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Arabinogalactan Protein from a Crude Cell Organelle Fraction of *Phaseolus vulgaris* L.[1]

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GERRIT-JAN VAN HOLST, FRANS M. KLIS, PIET J. M. DE WILDT, CORINNE A. M. HAZENBERG, JOPIE BUIJS, AND DURK STEGWEE
Plantenfysiologisch Laboratorium, Universiteit van Amsterdam, Kruislaan 318, 1098 SM Amsterdam, The Netherlands

ABSTRACT

Sonication of a crude cell organelle fraction from hypocotyl tissue of dark-grown bean seedlings, and from suspension-cultured cells released a hydroxyproline-containing protein. The purification of this protein is described. It was found to be an arabinogalactan protein composed of 90% carbohydrate and 10% protein. The major sugars are galactose, arabinose, and uronic acids, and the major amino acids are hydroxyproline, serine, and alanine. Its molecular weight was estimated at 1.4×10^5 daltons and the isoelectric point at pH 2.3. The molecule is soluble in 5% trichloroacetic acid and can be precipitated with β-galactosyl Yariv antigen. Pulse-chase experiments indicated that it was a secretory protein. The biosynthesis of arabinogalactan proteins is discussed.

Arabinogalactan proteins are widely distributed throughout the plant kingdom (8, 15). They are distinguished by their large carbohydrate moiety (usually more than 80%) in which galactose and arabinose are the principal sugars. Their protein moiety is rich in hydroxyproline, alanine, and serine. They can usually be precipitated with the colored artificial Yariv antigen containing β-D-glycosyl groups (8, 15). The biosynthesis of arabinogalactan proteins has not been studied in detail, but Chrispeels and coworkers (5, 12) have provided evidence that the Golgi apparatus is involved in the processing and transport of secretory hydroxyproline-containing proteins, supposedly extensin precursors. In this article we describe the isolation and properties of a secretory arabinogalactan protein from a crude organelle fraction of *Phaseolus*. Our results indicate that the way this protein is synthesized parallels that of the secretory hydroxyproline-containing proteins studied by Chrispeels and coworkers.

MATERIALS AND METHODS

Plant Material. In most experiments we used 6- to 9-cm-long hypocotyls of *Phaseolus vulgaris* L. cv. Prélude seedlings that had been grown in the dark under conditions as described by Sluiters-Scholten *et al.* (20). For some experiments, suspended cells obtained from bean hypocotyls and subcultured as described by Klis and Eeltink (16) were used.

Homogenization and Fractionation. Hypocotyl tissue was ground with pestle and mortar in 1.5 volume of grinding medium. The grinding medium contained 0.26 M sucrose, 50 mM maleate-Tris buffer (pH 6.5), 5 mM KCl, 2 mM $Na_2S_2O_5$, 1 mM $MgCl_2$, and 1 mM EDTA. The homogenate was filtered through 10-μm nylon gauze to remove cell walls. In the first experiment the filtrate was divided into three portions that were centrifuged for 30 min at 12,000, 48,000 and 200,000*g*, respectively (Table I); in the pulse-chase experiments the filtrate was centrifuged at 48,000*g* for 2 h (Table IV). For the isolation of arabinogalactan protein the filtrate was first centrifuged at 1,000*g* for 5 min to remove starch and nuclei and then at 48,000*g* for 30 min. Both supernatant and residue, after suspension in water, were dialyzed against water for 2 days. The residue was sonicated for 45 s and subsequently centrifuged at 48,000*g* for 2 h to give a pellet (firmly-bound material) and a soluble fraction of loosely-bound material. Firmly-bound material could be solubilized in 1% (w/v) SDS containing 1% (v/v) mercaptoethanol (1 h at 30 C). Suspended cells were homogenized in an equal volume of grinding medium and fractionated as described for hypocotyl tissue. The cell wall fraction was washed with grinding medium. The firmly-bound fraction did not completely dissolve in SDS because it was contaminated with cell wall material. All operations were carried out in the cold (4 C).

Pulse-Chase Experiments. The upper 2 cm below the hook of the hypocotyls were abraded with carborundum (120 mesh) and divided into 1-cm segments. One hundred thirty segments (corresponding with 9 g fresh weight) were incubated in a solution of 25 ml 1 μM IAA containing 15 μCi = 60 nmol [U-^{14}C]L-proline (New England Nuclear, UK) in the dark at 25 C. IAA was needed to keep the segments growing (4.3%/h). After 2 h, when about 10% of the label had entered the tissue, 0.6 mmol [^{12}C]proline was added. Samples of 20 segments were taken, homogenized and stored in ice until fractionation. The labeling experiment with suspended cells was carried out with the cells of a 7-day-old culture (9 g fresh weight) which were taken up in 90 ml of culture solution containing 25 μCi [U-^{14}C]L-proline (100 nmol) and were incubated at 30 C. After 17 min, when about 90% of the label had entered the cells, 1 mmol [^{12}C]L-proline was added. Samples containing 0.5 g of cells (fresh weight) were taken, filtered over 10-μm nylon gauze, washed with ice-cold 1 mM L-proline and stored in ice. The samples were fractionated as described above.

Purification. The loosely-bound fraction was applied on top of a Sephadex G-150 (Pharmacia, Sweden) column (82 × 2.6 cm) equilibrated with 25 mM ammonium acetate buffer (pH 4), plus 1 M NaCl. The hydroxyproline-containing fractions were pooled and dialyzed against distilled H_2O, and subsequently put in an isoelectrofocusing column (Ampholine column type 110 ml, LKB, Sweden) with a linear glycerol gradient (0–50% v/v) to prevent convection and with 0.5% w/v Ampholine (pH 2.5-4) (LKB, Sweden) as carrier ampholytes. The electrophoresis was carried out for 3 days at 350 v and at 10 C. Fractions of 3.8 ml were collected and their pH was measured. The pH increased linearly

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between pH 2.0 and 4.0. Then the fractions were dialyzed against several changes of distilled H_2O for at least 3 days. Before use in the isoelectrofocusing column, the ampholytes had to be freed from nondialyzable molecules by filtration over a Sephadex G-25 (Pharmacia, Sweden) column (30 × 1.5 cm) equilibrated with distilled H_2O.

Analyses. Protein was determined by the Lowry method after precipitation with 5% v/v trichloroacetic acid. Proteins were hydrolyzed in 6 N HCl for 3 h or 18 h at 120 C under N_2. The hydrolysates were used for amino acid analyses with a Beckman 121 M amino acid analyzer or for measuring the hydroxyproline content (11). Serine values declined during hydrolysis; the initial amount was determined by extrapolation to zero time.

Radioactivity in hydroxyproline was measured with the method of Blumenkrantz and Asboe-Hansen (3) using a Packard Tri-Carb liquid scintillation counter. Methanolysis of carbohydrate was carried out in 1 N methanolic HCl at 85 C for 24 h (7). Sugar compositions were determined by GC of the trimethylsilyl derivatives of the methanolyzed sugars (7). The amount of uronic acids was measured according to Blumenkrantz and Asboe-Hansen (2).

β-Lectin Assay. The substrate for the assays was prepared by coupling diazotized *p*-aminophenyl β-D-galactopyranoside (Sigma), to phloroglucinol to give 1,3,5 Tris (4-β-D-galactopyranosyl-oxyphenylazo) 2,4,6 trihydroxybenzene (24). The assays were carried out according to Jermyn and Yeow (15).

RESULTS

In the hypocotyl of a dark-grown bean seedling, cell division and cell elongation are spatially separated. Cell division occurs in the region adjacent to the cotyledons (*i.e.* the hook), whereas the next 2 cm of the hypocotyl are involved in cell elongation (23). The elongating part was homogenized and the cytoplasmic fraction of the homogenate was further fractionated by centrifugation at different forces. Table I shows that half of the hydroxyproline remained soluble even after centrifugation at 200,000*g*, indicating that this part of the cytoplasmic peptidyl hydroxyproline was not associated with membranes. The material that could be released from the residues by sonication was called loosely-bound. The residues obtained at all three centrifugal forces used contained equal amounts of loosely-bound hydroxyproline. The material in the residue that could not be released by sonication was called firmly-bound. The firmly-bound hydroxyproline-containing proteins could however be completely solubilized in SDS. Residues obtained at higher centrifugal forces contained more firmly-bound hydroxyproline. The distribution and the contents of hydroxyproline in slow- and nongrowing parts of the hypocotyl were the same as in the fast-growing part (data not shown).

Table I. *Fractionation of Cytoplasmic Peptidyl Hydroxyproline in Bean Hypocotyls*

A homogenate of elongating tissue was freed from cell walls by filtration and the filtrate was divided into three portions. The portions were centrifuged for 30 min at 12,000, 48,000, and 200,000*g*, respectively. The residues were subfractionated by sonication which released part of the hydroxyproline-containing material (loosely-bound). The amount of firmly-bound peptidyl hydroxyproline was calculated by subtracting the amount of loosely-bound hydroxyproline from the total amount.

Centrifugal force	Residue			Supernatant
	Loosely-bound	Firmly-bound	Total	
$g \times 10^{-3}$	*μg hydroxyproline/g fresh wt*			
12	2.9	1.5	4.4	11.1
48	2.8	3.9	6.7	10.5
200	2.8	5.0	7.8	7.8

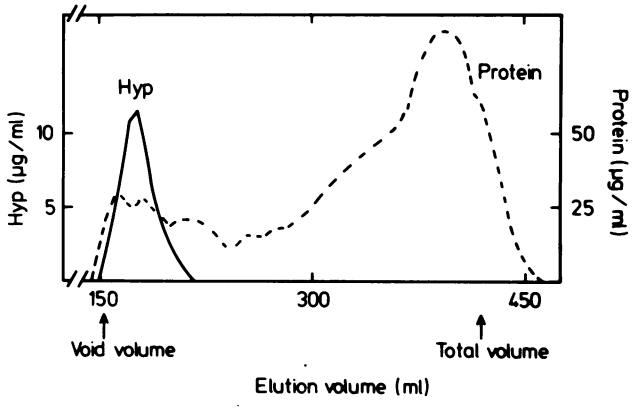

FIG. 1. Gel filtration of loosely-bound material on Sephadex G-150. A representative experiment.

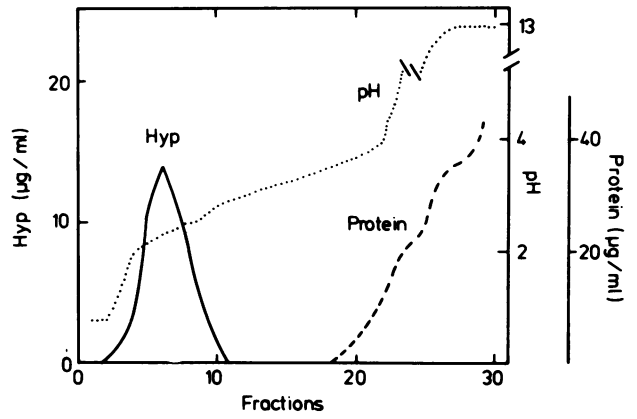

FIG. 2. Column electrofocusing of hydroxyproline-containing fractions from the Sephadex G-150 eluate. A representative experiment. Protein was determined by the Lowry method after precipitation with 5% trichloroacetic acid. Note that the hydroxyproline-containing protein is soluble in 5% trichloroacetic acid.

Table II. *Purification of Hydroxyproline-Containing Protein*

Fraction	Hydroxyproline	Protein Precipitated by 5% TCA[a]
	mg	
Loosely-bound material	1.34	49.9
Sephadex G-150 eluate: $K_{av} = 0.06$	0.64	2.05
Electrofocusing: pI = 2.3	0.43	0.00

[a] TCA, trichloroacetic acid. The hydroxyproline-containing protein is soluble in 5% trichloroacetic acid.

We purified the loosely-bound hydroxyproline-containing material further by a two-step procedure. In the first step the loosely-bound material was sieve-filtrated over Sephadex G-150 (Fig. 1). All the hydroxyproline-containing protein eluted in a single peak just after the void volume—the partition coefficient $K_{av} = 0.06$—together with a considerable amount of carbohydrate-containing material while most of the remaining protein eluted near the total volume. The hydroxyproline-containing material was subsequently electrofocused (Fig. 2) in the pH range 2 to 4. All hydroxyproline was detected in a single peak at pH 2.3, while some hydroxyprolineless protein was detected at and above pH 3. Table II shows that our two-step procedure led to considerable purification of the hydroxyproline-containing material; whereas the ratio of trichloroacetic acid-precipitable protein to hydroxyproline was 37 before purification, it fell to 3.2 after gel filtration

Table III. *Composition of Purified Hydroxyproline-Containing Protein*

Amino Acids		Sugars	
	mol/mol Arg		
Hyp	40	Gal	492
Asp	6	Ara	180
Thr	11	UA	112
Ser	28	including	
Glu	7	ManUA	34
Pro	4	GlcUA	16[a]
Gly	10	Glc	17
Ala	25	Rha	5
Val	5	Man	4
Cys	1	$GlcNH_2$	1
Met	1		
Ile	2		
Leu	5		
Tyr	1		
Phe	1		
Orn	1		
Lys	4		
His	1		
Arg	1		

[a] No other uronic acids were detectable; see "Discussion."

and became zero after electrofocusing. This latter also proves that the hydroxyproline-containing protein is soluble in 5% trichloroacetic acid. We assume that the losses of hydroxyproline during isolation (two-thirds of total) were largely due to irreversible binding of the hydroxyproline-containing protein to the Sephadex gel and the dialysis tubings. The purification procedure was repeated several times and appeared highly reproducible. Also, the chemical composition of the purified product was constant. The major amino acids in the isolated hydroxyproline-containing protein are hydroxyproline (26 mol %), serine (18 mol %) and alanine (16 mol %) (Table III). Also notable is the presence of an ornithine residue. The major sugars are galactose (61 mol %), arabinose (22 mol %), and uronic acids (14 mol %) (Table III). The total amount of sugars accounts for 90% w/w of the molecule. These data are characteristic of arabinogalactan proteins. Many arabinogalactan proteins show β-lectin activity and can be precipitated with β-D-glycosyl Yariv antigen (8). Indeed, our purified protein precipitated after adding β-D-galactosyl Yariv antigen. Precipitation also occurred in the Ouchterlony double diffusion assay. Therefore, we conclude that we have isolated an arabinogalactan-protein.

The presence of an arabinogalactan-protein in a crude cell organelle fraction raised the question whether it might be a secretory protein like other hydroxyproline-containing proteins (5, 10). Pulse-chase experiments with hypocotyl segments showed that in all intracellular fractions, peptidyl hydroxyproline was at least partly chaseable (Table IV). Pulse-chase experiments with hypocotyl segments have, however, the disadvantage that the cells close to the cut surfaces are more quickly labeled and chased than the cells farther away. In the following experiments, suspension-cultured bean cells were used. It was first demonstrated that sonication of a crude cell organelle fraction released a hydroxyproline-containing protein. Purification by the two-step procedure as described above showed that it eluted from the Sephadex G-150 column at the same elution volume; it also had the same isoelectric point and the same sugar composition as the arabinogalactan-protein from hypocotyls, and it precipitated with β-D-galactosyl Yariv antigen. Therefore, we believe that it was the same as the arabinogalactan protein from hypocotyls. In a pulse-chase experiment, labeled hydroxyproline appeared in the intracellular fractions within 5 min (Table IV). During the chase period, radioactive hydroxyproline disappeared from both the

Table IV. *Cellular Distribution of Radioactive Peptidyl Hydroxyproline in Elongating Hypocotyl Segments and in Suspension-Cultured Cells*

[^{14}C]Proline was added at time zero. The chase was added at 120 min (hypocotyl segments) or at 17 min (suspension-cultured cells). The cytoplasm was centrifuged at 48,000*g* for 2 h and the residue was sonicated to release loosely-bound protein, the remainder was extracted with SDS to free firmly-bound protein; the pellet, present only in the case of suspension-cultured cells, was cell-wall material and contained about 6% of the total cell wall hydroxyproline. The loosely-bound fraction of hypocotyl segments contained 4.2 μg hydroxyproline/g fresh wt, the firmly-bound fraction 5.5 μg hydroxyproline/g fresh wt, the supernatant 8.0 μg hydroxyproline/g fresh wt and the cell wall 42 μg hydroxyproline/g fresh wt. In suspended bean cells the corresponding values were 5.9, 2.9, 6.8 and 227 μg hydroxyproline/g fresh wt, respectively.

Time	Cytoplasm			Cell Wall
	Residue		Supernatant	
	Loosely-bound	Firmly-bound		
min	*[^{14}C]-hydroxyproline dpm × 10^{-3}/μg hydroxyproline*			
Hypocotyl segments				
120	1.1	2.7	0.9	0.6
140	1.1	1.2	0.9	0.7
160	0.7	0.8	0.7	0.6
240	0.6	0.6	0.5	0.7
Suspension-cultured cells				
5	1.0	2.7	0.3	0.00[a]
10	4.5	9.0	1.0	0.02
15	9.5	18.1	2.4	0.05
25	17.5	24.6	5.4	0.26
30	10.3	19.2	5.3	0.53
40	5.6	10.0	3.0	0.99
60	3.4	4.1	2.0	1.01
100	1.2	2.0	1.0	1.20

[a] Including SDS-insoluble radioactive hydroxyproline from the cytoplasmic fraction.

firmly-bound fraction, the loosely-bound fraction and the supernatant fraction with a half-life of about 10 to 15 min. The doubling time of [^{14}C]hydroxyproline in the cell wall fraction was comparable to the half-lives determined for the intracellular fractions. In these experiments, the decrease of radioactive hydroxyproline during the chase period in the combined intracellular fractions approximately equaled the increase of radioactive hydroxyproline in the cell wall fraction. This indicates that hydroxyproline-containing proteins, including the arabinogalactan-protein, leave the cytoplasm and become associated with the cell wall. Further evidence was obtained by extracting labeled cell walls. Cell walls were prewashed extensively with water and chloroform-methanol (1:1, v/v). Extraction with 1 M NaCl released radioactive hydroxyproline-containing protein that precipitated after adding β-D-galactosyl Yariv antigen.

DISCUSSION

For operational use we can divide the cytoplasmic hydroxyproline-containing proteins into three groups.

First, the hydroxyproline-containing proteins that could not be precipitated by centrifugation at 200,00*g* for 30 min. This material seemed to be mainly of cytosolic origin but might have been contaminated with some material from the following two groups.

Second, the hydroxyproline-containing protein that could be

released by sonication from the crude organelle fraction. This material was purified and appeared to be an arabinogalactan protein. Dashek (10) showed that the 12,000*g* residue obtained by centrifugation of the cytoplasm of suspended *Acer* cells contained the larger organelles, including the Golgi membranes. In bean hypocotyl tissue it contained all the loosely-bound hydroxyproline-containing protein; sonication of the residues obtained at higher centrifugal forces did not release more loosely-bound hydroxyproline (Table I). The arabinogalactan protein was chaseable and had a half-life of about 10 to 15 min in suspension-cultured cells. This equals the half-life time of hydroxyproline-containing proteins in the Golgi membranes (12). These data suggest that the arabinogalactan protein was derived from the Golgi-membrane system, although definitive proof is lacking and probably needs the use of marker enzymes.

Third, the hydroxyproline-containing proteins that could not be released by sonication of the crude organelle fraction, but could be extracted with SDS. In the pulse-chase experiments, the specific activity of the firmly-bound hydroxyproline was higher than and tended to decrease slightly prior to the specific activity of loosely-bound hydroxyproline. This may indicate that the fraction of firmly-bound hydroxyproline contains a precursor of the isolated arabinogalactan protein. On the other hand, the firmly-bound fraction might also contain precursors of the hydroxyproline-rich cell wall protein extensin.

The indications about the biosynthesis of the arabinogalactan protein are in agreement with results obtained with hydroxyproline-rich glycoproteins as reviewed by Chrispeels (5). In fact, it is not known whether the reviewed studies deal with extensin precursors, arabinogalactan proteins, or, possibly potato lectin-like proteins (1). The fact that a protein is hydroxyproline-rich and contains arabinose (6, 22) is not indicative for one of the hydroxyproline-containing proteins, nor is solubility in 5% trichloroacetic acid (4). An amino acid analysis of highly purified material is one of the few methods that are conclusive. The amino acid analysis shows that the arabinogalactan protein is rich in hydroxyproline and serine, like the cell-wall protein extensin (18, 21) and potato lectin (1), but unlike these proteins it is rich in alanine. It resembles the trichloroacetic acid-soluble hydroxyproline-rich cytoplasmic proteins isolated by Lamport (17), the extracellular hydroxyproline-rich glycoprotein isolated by Hori and Sato (14) and the β-lectins isolated by Gleeson and Jermyn (13) that were freed from contaminants with subtilisin.

Hydroxyproline is not the only unusual amino acid in the arabinogalactan protein. We also detected an amino acid that behaves chromatographically as ornithine. Ornithine has been detected in several arabinogalactan proteins (8).

The major sugars are galactose, arabinose, and uronic acids. The uronic acids were provisionally identified as mannuronic acid and glucuronic acid. Two major and two minor peaks of the derivatives obtained by trimethylsilylation of methanolyzed mannuronic acid (7) were detected, as well as two major and two minor peaks characteristic of the glucuronic acid derivatives. Also the ratios of their respective peak areas corresponded with those of mannuronic acid and glucuronic acid. There were no other uronic acids detectable. The total amount of mannuronic acid and glucuronic acid recovered by GC accounts for 45% of the amount of uronic acids measured by the colorimetric method (2) with glucuronic acid as standard. It seems likely that acid methanolysis is not able to free all uronic acids because of the relative stability of glycosiduronic acid linkages in acid conditions. The high content of uronic acids explains the low isoelectric point (pI 2.3) of the molecule. We can calculate the molecular weight of the arabinogalactan protein by supposing that the molecule contains at least one residue of each detected amino acid or sugar. This leads to a minimal estimate of 1.4×10^5 daltons, which corresponds well with the molecular weight estimate based on the elution volume on Sephadex G-150 (Sephadex G-150 has an exclusion limit of 1.5×10^5 daltons for polysaccharides).

The pulse-chase experiments indicate that arabinogalactan protein leaves the cytoplasm and becomes associated with the cell wall. This corresponds with the presence of β-lectin activity (supposed to be indicative of arabinogalactan protein) in a salt extract of cell walls. It certainly does not mean that arabinogalactan protein has to be a structural component of the cell wall. In fact, the following observations suggest that the association between arabinogalactan protein and cell walls is only temporary. First, Pope has shown that in pulse-chase labeled *Acer* cells, radioactive hydroxyproline appears in the cell walls before it enters the extracellular medium (Fig. 1 of ref. 19); second, the extracellular medium of suspension-cultured bean cells shows β-lectin activity (our observation); third, Clarke *et al.* (9) have detected β-lectin activity in the intercellular spaces of *Phaseolus vulgaris* cotyledons.

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