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A Novel Hydroxyproline-Deficient Arabinogalactan Protein Secreted by Suspension-Cultured Cells of *Daucus carota*[1]

Purification and Partial Characterization

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Arabinogalactan proteins (AGPs) are secreted or membrane-associated glycoproteins that have been operationally defined as binding to β-glucosyl Yariv artificial antigen, being rich in arabinose and galactose, and containing high levels of alanine, serine, and hydroxyproline. Using an anti-AGP monoclonal antibody (MAC 207) bound to cyanogen bromide-activated Sepharose 4B, we have purified by immunoaffinity chromatography an extracellular AGP from the culture medium of suspension-cultured cells of carrot (*Daucus carota*). The apparent molecular mass of this highly glycosylated proteoglycan is 70 to 100 kD as judged by sodium dodecyl sulfate-polyacrylamide gels. Although its sugar analysis, β-glucosyl Yariv binding, and high alanine, serine, and proline content are consistent with it being an AGP, the amino acid composition unexpectedly revealed this molecule to have no detectable hydroxyproline. This suggests that this glycoprotein is not a "classical" AGP, but represents the first example of a new class of hydroxyproline-poor AGPs. Deglycosylation of the AGP with anhydrous hydrogen fluoride revealed that the purified proteoglycan contains probably a single core protein with an apparent molecular mass of 30 kD. Direct visualization of the native AGP in the electron microscope showed ellipsoidal putative AGP monomers, approximately 25 nm by 15 nm, that showed a strong tendency to self assemble into higher-order structures. Upon desiccation, the glycosylated AGP formed paracrystalline arrays visible in the light microscope. Polarized Fourier transform infrared microspectroscopy of these arrays demonstrated a high degree of polarization of the sugar moieties under these conditions. These results put possible constraints on current models of AGP structure; a putative role for these novel AGPs as pectin-binding proteins is discussed.

Classically, the AGPs have been defined as a family of Hyp-rich glycoproteins and proteoglycans, originally associated with plant gums and exudates, that display β-lectin activity and are diagnostically precipitated by β-glucosyl Yariv reagent (Yariv et al., 1962, 1967). These molecules occur as glycoproteins associated with the plasma membrane and as proteoglycans in the extracellular matrix and intercellular spaces in all plant tissues examined from both higher and lower plant species (Clarke et al., 1978; Fincher et al., 1983; Samson et al., 1984; Basile and Basile, 1987; Pennell et al., 1989; Komavilas et al., 1991; Knox, 1992). The membrane-associated AGPs were first observed by Yariv-antigen staining of the interface between the plasma membrane and the cell wall (Clarke et al., 1975) and have recently been studied in more detail using monoclonal antibodies raised against carbohydrate epitopes present on AGPs (Norman et al., 1986, 1990; Knox et al., 1989, 1991; Pennell et al., 1989, 1991; Pennell and Roberts, 1990; Herman and Lamb, 1992).

Despite their abundance, no clear-cut function has yet been established for AGPs, but based upon localization studies, hypotheses abound. In root and floral meristems, membrane-associated epitopes have been shown to display developmentally regulated patterns of expression directly related to cell position and to cell fate, which suggests a role for these molecules in cell-cell interactions during plant development (Knox et al., 1989, 1991; Pennell and Roberts, 1990; Pennell et al., 1991). Moreover, a role for certain high-buoyant-density AGPs in actively suppressing local cell proliferation has been suggested. This is based on findings that the release of a high-buoyant-density fraction from the total bound AGPs in leafy liverworts and lettuce stem pith seems to be correlated with the experimental induction of cell division in the leaf primordia (Basile and Basile, 1992, 1993) and stem pith (Mignone and Basile, 1992).

Recently, studies involving the addition of embryogenic carrot cell line AGPs to nonembryogenic cell lines have demonstrated an apparent re-induction of embryogenicity in these lines (Kreuger and van Holst, 1993). Further, these intriguing results seem to support a developmental role for this family of proteins, and since, in angiosperms, secreted AGPs are a major constituent of the extracellular matrix of transmitting tissue, it has been proposed that they have a nutritive role in pollen tube growth and/or assist pollen adhesion to the stigma in species possessing "wet" stigmatic tissues (Labarca and Loewus, 1972; Clarke et al., 1979; Gell et al., 1986). There also is evidence of the accumulation of AGPs in response to wounding, which suggests a possible role for these molecules in plant defense (Fincher et al., 1983; Showalter and Varner, 1989). Lastly, it has been suggested that AGPs may use their lectin-like activity as cell-surface

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Abbreviations: AGP, arabinogalactan protein, FTIR, Fourier transform infrared.

extracellular matrix attachment proteins (Pennell et al., 1989; Roberts, 1990).

The secreted proteoglycan AGPs typically contain 2 to 10% protein by weight, which have an amino acid composition that is characteristically rich in Ser, Hyp, and Ala. N-terminal amino acid sequences of purified plasma membrane and secreted AGPs from *Lolium* and *Rosa* species have demonstrated the presence of a highly conserved N-terminal Ala-Hyp repeat sequence (Gleeson et al., 1989; Komalavilas et al., 1991). The carbohydrate moiety of these molecules typically consists of a 1,3-linked β-galactan backbone with side branches of 1,6-linked β-galactosyl residues that carry terminal residues typically of α-arabinofuranosides and minor amounts of uronic acids, Glc, rhamnose, Xyl, and Fuc.

Recently, however, the purification and characterization of several unusual proteins bearing similarities to but also displaying many differences from the "classical" AGPs have been reported (Qi et al., 1991; Fong et al., 1992; Kieliszewski et al., 1992). In their paper, Qi and colleagues reported the isolation of a high mol wt glycoprotein from gum arabic, which although containing polysaccharide side chains characteristic of AGPs, had an amino acid composition that more closely resembled extensins low in Ala but rich in Ser and His. Second, Kieliszweski et al. (1992) recently described a His-rich extensin isolated from *Zea mays* cell-suspension cultures that displayed other characteristics usually associated with AGPs. Amino acid sequences determined from chymotryptic digests of the deglycosylated protein revealed the presence of some repetitive motifs characteristic of extensins and others characteristic of AGPs. This protein was shown to be rich in Ala and Ser and was precipitated by β-glucosyl Yariv reagent. However, of most interest was the data obtained from β-elimination studies of the arabinogalactan component of the protein, which indicated that its *O*-linkage was to Ser rather than the characteristic *O*-Hyp linkage of other AGPs. The third "AGP-like" protein that has been described recently was a Hyp-rich glycoprotein isolated from salt eluates of Douglas fir (*Pseudotsuga menziesii*) cell-suspension culture, which was shown to contain both Ser-Hyp_4 and Ala-Hyp repeat units. Moreover, several other examples exist of proteins that share some characteristics of one "class" of cell wall protein while also possessing features of another "class" (reviewed in Showalter, 1993).

All these reports present data that suggest a blurring of the distinctions between the five classes of proteins associated with the cell surface, namely extensins, AGPs, Pro-rich proteins, solaneous lectins, and Gly-rich proteins. This indicates possible phylogenetic relationships between these various classes of wall protein (Kieliszewski et al., 1992), and that there may be many cell-wall-associated proteins yet to be described that do not strictly conform to one of the established classes of wall proteins, but may instead display features characteristic of two or more classes.

From the little that is currently known of the chemical and biophysical properties of the proteoglycan AGPs, a spherical structure for them termed the "wattle-blossom" model has been proposed (Fincher et al., 1983). In partial support of this hypothesis is the circular dichroism data from studies of the His-rich AGP-like protein previously cited, which displayed little polyproline II character, thus distinguishing it clearly from the extensins (Kieliszewski et al., 1992). Conversely, however, circular dichroism data from studies of purified AGP from *Lolium multiflorum* indicate some polyproline II helix character (van Holst and Fincher, 1984) in these molecules. In addition, recent studies of an AGP-like protein, isolated from gum arabic, that contains arabinogalactan glycan substituents but that does not possess an Ala-rich amino acid composition support a fibrous "hairy rope" model for the molecule (Qi et al., 1991).

In this study we present a preliminary characterization of the composition and shape of a novel immunoaffinity-purified Hyp-deficient AGP from the culture medium of suspension-cultured carrot (*Daucus carota*) cells. Deglycosylation of this protein has demonstrated the apparent presence of a 30-kD core protein. Results obtained from direct imaging of the native AGP tend to support the wattle-blossom model for proteoglycan AGP structure. Furthermore, polarized FTIR microspectroscopy has given further insight into the fine structure of this molecule. Preliminary functional analysis of this AGP suggests a possible role as a pectin-binding protein.

MATERIALS AND METHODS

Plant Material

The carrot (*Daucus carota*) cell line used in this study has been described previously (Lloyd et al., 1979; Knox et al., 1991).

Preparation of Culture Medium

Culture medium was prepared from carrot cell suspensions cultured for 7 d. The cells were removed by centrifugation, and the supernatant was filtered through filter paper (Whatman, Inc., Clifton, NJ), dialyzed overnight against sterile distilled water at 4°C, and lyophilized.

Immunoaffinity Purification of Secreted AGP from Culture Medium

The soluble secreted AGPs of the culture medium were precipitated with 80% (v/v) ice-cold ethanol from cell-free medium 7 d after subculture (Knox et al., 1991). After centrifugation at 25,000*g* for 20 min, the pellet was redissolved in water and lyophilized.

MAC 207, an anti-AGP monoclonal antibody known to recognize an Ara-containing AGP epitope (Pennell et al., 1989), was covalently bound to cyanogen bromide-activated Sepharose 4B as specified by the manufacturer (Pharmacia Fine Chemicals Ltd, UK); the swollen gel contained approximately 2 mg of antibody/mL. The MAC 207-Sepharose 4B was equilibrated in PBS/0.1% sodium azide.

Lyophilized ethanol precipitate from the culture medium (10 mg) was dissolved in 10 mL of PBS/azide and bound to a 40-mL column of MAC 207-Sepharose overnight at 4°C. Unbound material was removed by washing with 10 column volumes of PBS/azide, and the bound AGP was subsequently eluted with 0.1 M NaCl, 0.15 M Gly (pH 2.4) collected in 800-μL aliquots into 1.5-mL Eppendorf tubes containing 200 μL of 1 M Tris. Eluted fractions were tested for the presence of MAC 207-reactive AGP by an immunodot blot assay (see

Fig. 1). Fractions containing AGP were pooled and dialyzed overnight against sterile distilled water at 4°C and lyophilized.

Deglycosylation of Immunoaffinity-Purified AGP

A 50-mg sample of the immunopurified AGP was treated in 10 mL of anhydrous HF at 0°C for 30 min, the reaction was frozen in liquid nitrogen, and cold ether was added to complex the HF. The HF/ether complex was evaporated and the residue was suspended in 50 mM ammonium acetate buffer. The resultant sample was lyophilized prior to being used in subsequent experiments. The deglycosylation of the AGP was generously carried out by Professor Andrew Mort at Oklahoma State University (Stillwater, OK).

Immunochemistry

Western blots of SDS-PAGE gels were performed as described previously (Knox et al., 1989; Pennell et al., 1989) with the exception that anti-rat immunoglobulin G molecules conjugated to alkaline phosphatase (Sigma, UK) were used as secondary antibodies in the immunoblotting experiments. SDS-PAGE gels were silver stained according to Rabilloud et al. (1988).

β-Glucosyl Yariv Reagent Dot Blot

Samples of AGP (1–3 μg) dissolved in sterile distilled water were dotted onto nitrocelluose, air dried, blocked with 3% dried skimmed nonfat milk powder dissolved in PBS, pH 7.4, for 1 h, washed for 5 min in PBS, and subsequently immersed for 15 min in β-glucosyl Yariv reagent. The concentration of Yariv reagent was 0.15 mg cm^{-3} in 1% NaCl. The nitrocellulose was washed in PBS, pH 7.4, to decrease background staining before observations were made. The β-glucosyl Yariv reagent was a kind gift from Professor Dominick Basile (City University of New York).

Dot Blots to Study Interaction between AGP and Other Cell Wall Components

Polysaccharide samples (1 μg) dissolved or suspended in sterile distilled water (with the exception of polygalacturonic acid, which was dissolved in 20 mM PBS) were dotted onto nitrocellulose, air dried, blocked with 3% dried skimmed nonfat milk powder dissolved in PBS, pH 7.4, for 1 h, washed in PBS, and subsequently immersed in either PBS or purified AGP dissolved in various solvents at a concentration of 3 mg/mL for 1h. After incubation in the presence of AGP, blots were washed in PBS and incubated with MAC 207 and secondary antibody as for western blots.

Visualization

Immunoaffinity-purified AGPs were visualized in a Jeol 1200EX transmission electron microscope using three different methods.

Negative Staining

A small drop of AGP in water was placed on a carbon-coated filmed copper grid followed by a drop of either 2% uranyl acetate or 2% uranyl formate. After mixing, the excess liquid was blotted off and the grid was viewed. For immunogold labeling of negatively stained molecules, the immunoaffinity-purified AGP was adsorbed to plastic-coated gold grids and labeled with MAC 207, using a 5-nm colloidal gold anti-rat secondary antibody (Sigma, UK), as described by McCann et al. (1992).

Rotary Shadowing

A sample of 0.5 mL (concentration approximately 1 mg/mL) was mixed with 0.5 mL of glycerol and 100 μL of 1.5 M ammonium formate buffer, pH 6.8, and sprayed with an air brush onto a freshly cleaved mica surface (Shotton et al., 1979). The glycerol and buffer were then dried off under vacuum (10^{-5} torr) overnight, leaving the molecules dried onto the mica surface. A replica was made by rotary shadowing with platinum/palladium at a low angle, followed by a thin carbon coating (Tyler and Branton, 1980). The replica was floated off the mica onto the surface of distilled water and pieces were lifted onto uncoated copper grids. Tobacco mosaic virus particles were used as an internal marker for droplets in control experiments. The particles were sprayed onto mica in both the presence and absence of AGP to demonstrate that no aggregates or putative AGP monomers were present in the absence of the AGP sample.

Fast-Freeze, Deep-Etch, Rotary-Shadowed Replica Technique

A solution of AGP was mixed with an aqueous suspension of tiny flakes of mica and quick-frozen and deep-etched following the method of Heuser (1983). A rotary-shadowed replica of the molecules adsorbed to the mica flakes was floated onto the surface of distilled water and transferred to the surface of 70% hydrofluoric acid. After careful washing, pieces of replica were lifted onto plastic-filmed copper grids. Micrographs were printed onto reverse-contrast paper (Kodak Graphic Arts) to facilitate viewing of stereo pairs.

FTIR

FTIR spectra were obtained on a Spectra Tech instrument with microscope accessory. The introduction of two polarizers into the system permitted plane polarization of the IR beam before and after passing through the sample (McCann et al., 1992). A droplet of AGP in solution was dried down on a barium fluoride window to allow aperturing in the microscope. Solution and gel-state spectra were also obtained in horizontal Attenuated Total Reflectance mode. Samples were scanned for 256 co-added interferograms, with 4 cm^{-1} resolution and aperture dimensions of 10 × 10 μm.

Amino Acid Composition Analyses

Total amino acid composition analysis (excluding Hyp analysis) was performed on two 1.5-mg samples of lyophilized AGP. Each 1.5-mg sample was dissolved in 2 mL of 6 M HCl and hydrolyzed for 24 h at 110°C. A 0.1-mL sample of the resultant hydrolysate was derivatized with dansyl chloride and the amino acids were separated by HPLC and

analyzed as described by Knecht and Chang (1986). These analyses were performed at Unilever Research, Colworth Laboratory (Bedford, UK).

Hyp Analysis

The 1.9-mL remainders of the hydrolysates generated for the total amino acid composition analysis were lyophilized and then redissolved in 0.18 mL of sodium citrate, pH 2.2. Each sample was then analyzed for the presence of Hyp on an LKB 4151 alpha plus amino acid analyzer precalibrated with Hyp standards. These analyses were also performed at Unilever Research, Colworth Laboratory (Bedford, UK). Similar Hyp analyses were performed on a 3-mg sample of purified AGP by Dr. John Fox at the University of Birmingham, UK.

Three-milligram samples of AGP were analyzed for the presence of Hyp using a colorimetric assay as described by Lamport and Miller (1971), using Hyp standards to construct a calibration curve and potato lectin and BSA as positive and negative controls, respectively.

Sugar Analyses

AGP samples (1 mg) were subjected to methanolysis (1.2 M methanolic HCl, 24 h, 85°C) and neutralized with Ag_2CO_3, and the mixture was treated with 25 μL of acetic anhydride, stored in the dark for 2 h, and centrifuged. The supernatant was concentrated to dryness under reduced pressure at 40°C and dried over P_2O_5. The methyl glycosides were trimethyl silylated with 25 μL of hexamethyldisaline:chlorotrimethylsilane:pyridine (1:1:5, v/v/v) for 30 min at room temperature and analyzed by GLC on a capillary CpSil 5CB fused silica column (25 m/0.32 mm, Chrompack).

RESULTS AND DISCUSSION

Purification and Preliminary Characterization of Purified AGP

AGPs together with other proteoglycans and polysaccharides were selectively precipitated by ethanol from the medium of a 7-d-old carrot cell-suspension culture. The AGP present in this precipitate was then immunoaffinity purified by passing it down a MAC 207-Sepharose 4B affinity column. Figure 1, A and B, shows fractions collected from the column at various stages of the purification process immunoblotted using MAC 207 as the primary antibody. Although the column binding capacity for AGP has been exceeded, with with some MAC 207-reactive material being removed by initial washes, later washes contain no MAC 207-reactive material. Moreover, when low pH eluent is added, the bound AGP is selectively eluted as detected by MAC 207. MAC 207 was chosen as the affinity matrix to purify the AGP on several grounds. It has been shown to bind to a periodate-sensitive oligosaccharide epitope that is present on all AGPs that are also operationally defined by Yariv binding, but it does not bind to extensin, solaneous lectins, or any known cell wall polysaccharide (Pennell et al., 1989; Knox et al., 1991; Knox, 1992; M. Hahn, personal communication).

When the fractions eluted from the MAC 207 column are run on SDS-PAGE gels in parallel with the original culture medium and the gels are silver stained, the medium is shown to contain a wide spectrum of proteins, whereas the eluted fractions contain only a single "smeary" band of apparent molecular mass 70 to 100 kD (see Fig. 1C). Western blots of these gels probed using MAC 207 demonstrated the presence of two MAC 207-reactive bands of 180 to 200 kD and 70 to 100 kD, respectively, in the original medium, whereas the immunopurified material contains only a single MAC 207-reactive band at 70 to 100 kD, which corresponds to the single band seen on the silver-stained gels. The fact that

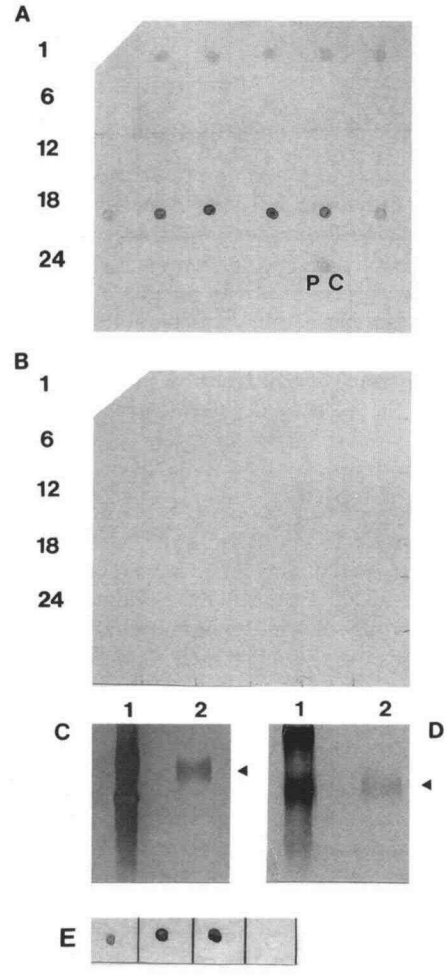

Figure 1. Immunoaffinity purification of secreted *D. carota* AGP. Immunoblots A and B were dotted with fractions collected during purification with a positive control (PC) onto which was dotted culture medium. Blot A was probed using MAC 207 as the primary antibody with an alkaline phosphatase-conjugated secondary antibody. Blot B was probed only with the secondary antibody. Fractions 18 to 23 were collected after the addition of elution buffer and are shown to contain MAC 207-reactive material. C, Silver-stained 10% SDS-PAGE gel run with culture medium in lane 1 and purified AGP in lane 2 (molecular mass 70–100 kD; see arrow). D, Western blot of a gel as shown in C but probed with MAC 207. E, Dot blot of purified AGP probed with β-glucosyl Yariv reagent. The dots contain, from left to right, 1, 2, and 3 μg of AGP followed by a negative control dotted with PBS, pH 7.4.

Table I. *Amino acid composition of immunopurified, secreted AGP from D. carota*

The results shown represent the mean of two HPLC runs.

Amino Acid	Mol %
Asx	10.7
Glx	9.2
Ser	7.4
Thr	6.1
Gly	11.3
Ala	12.2
Arg	4.4
Pro	5.0
Val	6.8
Met	0.0
Ile	4.6
Leu	8.7
Phe	3.7
Cys	0.0
NH_3	0.0
Lys	5.4
His	1.9
Tyr	2.8
Trp	Not analyzed
Hyp	Not detected

MAC 207 binds to the 70- to 100-kD band present in the immunopurified fractions confirms that the MAC 207-reactive AGP present in the culture medium has been successfully purified. The same 70- to 100-kD MAC 207-reactive band has been observed previously in ethanol-precipitated culture medium in the absence of the 180- to 200-kD band (Knox et al., 1991). The 180- to 200-kD MAC 207-reactive band is not present in the immunoaffinity-purified material because it is soluble in ethanol and is consequently not present in the material prepared for loading onto the immunoaffinity column.

The amino acid composition analysis presented in Table I shows that, for the MAC 207-immunopurified proteoglycan, the most abundant amino acid is Ala, followed by Gly, Asx, Glx, Leu, Ser, and Val. These data, with the marked exception of the absence of Hyp, are in general agreement with the amino acid compositions of previously studied AGPs (Fincher et al., 1983; Showalter et al., 1989). Sugar analyses of the MAC 207-reactive secreted proteoglycan shown in Table II show it to be rich in Ara and Gal and also to contain Xyl, Man, and other sugars, all of which are commonly associated with AGPs. Because there is no cell breakage involved in the preparation of the starting material and there is no starch present, therefore, in either the material loaded onto the column or the purified AGP, we believe the Glc content figure to be reliable. It also compares with levels in AGPs described by Jermyn and Guthrie (1985). Therefore, with the notable exception of the absence of Hyp, the sugar analyses, Yariv binding, the binding to MAC 207, and the total amino acid composition are all consistent with the idea that the purified proteoglycan is an AGP, as operationally defined (Jermyn, 1978). Because the absence of Hyp from such molecules is very unusual, we made a considerable effort to confirm this result several times in several laboratories using two methods of detection. In each case, the Hyp level was below the limits of detection.

Table II. *Monosaccharide sugar composition of immunopurified, secreted AGP from D. carota*

Monosaccharide	Mol %
Ara	18.7
Gal	40.2
Xyl	4.0
Man	6.0
Glu	5.9
Glc	25.1

Deglycosylation of Purified AGP Reveals the Core Protein

As shown in Figure 2, when deglycosylated AGP is run in parallel with native AGP, the deglycosylated protein runs as a single tight band of approximately 30 kD, as opposed to the 70- to 100-kD smear present in the lane loaded with the native glycosylated sample. This result suggests the presence of an AGP core protein whose apparent molecular mass in the native molecule varies with the length and composition of its carbohydrate moiety. There is also the variable appearance of a minor band (at approximately 60 kD) that we believe is either human cytokeratin or a partially deglycosylated core protein. These data support the previous study of a secreted "classical/Hyp-rich" AGP isolated from *Lolium multiflorum* culture media (Gleeson et al., 1989), whose molecular mass decreased from 200 to 35 kD, suggesting the possible presence of a single core protein.

The clear paradox in the observed molecular mass of the carrot AGP core protein, which would be 5 kD if based on a core protein that is 5% of a 100-kD native protein, may be explained by the anomalous migration of heavily glycosylated proteins in SDS-PAGE gels.

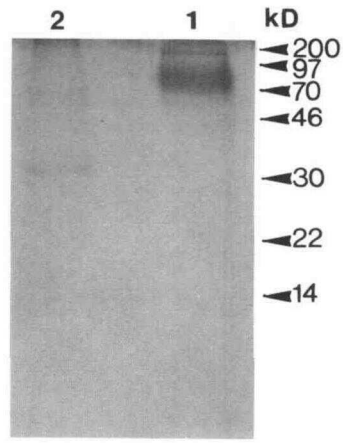

Figure 2. Ten percent SDS-PAGE gel of glycosylated and deglycosylated AGP. Glycosylated AGP ran as a smear from 70 to 100 kD as shown in lane 1, loaded with 10 μg of glycosylated AGP. HF-deglycosylated AGP ran as a single band as shown in lane 2, loaded with 1 μg of HF-deglycosylated AGP.

Visualization and Spectroscopic Studies of AGP

The purified AGP was visualized directly in the transmission electron microscope using a variety of techniques. The consistency of the images obtained, using three very different specimen-preparation methods and a wide variety of controls, gives us confidence that we are observing the molecules in their native state. By all methods used, the AGPs showed a strong tendency to self assemble into larger aggregates. Figure 3A shows such a higher-order structure by negative staining, whereas Figure 3, C and D, shows immunogold labeling of negatively stained aggregates using MAC 207, confirming that these aggregates are indeed composed of AGPs. Similar aggregates were also visualized by rotary-shadowing of molecules dried down on mica under vacuum (Fig. 3B) and by the fast-freeze, deep-etch, rotary-shadowed replica technique (Heuser, 1983) (Fig. 4). The stereo pair image in Figure 4 shows the three-dimensional structure of such an aggregate.

Images of the higher-order structures indicated that they might be composed of simple, small, ellipsoidal building blocks. Attempts were made to disrupt or to prevent aggregate formation in order to facilitate visualization of individual "monomers." Various chaotropic agents were used to attempt to disrupt aggregate formation, but use of the chaotropes made visualization of the AGP difficult (data not shown). However, when samples were visualized directly after dialysis against sterile distilled water, smaller aggregates and a number of monomers of 15 ± 5 nm × 25 ± 5 nm were successfully observed (Fig. 5).

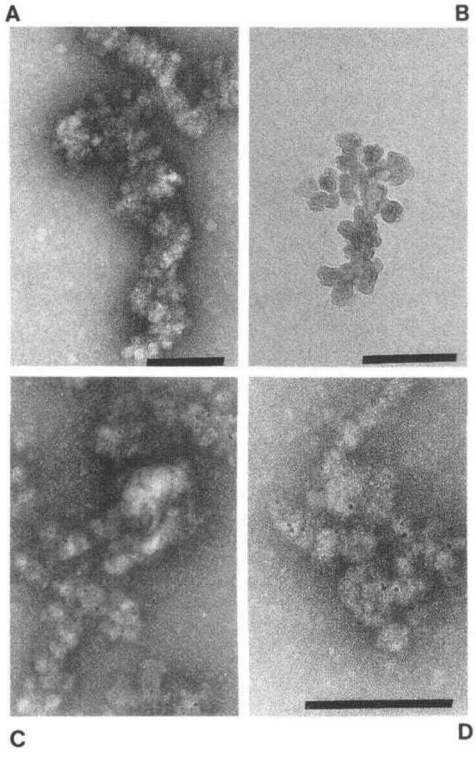

Figure 3. AGP aggregates imaged using various ultrastructural techniques. A, AGPs show a strong tendency to aggregate into higher-order structures, imaged here by negative staining with uranyl acetate. B, Similar aggregates of AGPs can be imaged by spraying them in glycerol onto a freshly cleaved mica surface, drying them in vacuo, and rotary shadowing them with platinum/palladium, followed by carbon. C and D, These aggregates can be immunogold-labeled with MAC 207 and negatively stained with uranyl acetate, confirming their identity as AGPs. The gold particles appear as small black dots. Scale bar represents 200 nm.

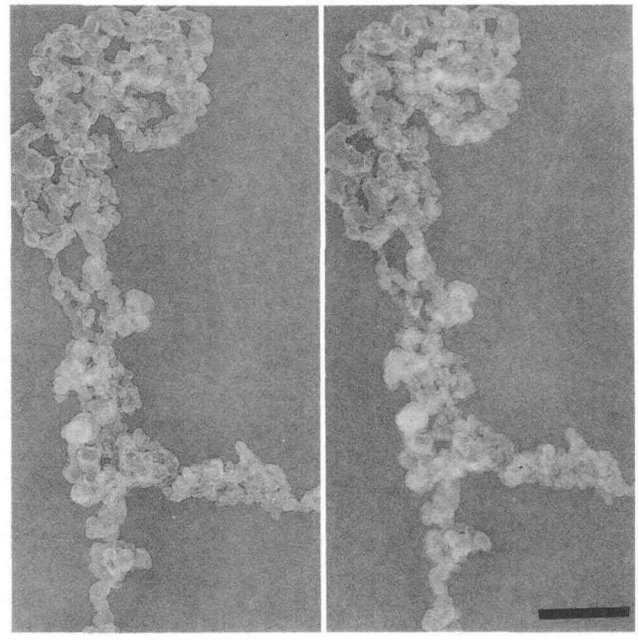

Figure 4. Stereo pair of micrographs showing the three-dimensional structure of a large aggregate of AGP imaged by the fast-freeze, deep-etch, rotary-shadowed replica technique. Scale bar represents 200 nm.

Each of these ellipsoidal subunits may represent a single AGP molecule. Although the apparent molecular mass is 70 to 100 kD as measured by SDS-PAGE, the presence of such high levels of uncharged carbohydrate means that the real molecular mass will be considerably underestimated (Pennell et al., 1989). However, the deglycosylated protein runs at around 30 kD, which suggests a polypeptide of around 270 amino acids. If this accounts for about 5% of the total molecule, it would mean the monomer would have a molecular mass of about 600 kD, implying that about 3200 sugar residues have to be accommodated. The amino acid composition suggests that no more than approximately 10% of the residues could possibly be glycosylated, which would mean that each branched oligosaccharide side chain contained on average a minimum 120 sugar residues. This would agree with estimates for side chain size in AGPs isolated from wheat endosperm (Fincher et al., 1974). From the dimensions of the imaged AGP, we can calculate a rough volume of 3000 nm^3, which, if we assume a typical proteoglycan occupancy of maybe 30%, would predict a mol wt for each monomer imaged of just under 10^6. This is consistent with the idea that the monomers imaged are indeed single AGP molecules.

Because there is no detectable Hyp present in this AGP,

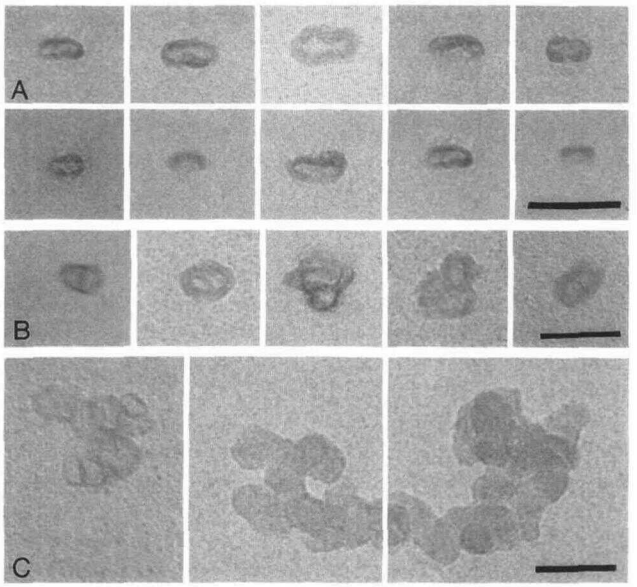

Figure 5. Rotary-shadowed images of putative monomers and small and large aggregates of AGP. A, Putative monomers are ellipsoids of approximately 25 nm × 15 nm. B, Small aggregates of two to four monomers. C, Larger aggregates also seem to be constructed from ellipsoidal building blocks. A-C, Scale bar represents 50 nm.

an obvious question that arises is to which amino acid is attached the large quantity of sugar present in this protein. In this connection, it is of interest to note that in the recent studies of a maize His-rich extensin/AGP, β-elimination studies demonstrated that the arabinogalactan moiety present was O-linked to Ser rather than to Hyp (Kieliszewski et al., 1992). This supports previous suggestions that some AGP carbohydrate may be O-linked to Ser, as well (Fincher et al., 1983). Therefore, it is not without precedent to suggest that the arabinogalactan constituent of the AGP under discussion is O-linked to the Ser residues present in the core protein.

To date, two models have been proposed to represent AGP and "AGP-like" molecules. The first, proposed by Fincher et al. (1983) and based upon the biophysical properties of AGP, predicts a spherical wattle-blossom model for monomers of AGP. The second, proposed by Qi et al. (1991) and based upon studies of an AGP-like glycoprotein isolated from gum arabic, indicates a twisted hairy rope model.

The direct imaging results presented in our study are consistent with the wattle-blossom model in that ellipsoidal rather than rod-like monomers were observed. However, upon desiccation, AGP dries down as a paracrystalline array. Data from FTIR polarized microspectroscopy (Fig. 6) suggest that, in the dehydrated state, the sugar side chains of the AGP have a very precise orientation, suggesting that a more ordered structure of the carbohydrate moiety is possible than in the model proposed by Fincher et al. (1983). Band stretches associated with particular molecular bonds of carbohydrates are sharply polarized, with the backbone of the sugar side chains at right angles to the direction of the "needles" in the paracrystalline arrays.

AGPs May Be Pectin-Binding Proteins

Because AGPs have been classically defined as β-lectins, it is important to inquire what, if any, are the endogenous ligands to which such molecules bind. Plasma-membrane-associated AGPs protrude from the membrane into the cell wall, and it seems logical to suggest that they are binding to glycans in the wall and consequently might be functioning

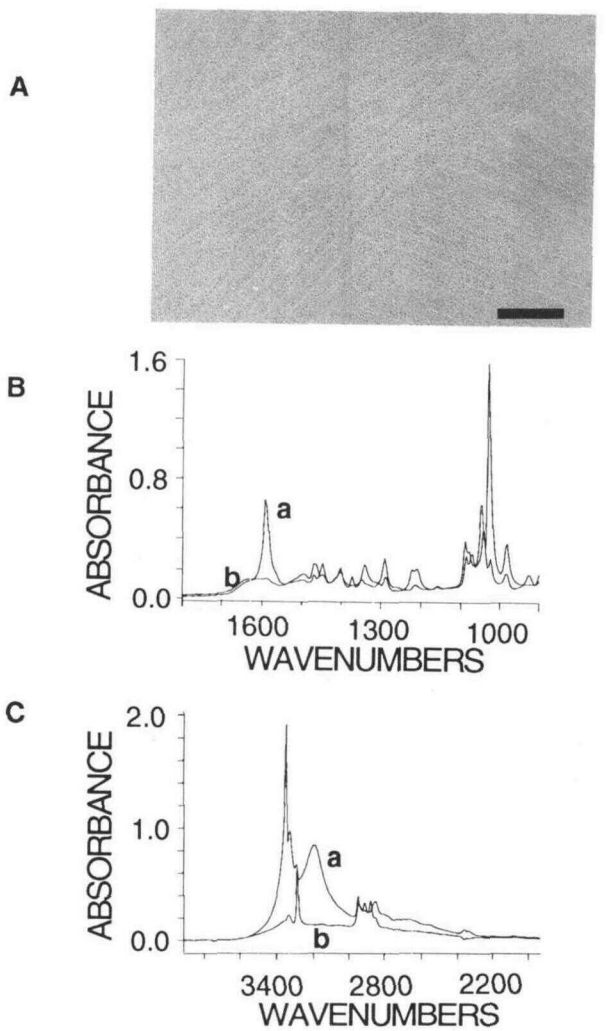

Figure 6. FTIR spectra of AGP paracrystalline arrays. A, Rotary-shadowed replica of paracrystalline array formed upon desiccation of AGP shows the presence of domains of needle crystals. Scale bar represents 100 nm. B, FTIR spectra in the region 1800 to 900 cm^{-1} of an area of the paracrystalline array with polarizers aligned parallel (a) and perpendicular (b) to the direction of the needles show strong polarization of band stretches associated with carbohydrates. Spectra were obtained from an area of 40 × 40 μm in a region of uniform crystal alignment. In the region 1200 to 900 cm^{-1}, there are clear differences in the peak ratios. Spectrum a has a sharply polarized free acid stretch at 1600 cm^{-1}, suggesting that the uronic acid component of the carbohydrate side chains is precisely orientated. C is the same as in B, but in the region 3800 to 2000 cm^{-1}. In this region, we can see that almost all of the peaks (largely attributable to C-H bond stretches) are highly polarized.

as cell surface extracellular-matrix binding proteins (Pennell et al., 1989). Moreover, the current study and other recent results provide several lines of evidence to suggest that the secreted AGPs may also bind to cell wall polysaccharides and in particular to pectic fractions of the wall. Crude pectic fractions have frequently been shown to be "contaminated" by tightly bound AGP (as detected by our panel of anti-AGP monoclonal antibodies), and we have also observed that Yariv reagent cross-reacts with pectin-containing cell wall fractions, as does MAC 207 (data not shown). Therefore, we performed some preliminary experiments in which sequentially extracted carrot cell-wall fractions were dried down on nitrocellulose and subsequently incubated, after blocking, with our purified soluble AGP. Fractions to which AGP bound were identified by subsequent incubation with MAC 207 and an enzyme-linked secondary antibody. The result was that only the pectic fractions showed enhanced MAC 207 labeling relative to dots incubated in the absence of added AGP. Commercial polygalacturonic acid (Sigma) did not bind to AGP, suggesting that a more complex molecule is the endogenous ligand. To determine whether these results were due to AGP binding to itself, control experiments were performed in which AGP dotted onto nitrocellulose was incubated in the presence and absence of AGP and subsequently incubated with MAC 207 as described above. The results of these experiments demonstrated that under the conditions used the AGP did not bind to itself.

The effect of calcium on the interaction between AGP and pectin was also indirectly examined (Fig. 7). In the presence of 1 mM EGTA, the apparent binding of AGP to pectin is reduced, which suggests that the interaction between AGP and pectin may be Ca^{2+} dependent and raises the intriguing possibility of calcium-dependent cell-matrix attachment. Two examples that also support the idea of their interaction are the dramatic co-localization of pectin and AGP in *Arabidopsis* pollen grains and tobacco pollen tube walls (Li et al., 1992; Van Aelst and Van Went, 1992). Van Aeslt and Van Went (1992) demonstrated that MAC 207 and JIM 5 (an anti-pectin monoclonal antibody) both specifically label the intine region of *Arabidopsis thaliana* pollen grains adjacent to the plasma membrane. Second, Li and co-workers, also using MAC 207, have identified discrete bands of AGP, which encircle growing tobacco pollen tubes (Li et al., 1992); furthermore, they also recently demonstrated that these bands cross-react strongly with an anti-pectin monoclonal antibody (personal communication, Sixth Cell Wall Meeting, Nijmegen, The Netherlands, 1992). The functional significance of this possible AGP-pectin interaction awaits future study.

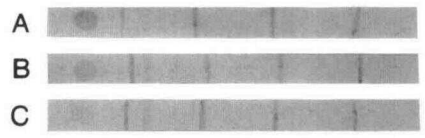

Figure 7. Dot blot showing the effect of calcium upon the binding of purified AGP to carrot and onion cell-wall fractions and polysaccharides. Samples on dot blot from left to right are unesterified pectin extracted from carrot cell walls, unesterified pectin extracted from onion parenchyma cell walls, rhamnogalacturonan I, rhamnogalacturonan II, and polygalacturonic acid (Sigma). Blot A was incubated in 3 mg/mL of AGP dissolved in 1% NaCl plus primary and secondary antibodies; B was incubated in 3 mg/mL of AGP dissolved in 1 mM EGTA plus primary and secondary antibodies; and C was incubated in primary and secondary antibodies only with no AGP incubation step.

CONCLUDING REMARKS

In conclusion, we draw particular attention to the unusual features of the molecule whose purification and initial characterization we have presented. Carrot cell cultures make and secrete large amounts of AGP, as well as polysaccharides and proteins, such as extensin. At least two AGPs are made in abundance, as defined by the fact that they are recognized by MAC 207. In the absence of either comprehensive amino acid sequence data or a functional assay, our definition of AGPs in general must, by default, be operational. We have purified to homogeneity one of these AGPs and find that it possesses many of the diagnostic features of classical AGPs: it contains high levels of Gal and Ara and abundant quantities of Ala and Ser, it binds to β-glucosyl Yariv reagent, and it reacts with MAC 207. The very unusual difference, however, is that it contains no detectable Hyp, previously thought to be a defining property of AGPs, or β-lectins (Jermyn, 1978). This forces us to expand our definition of AGPs. Just as the extensin family of cell-wall glycoproteins became broader and better defined when sequence data became available (Showalter, 1993), so we believe that it will become possible to make better sense of both this new, broader group of AGPs and the relationship between the secreted and membrane-associated AGPs only when molecular data eventually accumulate.

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