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Heterogeneity of Arabinogalactan-Proteins on the Plasma Membrane of Rose Cells[1]

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Arabinogalactan-proteins (AGPs) have been purified from the plasma membrane of suspension-cultured Paul's Scarlet rose (*Rosa* sp.) cells. The two most abundant and homogeneous plasma membrane AGP fractions were named plasma membrane AGP1 (PM-AGP1) and plasma membrane AGP2 (PM-AGP2) and had apparent molecular masses of 140 and 217 kD, respectively. Both PM-AGP1 and PM-AGP2 had β-(1–3)-, β-(1,6)-, and β-(1,3,6)-galactopyranosyl residues, predominantly terminal α-arabinofuranosyl residues, and (1,4)- and terminal glucuronopyranosyl residues. The protein moieties of PM-AGP1 and PM-AGP2 were both rich in hydroxyproline, alanine, and serine, but differed in the abundance of hydroxyproline, which was 1.6 times higher in PM-AGP2 than in PM-AGP1. Another difference was the overall protein content, which was 3.7% (w/w) in PM-AGP1 and 15% in PM-AGP2. As judged by their behavior on reverse-phase chromatography, PM-AGP1 and PM-AGP2 were not more hydrophobic than AGPs from the cell wall or culture medium. In contrast, a minor plasma membrane AGP fraction eluted later on reverse-phase chromatography and was more negatively charged at pH 5 than either PM-AGP1 or PM-AGP2. The more negatively charged fraction contained molecules with a glycosyl composition characteristic of AGPs and included at least two different macromolecules. The results of this investigation indicate that *Rosa* plasma membrane contains at least four distinct AGPs or AGP-like molecules. These molecules differed from each other in size, charge, hydrophobicity, amino-acyl composition, and/or protein content.

AGPs are a class of proteoglycans that are rich in galactosyl and arabinosyl residues (Clarke et al., 1979; Fincher et al., 1983; Bacic et al., 1988), and they appear to be present in all plant cells (Jermyn and Yeow, 1975; Clarke et al., 1978; van Holst and Clarke, 1986; Tsumuraya et al., 1988; Showalter and Varner, 1989). At the subcellular level, AGPs are found in cytoplasmic organelles (Anderson et al., 1977; van Holst et al., 1981; Herman and Lamb, 1992), on the plasma membrane (Knox et al., 1989; Pennell et al., 1989, 1991; Norman et al., 1990; Komalavilas et al., 1991; Kikuchi et al., 1993; Smallwood et al., 1996), in the cell wall (Basile and Basile, 1987; Schopfer, 1990; Serpe and Nothnagel, 1994, 1995; Schindler et al., 1995), in the medium of suspension-cultured cells (Bacic et al., 1987; Gleeson et al., 1989; Komalavilas et al., 1991; Chen et al., 1994), in stylar secretions (Gleeson and Clark, 1979; Gell et al., 1986; Du et al., 1994; Cheung et al., 1995), and in plant gums and mucilages (Akiyama et al., 1984; Osman et al., 1993).

The best-characterized AGPs are those found as soluble molecules, including AGPs isolated from the medium of suspension-cultured cells (e.g. Bacic et al., 1987; Komalavilas et al., 1991) and AGPs copiously exuded as tears from trees, such as gum arabic from *Acacia senegal* (Akiyama et al., 1984; Qi et al., 1991; Osman et al., 1993). These AGPs have molecular masses of 60 to 300 kD and consist of 90 to 95% carbohydrate. The remainder of the macromolecule is protein that is typically rich in Hyp, Ser, Ala, Thr, and Gly residues (Anderson et al., 1977; Clarke et al., 1979; Gleeson et al., 1989; Showalter and Varner, 1989; Komalavilas et al., 1991), although His-rich (Kieliszewski et al., 1992) and Hyp-deficient (Baldwin et al., 1993; Mollard and Joseleau, 1994) AGPs have also been reported. Carbohydrate-protein linkages have been identified in a few AGPs, and they include galactosyl linkages to Hyp or Ser (Hillestad et al., 1977; Strahm et al., 1981; Tsumuraya et al., 1984; Qi et al., 1991), arabinosyl linkages to Hyp (Pope, 1977; van Holst and Klis, 1981), and unidentified linkages to Thr (Tsumuraya et al., 1987; Saulnier and Brillouet, 1989). The carbohydrate portion of AGPs consists of a galactan framework having β-(1,3)-, β-(1,6)-, and β-(1,3,6)-linkages (Fincher et al., 1983; Bacic et al., 1987; Tsumuraya et al., 1988; Komalavilas et al., 1991; Carpita and Gibeaut, 1993). Ara and smaller amounts of other sugars such as GlcUA and Rha are attached, usually as terminal residues, to the galactan framework (Bacic et al., 1987; Komalavilas et al., 1991).

Much less is known about the biochemical structure of the AGPs that are associated with the plasma membrane or cell wall. These AGPs may play important roles during plant development, however, as suggested by the discovery of variations of PM-AGP or CW-AGP epitopes during somatic embryogenesis (Stacey et al., 1990; Pennell et al.,

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Abbreviations; AGP, arabinogalactan-protein; $(\beta\text{-D-Glc})_3$, $(\beta\text{-D-glucosyl})_3$ Yariv phenylglycoside; CM-AGPs, culture medium arabinogalactan-proteins; CW-AGPs, cell-wall arabinogalactan-proteins; CW-AGP1, cell-wall arabinogalactan-protein 1; *f* (in sugar derivatives), furanosyl; GGP, glucuronogalactan-protein; *p* (in sugar derivatives), pyranosyl; PM-AGPs, plasma membrane arabinogalactan-proteins; PM-AGP1, plasma membrane arabinogalactan-protein 1; PM-AGP2, plasma membrane arabinogalactan-protein 2; Rha, rhamnose; *t* (in sugar derivatives), terminal; TFA, trifluoroacetic acid.

1992), sexual development (Pennell and Roberts, 1990; Pennell et al., 1991), and tissue pattern formation (Knox et al., 1989; Schindler et al., 1995). Certain treatments that affect cell expansion or cell division have been observed to alter the expression of AGPs on the cell surface (Schopfer, 1990; Basile and Basile, 1993; Zhu et al., 1993), likewise suggesting a role of AGPs in development. Furthermore, we found that treatment of *Rosa* cells with $(\beta$-D-Glc$)_3$, which specifically binds to AGPs, had a strongly inhibitory but reversible effect on cell proliferation (Serpe and Nothnagel, 1994). Most of the $(\beta$-D-Glc$)_3$ was bound to the cell wall in the division-inhibited cells, although plasma membrane AGPs in *Rosa* also bind $(\beta$-D-Glc$)_3$ (Nothnagel and Lyon, 1986; Komalavilas et al., 1991).

Recently, we investigated the biochemical structure of $(\beta$-D-Glc$)_3$-binding molecules extracted from the cell wall of rose cells (Serpe and Nothnagel, 1995). These molecules were found to have typical AGP characteristics, including protein moieties rich in Hyp and Ala residues and carbohydrate frameworks rich in β-(1,3)-, β-(1,6)-, and β-(1,3,6)-Gal*p* residues. Although sharing these common characteristics, four CW-AGP fractions were distinguished on the basis of differences in charge, size, glycosyl composition, aminoacyl composition, and/or NMR spectrum. A major factor contributing to the heterogeneity of CW-AGPs was the GlcUA-to-Ara ratio, which was several times higher in one of the CW-AGPs than in the other rose CW-AGPs or CM-AGPs.

Information regarding structural characteristics of plasma-membrane-bound AGPs has been limited. Macromolecules with carbohydrate and amino acid compositions characteristic of AGPs have been purified from homogenized tobacco cells by immunoaffinity with a monoclonal antibody directed against the plasma membrane (Norman et al., 1990) or from purified rose plasma membranes by $(\beta$-D-Glc$)_3$-induced precipitation (Komalavilas et al., 1991). These preparations appeared heterogeneous, perhaps containing several AGP-like molecules (Norman et al., 1990; Komalavilas et al., 1991). Other studies involving monoclonal antibodies have also suggested that the plasma membrane contains several AGP-like molecules. Pennell et al. (1989) used MAC 207, a monoclonal antibody recognizing an epitope containing L-Ara and D-GlcUA, to identify a family of about 15 AGP-like molecules in microsomal fractions from carrot cell suspensions. The MAC 207 antibody also recognized four different macromolecules on sugar beet leaf plasma membranes (Pennell et al., 1991).

In the present study we investigated the PM-AGP complement of suspension-cultured rose cells. Our aim was to ascertain the degree of heterogeneity of PM-AGPs and to analyze the biochemical structure of these molecules. Biochemical characterization of PM-AGPs may facilitate further analysis of their possible role in plant development. Moreover, structural comparison of PM-AGPs with CW-AGPs and CM-AGPs would reveal the extent of similarities between these molecules and may suggest specific precursor-product relationships among them. The possibility of a precursor-product relationship between plasma membrane-bound AGPs and soluble AGPs is particularly intriguing in view of the recent use of partial amino acid sequences from five soluble AGPs to obtain corresponding cDNAs (Chen et al., 1994; Du et al., 1994, 1996; Mau et al., 1995). For two of these AGPs, one from the medium of a pear cell-suspension culture (Chen et al., 1994) and another from styles of *Nicotiana alata* (Du et al., 1994), the cDNAs encode polypeptides that include a carboxy-terminal hydrophobic sequence that was predicted to be a transmembrane helix. In neither case, however, was it certain if the carboxy-terminal hydrophobic sequence was present in the mature, soluble AGP.

MATERIALS AND METHODS

Purification of PM-AGPs

Suspension-cultured cells of Paul's Scarlet rose (*Rosa* sp.) were grown as described by Nothnagel and Lyon (1986). Seven days after transfer, cells were collected and thoroughly washed with distilled water on a nylon net with 64-μm openings. All subsequent manipulations were conducted at 0 to 4°C. After washing, the cells were resuspended in a homogenizing buffer (50 mM Tris-HCl [pH 7], 10 mM KCl, 1 mM EDTA, 0.1 mM $MgCl_2$, 8% [w/v] Suc, and 1 mM PMSF), and then broken using a decompression bomb (Parr Instrument, Moline, IL) at 10,000 to 15,000 kPa of N_2 pressure. The homogenate was stirred under vacuum for 10 min and then filtered through a nylon net (64-μm openings). The filtrate was centrifuged at 1,000*g* for 15 min. The pellet was discarded and the supernatant was centrifuged at 40,000*g* for 1 h. The resulting microsomal pellet was resuspended in water containing 5 μg mL^{-1} chymostatin (Sigma). A preparation enriched in plasma membrane vesicles was obtained from the suspension of microsomes by aqueous two-phase partitioning, optimized for rose membranes as described by Komalavilas et al. (1991).

Plasma membrane vesicles were incubated overnight in 1% (w/w) Triton X-100 and then centrifuged at 40,000*g* for 1 h. The resulting pellet was set aside for further analysis. To the supernatant were added $(\beta$-D-Glc$)_3$ and NaCl to final concentrations of 0.08 mg mg^{-1} protein and 1% (w/v), respectively. Following overnight incubation, the mixture was centrifuged at 1,700*g* for 10 min. The resulting pellet was further purified by two cycles of resuspension in water and precipitation in 1% (w/v) NaCl. The final pellet was completely dissolved in DMSO. To each milliliter of this solution in DMSO were added 3 mL of acetone and 80 μL of 1% (w/v) NaCl solution to form a flocculate that was precipitated by centrifugation at 700*g* for 10 min. Addition of acetone alone did not always result in the formation of a flocculate, and this phenomenon was also observed with other AGPs, including gum arabic. Increasing the amount of acetone added to the DMSO resulted in precipitation of $(\beta$-D-Glc$)_3$ and thus could not be used to ensure flocculation of $(\beta$-D-Glc$)_3$-free AGPs. By contrast, the addition of very small amounts of NaCl or other electrolytes resulted in flocculation of AGPs without precipitation of $(\beta$-D-Glc$)_3$. Based on this result, AGPs appear to behave as charged lyophobic colloids in the DMSO:acetone (1:3) mixture. Such colloids are very sensitive to the addition of a small amount

of electrolyte, which allows the charged particles to make a closer approach to each other, thus inducing flocculation (Kemp, 1979). The pellet obtained following centrifugation was similarly redissolved in DMSO and precipitated with acetone and NaCl two more times to remove all but a trace of the (β-D-Glc)$_3$ from the pellet.

Fractionation of PM-AGPs by Anion-Exchange Chromatography

The AGPs obtained from plasma membrane vesicles were fractionated by anion-exchange chromatography on an HPLC system (Serpe and Nothnagel, 1995). The AGP preparation was loaded onto a 1-mL anion-exchange column (Resource Q, Pharmacia LKB) in 20 mM sodium acetate (pH 5.0), and then the column was washed with 5 column volumes of the same buffer. Bound material was eluted with a gradient of sodium acetate (pH 5.0), which increased at a rate of 2.42 mM mL^{-1} to a concentration of 500 mM and then further increased at a rate of 50 mM mL^{-1} to 1000 mM. A flow rate of 0.93 mL min^{-1} was used throughout the elution. Fractions (2 mL) were collected and aliquots were analyzed for carbohydrate by the 2-aminothiophenol fluorometric assay (see below). The fractions were pooled accordingly into five major fractions that were subsequently concentrated and desalted by ultrafiltration with Centricon-30 devices (Amicon, Beverly, MA).

Reverse-Phase Chromatography of PM-AGPs

Reverse-phase chromatography was performed on a 3-mL column (Resource RPC, Pharmacia LKB). After the fractions obtained from the Resource Q column were concentrated and desalted, they were prepared separately in aqueous 0.1% (v/v) TFA and loaded onto the reverse-phase column. The column was washed with 5 column volumes of aqueous 0.1% TFA. Bound material was eluted with a two-stage gradient of 0 to 60% (v/v) acetonitrile in aqueous 0.1% TFA. The acetonitrile concentration was increased at a rate of 0.2% min^{-1} from 0 to 9% and then at 1.13% min^{-1} from 9 to 60%. A flow rate of 1.1 mL min^{-1} was used throughout the elution. Fractions (1.1 mL) were collected, and aliquots were analyzed for carbohydrate by the 2-aminothiophenol fluorometric assay (see below). The fractions were pooled accordingly and then concentrated by evaporation under vacuum at 30°C.

Gel-Permeation Chromatography of PM-AGPs

Gel-permeation chromatography was performed on a Sepharose CL-6B column (116 cm × 0.785 cm^2) equilibrated with a buffer consisting of 0.05 M ammonium acetate (pH 6.9) and 0.02% (w/v) sodium azide. The void and included volumes of the column were determined using 853-kD pullulan (Alltech Associates, Deerfield, IL) and Suc, respectively, as standards. The column was calibrated with pullulan or dextran (Polysciences, Warrington, PA) standards of sizes between 10 and 600 kD (Serpe and Nothnagel, 1995). Aliquots of the concentrated and desalted fractions obtained from the anion-exchange column or aliquots of the polysaccharide standards were prepared separately in 0.02% sodium azide, loaded on the gel-permeation column, and eluted with the ammonium acetate/sodium azide buffer at a flow rate of 6 mL h^{-1}. Fractions (1.0 mL) were collected and aliquots were analyzed for carbohydrate by the 2-aminothiophenol fluorometric assay (see below).

Estimation of Total Carbohydrate

Total carbohydrate was estimated by a modification of the fluorometric procedure described by Zhu and Nothnagel (1991). In brief, 2-aminothiophenol (Aldrich) was first mixed with an equal volume of 100% ethanol. An aliquot of this mixture was then added to 36% (w/v) H_2SO_4 to obtain a concentration of 0.02% (v/v) 2-aminothiophenol in the H_2SO_4 solution. This solution was then diluted with water to obtain a stock solution containing 0.0093% (v/v) 2-aminothiophenol in 17% (w/v) H_2SO_4. When stored in a dark bottle at room temperature, this stock solution gave consistent results for at least 3 months. In a 4-mL glass vial, 1.5 mL of this stock solution was combined with an aliquot (0.5 mL, or diluted with water to 0.5 mL) of the solution of carbohydrate to be measured. The vial was tightly closed with a screw cap having a Teflon-faced liner and then incubated in a dry block heater set at 150°C. After heating for 1 h, the vial was cooled to room temperature, and fluorescence was measured as previously described (Zhu and Nothnagel, 1991). This modified method gives results comparable to those obtained by Zhu and Nothnagel (1991) but it is simpler and more reproducible. In particular, the modified method affords better solubility and stability than the use of an intermediate solution of 2-aminothiophenol in diluted HCl, as described by Zhu and Nothnagel (1991).

Electrophoretic Methods

One-dimensional SDS-PAGE was conducted according to Laemmli (1970). Gels were silver-stained for both protein and carbohydrate by using a slight modification of the method described by Tsai and Frasch (1982).

Determination of Glycosyl Composition

Glycosyl composition was determined by GLC of the trimethylsilyl derivatives of the methyl glycosides (Komalavilas et al., 1991). The glycosyl composition of AGPs was also determined by high-performance anion-exchange chromatography with pulsed amperometric detection following methanolysis and TFA hydrolysis (Lampio and Finne, 1991; De Ruiter et al., 1992).

Analysis of Glycosyl Linkage Composition

Dry samples (approximately 200 μg) of PM-AGPs were processed for methylation and GLC-MS analysis by the procedures described for CW-AGPs (Serpe and Nothnagel, 1995).

Analysis of AGPs by NMR

Samples (0.5–1.0 mg) of PM-AGPs were prepared in deuterium oxide, and ^{1}H-NMR spectra were recorded with a 500-MHz NMR spectrometer (General Electric) as described for CW-AGPs (Serpe and Nothnagel, 1995). The identities of the peaks were assigned by comparison with published spectra (Cavagna et al., 1984; Cartier et al., 1987; Komalavilas et al., 1991; Agrawal, 1992; Saulnier et al., 1992; Gane et al., 1995).

Analysis of Aminoacyl Composition

Amino acid analysis was performed as described for CW-AGPs (Serpe and Nothnagel, 1995).

RESULTS

After incubation of plasma membrane vesicles in 1% (w/w) Triton X-100 and subsequent centrifugation, the total membrane protein was approximately equally distributed between the pellet and the supernatant. As judged by rocket electrophoresis in (β-D-Glc)$_3$-containing gels, AGPs were present only in the supernatant (not shown). Purification of the AGPs from this supernatant resulted in an overall yield of 15 to 30% of the total amount of AGPs on the plasma membrane, an amount that was previously estimated to be about 6.7% (w/w) AGPs per total plasma membrane protein (Komalavilas et al., 1991). The crossed-electrophoresis pattern (not shown) of the total AGP fraction purified from the plasma membrane vesicles was similar to the pattern observed with Triton X-100-solubilized plasma membrane vesicles (Komalavilas et al., 1991). This result suggests that the total PM-AGPs recovered were not a subpopulation of PM-AGPs but rather were representative of the whole PM-AGP population.

The molecules (total PM-AGPs) purified from the Triton X-100 extract of plasma membrane vesicles by (β-D-Glc)$_3$ precipitation were loaded onto an anion-exchange column. From this column PM-AGPs eluted in two major peaks and one minor peak (Fig. 1). The first peak eluted between 50 and 140 mM sodium acetate (fraction 1) and accounted for 21% (w/w) of the carbohydrate recovered from the column. The second peak eluted at 200 to 350 mM sodium acetate (fraction 2) and accounted for 50% of the carbohydrate recovered from the column. The minor peak eluted at more than 500 mM sodium acetate (fraction 3) and accounted for 7.6% of the carbohydrate recovered. The remainder of the carbohydrate recovered from the column eluted between these three fractions.

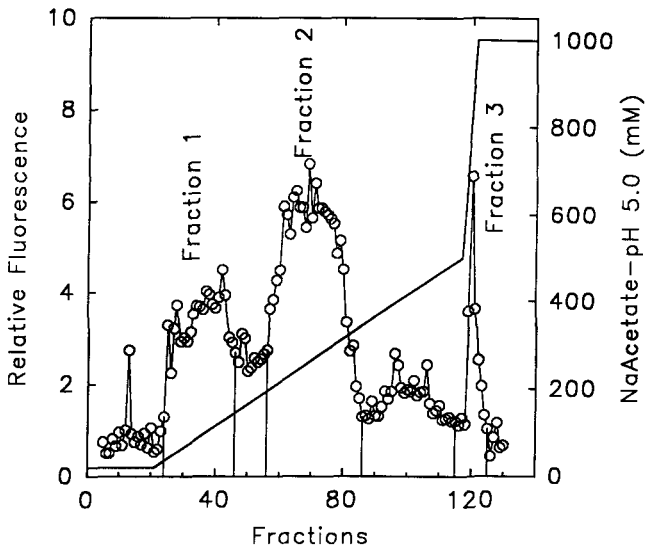

Figure 1. Fractionation of total PM-AGPs by anion-exchange chromatography. Fractions were analyzed for carbohydrate by the 2-aminothiophenol fluorometric assay and pooled as indicated.

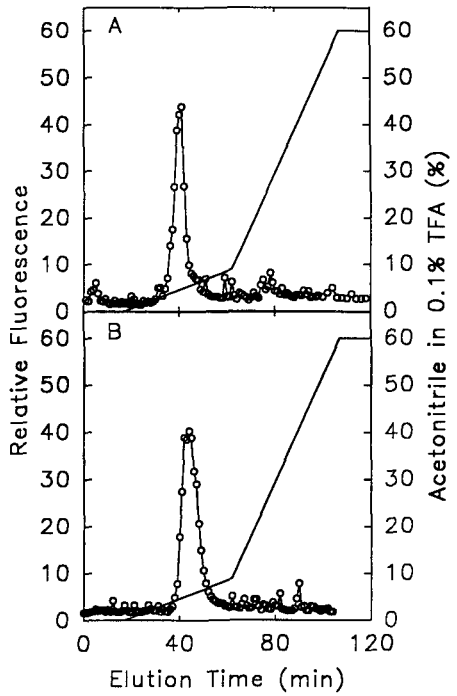

Figure 2. Reverse-phase chromatography of PM-AGPs previously separated by anion-exchange chromatography. A, Pooled fraction 1 from Figure 1. B, Pooled fraction 2 from Figure 1. Fractions were analyzed for carbohydrate by the 2-aminothiophenol fluorometric assay.

Further fractionation of the major PM-AGP fractions 1 and 2 was attempted by reverse-phase chromatography. When fractions 1 and 2 from the anion-exchange column were separately loaded onto the reverse-phase column, they eluted as single peaks at 5.1% (v/v) acetonitrile (Fig. 2A, retention time 40.5 min) and 5.8% acetonitrile (Fig. 2B, retention time 44 min), respectively. Although fractions 1 and 2 eluted separately as single peaks from the reverse-phase column, total PM-AGPs (not previously fractionated by anion-exchange chromatography) eluted from this column in one major peak and two minor peaks (Fig. 3). The major peak eluted at about 5.5% acetonitrile (retention time 42.5 min) and contained 73% of the carbohydrate recovered from the column. The minor peaks eluted at 27.2% acetonitrile (retention time 84 min) and 34.6% acetonitrile (retention time 90.5 min) and contained 15.7 and 4.8% of the carbohydrate recovered, respectively. As judged by reten-

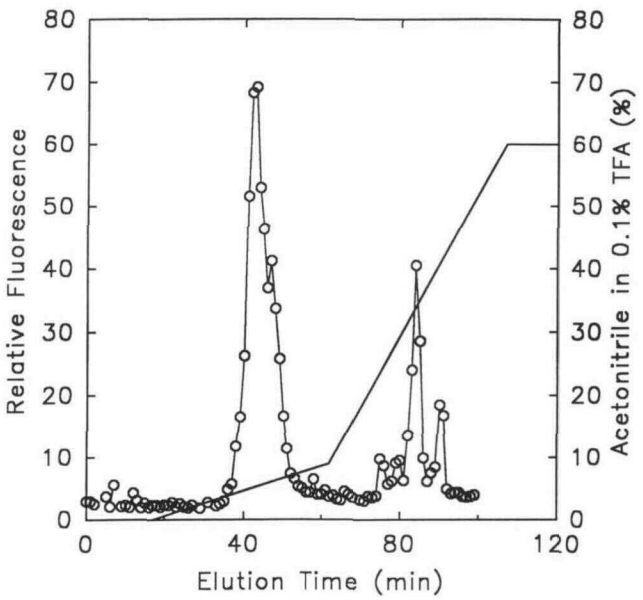

Figure 3. Fractionation of total PM-AGPs by reverse-phase chromatography. Fractions were analyzed for carbohydrate by the 2-aminothiophenol fluorometric assay.

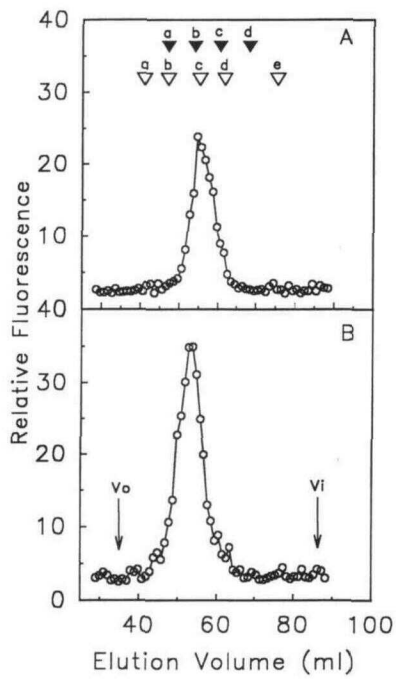

Figure 4. Gel-permeation chromatography of PM-AGPs previously separated by anion-exchange chromatography. A, Pooled fraction 1 from Figure 1. B, Pooled fraction 2 from Figure 1. Fractions were analyzed for carbohydrate by the 2-aminothiophenol fluorometric assay. The void (Vo) and included (Vi) volumes of the column are marked. Arrowheads indicate positions of elution of molecular mass standards (pullulans and dextrans). For pullulans (▽), a, b, c, d, and e indicate 380, 180, 100, 48, and 12 kD, respectively. For dextrans (▼), a, b, c, and d indicate 600, 175, 75, and 40 kD, respectively.

tion times, the major peak in Figure 3 appeared to contain fractions 1 and 2 (Figs. 1 and 2). A similarity between fraction 3 from the anion-exchange chromatography (Fig. 1) and either or both of the two minor peaks in Figure 3 remained possible but uncertain.

Fractions 1, 2, and 3 were also analyzed by gel-permeation chromatography. When fraction 1 was passed through the Sepharose CL-6B column, a single peak eluted at a volume corresponding to a molecular mass of 77 or 140 kD, depending on whether it was compared with pullulan or dextran standards, respectively (Fig. 4A). Similarly, fraction 2 eluted as a single, symmetrical peak at a volume corresponding to a molecular mass of 108 or 217 kD, depending on whether it was compared with pullulan or dextran standards, respectively (Fig. 4B). By contrast, fraction 3 eluted as a broad, asymmetrical peak, which was indicative of heterogeneity in this material (not shown).

Using SDS-PAGE, fractions 1 and 2 migrated as broad bands with apparent molecular masses between 122 and 220 kD and between 115 and 187 kD, respectively, compared with protein standards (Fig. 5). These molecular mass estimates were not consistent with those obtained by gel-permeation chromatography. Fraction 1 appeared to be of higher average molecular mass than fraction 2. With gel-permeation chromatography, however, the molecular mass of fraction 1 was lower than that of fraction 2 (Fig. 4). These dissimilar results might be due to an inherent electrical charge difference between fractions 1 and 2. As judged by their order of elution from the anion-exchange column (Fig. 1), fraction 1 was less negatively charged than fraction 2. If the negative charges acquired through binding of SDS to the AGPs were not sufficient to overwhelm this inherent charge difference, then fraction 2 could have migrated faster than fraction 1 in an electric field, even though fraction 2 was of higher molecular mass than fraction 1.

Analysis by SDS-PAGE showed that fraction 3 (Fig. 1) contained molecules with electrophoretic mobilities distinct from those observed for fractions 1 and 2 (Fig. 5). Fraction 3 separated into two major bands of apparent molecular masses between 176 and 253 kD and between 96 and 127 kD. In addition, a dark-staining band appeared at

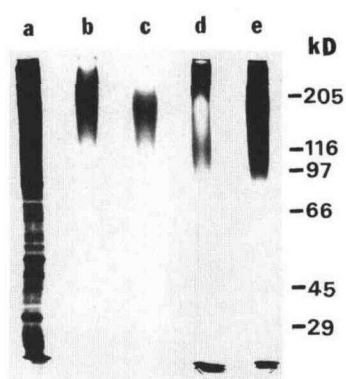

Figure 5. Analysis of PM-AGPs by SDS-PAGE. Samples were fractionated on 7% (w/v) polyacrylamide gels. Lane a, Total plasma membrane protein (3 μg). Lanes b, c, and d correspond to fractions 1 (30 μg), 2 (40 μg), and 3 (11 μg) from Figure 1. Lane e, Total PM-AGPs (40 μg). The gel was silver-stained for protein and carbohydrate. Protein molecular mass standards are indicated in kD on the right.

the dye front. It seemed possible that the two major electrophoretic bands might correspond to the two minor peaks that eluted late (retention times 84 and 90.5 min) in the reverse-phase chromatography of total PM-AGPs (Fig. 3). Thus, these two minor peaks were collected separately and analyzed by SDS-PAGE. The result (not shown) was that both the 84- and the 90.5-min peaks contained components with electrophoretic mobilities similar to those of the two major bands in Figure 5, lane d. Although this result suggests the existence of a similarity between fraction 3 (Fig. 1) and the 84- and 90.5-min peaks (Fig. 3), the heterogeneity of these fractions prevented a clear identification of that similarity.

Fractions 1 and 2 (Fig. 1) contained molecules with sugar compositions that were characteristic of AGPs (Table I). Both fractions had high levels of Gal and Ara and minor levels of GlcUA, Rha, and other sugars. The glycosyl compositions of fraction 1 and 2 were similar except for small differences in abundances. Fraction 2 had higher levels of GlcUA, 4-*O*-methyl GlcUA, and Rha, whereas fraction 1 had a higher level of Glc. The analysis of fraction 2 directly after anion-exchange chromatography revealed a low level (5.4 mol%) of GalUA. The composition of fraction 2 as reported in Table I was obtained after reverse-phase chromatography (Fig. 2B) because this step removed the contaminating GalUA.

Although electrophoresis (Fig. 5) clearly showed that fraction 3 (Fig. 1) was heterogeneous, it seemed worthwhile to determine the glycosyl composition of this mixture. Fraction 3 contained typical AGP sugar residues such as GlcUA, Gal, and Ara, but the abundances of these sugars were notably different from those found in fractions 1 and 2 (Table I). For example, the abundance of total GlcUA (GlcUA plus 4-*O*-methyl GlcUA) in fraction 3 was 2.8 and 4 times higher than in fractions 2 and 1, respectively. Conversely, the abundance of Gal in fraction 3 was less than one-half of that in either fraction 1 or 2. Another unusual characteristic of fraction 3 was its high content of GalUA. Compositional analyses of materials from the 84- and 90.5-min peaks in Figure 3, however, revealed high levels of Gal and GlcUA but no GalUA (results not shown). The small amount of fraction 3 that we were able to obtain, together with its considerable heterogeneity, discouraged us from further investigation of this material.

Table I. *Mol% of glycosyl residues in PM-AGPs*

Fraction numbering corresponds to the pooled fractions shown in Figure 1. Data presented here were obtained by GLC of the trimethylsilyl derivatives of the methyl glycosides. Similar results were obtained by high-performance anion-exchange chromatography with pulsed amperometric detection following methanolysis and TFA hydrolysis (data not shown). Data presented for fraction 2 were obtained after reverse-phase chromatography (Fig. 2B).

Sugar	Fraction 1	Fraction 2	Fraction 3
Ara	24.6	25.7	18.7
Rha	4.1	7.7	3.9
Fuc	0.0	0.0	0.0
Xyl	1.2	1.3	0.0
GlcUA	5.7	7.9	24.5
GalUA	0.0	0.0	17.1
4-*O*-Methyl GlcUA	0.9	1.6	1.7
Man	0.5	0.5	8.2
Gal	56.5	54.1	22.1
Glc	6.7	1.1	3.8
GalN[a]	0.0	0.0	0.0
GlcN[b]	0.0	0.0	0.0

[a] GalN, Galactosamine. [b] GlcN, Glucosamine.

Table II. *Mol% of aminoacyl residues in PM-AGP1 and PM-AGP2*

Amino Acid	PM-AGP1	PM-AGP2
Asx[a]	7.8	3.5
Hyp	20.0	32.2
Glx[b]	4.9	3.5
Ser	11.4	13.4
Gly	7.2	3.7
His	0.9	0.4
Arg	1.0	0.3
Thr	5.4	11.6
Ala	21.6	18.8
Pro	2.8	2.2
Tyr	0.3	0.2
Val	5.8	3.5
Met	0.6	0.6
Ile	1.6	1.0
Leu	4.8	1.7
Phe	0.9	0.6
Lys	2.1	2.7
Cys	0.7	ND[c]
Trp	ND	ND

[a] Asx, Asn or Asp. [b] Glx, Gln or Glu. [c] ND, Not determined.

Further biochemical analyses were performed with the more abundant and homogeneous fractions 1 and 2, which were now designated PM-AGP1 and PM-AGP2, respectively. As estimated by analyses of carbohydrate and amino acid compositions, the protein proportion in PM-AGP1 was 3.7% (w/w), whereas the protein proportion in PM-AGP2 was much higher at 15%. The most abundant amino acids in both PM-AGPs were Hyp, Ala, and Ser (Table II). The abundance of Hyp, however, was 1.6 times greater in PM-AGP2 than in PM-AGP1. The fourth most abundant amino acid in PM-AGP2 was Thr, which was 2.1 times more abundant than in PM-AGP1.

Methylation analysis showed that PM-AGP1 and PM-AGP2 contained glycosyl linkages that were characteristic of AGPs (Table III). Both PM-AGPs were rich in β-(1,3)-, β-(1,6)-, and especially β-(1,3,6)-Gal*p* residues. Thus, both AGPs were highly branched molecules. The methylation analysis likewise showed that Ara residues were predominantly present in *t*-Ara*f* form, although some (1,3)-Ara*f* residues were also present (Table III). The Rha residues were detected only in *t*-Rha*f* form, whereas GlcUA residues were found in both the *t*-Glc*p*UA and (1,4)-Glc*p*UA forms.

The ^{1}H-NMR spectra of PM-AGP1 and PM-AGP2 showed strong signals for H-1 of α-Ara and β-Gal residues (Fig. 6, A and B), indicating that these anomeric configurations were dominant in PM-AGPs, as they were in the CM-AGPs (Komalavilas et al., 1991) and CW-AGPs (Serpe and Nothnagel, 1995) of rose. The spectra for PM-AGP1

Table III. *Glycosyl linkage compositions of PM-AGP1 and PM-AGP2*

Glycosyl Linkage	ecr[a] Response Factor	PM-AGP1	PM-AGP2
		relative mol%	
t-Ara*f*	0.60	15.9	26.7
3-Ara*f*	0.66	4.5	4.0
t-Rha*p*	0.61	3.7	8.2
t-Gal*p*	0.70	6.9	5.7
3-Gal*p*	0.74	10.2	5.6
6-Gal*p*	0.75	7.1	10.2
3,6-Gal*p*	0.80	41.3	26.0
t-Glc*p*UA	0.75	4.0	3.2
4-Glc*p*UA	0.80	6.5	10.4

[a] ecr, Equivalent carbon response (Sweet et al., 1975), used to correct for differential responses of various linkages in flame ionization detection.

and PM-AGP2 were very similar except for the relative intensities of certain signals. Consistent with the relative abundances of Rha residues in the two AGPs (Table I), the signal for the methyl hydrogens of Rha residues was larger in PM-AGP2 than in PM-AGP1 (Fig. 6, A and B). Similarly, the signal at 3.30 parts per million, which was assigned to the methyl hydrogens of 4-*O*-methyl GlcUA (Cavagna et al., 1984), was larger in PM-AGP2 than in PM-AGP1. The spectra revealed no evidence of acetylated sugars in either PM-AGP1 or PM-AGP2, leaving GGP as the only rose proteoglycan thus far found to contain acetylated sugars (Serpe and Nothnagel, 1995).

DISCUSSION

The plasma membrane of rose cells contains several macromolecules that display typical AGP characteristics. These macromolecules have a binding affinity for $(\beta$-D-Glc$)_3$ and a carbohydrate composition that is rich in Gal, Ara, and GlcUA residues (Table I). Moreover, the two most abundant PM-AGP fractions, PM-AGP1 and PM-AGP2, contained the α-*t*-Ara*f* residues and β-(1–3)-, β-(1,6)-, and β-(1,3,6)-Gal*p* residues (Table III) that form the characteristic framework of type II arabinogalactans (Aspinall, 1973). Both PM-AGP1 and PM-AGP2 contained *t*- and (1,4)-Glc*p*UA residues, which are found in many but not all AGPs (Clarke et al., 1979; Bacic et al., 1987; Gane et al., 1995). Also, the protein moieties of PM-AGP1 and PM-AGP2 were rich in Hyp, Ala, and Ser (Table II), which are abundant amino acids in most AGPs (Anderson et al., 1977; Clarke et al., 1979; Gleeson et al., 1989; Komalavilas et al., 1991).

The two major PM-AGP fractions purified from rose plasma membrane could be distinguished from each other by several features. Based on their elution profile in anion-exchange chromatography (Fig. 1), PM-AGP1 was less negatively charged than PM-AGP2 at pH 5.0. Gel-permeation chromatography showed that PM-AGP1 was smaller than PM-AGP2. The respective molecular masses of PM-AGP1 and PM-AGP2 were 140 and 217 kD, as estimated from dextran standards, which, given the highly branched nature of these AGPs, probably provided more accurate estimates than pullulan standards. The protein proportions of PM-AGP1 and PM-AGP2, 3.7 and 15% (w/w), respectively, also distinguished the molecules, as did their aminoacyl compositions. The abundances of Hyp and Thr were considerably higher in PM-AGP2 than in PM-AGP1. Taken together, the four amino acids Hyp, Ala, Ser, and Thr accounted for 76% of the amino acids in PM-AGP2 and 58% of the amino acids in PM-AGP1 (Table II).

Although PM-AGP1 and PM-AGP2 were similar in their proportions of total Gal and total Ara residues (Table I), dissimilarities were also evident in the finer details of their carbohydrate components. The levels of GlcUA, 4-*O*-methyl GlcUA, and Rha were higher in PM-AGP2 than in PM-AGP1. Another important distinction was the ratio of total-terminal to total-branched sugars. Based on the residues listed in Table III (Glc, Xyl, and Man were also present [Table I] but were not identified in the methylation analysis), these ratios were 0.74 and 1.68 for PM-AGP1 and PM-AGP2, respectively. This difference suggests that fewer but more highly branched polysaccharide chains originated from the polypeptide backbone of PM-AGP1 than

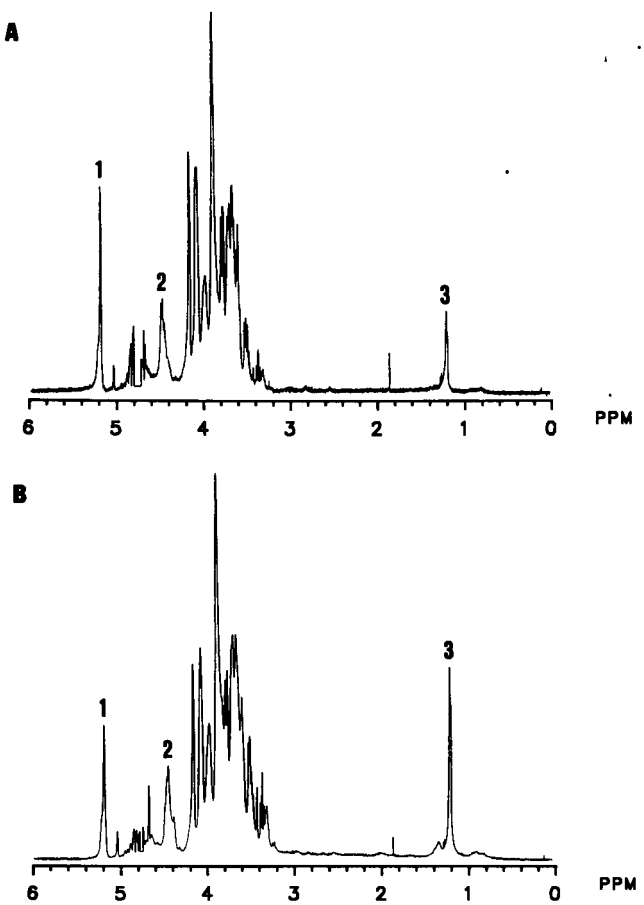

Figure 6. The ^{1}H-NMR spectra of PM-AGPs. A, PM-AGP1. B, PM-AGP2. Peaks were identified as follows: 1, H-1 of α-Ara residues; 2, H-1 of β-Gal residues; 3, CH_3 of Rha residues. Signals in the range of 3.2 to 4.2 parts per million (PPM) were due primarily to ring ^{1}H resonances of Gal, Ara, and GlcUA residues. The signal at 1.86 parts per million was due to residual acetate in the samples.

from the polypeptide backbone of PM-AGP2 (Fincher et al., 1983; Qi et al., 1991). The extent to which this difference causes dissimilarities in the shapes of these molecules might be investigated by biophysical methods such as transmission electron microscopy (Qi et al., 1991; Baldwin et al., 1993) or by Fourier transform IR polarized microspectrometry (Baldwin et al., 1993).

The structural characteristics of PM-AGP1 were similar to the previously characterized CW-AGP1 (Serpe and Nothnagel, 1995) and especially CM-AGPb (Komalavilas et al., 1991) of rose. As judged by gel-permeation chromatography, PM-AGP1, CM-AGPb, and CW-AGP1 had similar molecular masses (140-, 141-, and 130-kD, respectively, compared with dextran standards). These three AGPs also showed analogous native-electrophoretic mobilities in agarose gels (not shown) and similar migration patterns on SDS-PAGE (Fig. 5; cf. Komalavilas et al., 1991; Serpe and Nothnagel, 1995). Furthermore, the protein proportions, glycosyl compositions, and aminoacyl compositions of PM-AGP1 were similar to those of CM-AGPb and CW-AGP1 (Tables I and II; cf. Komalavilas et al., 1991; Serpe and Nothnagel, 1995). Some contrast, especially for CW-AGP1, was evident among the extents of branching in the carbohydrate portions of these molecules. The proportions of β-(1,3,6)-Gal*p* residues that were expressed relative to all sugar residues present were approximately 36, 33, and 25 mol% for PM-AGP1, CM-AGPb, and CW-AGP1, respectively. Based on multifaceted structural similarities, however, a viable hypothesis is that PM-AGP1 is released from the plasma membrane to give rise to CM-AGPb and possibly CW-AGP1.

Several structural differences were evident between PM-AGP2 and the CW-AGPs or CM-AGPs of rose. For example, PM-AGP2 was readily distinguishable from GGP, one of the CW-AGPs (Serpe and Nothnagel, 1995), on the bases of differences in electrical charges, glycosyl compositions, and aminoacyl compositions. Similarly, PM-AGP2 was distinguishable from CW-AGP1, CM-AGPa, and CM-AGPb on the bases of differences in molecular mass, protein proportions, and aminoacyl compositions (Fig. 4; Tables I and II; cf. Komalavilas et al., 1991; Serpe and Nothnagel, 1995). The molecular masses of PM-AGP2, CW-AGP1, CM-AGPa, and CM-AGPb were 217, 130, 109, and 141 kD, respectively, compared with dextran standards. The protein proportions of PM-AGP2, CW-AGP1, CM-AGPa, and CM-AGPb were 15, 4.5, 3.6, and 5.6% (w/w), respectively. At 32.2 mol% (Table II), the abundance of Hyp in PM-AGP2 was from 1.35 to 2.71 times higher than in any of the other rose AGPs characterized so far (Komalavilas et al., 1991; Serpe and Nothnagel, 1995).

In addition to PM-AGP1 and PM-AGP2, the plasma membrane components that precipitated with $(\beta\text{-D-Glc})_3$ included minor fractions that eluted late in the anion-exchange (Fig. 1) and reverse-phase (Fig. 3) chromatographies. The precipitability of these materials with $(\beta\text{-D-Glc})_3$ and the abundances of Gal, Ara, and GlcUA residues (Table I, fraction 3) strongly argued that AGPs were present. Several analyses (Figs. 3 and 5, and native electrophoresis in agarose gels [not shown]), however, clearly demonstrated heterogeneity in these minor fractions. Electrophoresis of fraction 3, for example, demonstrated the presence of at least two macromolecules (Fig. 5). The band nearest to the top of the gel might have contained a molecule similar to GGP. This material and GGP had similar electrophoretic mobilities in SDS-PAGE and similar elution characteristics in anion-exchange chromatography (Figs. 1 and 5; cf. Serpe and Nothnagel, 1995). Furthermore, for comparable amounts of carbohydrate, silver-staining was more intense in fraction 3 than in the other AGP fractions (Fig. 5). A similar trend was observed with GGP (Serpe and Nothnagel, 1995).

Other characteristics of fraction 3 remained puzzling. An unusually high proportion of GalUA residues (17.1 mol%; Table I) was present, as was a silver-staining component that migrated at the dye front in SDS-PAGE (Fig. 5). Whether this fast-electrophoresing band contained short uronides or some other small, anionic molecules remains unknown. Although we were unable to prepare enough of these various minor components to enable fuller characterization, the available data indicate the presence of at least two minor PM-AGPs in addition to the two major PM-AGPs, PM-AGP1 and PM-AGP2. Thus, it appears that the rose plasma membrane contains at least four distinct AGPs.

The mechanism by which PM-AGP1 and PM-AGP2 associate with the plasma membrane is not fully understood. Compared with rose CM-AGPs (Komalavilas et al., 1991) and CW-AGPs (Serpe and Nothnagel, 1995), neither PM-AGP1 nor PM-AGP2 contained a greater abundance of hydrophobic amino-acyl residues (Table II), which would suggest a greater likelihood of the presence of a membrane-embedded protein domain. Furthermore, both PM-AGP1 and PM-AGP2 eluted from the reverse-phase column (Fig. 2) at low acetonitrile concentrations (5–6%), which were comparable to the acetonitrile concentrations that eluted nonmembrane-bound AGPs, such as rose CW-AGP1 (retention time 40.5 min) and a secreted AGP from pear (Chen et al., 1994). Thus, based on their behavior on reverse-phase chromatography in 0.1% (v/v) TFA, PM-AGP1 and PM-AGP2 did not seem more hydrophobic than CW-AGPs or CM-AGPs.

Greater hydrophobicities were evident for the two minor peaks (elution times 84 and 90.5 min) in the reverse-phase chromatogram of total PM-AGPs (Fig. 3). These two peaks eluted late in the gradient at markedly higher acetonitrile concentrations than did PM-AGP1 and PM-AGP2. The greater hydrophobicities of these minor PM-AGPs indicate the potential for a tighter interaction with the hydrophobic portion of the membrane, although hydrophobicity that is apparent in 0.1% (v/v) TFA may disappear at physiological pH. Although these results point to variation in hydrophobicity among rose PM-AGPs, other investigators have used the technique of phase partitioning in Triton X-114 to obtain evidence of interspecies differences in PM-AGP hydrophobicities. In particular, rice PM-AGPs have recently been reported to be more hydrophobic than PM-AGPs from carrot (Smallwood et al., 1996), *Nicotiana* (Norman et al., 1990), and sugar beet (Pennell et al., 1991). Differences

in hydrophobicities among PM-AGPs may indicate different mechanisms of association with the plasma membrane.

Variation in mechanism of membrane association is not without precedent among plasma membrane-bound proteoglycans. Three types of association between heparan sulfates and animal plasma membranes have been characterized and include noncovalent binding of the heparan sulfate to another membrane component, intercalation of a hydrophobic domain of a heparan sulfate core protein into the lipid bilayer, and intercalation of a heparan sulfate-linked glycosylphosphatidylinositol anchor into the lipid bilayer (Yanagishita and Hascall, 1992). In the latter type the heparan sulfate core protein is synthesized with a carboxy-terminal hydrophobic sequence that initially anchors the protein in the ER membrane but is then cleaved off and replaced by a glycosylphosphatidylinositol anchor before departure from the ER (David, 1993).

Since cDNAs for two AGPs have been shown to encode carboxy-terminal hydrophobic sequences that may or may not be present in the core proteins of the mature AGPs (Chen et al., 1994; Du et al., 1994), the question arises whether any PM-AGPs are linked to glycosylphosphatidylinositol or to other lipid anchors. The ^{1}H-NMR spectrum of PM-AGP2 (Fig. 6B) is interesting in this regard because it contained a small peak centered at 1.33 parts per million near the much larger 1.19-parts-per-million band that was due to methyl hydrogens of Rha residues. The position of the 1.33-parts-per-million peak was within the range expected for signals from hydrocarbon chains, such as in palmitic acid (Sadtler Research Laboratories, 1967, 1974), and the intensity of the peak corresponded to approximately 1.7 hydrocarbon chains per PM-AGP2 molecule. Alternatively, the 1.33-parts-per-million peak might have arisen from methyl hydrogens in side chains of amino acids such as Thr (Sadtler Research Laboratories, 1970), although resonances due to amino-acyl residues have generally not been observed in AGPs because restricted mobility in the inner regions of AGP molecules causes severe broadening and flattening of NMR signals (Saulnier et al., 1992; Gane et al., 1995). Additional studies are needed to determine if lipid anchors are present on any PM-AGPs.

It has been suggested that in membrane-associated AGPs the protein portion is larger than the carbohydrate portion (Chasan, 1994). The results in rose PM-AGPs, however, do not support such a notion. The protein content of total rose PM-AGPs was 10% (w/w) (Komalavilas et al., 1991), and the protein contents of PM-AGP1 and PM-AGP2 were 3.7 and 15% (w/w), respectively. Consequently, as with AGPs from the cell wall and the culture medium, rose PM-AGPs are molecules with a high ratio of carbohydrate to protein. The protein contents of PM-AGPs from other species, however, may be higher. For example, in *Nicotiana glutinosa* the protein content of a PM-AGP fraction was between 27 and 37% (Norman et al., 1990).

The results of this and previous studies (Komalavilas et al., 1991; Serpe and Nothnagel, 1994, 1995) reveal significant AGP heterogeneity on the surface of rose cells in suspension culture. Structural similarities between some of these AGPs, such as between PM-AGP1, CM-AGPb, and CW-AGP1, suggest a possible precursor-product relationship between these molecules. In contrast, other cell-surface AGPs, such as PM-AGP2 and GGP, are found predominantly at a particular subcellular location. The structural details that determine retention of certain AGPs on the plasma membrane or in the cell wall are still uncertain. Attachment of AGPs to the plasma membrane through lipid anchors, through hydrophobic protein domains, or through other membrane proteins all remain viable hypotheses. Localization at the cell surface may also involve differences in molecular size and electrical charge. Secreted AGPs are smaller and less negatively charged than PM-AGP2 or GGP, which were not detected in the culture medium. A better understanding of the mechanisms responsible for the localization of AGPs on the cell surface may come from the identification of AGP-binding molecules in the plasma membrane or the cell wall.

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