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ARABINOGALACTAN PROTEIN IN THE EXTRACELLULAR SPACE OF *PHASEOLUS VULGARIS* HYPOCOTYLS

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Key Word Index—*Phaseolus vulgaris*; bean; Leguminosae; hydroxyproline; glycoprotein; arabinogalactan protein; β-lectin; extracellular space.

Abstract—Most hydroxyproline in the soluble fraction (cytosol, extracellular fluid and the contents of ruptured organelles) of homogenized bean hypocotyls originated from arabinogalactan protein. Using a vacuum infiltration–centrifugation technique, we extracted hydroxyproline-containing compounds from the extracellular space, accounting for about 25% of hydroxyproline in the soluble fraction. The bulk of this material was soluble in 5% trichloroacetic acid and could be precipitated with β-Gal-Yariv reagent. Isoelectrofocusing of the extracellular solution showed a major hydroxyproline peak at low pH, and minor peaks at pH 5 and 9, respectively. We conclude that arabinogalactan protein accounts for most of the salt-soluble, extracellular hydroxyproline-containing compounds.

INTRODUCTION

Arabinogalactan proteins (AGPs) are widely distributed throughout the plant kingdom [1, 2]. They are highly glycosylated (usually more than 80%) glycoproteins; their protein moiety is rich in hydroxyproline, alanine and serine, and the principal sugars are arabinose, galactose and uronic acids, the latter probably being responsible for their isoelectric point at low pH. The high degree of glycosylation of AGPs presumably explains why they are usually soluble in 5% TCA. Many AGPs show β-lectin activity and can be precipitated with β-D-glycosyl-Yariv reagent, a red coloured dye [2]. No general function can yet be ascribed to AGPs.

Van Holst *et al.* [3] purified AGP from a crude cell organelle fraction of *Phaseolus vulgaris* hypocotyls; they found that about 50% of hydroxyproline-containing material remained soluble even after high-speed centrifugation. The soluble fraction is cell wall- and (almost) membrane-free and contains cytosol, contents of ruptured organelles and extracellular liquid. Recently Terry *et al.* [4] found soluble hydroxyproline-containing compounds in the extracellular space of pea stems, but they did not determine the nature of these compounds. This raised the question whether the soluble hydroxyproline-containing molecules in bean hypocotyl homogenates are (partly) of extracellular origin, and whether they are soluble extensin-like compounds like those found in cell walls of aged carrot slices [5]. In this paper we demonstrate that a considerable part of the hydroxyproline-containing molecules in hypocotyl homogenates is of extracellular origin and mainly consists of AGP.

RESULTS AND DISCUSSION

Isoelectrofocusing of the soluble fraction of bean hypocotyl homogenates

Van Holst *et al.* [3] isolated AGP from a crude membrane fraction of *Phaseolus vulgaris* hypocotyls and found that AGP has an isoelectric point at pH 2.3. We isoelectrofocused the soluble fraction of bean hypocotyls and analysed the gradient for hydroxyproline (Hyp). As shown in Fig. 1 most Hyp was found at very low pH; minor peaks were present at pH 5.7 and at pH 8.5. Hyp-containing material with an isoelectric point below pH 3.8 could be precipitated with β-Gal-Yariv reagent; also there was some indication that this material is heterogeneous. From these data we conclude that most Hyp in the soluble fraction is incorporated in AGP.

Isolation of extracellular water-soluble compounds

Sections of bean hypocotyls were infiltrated under

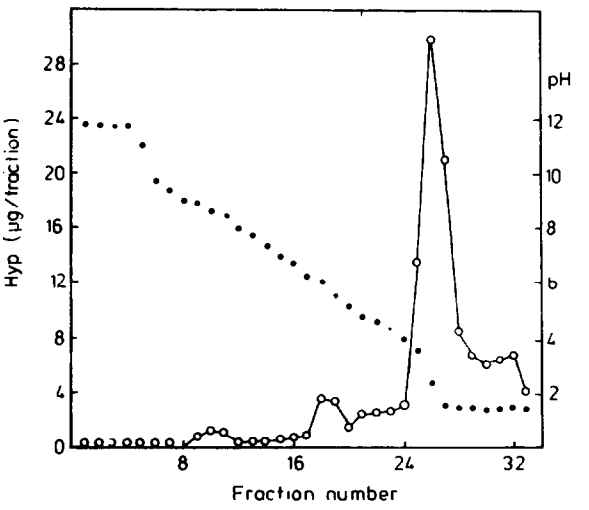

Fig. 1. Isoelectrofocusing of the soluble fraction of bean hypocotyls. (● ● ●) pH; (O——O) µg Hyp/fraction. Total amount of Hyp was 6 µg/g fr. wt.

vacuum with water and centrifuged at different centrifugal forces. Figure 2 shows the increase of the activity in the centrifugate of glucose-6-phosphate dehydrogenase, a cytosolic enzyme, with centrifugal force. At low forces no glucose-6-phosphate dehydrogenase activity was detectable, indicating that cell rupture did not occur. Above 1800 *g*, glucose-6-phosphate dehydrogenase activity increased sharply, and at 4000 *g* 12% of the total activity of the tissue was found, indicating that considerable cell damage had occurred. Further centrifugations were done at 1000 *g*. To optimize the extraction of Hyp-containing compounds, moderately concentrated salt solutions were used instead of water. Table 1 shows the effect of vacuum infiltration with several salt solutions on the amount of Hyp in the released liquid. When the hypocotyl sections were extracted with salt solutions instead of water, the amount of Hyp in the centrifugate increased. Repeating the infiltration–centrifugation sequence with 100 mM potassium chloride twice, increased the recovery by another 70%. So, in most subsequent isolations bean hypocotyl sections were infiltrated and centrifuged three times. Prolonging the centrifugation time did not change the recovery of Hyp, and thus we always used a 10-min-period. With this procedure we could extract from the extracellular space an amount of Hyp equivalent to about 25% of the amount of Hyp present in the soluble fraction. Our results indicate that the method of Terry and Bonner [6] is applicable to bean hypocotyls when the centrifugal force is not increased above 1000 *g*.

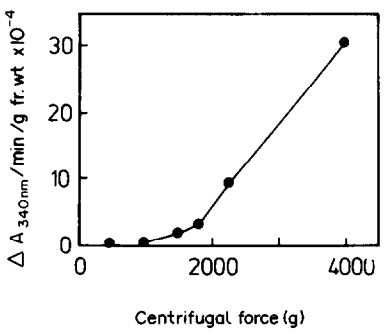

Fig. 2. Effect of centrifugal force on the release of glucose-6-phosphate dehydrogenase activity from bean hypocotyl sections infiltrated under vacuum with water. Total cytosolic acitivity was 0.025 $A_{340\,nm}$/min/g fr. wt.

Table 1. Hydroxyproline released from bean hypocotyl sections infiltrated with different salt solutions

Infiltration solution	Hydroxyproline
	μg/g fr. wt
None (1)	0.07
H_2O (1)	0.63
20 mM Na_3PO_4 (1)	0.83
50 mM $CaCl_2$ (1)	1.0
50 mM KCl (1)	0.65
100 mM KCl (1)	0.97
100 mM KCl (3)	1.7

The number of vacuum infiltration–centrifugation cycles is given in brackets. No glucose-6-phosphate dehydrogenase activity was detectable except when sections were extracted with 0.2 M KCl; in that case, trace amounts of activity became measurable and therefore Hyp content was not determined.

Analysis of extracellular hydroxyproline-containing compounds

As indicated in Table 1 we found Hyp-containing molecules in the extracellular space of hypocotyls of dark-grown bean seedlings. We also found comparable amounts of Hyp in hypocotyls and epicotyls of light-grown plants (1.6 and 1.0 μg Hyp/g fr. wt, respectively, after one vacuum infiltration). We assayed the extracellular extract of etiolated bean hypocotyls for total carbohydrate, uronic acid, solubility of Hyp-containing molecules in 5% TCA and the precipitability of these compounds with β-Gal-Yariv reagent. We determined in the extract 0.31 mg total carbohydrate/g fr. wt, 21 μg uronic acids/g fr. wt, and 90% of Hyp-containing compounds appeared to be soluble in 5% TCA. AGPs are known to bind β-Gal-Yariv reagent and form precipitable complexes with this reagent in the presence of 1% sodium chloride. We were able to precipitate 90% of the extracellular Hyp-containing molecules with Yariv reagent. The presence of extracellular AGP was confirmed by isoelectrofocusing experiments. Figure 3 shows the results of isoelectrofocusing of extracellular fluid: most hydroxyproline is detectable at very low pH values.

To show that compounds with a very low isoelectric point bind β-Gal-Yariv reagent, we isoelectrofocused extracellular extract and soluble fraction on polyacrylamide gels and stained the gels with β-Gal-Yariv reagent. Bands became visible at low pH (pH 2.5–3) for both the extract and the soluble fraction. We could show chemically that Hyp was present in the coloured bands. In polyacrylamide gels the isoelectric points seem to be at higher pH values than in the isoelectrofocusing column but these differences can presumably be attributed to the different separation systems used. Finally, we quantified

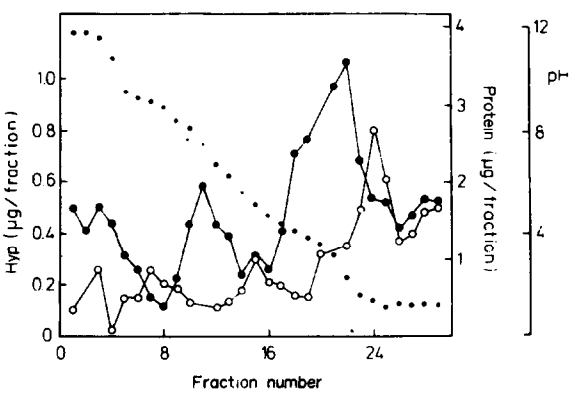

Fig. 3. Isoelectrofocusing of extracellular extracts from bean hypocotyls vacuum-infiltrated with 0.1 M KCl. (● ● ●) pH; (○——○) μg Hyp/fraction; (●——●) μg protein/fraction.

the amount of extracellular β-lectin activity as compared with the β-lectin activity in the soluble fraction. To estimate the relative amount of β-lectin activity, we developed a modification of the β-lectin assay described by Jermyn and Yeow [2]. β-Lectins can be precipitated with the intensely coloured β-D-glycosyl-Yariv reagent; by solubilizing the precipitate and determining the absorbance at 395 nm (for β-Gal-Yariv) one has an indication of the amount of β-lectin bound. Unfortunately, this assay could not be used in our circumstances, because β-galactosidase activity is disturbing the test by hydrolysing the sugar bonds and so generating water-insoluble, coloured molecules. We used tritium-labelled Yariv reagent in which the label is incorporated exclusively in the sugar residue; with this approach labeled precipitate represents a real Yariv-β-lectin complex. Using this radioactive β-lectin assay we could calculate that about 15% of total β-lectin activity in the soluble fraction of homogenized hypocotyls is detectable in the extracellular solution. Van Holst *et al.* [3] found that AGP, isolated from a crude membrane fraction by sonication, had an isoelectric point at pH 2.3. We found Hyp-containing molecules with a larger range of low isoelectric points, suggesting that more than one AGP is present in the extracellular space. The extreme acid character of a part of the Hyp-containing molecules may be due to very strong acid groups in their structure, such as sulphate groups; these have been demonstrated in another Hyp-rich glycoprotein [7], that is found in *Chlamydomonas reinhardtii*, and that shows some resemblance to AGP [8]. The isoelectrofocusing experiments further show two minor peaks at about pH 9 and at pH 5. The Hyp-containing peaks at pH 9 might represent extensin-like compounds, since extensin is known to have a high isoelectric point [5]; they might resemble the salt-extractable Hyp-rich glycoprotein as described by Stuart and Varner [5]. Hyp at pH 5 could not be attributed to any known compound.

In this study we show that in the soluble fraction of homogenized bean hypocotyls most Hyp is incorporated in AGP. The soluble fraction contains Hyp-compounds of extracellular origin, mostly AGP. The preceding results also indicate that in bean hypocotyls a probably heterogeneous group of AGPs is present in the extracellular space. Van Holst *et al.* [3] showed that the amount of AGP covalently linked with the cell wall is negligible. Our work shows that a part of AGP is possibly attached to the cell wall by ionic bonds but we cannot exclude the possibility that AGP is loosely associated with the plasma membrane surface or occurs in the intercellular liquid since we could extract reasonable amounts of Hyp-containing compounds with water alone.

EXPERIMENTAL

Plant material. In most experiments we used 6-day-old seedlings of *Phaseolus vulgaris* L. cv. Prélude (Royal Sluis, The Netherlands) that had been grown in the dark. Growth conditions were as follows: 75 g of beans were sown in a plastic tray (40 × 30 × 8 cm) filled with 5 l. Perlite wetted with 1.7 l. tap water; during growth the temp. was maintained at 25° and the relative humidity at 65%. 5-cm sections cut directly from below the hook were used. When light-grown plants were used, we isolated the upper 5 cm of epicotyl and hypocotyl of 8-day-old plants grown in the glasshouse.

Soluble fraction. In some experiments we used the soluble fraction of homogenized dark-grown hypocotyls. Hypocotyl tissue was ground with pestle and mortar in homogenization buffer (40 mM Tris–HCl, 1 mM dithiothreitol, 1 mM EDTA, 10 mM KCl, 0.1 mM $MgCl_2$, pH 7.8; 1 ml buffer per g fr. wt) for 3 min. The homogenate was filtered through 10-μm nylon filter to remove cell walls, and centrifuged for 2 hr at 48000 *g*. The supernatant was called the soluble fraction.

Isolation of extracellular, water-soluble components. 5-cm sections of etiolated hypocotyls excised directly below the hook were packed vertically into a glass holder with their basal ends placed on a coarse glass filter bottom (about 10 g fr. wt per holder). The sections were thoroughly washed with H_2O to remove cytoplasmic contamination from the cut surfaces, and, unless specified otherwise, infiltrated with 100 mM KCl by submerging them for 3 min without vacuum, followed by 3 min with vacuum. Then the holder was placed in a plastic centrifuge tube and centrifuged at 1000 *g* for 10 min. This sequence was repeated twice. About 0.35 ml of centrifugate was collected per g fr. wt of dark-grown hypocotyls. Epicotyl and hypocotyl tissue of light-grown plants was treated in the same way. Cell rupture was checked immediately by measuring the activity in the extract of the cytosolic enzyme glucose-6-phosphate dehydrogenase.

Isoelectrofocusing in an electrofocusing column. An Ampholine column (type 110 ml, LKB, Sweden) was used with a linear glycerol gradient (0–50%, v/v) and with 0.5% (w/v) Servalyt, pH 2–11, (Serva, GFR) as carrier ampholyte. The column was run at 350 V and at 10° for 3 days; 4 ml fractions were collected and their pH was determined. From each fraction 2 ml aliquots were dialysed against 50 mM Tris–HCl, pH 7.5, and then hydrolysed in 6 M HCl at 120° for 3 hr to release Hyp.

Isoelectrofocusing on polyacrylamide gels. Polyacrylamide slabgels (5% polyacrylamide) were made according to ref. [9] with 2.4% (w/w) Ampholines, pH 2.5–4, (LKB, Sweden). The 0.7 mm-thick gels were run on a LKB Multiphor Apparatus with 1 M glycine and 1 M H_3PO_4 as cathode and anode buffer, respectively; the cooling temp. was 8°. Samples were applied on small paper filters placed on the middle of the gel. After 5 hr at 1000 V, 1 cm pieces were excised at one side of the gel to determine the pH and the remainder of the gel was stained directly with β-Gal-Yariv reagent [0.1 mg/ml 10% (v/v) DMSO in H_2O] overnight at 40°. The gel was destained with several changes of H_2O.

Glucose-6-phosphate dehydrogenase. A 0.1 ml sample was incubated with 0.8 ml buffer (100 mM Tris–HCl, 10 mM $MgCl_2$, pH 8), 0.025 ml 25 mM $NADP^+$ and 0.1 ml 20 mM glucose-6-phosphate. The increase of $A_{340\,nm}$ was measured at 25°. Blanks were run without glucose-6-phosphate.

Chemical analyses. Hyp was determined according to the method of ref. [10]. Uronic acids were measured with the method of ref. [11] with galacturonic acid as a standard, and total carbohydrate was determined according to ref. [12] with glucose as a standard. Protein was quantitatively precipitated with MeOH (final concn 84%, v/v; 2 hr at 4°) and its concn was measured according to ref. [13] using bovine serum albumin as a standard.

β-Lectin activity. The unlabeled substrate for the assay was prepared by coupling diazotized *p*-aminophenyl-β-galactopyranoside (Sigma), to phloroglucinol to give 1,3,5,tris-(4-β-D-galactopyranosyl-oxyphenylazo)2,4,6-trihydroxybenzene (β-Gal-Yariv) [14]. ^3H-labeled substrate was prepared by incubating 0.1 mg β-Gal-Yariv in 1 ml 0.1 M NaPi buffer, pH 7, with 1 unit galactose oxidase (EC 1.1.3.9., Sigma) at 37° for 24 hr. In order to remove the enzyme, 2 ml 0.1 M NaH_2PO_4 was added, the mixture was boiled for 5 min and denaturated protein was spun down at 1000 *g* for 20 min. Then the pH of the supernatant was adjusted to 8 with 0.1 M NaOH and a few grains of $[^3H]KBH_4$ (2.48 GB/mmol: Amersham) were added; after 16 hr at room

temp. excess cold $NaBH_4$ was added and the mixture was allowed to stand at room temp. for 1 hr. The reaction was stopped by lowering the pH to 3 with 2 M HCl. Sp. act. of β-Gal-Yariv was 1.44 TBq/mol. β-Lectin activity was measured as follows: a 0.5 ml sample was incubated in a glass tube with 0.025 ml 1 mM β-Gal-Yariv and 0.0125 ml (25 kBq) ^{3}H-labeled β-Gal-Yariv in the presence of 1% NaCl at 40° for 1 hr. Then the tubes were centrifuged at 1500 *g* for 10 min, the pellets were washed × 3 with 0.5 ml 1% NaCl and then dissolved in 0.5 ml 0.1 M NaOH. A linear relationship between radioactivity and sample dilution was found for both the soluble fraction of homogenized hypocotyls and the extracellular solution (data not shown).

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