (25.8)	62.0 ± 2.6 (2.9)	863.7 ± 42.0 (40.7)
	+	2001.5 ± 172.1	643.0 ± 29.1 (31.2)	523.6 ± 13.6 (25.4)	63.3 ± 5.7 (3.1)	828.6 ± 48.3 (40.3)
[U-^{14}C]tyrosine:						
2 d	−	986.2 ± 122.9	420.9 ± 28.5 (52.9)	269.9 ± 41.8 (33.9)	19.5 ± 2.1 (2.5)	85.5 ± 49.4 (10.7)
	+	1058.0 ± 43.1	517.0 ± 36.0 (52.9)	295.5 ± 44.9 (30.2)	21.1 ± 0.8 (2.2)	143.4 ± 13.5 (14.7)
3 d	−	1164.6 ± 58.9	441.6 ± 13.9 (51.0)	238.5 ± 15.8 (27.5)	36.3 ± 0.1 (4.2)	149.5 ± 11.8 (17.3)
	+	1271.4 ± 213.0	443.0 ± 49.2 (48.3)	247.9 ± 33.4 (27.0)	45.2 ± 6.8 (4.9)	181.0 ± 8.8 (19.7)
4 d	−	1535.7 ± 59.1	324.6 ± 19.6 (35.5)	254.0 ± 11.0 (27.8)	42.7 ± 2.6 (4.7)	291.8 ± 15.6 (32.0)
	+	1570.0 ± 58.1	414.4 ± 13.9 (41.0)	272.3 ± 8.1 (27.0)	39.5 ± 3.1 (3.9)	283.7 ± 14.1 (28.1)
[U-^{14}C]proline:						
2 d	−	1069.1 ± 78.2	740.4 ± 45.7 (62.7)	422.9 ± 25.9 (35.8)	2.0 ± 0.2 (0.2)	16.2 ± 2.2 (1.4)
	+	1255.3 ± 79.0	898.3 ± 50.0 (64.0)	485.3 ± 12.0 (34.6)	2.4 ± 0.2 (0.2)	16.8 ± 1.6 (1.2)
3 d	−	1856.3 ± 52.7	608.3 ± 27.7 (51.2)	547.7 ± 22.2 (46.1)	5.0 ± 0.3 (0.4)	28.0 ± 1.2 (2.4)
	+	1593.8 ± 111.0	580.3 ± 8.9 (53.3)	478.2 ± 41.6 (43.9)	4.2 ± 0.5 (0.4)	26.2 ± 2.3 (2.4)
4 d	−	1437.1 ± 64.2	1077.2 ± 52.0 (71.8)	391.4 ± 16.4 (26.1)	3.9 ± 0.1 (0.3)	27.9 ± 3.1 (1.9)
	+	1254.4 ± 39.5	937.7 ± 61.5 (72.0)	338.5 ± 14.4 (26.0)	3.5 ± 0.2 (0.3)	22.2 ± 4.1 (1.7)

Table II. *Incorporation of Radioactivity from ^{14}C-Amino Acids into Cell Wall Fractions*

Cell wall material was fractionated sequentially in anhydrous DMSO, hot 0.5 M ammonium oxalate, and step-wise with increasing concentrations of 0.01 N to 4 N KOH (Fig. 2). Samples of each were assayed for radioactivity by liquid scintillation spectroscopy. Values represent the mean ± SD of three experiments of 17 to 20 coleoptile sections each; values in parentheses are percent of radioactivity in each fraction.

Amino Acid: Coleoptile Age	IAA	DMSO	Ammonium Oxalate	KOH	α-Cellulose
		cpm/mg cell wall (× 10^{-3})			
[U-^{14}C]phenylalanine					
2 d	−	7.03 ± 4.68 (2.6)	13.98 ± 0.20 (5.1)	221.38 ± 7.13 (81.2)	30.34 ± 5.56 (11.1)
	+	6.39 ± 2.00 (2.3)	15.34 ± 1.71 (5.6)	228.60 ± 11.98 (84.0)	21.75 ± 1.78 (8.0)
3 d	−	24.77 ± 6.25 (4.7)	24.30 ± 1.04 (4.6)	428.79 ± 24.55 (80.6)	53.82 ± 5.50 (10.1)
	+	27.57 ± 2.78 (5.1)	27.26 ± 3.04 (5.0)	446.95 ± 36.10 (82.4)	40.56 ± 10.62 (7.5)
4 d	−	42.00 ± 7.65 (4.9)	43.00 ± 1.66 (5.0)	698.01 ± 80.15 (80.8)	80.66 ± 3.24 (9.3)
	+	19.53 ± 6.82 (2.4)	38.11 ± 1.74 (4.6)	690.09 ± 43.14 (83.3)	80.91 ± 13.04 (9.8)
[U-^{14}C]tyrosine					
2 d	−	8.98 ± 1.82 (10.5)	8.92 ± 1.71 (10.4)	47.68 ± 4.91 (55.8)	19.92 ± 3.35 (23.3)
	+	17.18 ± 10.48 (12.0)	18.80 ± 3.12 (13.1)	80.07 ± 36.70 (55.7)	27.37 ± 5.37 (19.1)
3 d	−	11.13 ± 1.40 (7.4)	12.02 ± 1.74 (8.0)	103.64 ± 20.28 (69.3)	22.70 ± 2.93 (15.2)
	+	12.82 ± 3.71 (7.1)	21.00 ± 3.21 (11.6)	120.99 ± 17.39 (66.9)	26.15 ± 10.95 (14.5)
4 d	−	28.79 ± 5.04 (9.9)	33.87 ± 5.63 (11.6)	176.14 ± 11.56 (60.4)	53.00 ± 11.27 (18.2)
	+	23.87 ± 1.78 (8.4)	34.50 ± 4.44 (12.2)	180.34 ± 19.88 (63.6)	45.02 ± 12.55 (15.9)
[U-^{14}C]proline					
2 d	−	1.24 ± 0.51 (7.6)	1.59 ± 0.37 (9.8)	12.82 ± 6.04 (79.1)	0.56 ± 0.42 (3.5)
	+	0.66 ± 0.32 (3.9)	2.05 ± 0.26 (12.2)	13.46 ± 5.30 (80.0)	0.66 ± 0.46 (3.9)
3 d	−	2.23 ± 0.37 (8.0)	2.28 ± 0.10 (8.2)	21.97 ± 8.10 (78.5)	1.49 ± 0.58 (5.3)
	+	1.59 ± 1.36 (6.1)	2.87 ± 0.82 (10.9)	20.73 ± 7.91 (79.0)	1.00 ± 0.29 (3.8)
4 d	−	1.93 ± 0.60 (6.9)	2.27 ± 0.18 (8.1)	22.68 ± 4.73 (81.2)	1.06 ± 0.40 (3.8)
	+	2.24 ± 0.41 (10.1)	1.73 ± 0.21 (7.8)	17.28 ± 2.49 (77.8)	0.97 ± 0.35 (4.4)

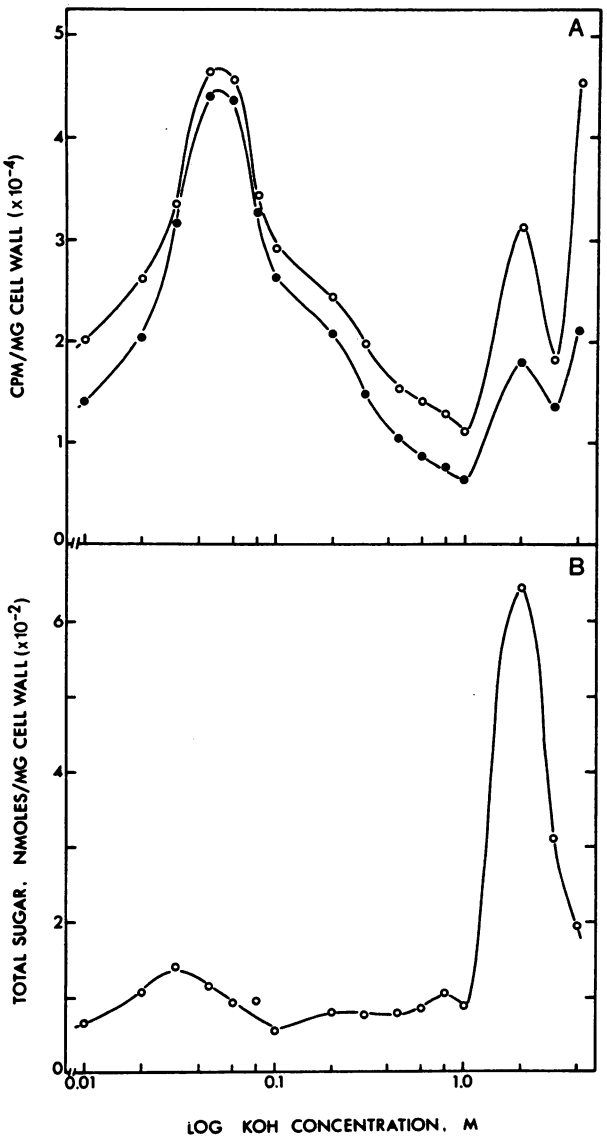

FIG. 2. Comparison of the fractionation of hemicellulosic sugar and aromatic material with increasing concentration of alkali. Hemicellulosic material from sections from 3-d coleoptiles (without IAA) was fractionated by increasing concentration of alkali, and samples of each soluble fraction were neutralized and total sugar determined by the phenol-H_2SO_4 method (11). The remainder was brought to 5 N NaOH under N_2 to complete saponification, and after acidification, aromatic material was partitioned in butanol. A, Alkali fractionation of total radioactivity (O) and butanol-soluble radioactivity (●); B, fractionation of hemicellulosic sugar.

Injector and interface oven were at 250°C and 2-μl samples were injected with a split ratio of about 100:1. He carrier flow was 1.5 ml/min, and spectra were obtained at 70 eV and at a source temperature of 300°C.

Extraction and Quantitation of Amino Acids. Coleoptile sections (0.5–4.5 g) from 2 d- through 4 d-old seedlings were homogenized in 2 volumes of ice cold 10% (w/v) TCA. One-tenth μCi each of [U-^{14}C]proline and [U-^{14}C]tyrosine were added to monitor recovery. The homogenates were centrifuged at 13,000*g* for 10 min at 0 to 4°C. The supernatant was partitioned 2 times against an equal volume of Freon:tri-octyl amine (3:1, v/v) to remove the TCA. The aqueous layers were loaded onto 0.9-cm × 3.0-cm columns of precycled Dowex 50W-×8 (H^+) equilibrated with H_2O. The columns were washed with 10 ml of H_2O, and the amino acids were eluted with 10 ml of 6 N NH_4OH. The NH_4OH was evaporated under reduced pressure; the residue was dissolved in 2.0 ml of 6 N NH_4OH, and 200 μl samples were evaporated to dryness in a stream of N_2 in 1-dram vials sealed with Teflon-lined caps. The *N*-heptafluorobutyryl isobutyl derivatives were prepared as described by Rhodes *et al.* (24) and separated on a 0.2-mm × 25-m WCOT vitreous silica capillary column of OV-11 (Supelco) temperature programmed from 120 to 250°C at 5°C/min. Samples (2 μl) were injected with a split ratio of 100:1 and a N_2 carrier flow of about 1.0 ml/min. Injector and flame ionization detector were at 260°C.

Paper Chromatography of Amino Acids. Aqueous soluble material after butanol extraction was neutralized, dialyzed against running deionized H_2O for at least 18 h, and lyophilized. The dry material was hydrolyzed in 1 ml of 6 N HCl under N_2 in sealed 1-dram vials for 24 h at 121°C. The HCl was evaporated in a stream of N_2, and the residues were dissolved in 6 N NH_4OH and spotted on strips of Whatman 3MM filter paper. Amino acids were separated by descending chromatography in 1-butanol:H_2O:acetic acid (15:5:3; v/v/v) (14). Spots of reference strips were detected by ninhydrin; proline and hydroxyproline were detected with an isatin spray reagent (Sigma). Strips with radioactive samples were cut into 1.5-cm sections and counted by liquid scintillation spectroscopy.

RESULTS AND DISCUSSION

Maize coleoptiles elongate for about 5 d before senescence begins. Rapid elongation begins about the 2nd d when the coleoptile ruptures the pericarp; elongation rates are maximal between 2 and 3 d incubation, but rates decrease between 3 and 4 d when the etiolated leaves emerge through the coleoptile tip (Fig. 1, inset). By the 2nd d, the coleoptiles are large enough to excise sections, and IAA induces elongation rates twice that in control sections (Fig. 1). As the coleoptiles age, the response to IAA diminishes, and by 4 d, elongation rates of excised sections are minimal. Why auxin responsiveness is lost is not clear, but a reasonable possibility is that upon cell elongation there are irreversible changes in cell wall structure that render them unresponsive to auxin action and, therefore, limit the extent of wall expansion. Avery and Burkholder (2) have shown that cell divisions cease early during coleoptile development, so the proportion of fully elongated cells in 5-mm sections would become increasingly larger and, hence, unresponsive to IAA as the coleoptiles age.

Amounts of labeled phenylalanine, tyrosine, and proline taken up by the coleoptile sections in 12 h were similar (Table I); uptake of the aromatic amino acids was markedly greater in sections from older coleoptiles, even though elongation was reduced (Table I; Fig. 1). Auxin had insignificant effect on uptake and incorporation of radioactivity from any amino acid into any cellular pool (Tables I and II).

A major consideration in interpreting data on uptake and incorporation of the labeled amino acids is the endogenous pool size and, hence, the extent of isotope dilution. Quantitation of amino acids demonstrated that Asx (aspartic acid and asparagine) and Glx (glutamic acid and glutamine) were the major constituents. Levels of Asx and Glx were initially 17.2 and 7.0 μmol/mg fresh weight, respectively, in sections from 2 d coleoptiles and decreased to 11.9 and 0.9 μmol/mg fresh weight in 4 d coleoptides. Levels of proline, phenylalanine, and tyrosine were much lower. Amounts of proline were initially 1.42 μmol/mg fresh weight and fell to 0.76 μmol/mg fresh weight in 3-d coleoptiles and to 0.54 μmol/fresh weight in 4 d coleoptiles; levels of phenylalanine and tyrosine were initially 0.47 and 0.49 μmol/mg fresh weight, increased slightly to 0.75 and 0.60 μmol/mg fresh weight in 3-d coleoptiles, and decreased to only 0.37

Table III. *Distribution of Radioactivity from ^{14}C-Amino Acids Incorporated into Hemicellulosic Material*

Hemicellulosic material was fractionated in increasing concentrations of alkali as represented in Figure 2. Values were grouped in dilute alkali- (0.01–0.2 N KOH: GAX I), moderate alkali- (0.3–1 N KOH; GAX II), and strong alkali-extractable material (2–4 N KOH: MG-GAX). Values represent the mean of three experiments with 17 to 20 coleoptile sections each; values in parentheses are the percent distribution of radioactivity in each fraction.

Fraction: Coleoptile Age	[U-^{14}C]Phenylalanine		[U-^{14}C]Tyrosine		[U-^{14}C]Proline	
	Butanol	Aqueous	Butanol	Aqueous	Butanol	Aqueous
	cpm/mg cell wall (× 10^{-3})					
GAX I						
2 d	133.1 (60.1)	9.7 (4.4)	31.2 (65.4)	3.1 (6.5)	1.4 (11.0)	1.7 (13.4)
3 d	232.5 (54.2)	17.1 (4.0)	65.9 (63.5)	4.4 (4.2)	2.3 (10.5)	2.6 (11.8)
4 d	363.2 (52.0)	25.9 (3.7)	83.8 (47.6)	9.6 (5.5)	2.6 (11.5)	1.6 (7.0)
GAX II						
2 d	33.1 (14.9)	9.4 (4.2)	6.1 (12.8)	1.1 (2.3)	1.0 (7.9)	1.8 (14.2)
3 d	59.0 (13.8)	19.2 (4.5)	14.3 (13.8)	2.5 (2.4)	1.5 (6.8)	2.9 (13.2)
4 d	119.8 (17.2)	28.7 (4.1)	24.5 (13.9)	7.4 (4.2)	1.2 (5.3)	2.0 (8.8)
MG-GAX						
2 d	22.0 (9.9)	14.2 (6.4)	3.8 (8.0)	2.4 (5.0)	2.8 (22.0)	4.0 (31.5)
3 d	59.5 (13.9)	41.4 (9.7)	10.1 (9.7)	6.5 (6.3)	5.0 (22.7)	7.7 (35.0)
4 d	80.6 (11.5)	79.8 (11.4)	28.0 (15.9)	22.8 (12.9)	6.9 (30.4)	8.4 (37.0)

and 0.34 μmol/mg fresh weight respectively, in 4-d coleoptiles. Thus, with the exception of slightly higher proline levels in 2 d coleoptiles, apparent concentrations of each of the amino acids of interest in this study were similar, and estimations of uptake and incorporation of labeled amino acid should reasonably reflect the relative partitioning of endogenous pools.

When sections from young coleoptiles were pulse-labeled with proline, at least one-half of the radioactivity remained soluble in 80% ethanol. In sections from older coleoptiles, much less radioactivity from the aromatic amino acids remained in this ethanol-soluble fraction, while more than 70% of the radioactivity from proline remained soluble (Table I). Regardless of coleoptile age, sections incorporated about 25 to 30% of the radioactivity from phenylalanine into ethanol-precipitable material containing most of the protein of the cells. Sections from young coleoptiles incorporated about 20% of the radioactivity from phenylalanine into the cell wall fraction, and sections from mature coleoptiles incorporated about 40% of this label into the wall—about 65% more than that incorporated into the major protein fraction (Table I). The fate of radioactivity from uptake of tyrosine was similar, and despite the fact that uptake was generally slower than that of phenylalanine, slightly more remained soluble in 80% ethanol at the expense of incorporation into the cell wall. Both phenylalanine and tyrosine apparently accumulate at rates faster than they are used, and some caution must be exercised in interpreting these data; artificial increases in cytoplasmic pools may induce alternative "detoxification" pathways. Fry (16) also expressed concern over this possibility in his experiments in which [^{14}C]cinnamate was fed to spinach cell suspension cultures, but providing cinnamate as a single pulse or slowly via a peristaltic pump made little difference in its metabolic fate.

The fate of radioactivity from uptake of proline was drastically different than that of the aromatic amino acids. Much more remained soluble in 80% ethanol, and less than 3% of the radioactivity was incorporated into cell wall material regardless of the age of the coleoptile from which sections were taken (Table 1). Hence, the aromatic amino acids are precursors of predominant components of the cell wall, and proline and hydroxyproline in wall protein are obviously minor constituents.

When the cell wall material was fractionated further, most of the radioactivity from any amino acid was in the hemicellulose fraction (Table II). Much of the remainder was not extracted by as much as 4 N KOH and remained in the α-cellulose fraction. The hemicellulosic material was extracted step-wise in increasing concentrations of alkali, and esterified phenolics were saponified in 5 N NaOH; the radioactivity was assayed in both the butanol-soluble fractions of the saponification mixture and in the aqueous-soluble fractions. An example of a fractionation of hemicellulosic material from sections of 3 d coleoptiles demonstrated that radioactivity from [^{14}C]phenylalanine was mostly incorporated into dilute alkali-extractable material, and most of this radioactivity was soluble in butanol (Fig. 2). Most of the butanol-soluble radioactivity was released by 0.03 N to 0.06 N KOH, coincident with extraction of GAX I (Fig. 2), shown previously to comprise a highly substituted GAX (5). Although the data indicated that the highly substituted GAX contained most of the esterified phenolics and may be cross-linked by these aromatics, dilute alkali could de-esterify aromatics attached to polysaccharide attached more firmly to the matrix through other linkages. A smaller, but significant amount of radioactivity was extracted by more concentrated alkali, but more of this radioactivity remained in the aqueous-soluble fraction after butanol extraction (Fig. 2). After extraction in 3 N KOH, extraction with 4 N KOH for 12 h released more aqueous-soluble radioactivity but little additional polysaccharide (Fig. 2). The butanol-soluble material from moderate and strong alkali extractions was not ferulic acid or other hydroxycinnamates but was not characterized further. An even larger amount of radioactivity remained in the α-cellulose fractions (Table II).

Comparisons of the incorporation of radioactivity from the amino acids into these butanol and aqueous fractions in similar extractions were summarized in Table III and demonstrated that carbon from aromatic acids was primarily in the butanol-soluble fractions from dilute alkali extracts. Although there were substantial increases in the amounts of radioactivity from phenylalanine and tyrosine incorporated into each fraction in sections from older coleoptiles, the proportions of radioactivity in the butanol-soluble fractions decreased slightly in dilute alkali extracts and increased concomitantly in the more concentrated alkali fractions (Table III). The small amount of radioactivity from [^{14}C]proline was distributed in both butanol- and aqueous-soluble fractions, but in contrast to the distribution of carbon from aromatic amino acids, most of the label from proline was in material extracted by the concentrated alkali (Table III).

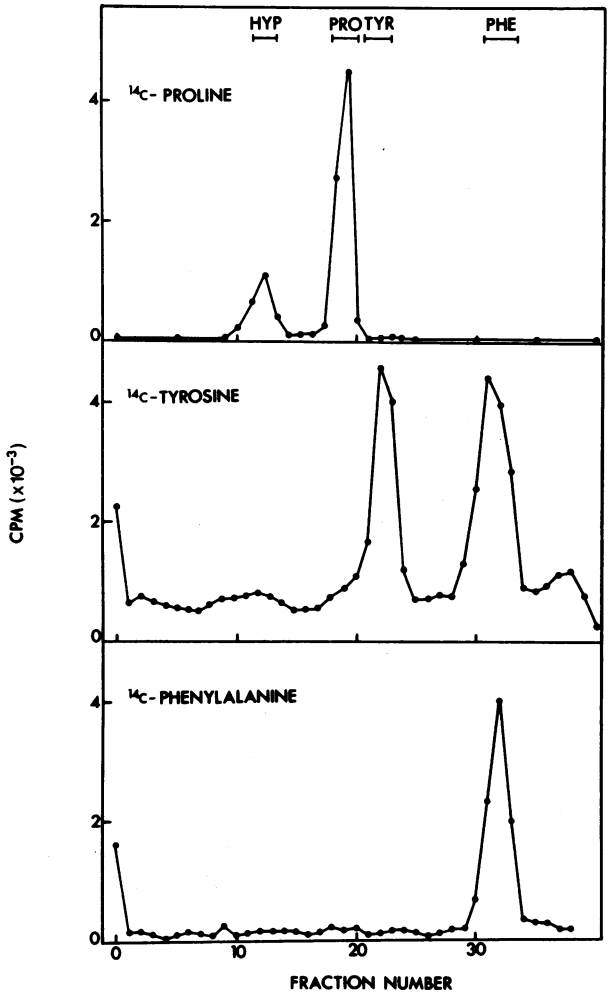

FIG. 3. Separation of radioactive amino acids from cell walls of maize coleoptiles. After butanol extraction, the aqueous soluble material from the strong-alkali extractions of the cell wall material from sections from 3 d-old coleoptiles (without IAA) was dialyzed and lyophilized, and the dry material was hydrolyzed in 6 N HCl under N_2 for 24 h at 121°C. The HCl was evaporated under a stream of N_2, and residue was dissolved in 6 N NH_4OH and spotted on Whatman 3MM paper strips. Amino acids were separated by descending chromatography 18 h in 1-butanol:H_2O:acetic acid (15:5:3, v/v/v) (14). Reference spots were detected by ninhydrin or an isatin spray reagent (Sigma); radioactivity was assayed in 1.5-cm sections by liquid scintillation spectroscopy.

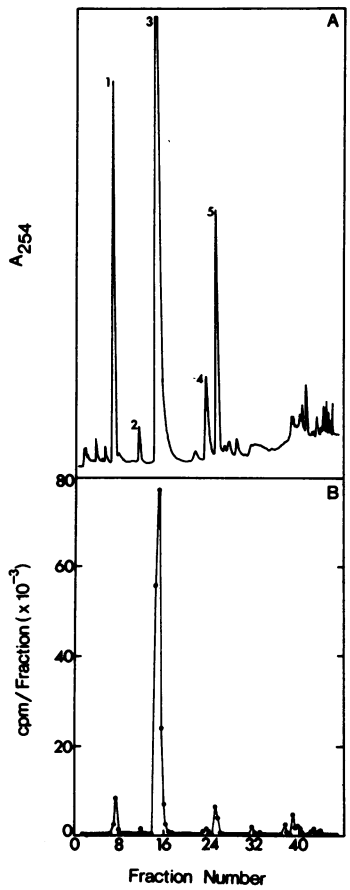

FIG. 4. Separation of hydroxycinnamic acids by reverse-phase HPLC. Butanol-soluble material from dilute alkali extracts from cell walls of sections from 3 d-coleoptiles (without IAA) was evaporated in a stream of N_2, and the residue was dissolved in methanol and brought 15% methanol in 85% 0.05 M Na-acetate (pH 4.7). Hydroxycinnamic acids were resolved in a methanol gradient. A, UV -absorbing material; peaks: 1, an unidentified derivative from ferulic acid; 2, *p*-coumaric acid; 3, ferulic acid; 4, *o*-coumaric acid (internal standard); 5, diferulic acid. B, Radioactivity in 1-ml fractions.

Analysis of acid hydrolysates of the insoluble material remaining after butanol extraction demonstrated that much of the radioactivity remaining was in amino acid. About 24% of the radioactivity from [^{14}C]proline was in hydroxyproline and the rest in proline. Radioactivity from both [^{14}C]phenylalanine and [^{14}C]tyrosine was incorporated into much more unidentified material. Substantial radioactivity from [^{14}C]phenylalanine remained primarily in phenylalanine while radioactivity from [^{14}C]tyrosine was in both tyrosine and phenylalanine, an unavoidable artefact of the saponification (Fig. 3).

Further fractionation of the butanol-soluble material from dilute alkali extracts by HPLC demonstrated that over 90% of the radioactivity was associated with the major UV absorbing peak identified by retention time as ferulic acid (Fig. 4). Small amounts of radioactivity were found in a void fraction, *p*-coumaric and, possibly, diferulic acid. Similar fractions from material from unlabeled coleoptile sections were acidified after HPLC, and these hydroxycinnamic acids were partitioned into ethyl acetate; solid probe MS of residues after evaporation of the ethyl acetate demonstrated that ferulic acid (m/z [relative intensities]): 195 [M^+ +1; 4], 194 [M^+; 100], 179 [M^+ −15; 20], 177 [M^+ −17; 8], 134 [M^+ −60; 6], 133 [M^+ −61; 23], 107 [M^+ −87; 7], 105 [M^+ −89; 12], 77 [M^+ −117; 15]) and *p*-coumaric acid (m/z [relative intensities]: 165 [M^+ +1; 5], 164 [M^+; 100], 147 [M^+ −17; 39], 119 [M^+ −45; 22], 118 [M^+ −46; 22], 107 [M^+ −57; 8], 91 [M^+ −73; 26], 89 [M^+ −75; 10], 65 [M^+ −99; 19]) were the major hydroxycinnamic acids. From GLC-MS analysis of TMSi-derivatives, identification of these hydroxycinnamic acids was verified. Diferulic acid was tentatively identified by retention time with the synthesized diferulic acid, but insufficient amounts were recovered to verify its identity by MS. In preliminary experiments, $NaBH_4$ was added to the KOH during extractions of cell walls to prevent end elimination of polysaccharides; this treatment resulted in an increase in radioactivity incorporated into a substance, not absorbing 254 nm light, that eluted just before *p*-coumaric acid on HPLC. Solid probe MS of a residue prepared from a similar fraction from unlabeled coleoptile sections yielded (m/z [relative intensities]: 196 [M^+; 32], 179 [M^+ −17; 1], 164 [M^+ −32; 2], 137 [M^+ −59; 100], 122 [M^+ −74; 13], 107 [M^+ −89; 9], 91 [M^+ −105; 7], and 77 [M^+ −119; 8]). A substance with virtually identical chromatographic and MS spectrum could be synthesized from authentic ferulic

acid treated with $NaBH_4$ in alkali, indicating that $NaBH_4$ reduced the α,β-unsaturated carbonyl side-chain of the ferulic acid. The molecular ion increased by 2 m/z, and the facile α-cleavage of the hydrocarbon side-chain yielded the base peak m/z 137.

Consistent with observation of increases in incorporation of radioactivity from aromatic amino acids into the cell walls of older coleoptiles, quantitation of the hydroxycinnamic acids after HPLC and GLC of TMSi derivatives yielded only 9 nmol of ferulic acid per mg of cell wall material from 2 d-old coleoptiles. Amounts increased substantially to 25 nmol/mg by the 3rd d and 42 nmol/mg by the 4th d. These values are consistent with those from wall preparations of barley coleoptiles at similar stages of development (31).

Only a smaller fraction of the saponifiable pool of hydroxycinnamic acids was found in diferulic acid indicating that polysaccharide extracted by dilute alkali is cross-linked by a small fraction of esterified ferulic acid derivatives. The amounts of ferulic acid increase in walls of older coleoptiles rather than being directed toward synthesis of diferulic acid. Pulse-chase studies are obviously needed to determine the proportion of ferulic acid that turns over to more recalcitrant material. Thus, ferulic acid may serve as initiation sites for lignification (12, 19), cross-linking molecules between hemicellulose and lignin (26), or even as allelopathic agents (25).

Substantial amounts of aromatic material are not saponifiable (Fig. 2; Table III), and in studies where phenolics were oxidized by acidic $NaClO_2$, hemicelluloses were rendered soluble in dilute alkali (6). Buchala *et al.* (4) showed that $NaClO_2$ induced no gross changes in polysaccharide structure. Although molecular size of MG-GAX and GAX II fractions of the maize coleoptile is reduced substantially by treatment with $NaClO_2$, the polysaccharide structure appears unaffected (data not shown). Because so much material is solubilized by concentrated alkali after saponifiable components have been removed, nonsaponifiable phenolics must constitute a major cross-bridging function. The chemical nature of these phenolic linkages was not examined here. Others such as Scalbert *et al.* (26) demonstrated by ^{13}C-NMR spectroscopy that wheat straw contains both esterified and etherified ferulic acid.

In summary, walls of maize coleoptiles contain substantial amounts of aromatic substances compared to proline or hydroxyproline, but absolute amounts are actually quite small. Based on quantitation of levels of ferulic acid and the partitioning of the radioactivity from the aromatic amino acids into all fractions, the amounts of aromatic material constitute only 0.3 and 1.2% cell wall weight of 2 and 4 d coleoptiles, respectively. These amounts are far lower than those of developed tissue from other Gramineae where lignin constitutes 15 to 26% of the cell wall (20). Little carbon from proline is incorporated into any cell wall fractions, and thus, hydroxyproline-rich glycoproteins constitute a minor component. While most of the aromatic components are saponifiable, most of this material is ferulic acid; only a small amount of diferulic acid was detected, and furthermore, only a small fraction of the hemicellulosic GAX is immobilized by diferulic esters. A much more significant fraction of hemicellulosic material is enmeshed by aromatic cross-linkages less susceptible to alkali extraction. Finally, auxin has no effect on the uptake of these amino acids or the incorporation of their carbon into any fraction, cytoplasmic or cell wall, at any stage of development. This observation may underscore the fact that substantial incorporation of aromatic material into the cell wall occurs well after cell elongation, and these aromatics participate in irreversible cross-linking of the primary wall during differentiation.

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