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Hydroxyproline Arabinosides in the Plant Kingdom[1]

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ABSTRACT

The hydroxyproline-O-arabinosyl linkage is present in cell walls of selected tissues representing green plants from algae to angiosperms.

Primary cell walls of flowering plants contain the hydroxyproline-rich glycoprotein, extensin (4). The carbohydrate-protein linkage which involves the hydroxyproline hydroxyl group in a glycosidic linkage with arabinose is stable to alkali (5, 6). This alkali stability has been exploited to provide a procedure for the detection and determination of the hydroxyproline glycosidic linkage.

Here we show that this linkage is widely distributed in the plant kingdom as evidenced by its occurrence in green algae, liverworts, horsetails, mosses, and ferns, as well as in flowering plants. We were not able to detect the presence of this linkage in animal protein.

Demonstration of the hydroxyproline-O-glycosidic linkage involves mild alkaline hydrolysis. Under these conditions racemization of the hydroxyproline glycosides occurs. Therefore we describe chromatographic methods for the complete resolution of the racemic hydroxyproline arabinoside mixtures and for the estimation, by directly monitoring column eluates for hydroxyproline or arabinose, of the relative amounts of each arabinoside.

METHODS

Cell Wall Preparations. These were obtained as previously described (4) except that some of the tougher materials were ground in liquid nitrogen before sonication in cold water.

Alkaline Hydrolysis of Cell Wall Preparations. Wall preparations were hydrolyzed by refluxing for 6 hr in excess 0.22 M $Ba(OH)_2$. The hydrolysate was neutralized with H_2SO_4, centrifuged, and evaporated to a small volume at 40 C.

Chromatographic and Electrophoretic Identification of Sugars and Amino Acids. These identifications were accomplished by methods detailed previously (4).

Chromatographic Separation of Hydroxyproline Arabinosides. We achieved best resolution of the racemic mixtures by chromatography on a 75- × 0.6-cm column of Technicon Chromobeads B resin eluted with a pH gradient (Fig. 1) at room temperature.

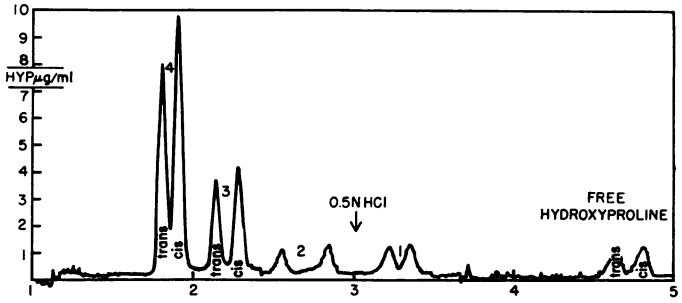

FIG. 1. Separation of hydroxyproline arabinosides on Chromobeads B. A sample containing not less than a total of 200 μg of hydroxyproline in about 0.5 ml of H_2O at pH 3 to 7 is placed on a 0.6- × 75-cm column of Chromobeads B H^+ form washed with water (resin from Technicon Corporation). The column is eluted with a pH gradient produced in a two-chambered vessel. Initially the mixing chamber contained 100 ml of water and the reservoir contained 100 ml of 0.5 N HCl. After pumping at 60 ml/hr (about 700 psi) for 3 hr, the column was pumped with 0.5 N HCl for another 2 hr. The eluate was fed into either the hydroxyproline analyzer or the pentose analyzer. The chromatogram depicted was obtained with the hydroxyproline analyzer and shows the hydroxyproline arabinoside profile of cell walls prepared from cultured *Acer pseudoplatanus* cells. The double peaks labeled 4, 3, 2, and 1 correspond to the *trans* and *cis* hydroxyproline tetra-arabinosides, triarabinosides, diarabinosides and monoarabinosides, respectively.

Continuous Hydroxyproline Assay. We modified the method of Kivirikko (3) to make it suitable for continuous assay of the column eluate (Fig. 2). The reagents were prepared as described previously (6). Standard solutions containing 10 μg of hydroxyproline per ml gave an absorbancy at 560 nm of 0.6 in a 15-mm flow cell. Hydroxyproline in the presence of HCl gave a similar absorbance, provided that the HCl normality did not exceed 0.5 N. *Cis* and *trans* hydroxyprolines and their arabinosides all gave similar absorbancies per mole of hydroxyproline.

Continuous Arabinose Assay. We modified the ferric chloride orcinol method of Dische (1) to make it suitable for continuous assay of the column eluate (Fig. 3). The reagent consisted of 3330 ml of concentrated HCl, 670 ml of H_2O, 4 g of orcinol, and 4 g of $FeCl_3 \cdot 6H_2O$.

RESULTS

Alkaline hydrolysis of the cell walls released the racemic mixtures of free hydroxyproline and hydroxyproline arabinosides. The profiles for any given species became constant after a 4-hr hydrolysis, remained constant even after a 24-hr hydrolysis, and was characteristic for each species (Table I). Thus the flowering plants examined showed a preponderance of hyp ara_4 (hydroxyproline tetra-arabinoside) with lesser

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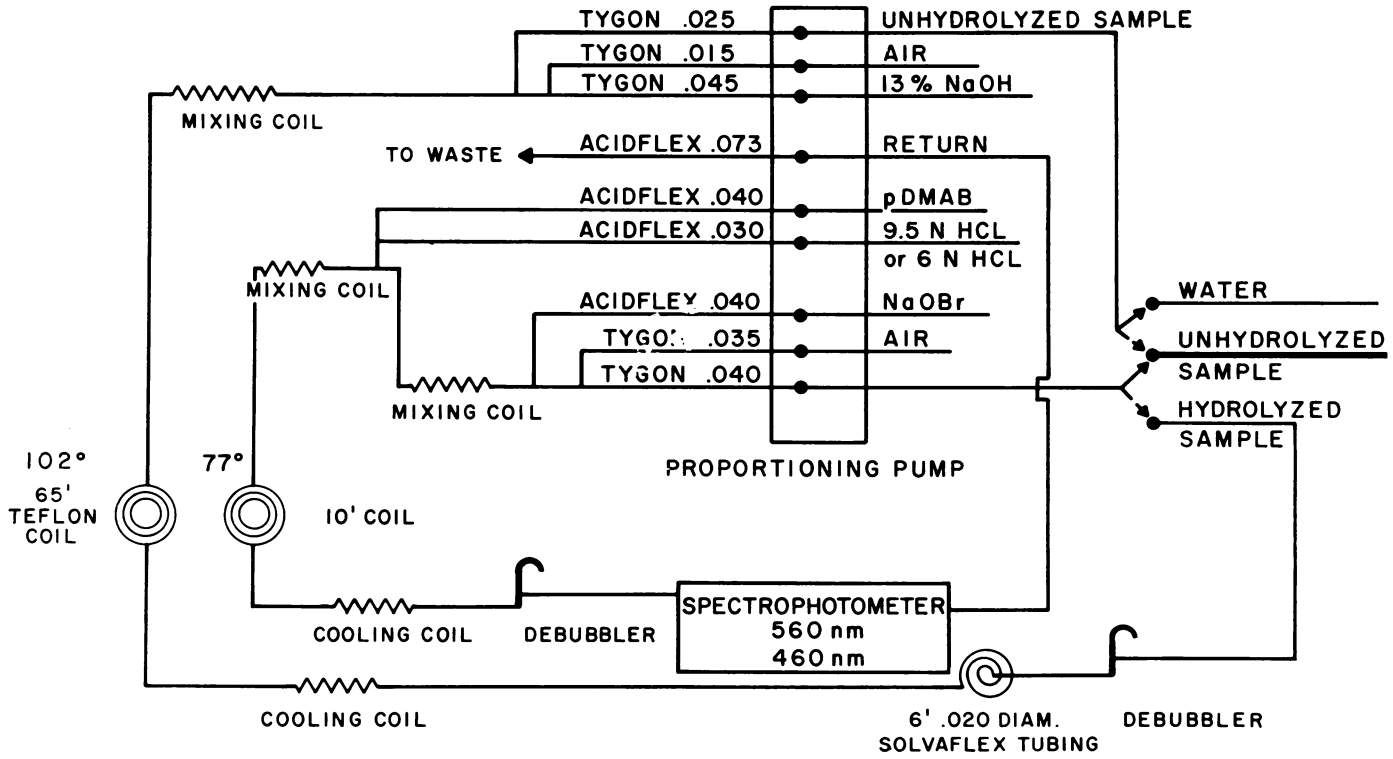

FIG. 2. Hydroxyproline analyzer flow diagram. This flow diagram shows the pump tube arrangement used for estimation of free hydroxyproline or hydroxyproline arabinosides and of peptide-bound hydroxyproline after alkaline hydrolysis.

The hydroxyproline analyzer can be used for direct monitoring of column eluates or for estimation of hydroxyproline in discrete samples. In the latter instance, the sampler takes a sample every 2 min. This requires a minimal sample of 1.3 ml for direct hydroxyproline estimation and 0.5 ml for hydroxyproline estimation after alkaline hydrolysis.

Absorbancies obtained with free and peptide bound hydroxyproline are summarized as follows.

Hydroxyproline	Absorbancy (560 nm in 15-mm Flow Cell)	
	A. Hydrolysis omitted	B. After alkaline hydroysis
Free hyp, 10 μg/ml (or O-substituted)	0.6	0.1
Peptidyl hyp, 10 μg/ml	0.0, no reaction	0.025

Note that the lower absorbancy of free hydroxyproline after passage through the hydrolysis coil is not due to degradation but results from the smaller initial sample taken as well as the dilution involved in the addition of NaOH to the sample. The difference in absorbancy (column B) between free and peptidyl hydroxyproline shows the amount of hydroxyproline which is released as the *free* amino acid. This is somewhat dependent on the type of peptides hydrolyzed; we found 25% hydroxyproline release from the tryptic peptides of acid-stripped walls (these peptides are described in Ref. 7). When many samples are assayed, especially those needing hydrolysis, the time delay from samples to spectrophotometer shows up to 5-min variations. This cannot be tolerated when analyzing fraction collector samples and we therefore add 10 μl of 10% (w/v) tartrazine to every (0.5 ml) 10th sample to act as an *internal* index and monitor the absorbancy at 460 nm. This is zero for the hydroxyproline chromagen but about 0.13 absorbance unit at the level of tartrazine used. When internal indexing is required for samples omitting hydrolysis, we add 5 μl of 10% (w/v) tartrazine to every (1.5 ml) 10th sample. Switching from *direct* hydroxyproline estimation to estimation *after hydrolysis* was achieved by the use of two Hamilton distribution valves. Similar distribution valves were also used to facilitate switching the reagent lines from reagent to wash or a different reagent concentration; *e.g.*, 6 N HCl for direct assay or 9.5 N HCl for assay after alkaline hydrolysis. Teflon coil connections were made with Chromatronix fittings. Tubing to glass connections were secured with $\frac{3}{16}$-inch hose clamps from Rho Scientific Corporation. Standard size tubing and proportioning pump were obtained from the Technicon Corporation.

amounts of hyp ara_8. The fern *Onoclea* and the horsetail, *Equisetum*, showed about equal amounts of hyp ara_4 and hyp ara_8. The liverwort *Sphaerocarpos* showed hyp ara_8 and hyp ara_3 predominating with traces of hyp ara_4. The alga *Chlorella vulgaris* showed hyp ara_3 predominating. The data also show that the hydroxyproline hydroxyl group is nearly completely glycosylated in the dicotyledonous plants studied (*cf.* 5 and 6) but that in the lower groups up to 74% of the hydroxyproline may be unsubstituted. The chromatograms generally show a slight excess of the *cis*-D-hydroxyproline arabinosides compared with the *trans*-L forms. Neither an insoluble commercial preparation of collagen nor the cellulosic tunic of the tunicate *Ciona intestinalis* yielded hydroxyproline glycosides on alkaline hydrolysis. We were also unable to detect hydroxyproline in acid hydrolysates of *Acanthamoeba* sp. cellulosic cyst walls.

DISCUSSION

The idea that the structural cell wall protein extensin might provide cross links in a polysaccharide network originated from the discovery of hydroxyproline in primary cell walls of higher plants. The isolation of hydroxyproline arabinosides supported this idea, and their occurrence in lower plants as reported here and their probable occurrence in some

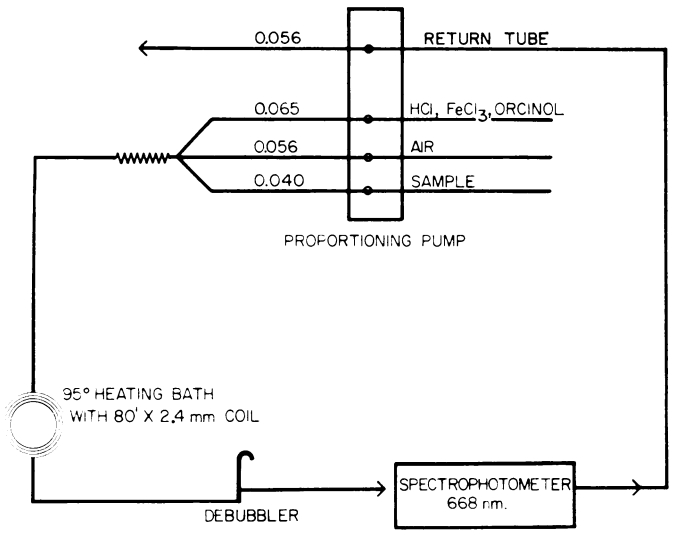

FIG. 3. Pentose analyzer flow diagram. This flow diagram shows the pump tube arrangement used for estimation of free or combined arabinose. When used with the sampler, minimal sample volume is 1.3 ml; sampling time is 2 min.

The absorbancy obtained with either free or combined arabinose, 10 μg/ml, was 0.22 absorbance unit at 668 nm with a 15-mm flow cell.

Table I. *Hydroxyproline Arabinosides Released by Alkaline Hydrolysis of Cell Walls of Different Plants*

Group	Species	Hydroxyproline Arabinosides				Unsubstituted Hydroxyproline
		Hyp ara_4[1]	Hyp ara_3	Hyp ara_2	Hyp ara	
		%				
Algae	*C. vulgaris*	0	4	16	6	74
Liverworts	*Sphaerocarpos*	1	30	26	3	40
Mosses	*Funaria hygrometrica*	3	8	18	8	63
Ferns	*Onoclea sensibilis* (leaves)	21	32	6	8	33
Horsetails	*Equisetum* sp. (sporophyte)	19	19	5	5	52
Gymnosperms	*Ginko biloba* (culture)	33	44	6	4	13
	Cupressus sp. (culture)	26	34	8	6	26
	Ephedra sp. (culture)	27	37	4	6	26
Monocotyledons	*Zea mays* (pericarp)	4	13	2	15	66
	Avena sativa (coleoptile)	6	11	3	5	75
	Iris kaempferi (pericarp)	10	11	3	5	71
	Allium porrum (pericarp)	6	13	3	5	73
Dicotyledons	*A. pseudoplatanus* (culture)	75	17	3	2	3
	Lycopersicon esculentum (culture)	52	28	4	6	10
	Convolvulus arvensis (culture)	63	22	6	4	5
	Vicia tetrasperma (culture)	52	31	5	4	8
	Pisum sativum (root)	33	41	7	6	13

[1] Hyp ara_4 is hydroxyproline tetra-arabinoside.

algae which lack cellulose (8) support the far reaching generalization that the cell walls of green plants possess a hyp-rich *protein*-glycan network analogous to and perhaps homologous with the peptido-glycan network of bacteria and blue green algae. To understand the significance of this drastic changeover in the organization of the cell wall framework requires some knowledge of unknown intermediate species, assuming of course that the two cell wall types are in fact phylogenetically related. However, as a working hypothesis we suggest that the greatly increased potential for morphogenesis of the cell wall, so evident in the postbacterial types, probably stems from the increased information (amino acid sequences) built into the wall. Because sequence generates the tertiary structure of the polypeptide this must determine the orientation of the polysaccharide chains attached to hydroxyproline. Thus the protein component would allow exceedingly fine control of the three-dimensional molecular architecture of the cell wall.

Significantly the hyp-ara linkage is found only in rather closely phylogenetically related groups; for example, it must be absent from the red algae as Gotelli and Cleland (2) report that this group is devoid of hydroxyproline. It seems quite remarkable that the sugar is invariably arabinose. On examination of the related groups there appears to be a trend towards a longer arabinose oligosaccharide substituent in the more advanced groups (Table I). There may also be some correlation between the extent of hydroxyproline glycosylation and phylogenetic position (Table I).

Because previous work (6) with tomato showed that enzymic degradation of cell walls released hydroxyproline-rich glycopeptides which contained *galactose* as well as arabinose, we must consider the possibility that galactose is attached by an *alkali-labile linkage* to the terminal arabinose of the hydroxyproline arabinosides. If, as seems likely, a polysaccharide chain is attached to the hydroxyproline arabinoside by this "galactosyl" linkage, its alkali lability, in *all* groups studied so far, is a feature which has been conservatively retained throughout evolution of the green plants. This leads us to speculate that it has a special significance: for example it might be an *enzymically*-labile linkage of a polysaccharide-protein network. Cleavage or resynthesis of such linkages could account for changes in cell wall plasticity.

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