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The Role of Carbohydrate in Maintaining Extensin in an Extended Conformation[1]

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ABSTRACT

Monomers of the plant cell wall glycoprotein extensin are secreted into the wall where they become cross-linked to each other to form a rigid matrix. Expression of the extensin matrix is correlated with the inhibition of further cell elongation during normal development, with increased resistance to virulent pathogens and with other physiological responses characterized by wall strengthening. Carbohydrates make up about two-thirds of the mass of extensin. Arabinose oligomers linked to hydroxyproline residues represent 95% of the total carbohydrate with the remainder occurring as single residues of galactose linked to some serine residues. Electron microscopy of shadowed extensin shows the glycosylated form to be an easily visualized and highly elongated molecule. In contrast, extensin that has been deglycosylated with anhydrous hydrogen fluoride is difficult to resolve in the EM. Glycosylated extensin elutes from a gel filtration column much more rapidly than does the deglycosylated form, and from this analysis we have calculated respective Stokes' radii of 89 and 11 Ångstroms for these molecules. Others have shown that inhibition of extensin glycosylation has no effect on its secretion or insolubilization in the cell wall, but that this extensin cannot inhibit cell elongation. It is likely that carbohydrate moieties keep extensin in an extended conformation and that extensin must be in this conformation to form a cross-linked matrix that can function properly *in vivo*.

Extensin, a HRGP,[3] is a major structural component of the primary cell wall of dicots (16, 19). Extensin monomers are secreted into the wall and 'insolubilized' there, presumably by the formation of covalent isodityrosine (IDT) cross-links between molecules (see Ref. 25 for references). The cross-linked extensin matrix is rigid and highly insoluble, and its major function appears to be to strengthen cell walls. The presence of extensin in the wall makes cells resistant to lysis in hypotonic medium (12), inhibits further elongation growth in pea epicotyl sections and root tips (6, 21, 28), renders cells more resistant to virulent pathogens (10, 11), and appears to play a role in the morphogenesis of hepatophytes (2). Carbohydrate constitutes about two-thirds of the glycoprotein mass, most of which is arabinose linked to hydroxyproline as tetra-arabinosides (13, 27). A detailed linkage analysis of extensin arabinosides has been published (1). Single residues of galactose are linked to some serine residues (4, 17).

Molecular models of extensin demonstrate that tetra-arabinosides may be able to hydrogen bond to the protein backbone and thereby stabilize it in an extended polyproline II conformation (15). It is known from circular dichroism spectroscopy that the entire backbone of glycosylated extensin is in this conformation *versus* 50% for the deglycosylated form (27). *In vivo*, proline hydroxylation and subsequent arabinosylation can be inhibited with the ferrous ion chelator α,α'-dipyridyl (5). Under these conditions, extensin is still secreted and insolubilized in the wall (23), but it is no longer capable of inhibiting cell elongation in pea epicotyl sections and root tips (21, 28). These observations suggest that carbohydrate moieties contribute to the structure of extensin and that this structure is important to its normal function. We showed in the preceding report that glycosylated extensin is a highly elongated molecule when examined in the electron microscope, and discussed how substructures representing intramolecular and intermolecular isodityrosine cross-links might affect the structure of the entire extensin matrix (25). We also presented preliminary evidence for two distinct extensin-like HRGPs from carrot which we refer to as extensin-1 and extensin-2 (24, 25). All of the experiments described here concern extensin-1, which has been characterized in detail at both the protein and nucleic acid levels (3, 25, 27). We have used EM together with gel filtration chromatography to examine how carbohydrates affect the structure of individual extensin-1 molecules.

MATERIALS AND METHODS

Most of the methods used for this work are as described in the preceding report (25), including: purification and characterization of extensin-1, gel electrophoresis, EM, gel filtration chromatography, and protein and carbohydrate assays.

Extensin-1 was deglycosylated with anhydrous HF by Van Holst and Varner's (27) modification of the method of Mort and Lamport (20). Less than 1.0 mg of purified protein was lyophilized in a plastic microfuge tube and then further dried overnight under reduced pressure in the presence of P_2O_5. The dried extensin was treated with 30 μl of dry methanol and 270 μl anhydrous HF in pyridine (Aldrich) for 90 min at room temperature. The reaction was quenched with 1.0 ml of water and the HF was removed by dialysis.

Stokes' radii were determined by gel filtration chromatography. A standard curve was drawn by plotting the Stokes' radii of standard proteins against a function of their elution volumes ($[\text{Log } K_{av}]^{1/2}$, where $K_{av} = [V_e - V_o]/[V_t - V_o]$) (22). Stokes' radii for these proteins (fibrinogen, thyroglobulin, immunoglobulin G, ovalbumin, and Cyt *c*) were calculated by the Stokes-Einstein equation,

$$\text{Stokes' radius (cm)} = kT/6\pi\eta D,$$

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[3] Abbreviations: HRGP, hydroxyproline-rich glycoprotein; HF, anhydrous hydrogen fluoride; pp II helix, polyproline II helix.

using published values for the diffusion coefficient (D) of each (9).

RESULTS

Extensin-1 was purified from aerated carrot phloem parenchyma slices as described in the previous paper (25) and deglycosylated with anhydrous HF. Deglycosylation is quantitatve and yields molecules of noticeably smaller size on an acid-urea gel (Fig. 1). Although there are no reliable size markers for this gel system, we are able to estimate the apparent mol wt of deglycosylated extensin-1 to be 30 kD based on its migration relative to glycosylated monomers (86 kD), dimers (172 kD), and trimers (258 kD). This value is consistent with a mol wt of about 33 kD for the protein backbone of extensin-1 based on its amino acid composition as derived from a genomic clone (3). Alditol acetate analysis indicates that glycosylated extensin-1 contains 95% arabinose and 5% galactose (25). HF removes all of the arabinose and about half of the galactose, so less than 3% of the original carbohydrate remains.

Molecules of glycosylated extensin-1 that have been shadowed with platinum are easily visualized in the electron microscope and are characterized by distinct substructural features such as kinks and cross-links (Fig. 2a; Ref. 25). In sharp contrast with this result, deglycosylated molecules are difficult to resolve by this technique and lack these substructural features (Fig. 2b); they can be distinguished from samples prepared from glycerol alone, however (Fig. 2c). To assure that we were photographing fields that contained molecules, all micrographs were taken in grid squares that contained a particular type of dirt spot which results when glycerol droplets dry (26) and were found to always contain glycosylated extensin molecules (25).

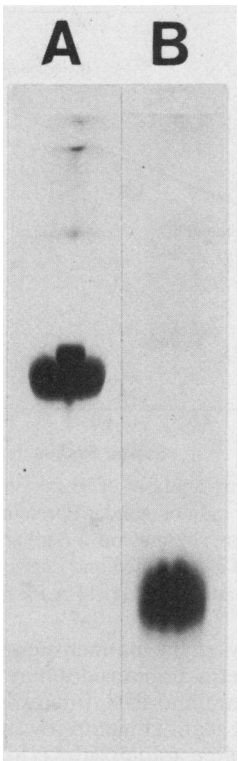

FIG. 1. Acid-urea PAGE of glycosylated and deglycosylated extensin-1. A, Preparation of purified glycoprotein containing predominantly monomers (about 86 kD) and much smaller amounts of oligomers; B, treatment of the glycoprotein with anhydrous HF in pyridine removes nearly all of its sugars and decreases its mol wt to about 30 kD (R_F values were determined using the relative migration of glycosylated monomers, dimers, and trimers as standards). The gel contained 6% acrylamide and was stained with silver.

Chromatography of glycosylated and deglycosylated extensin-1 on Sephacryl S-400 shows that the glycosylated form elutes much sooner than the other (Fig. 3). Elution from a gel filtration column has been shown to be a function of molecular size or Stokes' radius; Stokes' radius will be proportional to mol wt for globular proteins but not for highly elongated fibrous proteins (22). The glycoprotein elutes just after two proteins of very high mol wt and large Stokes' radius, fibrinogen, and thyroglobulin, which suggests that it too has a very large Stokes' radius, despite a mol wt of only 86 kD. Deglycosylated extensin-1 (30 kD) elutes according to its mol wt, that is, between ovalbumin (45 kD) and Cyt c (17 kD), which suggests that it may be globular or in the form of a random coil. The Stokes' radius for each form of extensin-1 was calculated by comparing its elution volume to those of proteins of known Stokes' radius (Fig. 4). The Stokes' radius of glycoprotein monomers is 89 Å and that of the deglycosylated form is 11 Å.

DISCUSSION

Validity of the Electron Microscopic Images. Deglycosylation of extensin-1 with anhydrous HF decreases its mol wt by a factor of two-thirds (Fig. 1), yet the structural differences that accompany this modification are disproportionately greater. Van Holst and Varner (27) have shown that about 50% of the protein backbone of deglycosylated extensin is in the polyproline II conformation compared to 100% for the glycoprotein. Since deglycosylated molecules are so similar to glycosylated ones by this criterion, it is surprising that the two forms are so drastically different when assayed by EM (Fig. 2). It is possible that the polyproline II conformation is maintained over the repeated Ser-Hyp_4 domains, which are fairly evenly spaced along the molecule and represent nearly half of the total amino acids (3), but not over the sequences that span them. Despite a large number of short linear domains, the invisibility of deglycosylated extensin suggests that the molecule as a whole is not elongated. It is also possible, however, that stripping the molecule of its carbohydrate renders it too small or thin to be resolved by our techniques. To distinguish between these possibilities, we determined the molecular size of each form of extensin by gel filtration chromatography (Figs. 3 and 4). This analysis indicates that the glycoprotein is an extended rod with a Stokes' radius of 89 Å, and that deglycosylated extensin-1 is in the form of a random coil with a Stokes' radius of only 11 Å. Thus, the electron micrographs appear to provide a true representation of the structural configurations of these molecules in solution.

Stabilization of the Polyproline II Helix by Oligosaccharide Sidechains. Lamport (15) has shown by molecular model building that hydroxyproline arabinosides (especially trimers and tetramers) should be able to form hydrogen bonds to a polyhydroxyproline backbone that is in the polyproline II helical conformation. The importance of carbohydrate in constraining such a protein backbone in a pp II helix is moot, however, since polyhydroxyproline alone can assume this conformation spontaneously. Similarly, 50% of the backbone of deglycosylated extensin, presumably in the hydroxyproline-rich domains, is also organized into a pp II helix (27). Thus, the more important interactions are probably those which occur between arabinose oligomers and the regions which span the hydroxyproline-rich domains, thereby constraining these domains, and in turn the entire molecule, in an extended pp II helix. Significantly, hydroxyl groups on arabinose residues three and four are shown to be capable of forming hydrogen bonds with carbonyl groups of the protein backbone (15), suggesting that the stabilization provided by these bonds is independent of amino acid sequence.

A tentative model for the interaction of arabinosides with the

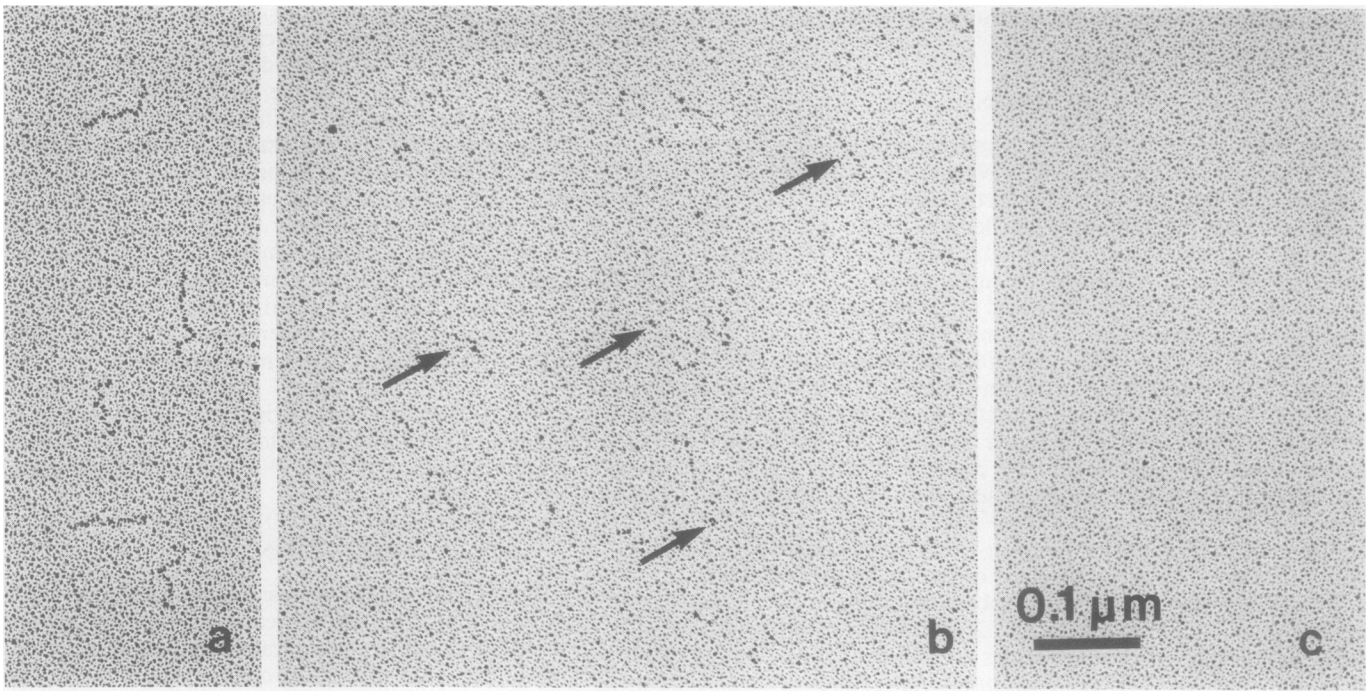

FIG. 2. Electron micrographs of fields of extensin-1 molecules. a, Glycosylated molecules are distinct, rod-like structures which may show substructural features such as kinks and cross-links; b, deglycosylated molecules are difficult to discern as discrete entities, yet may be globular or somewhat elongated (arrows); c, preparations containing glycerol alone show a homogeneous background and do not contain structures that resemble either glycosylated or deglycosylated extensin-1 (×140,000).

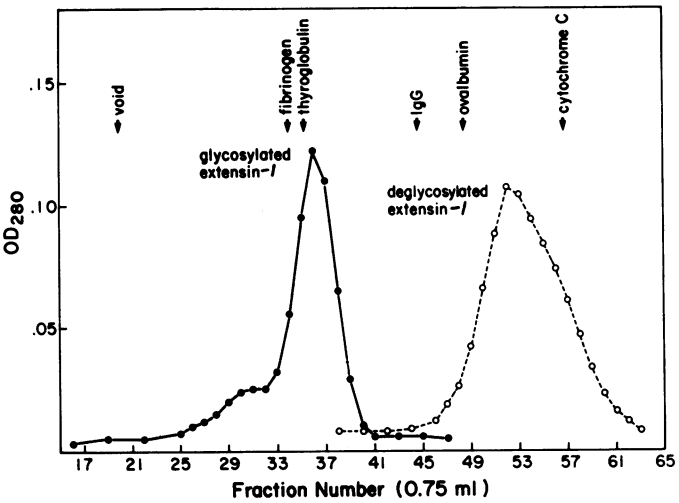

FIG. 3. Chromatography of glycosylated and deglycosylated extensin-1 on a Sephacryl S-400 gel filtration column. Glycosylated monomers elute far in advance of deglycosylated molecules. A faster eluting shoulder is enriched in glycosylated oligomers. The elution positions of standard proteins also are shown. The column (1.1 × 48 cm) was equilibrated in 150 mM Tris-HCl (pH 8.0) and protein was eluted at 9.0 ml/h.

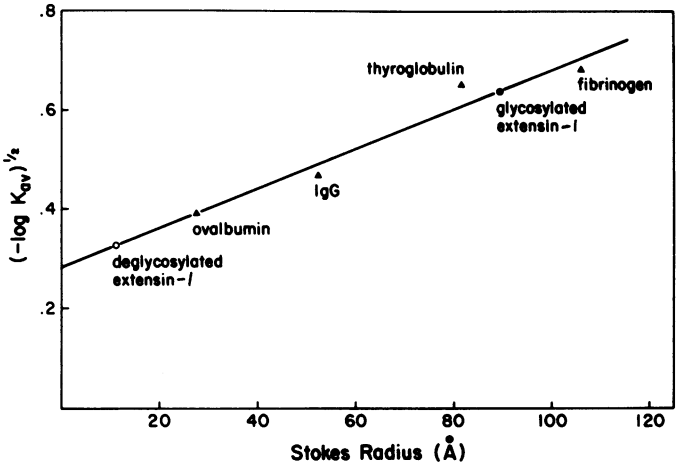

FIG. 4. Stokes' radius analysis of glycosylated and deglycosylated extensin-1. The Stokes' radii of standard proteins were plotted against a function of their elution volumes on a Sephacryl S-400 column (22). The elution volumes of glycosylated and deglycosylated extensin-1 suggest respective Stokes' radii of 89 and 11 Å for these molecules.

protein backbone of extensin to maintain it in an extended conformation is presented in Figure 5. This model accounts for the Stokes' radius and electron microscopic data we have presented here for glycosylated and deglycosylated extensin-1 molecules, and for the pp II data of Van Holst and Varner (27). Nearly all prolines in extensin-1 are hydroxylated (25, 27), and nearly all of these hydroxyls are substituted with tri- or tetra-arabinosides (in a ratio of about 1:3) (27). Ser-Hyp_4 sequences account for 125 of 274 residues in extensin-1 (3), so these domains are spanned by an average of six residues. These spanning domains could be stabilized in a pp II by hydrogen bonding to arabinose oligomers in the manner suggested by Lamport (15). It seems likely that tetra-arabinosides may provide longer range stabilization than triarabinosides. Interestingly, there is an evolutionary trend in the plant kingdom toward increasing amounts of longer hydroxyproline arabinosides (18).

Our model ignores or generalizes certain data for the sake of simplicity. For example, the actual number of spanning residues varies from 2 to 15. A region of the protein backbone which contained neither Ser-Hyp_4 domains nor carbohydrates might be expected to form bends readily. However, the distribution of kinks in extensin-1 monomers (25) does not coincide with the four long spanning domains of 13 to 15 residues (3). It is quite possible, therefore, that hydroxyprolines in these regions are

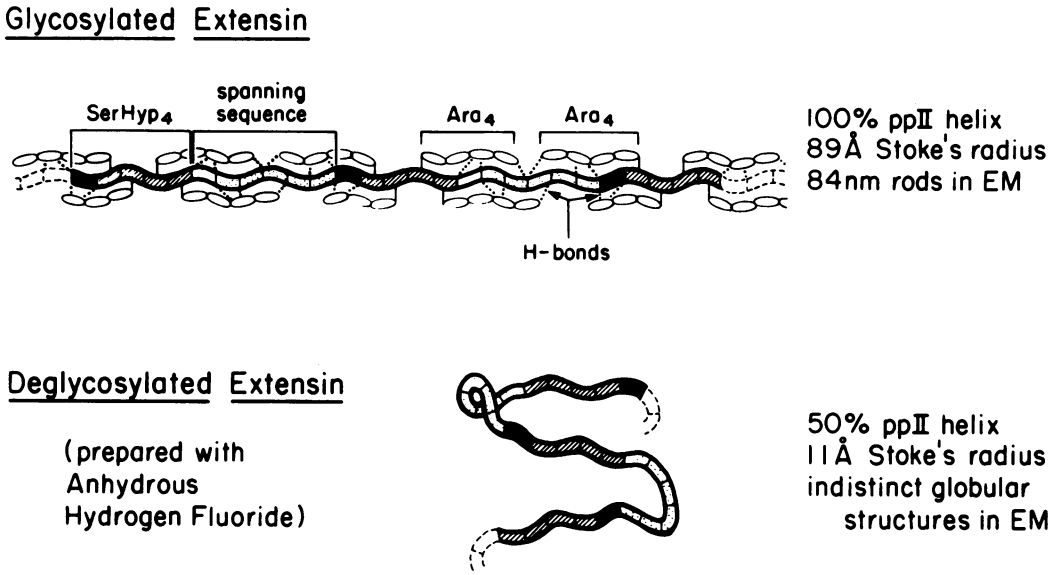

FIG. 5. Model depicting the role of hydroxyproline-linked arabinosides in maintaining extensin in an elongated conformation. The model accounts for the Stokes' radius and electron microscopic data for glycosylated and deglycosylated extensin-1 molecules presented in this report and the pp II data of Van Holst and Varner (27). See text for further details.

glycosylated and that these arabinosides effect local stabilization of the protein backbone. In addition, we have no evidence for arabinosides being directed toward both amino and carboxy termini. Other configurations could be envisioned, but they would be more difficult to render in two dimensions. Galactose residues attached to serine also could affect extensin structure, but since they represent only about 5% of the total carbohydrate and are not removed efficiently by HF, we have not considered them here.

Importance of Glycosylation for Extensin Function. The major function of extensin-like HRGPs appears to be to strengthen cell walls through the formation of a rigid, cross-linked matrix (2, 6, 10–12, 21, 28). Additional experimental evidence suggests that extensins must be glycosylated to perform these functions. Hydroxylation of proline and subsequent arabinosylation can be inhibited *in vivo* with α,α'-dipyridyl, a ferrous ion chelator (5). Under such conditions, extensin is still secreted and covalently bound to the cell wall (23), but the cross-linked matrix that it forms can no longer inhibit cell elongation in pea epicotyl sections or root tips (21, 28). The importance of carboyhdrate in the normal function of HRGPs also is seen in the development of the leafy liverworts. Basile (2) has noted that dipyridyl promotes the formation of adventitious ventral leaves on these plants, and has suggested that these phenovariants arise because the normal inhibitory function of wall-bound HRGPs has been abolished. The proline analog 3,4-dehydroproline is a potent and specific inhibitor of proline hydroxylation and subsequent glycosylation (8). This reagent inhibits *Chlamydomonas* cells of opposite mating types from agglutinating, presumably because the hydroxyproline-rich agglutinin, which is a highly elongated molecule, must be glycosylated to function properly (7). L-Hydroxyproline is a relatively nonspecific inhibitor of proline hydroxylation, but nonetheless, plants treated with it are susceptible to pathogen attack where they otherwise would have been resistant (10).

Our gel filtration and electron microscopic data show that carbohydrate is essential for maintaining extensin-1 in an extended conformation. We must now ask whether the noted correlations between glycosylation and extensin function are due to glycosylation *per se* (*e.g.* the interaction of sugar moieties with other cell wall polysaccharides or perhaps the protection of the backbone from proteolysis; 4, 14), or due to the structural rigidity that carbohydrates confer on these molecules. We demonstrated in the preceding paper that intermolecular cross-links commonly involve the ends of glycosylated extensin-1 molecules, that the two ends of a molecule are separated by about 84 nm and that a matrix formed of such molecules would be relatively open or porous (25). Taken together these data suggest that cross-linking sites must be widely separated to form a matrix that functions properly *in vivo*, and that molecules must be fully glycosylated (*i.e.* elongated) to achieve this separation. Pea epicotyl sections and root tips treated with dipyridyl continue to elongate despite the accumulation and cross-linking of extensin in their walls (21, 28). The extensin matrix in these walls might be formed from molecules cross-linked in a standard pattern (*i.e.* involving the ends of molecules; 25), but might be collapsed or improperly located in the wall. It would be interesting to examine the structure of the matrix formed by each type of extensin, but it is evident that deglycosylated extensin cannot be resolved in the electron microscope (Fig. 2) and that the glycosylated extensin matrix is too complicated to analyze by available electron microscopic techniques (*cf.* Fig. 6l in Ref. 25). Alternatively, localization of each type of molecular in intact cell walls with specific antibodies could provide some clues about how they actually function. We have raised antisera that are quite specific for each form of extensin-1, but so far, we have succeeded at labeling epoxy-embedded thin sections only with antibodies directed against the glycosylated form (24).

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