A Second Extensin-Like Hydroxyproline-Rich Glycoprotein from Carrot Cell Walls'

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ABSTRACT

The insoluble extensin matrix of dicot cell walls has been studied most fruitfully by examining the salt-extractable precursors to this matrix. Multiple extensin-like hydroxyproline-rich glycoproteins (HRGPs) have been isolated, or their existence inferred, from tomato, potato, bean, soybean, melon, carrot, and other plants. We and others previously have studied a carrot extensin which we call extensin-1. Here we report on the properties of extensin-2, a second salt-extractable hydroxyproline-rich glycoprotein from carrot. Like extensin-1, extensin-2 contains large amounts of hydroxyproline, serine, histidine, and lysine. In contrast, its tyrosine content is only about one-third that of extensin-1. Arabinose and galactose are the most abundant neutral sugars in both proteins, and nearly identical buoyant densities in CsCl suggest a similar proportion of carbohydrate in each. The size of extensin-2 is about half the size of extensin-1 based on: (a) the measured lengths of shadowed molecules (about 40 versus 84 nanometers); (b) the migration of extensin-2 in acid-urea gels relative to monomers, dimers, and trimers of extensin-1; and (c) the Stokes' radii of these molecules as determined by gel filtration chromatography. Electron microscopy of shadowed extensin-2 molecules indicates that they contain kinks, which may indicate the presence of intramolecular isodityrosine cross-links, but intermolecular cross-links, either with other extensin-2 molecules or extensin-1 molecules, are observed rarely if ever.

Recent evidence indicates that dicot cell walls probably contain a family of related HRGPs3 that previously have been lumped together under the single name 'extensin' (for reviews see Refs. 21, 25, 32). Common features of these proteins include a large amount of Hyp, which generally occurs together with Ser in the characteristic sequence Ser-Hyp, as well as considerable amounts of Tyr, Lys, His, and Val. Carbohydrate constitutes greater than half of the mass of the glycoprotein, with Ara representing about 90% of the sugar.

Tomato suspension culture cells were reported to contain two precursors to the cross-linked extensin matrix, called P1 and P2, that have similar but distinct amino acid and carbohydrate compositions; these proteins were isolated in equimolar amounts and were insolubilized in the wall with identical kinetics (26).

Improved separation techniques and subsequent analysis indicated that P1 was actually comprised of two highly related proteins, Pla and Plb (27). Peptide sequencing of these three proteins has confirmed the presence of unique sequences in each, and a similar analysis of tryptic fragments of the insoluble extensin matrix has suggested the presence of a fourth protein, P3, which has not been isolated in soluble form (27). Thus, tomato cell walls appear to contain at least four distinct extensin-like proteins.

Two cDNAs have been synthesized from wounded carrot tissue that encode distinct proline-rich in vitro translation products (7), but only one of these appears to contain the sequence Ser-Pro γ (8). A tomato genomic clone, which was isolated using the carrot probe and contains Ser-Pro γ coding domains, was shown to hybridize to at least three mRNAs on Northern blots from pathogen-infected bean plants (24). Bolwell and co-workers (2, 3), who also were studying pathogen infected bean plants, have found that there are at least two proteins made under these conditions that are rich in hydroxyproline and arabinose.

A single extensin-like HRGP from the cell walls of aerated carrot root slices has been isolated and described (8, 9, 30, 31). We now refer to this protein as "extensin-2" to distinguish it from "extensin-1," a similar but distinct protein from carrot (28). In this paper we describe extensin-2 more fully.

MATERIALS AND METHODS

Purification of Extensin-2. Chrispeels and co-workers (9) pioneered the use of aerated carrot root slices for isolating large quantities of extensin. Most of the methods used to isolate and characterize Ext-2 are identical to ones described previously for Ext-1 (28, 29). Extensin was extracted with 0.2 M CaCl₂ from carrot root slices that had been aerated for 3 d. The salt extract was concentrated by ultrafiltration and contaminating proteins were precipitated with 10% TCA. The supernatant was dialyzed to remove TCA and then lyophilized to yield the crude extensin fraction.

Crude extensin was chromatographed on CM-cellulose in 25 mM Tris-HCl (pH 8.0) to give a major peak at 25 mM NaCl, which contained nearly pure Ext-1, and a second peak at 90 mM NaCl, which contained Ext-2 and other proteins. Chromatography on Sephacryl S-400 in 150 mM Tris-HCl (pH 8.0) was used to separate Ext-2 from these proteins. Purification was monitored by SDS-PAGE (16).

Analysis of Extensin-2. Amino acid analysis of purified Ext-2 was performed for us by G. Cassab and J. Varner (Washington University, St. Louis) as described (6). Neutral sugar composition was determined from alditol acetate derivatives with a 10 m SP-2330 column on a Hewlett-Packard 5880 gas chromatograph (20). Electron microscopy of shadowed molecules and Stokes' radius analysis using gel filtration chromatography were performed as described (28, 29).

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3 Abbreviations: HRGP, hydroxyproline-rich glycoprotein; AGP, arabino-galactan protein; Ext-1, extensin-1; Ext-2, extensin-2; pp II helix, polyproline II helix; CM, carboxymethyl.
The buoyant densities of Ext-1 and Ext-2 were determined by isopycnic centrifugation in CsCl. Purified protein was added to a solution of CsCl in 25 mM Tris-HCl (pH 8.0) to give a final density of 1.45 g/cm³. Gradients were formed by centrifugation in a Beckman SW 50.1 rotor for 49 h at 40,000 rpm (150,000g) at 10°C. The gradients were separated into 6 drop fractions by pumping through a capillary from the bottom of the tubes. The fractions were analyzed for density by refractive index and for protein by A at 280 nm.

**Peroxidase Assays.** Fractions were assayed for peroxidase activity in a mixture containing 50 mM Na-acetate (pH 5.2), 0.5 mM o-dianisidine and 15 mM CaCl₂, to which 5 mM H₂O₂ was added to begin the reaction (D Church, personal communication). A calibrated chart recorder was used to measure A at 460 nm. One unit of activity was defined as 1.0 A₄₆₀/min over the linear range of the curve (large amounts of product inhibited further reaction). Specific activity is listed as units/µg of protein.

The crude extensin fraction was assayed further for peroxidase activity by non-denaturing PAGE. The gel contained 3 to 12% acrylamide in a buffer consisting of 0.38 M acetic acid and 0.06 M KOH (pH 4.3). A 3% well-forming gel in the same buffer was added after the resolving gel polymerized. The reservoir buffer contained 0.014 M acetic acid and 0.035 M β-alanine. A 6X sample buffer contained 30 ml glycerol, 0.9 ml 4 N KOH, 1.29 ml acetic acid and 0.04 g methyl green per 50 ml. The gel was run at 4°C. toward the cathode at constant voltage for 3 h at 100 V and 4 h at 200 V. The gel was stained for peroxidase in the same solution used for the soluble assay. The reaction was stopped by several water washes.

**RESULTS**

**Purification of Ext-2.** The TCA-soluble fraction of salt-extractable proteins from carrot cell walls (crude extensin) contained a characteristic set of proteins when analyzed by SDS-PAGE (Fig. 1, lane A). Ext-1 remained in the stacking gel or just barely entered the resolving gel, and Ext-2, which is a minor component of this fraction and is hardly detectable, migrated with an apparent mol wt of about 145,000. The two extensins could be separated by chromatography of crude extensin on CM-cellulose (28). The major peak from this column eluted at 25 mM NaCl and, based on SDS-PAGE, was nearly homogeneous for Ext-1 (lane B). A smaller peak eluted at 90 mM NaCl and contained Ext-2 and several low mol wt proteins (lane C). The Ext-2 peak contained a small amount of Ext-1, which was eliminated by a second round of chromatography on CM-cellulose (not shown).

Extensin-2 was separated from the contaminating low mol wt proteins by gel filtration chromatography on Sephacryl S-400 (Fig. 2). Two peaks were resolved on this column. The faster eluting peak appeared to contain only Ext-2 (Fig. 1, lane D) and the slower one the low mol wt proteins (lane E). We consider Ext-2 isolated in this manner to be operationally pure. It should be noted that there is no evidence for a faster eluting shoulder on the Ext-2 peak from this column. The presence of such a shoulder was used as partial evidence for the existence of cross-linked oligomers of Ext-1 (28).

The extensins migrated in an acid-urea PAGE system as distinct bands, even though other proteins were not well resolved (see Fig. 3 in Ref. 28). The migration of Ext-2 in this gel system was compared to that of monomers, dimers and trimers of Ext-1 (86, 172, and 258 kD, respectively). Analysis of Rₜ values from three different gels suggested an apparent mol wt of 40,400, slightly less than half that of Ext-1 monomers.

**Biochemical Properties of Extensin-2.** The amino acid compositions of Ext-2 and Ext-1 are shown in Table I. Hyp is the most abundant amino acid in both proteins even though Ext-2 contains only about 60% of the mol % Hyp found in Ext-1. Ser is equally abundant in each, but Ext-2 contains a greater propor-
Fig. 2. Protein from the Ext-2 peak of a CM-cellulose column (twice purified) was chromatographed on Sephacyr S-400. The peak at 3.9 h contained pure Ext-2. The peak at 5.0 h contained the low molecular mass proteins (42 kD). The column measured 1.1 x 48 cm, and protein was eluted at 9.0 ml/h in 150 mM Tris-HCl (pH 8.0). Blue dextran and Cyt c were used as markers for the void volume and included volume, respectively.

Table I. Amino Acid Composition of Carrot Ext-2 and Ext-1

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Ext-2 Residues/Molecule a</th>
<th>Ext-1 b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyp</td>
<td>mol %</td>
<td>mol %</td>
</tr>
<tr>
<td>Asx</td>
<td>27.5</td>
<td>35.8</td>
</tr>
<tr>
<td>Thr</td>
<td>2.9</td>
<td>3.8</td>
</tr>
<tr>
<td>Ser</td>
<td>4.1</td>
<td>5.4</td>
</tr>
<tr>
<td>Gly</td>
<td>16.9</td>
<td>22.0</td>
</tr>
<tr>
<td>Pro</td>
<td>6.0</td>
<td>7.8</td>
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</tr>
<tr>
<td>Asx</td>
<td>3.4</td>
<td>4.4</td>
</tr>
<tr>
<td>Cys</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Val</td>
<td>3.7</td>
<td>4.9</td>
</tr>
<tr>
<td>Met</td>
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<td>0.0</td>
</tr>
<tr>
<td>Ile</td>
<td>1.0</td>
<td>1.3</td>
</tr>
<tr>
<td>Leu</td>
<td>1.2</td>
<td>1.6</td>
</tr>
<tr>
<td>Tyr</td>
<td>3.9</td>
<td>5.1</td>
</tr>
<tr>
<td>Phe</td>
<td>10.5</td>
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<tr>
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<tr>
<td>Trp</td>
<td>ND a</td>
<td>ND</td>
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</tbody>
</table>

*a Assuming 130 residues per molecule. See "Discussion." b From Stafstrom and Staelelin (28). c Less than 0.05 mol %. d Not determined.

glycoproteins is a function of their carbohydrate content (30), we conclude that Ext-2 is also comprised of about 65% carbohydrate by weight.

Alditol acetate analysis was performed on protein from the Ext-2 peak of a CM-cellulose column (run twice) before it was known that this fraction contained proteins other than Ext-2 (Fig. 1). The neutral sugar composition of this fraction is: 87.3% Ara, 8.2% Gal, 3.5% Glu, 0.8% Man, and 0.3% Xyl. Since extensins are known to contain large proportions of Ara and lesser amounts of Gal, this profile is probably fairly representative of Ext-2. The highly glycosylated AGPs are not a likely source of contamination since they are acidic and elute in the void volume of a CM-cellulose column. The neutral sugar composition of this latter fraction is 66.3% Gal, 24.3% Ara, plus small amounts of Rha, Man, Glu and Xyl, which is consistent with a high content of AGPs (14).

Analytical gel filtration chromatography on Sephacyr S-400 was used previously to determine the Stokes' radius of Ext-1 to be 89 Å (29). Analysis of Ext-2 by the same method suggested a Stokes' radius of 70 Å (data not shown). Ext-1 and Ext-2 cannot be separated by this method. The two proteins elute in the same peak, although Ext-2 occurs only in the fractions on the trailing side of the peak (not shown).

Peroxidase Assays. Several of the fractions described in Figure 1 were assayed for peroxidase activity (Table II). The specific activity of the crude exten fragment (Fig. 1, lane A) is vastly greater than partially purified Ext-1 (lane B) or Ext-2 (lane C), or the low mol wt contaminants of the Ext-2 peak (lane E). The crude exten fragment was further purified for peroxidase activity by affinity chromatography on a non-denaturing cationic gel system (Fig. 4). At least six distinct peroxidase-positive bands were revealed by this technique. Most of these bands do not have distinct silver-stained counterparts. A two-dimensional gel electrophoresis technique was used to confirm the identities of Ext-1 and Ext-2 (not shown).

Electron Microscopy. Ext-2 molecules were rotary shadowed with platinum and examined in the electron microscope (Fig. 5A). These molecules are highly elongated and measure 40.7 ± 2.9 nm (n = 12), which is just less than half the length of Ext-1 molecules (84.4 ± 7.5 nm) (28). Distinct oligomers of Ext-2 were not detected, although suggestive interactions were seen occasionally. Some molecules appeared to contain kinks.

Protein samples isolated from the Ext-2 peak of a CM-cellulose column (Fig. 1C) occasionally contained structures with a large central granule and a variable number of radiating or entangled exten-like molecules (Fig. 5, B and C). The elongated molecules in these structures appear to be too long to be Ext-2 and therefore may be Ext-1. The central granule may be composed of one or more of the low mol wt proteins in this fraction. The protein molecules found in the void fraction of the ion exchange column (putative AGPs) are shown in Figure 5D. They are broader, shorter and more irregular than either of the extensins. One structure in this field resembles a free central granule.
DISCUSSION

Purification and Properties of Extensin-2. Multiple extensin-like proteins have been isolated, or their existence inferred, from a number of dicot species (25). We have described here a second extensin-like HRGP isolated from cell walls of aerated (wounded) carrot root slices. We call this protein extensin-2 to highlight its structural and biochemical similarities to extensin-1 but demonstrate that it is clearly a different protein.

The only salt-extractable cell wall proteins that are found exclusively in a TCA-soluble fraction are the extensins. These proteins can be separated from each other by two rounds of ion exchange chromatography (28). Ext-2 is separated from several low mol wt contaminants, and purified to apparent homogeneity, by subsequent gel filtration chromatography (Figs. 1 and 2). Ext-2 purified in this manner has a buoyant density in CsCl nearly the same as that of Ext-1 (Fig. 3), which suggests that each contains about 65% carbohydrate by weight (31). Furthermore, both proteins are especially rich in arabinose.

Additional comparison of the two proteins accentuates their dissimilarity. Hyp is the most abundant amino acid in each, but Ext-2 contains about 28 mol % of this amino acid versus 47 mol % in Ext-1 (Table I). Although reduced, the Hyp content in Ext-2 is still far greater than that in hydroxyproline-poor glycoproteins from wounded bean leaves (4, 5). In fact, the Hyp content of Ext-1 and Ext-2 are approximately within the range found in HRGPs from other species (25). Whether Hyp in Ext-2 occurs in the characteristic sequence Ser-Hyp, is not known. Soluble HRGPs are typically rich in Ser and the basic amino acids Lys and His. These amino acids constitute 36.5% of the total in Ext-2, which is similar to but slightly greater than other soluble HRGPs. A striking difference between Ext-2 and these proteins, including Ext-1, is a reduced proportion of Tyr (3.9 mol % versus an average of 9.8 mol %). Since tyrosine is presumed to be involved in intra- and intermolecular cross-linking through the formation of isodityrosine residues (12, 15), the reduced tyrosine content in Ext-2 may affect this property (see below). The relative abundance of most other amino acids also varies between the two extensins, but it would be more risky to speculate on the relevance of these differences.

One approach for approximating the mol wt of Ext-2 utilizes its migration in acid-urea gels relative to monomers, dimers, and trimers of Ext-1 (see Fig. 3 in Ref. 28). Based on analysis of several such gels, the total mol wt of the Ext-2 is suggested to be 40,400. Since Ext-2 contains about 65% carbohydrate (Fig. 3), its protein mol wt is about 14,100. A protein of this mass and the amino acid composition of Ext-2 (Table I; 10.96 kD/100 residues) should contain about 129 amino acid residues. A caveat of this method is the unproven assumption that migration in this gel system is proportional to mol wt. A second approach is to divide the length measured in the EM by the axial repeat distance between amino acids residues in a pp II helix (0.312 nm/residue) (18). This method works well for Ext-1, which has a measured length of 84.4 nm and a predicted length of 85.5 nm based on 274 amino acid residues (28). Ext-2 measured 40.7 nm and would therefore be expected to contain about 130 residues. Although Ext-2 is a highly elongated molecule (Fig. 5A), it has not been demonstrated that its protein backbone occurs in a pp II helical conformation. Nonetheless, the fact that we arrive at nearly identical values based on completely different assumptions suggests that Ext-2 may indeed contain about 130 amino acid residues.

Analytical gel filtration chromatography revealed that glycosylated Ext-1 has a Stokes’ radius of 89 Å, which was further evidence that this 86 kD protein was highly asymmetric (29). An identical analysis of Ext-2 suggested a Stokes’ radius of 70 Å (not shown). This data confirms and extends the electron microscopy evidence that Ext-2 is also highly asymmetric (Fig. 5A). It has been suggested that these hydroxyproline-linked arabinosides hydrogen bond to the protein backbone of Ext-1 to maintain it in an extended pp II conformation (18, 29, 31). It is quite possible that carbohydrate on Ext-2 serves the same function.

Chen and Varner (7) synthesized a second cDNA from wounded carrot tissue that did not correspond to extensin (Ext-1). Although it hybrid-selected message for a 33 kD proline-rich in vitro translation product, sequencing showed that Pro occurred in the sequence Pro-Pro-Val instead that Ser-Pro-Val, was absent. We feel that this cDNA does not correspond to Ext-2 either. First, the protein mol wt of Ext-2 is only 14,100, although processing could reduce the size of the primary translation product. And second, the partial amino acid content corresponding to this cDNA is not consistent with that of Ext-2 (Table I).

Possible Functions of Ext-2. Synthesis of extensin precursors...
and their cross-linking in the cell wall has been correlated with: protection of cells from lysis in hypotonic medium (17); inhibition of further cell elongation during normal development (23); protection from pathogen attack (13); and regulation of morphogenesis (1). We previously have considered how different positions and proportions of intra- and intermolecular cross-links in Ext-1 might affect these wall strengthening functions (28). Our present information suggests that Ext-1 and Ext-2 are expressed coordinately, but differential expression of more than one extensin-like HGRP also might affect the properties of the cross-linked matrix.

Oligomers of Ext-1, which can be detected by acid-urea PAGE, EM, and gel filtration chromatography, are probably transient intermediates between secreted monomers and the fully cross-linked extensin matrix (28). We were unable to detect distinct oligomers of Ext-2 either directly by electron microscopy (Fig. 5A) or indirectly by the presence of a faster-eluting shoulder on a gel filtration column (Fig. 2). Furthermore, in our analysis of Ext-1 oligomers (28) we did not observe structures that appeared to be hybrids between the two extensins. Thus it is not clear whether Ext-2 is covalently cross-linked in the cell wall. The lower proportion of tyrosine in Ext-2 (Table I) is consistent with

FIG. 5. Electron microscopy of rotary shadowed protein molecules. A, Purified Ext-2 molecules are elongated rods which measure 40.7 ± 2.7 nm. Distinct bends or kinks are quite common on these molecules (arrowheads). A rare, putative oligomer is indicated (arrow). B and C, Protein samples from the Ext-2 peak of a CM-cellulose column (but not further purified by gel filtration chromatography) sometimes contain 'spider-like' structures composed of a large central granule plus several radiating or entangled extensin-like molecules. These molecules are too long to be Ext-2. D) The void volume of a CM-cellulose column contains arabinogalactan proteins. A single central granule-like structure is seen (arrow). (A and D, x137,000; B and C, x195,000.)
a reduced ability to form isodityrosine cross-links. If the kinks we observe in Ext-2 molecules are indeed intramolecular IDT cross-links (Fig. 5A), as we suggested for kinks in Ext-1, then still fewer tyrosine residues would be available for intermolecular cross-linking.

One possible alternative function for Ext-2 is that it has carbohydrate binding properties similar to the solanaceous lectins. Lectins from potato and tobacco are suggested to be involved in disease resistance since they can agglutinate avirulent but not virulent strains of bacteria (19, 22). Van Holst and Varner (31) reported that carrot extensin nonspecifically agglutinates Pseudomonas solanacearum strain B1 at the same titer as the tobacco and potato agglutinins. Since these authors relied on isotypic centrifugation in CsCl to purify extensin, it is likely that their preparations contained both Ext-1 and Ext-2 (Fig. 3). This conclusion is supported by the fact that electron microscopy of these preparations showed them to contain molecules that measured 80 nm (Ext-1) and 40 nm (Ext-2). It would be interesting to repeat the agglutination experiments using more highly purified carrot extensins.

Central Granules and Peroxidase Activity. The “spider-like” structures observed in some Ext-2 preparations are enzymatic (Fig. 5, B and C). They are the only higher order structures that co-elute with Ext-2 from an ion exchange column yet the elongated molecules associated with them show greater resemblance to Ext-1. The central location of the granule in these structures suggests that it might serve a structural or enzymatic role in the assembly or function of the cross-linked matrix. Several lines of indirect evidence suggest that extensin molecules are cross-linked to each other by peroxidase-catalyzed isodityrosine formation (10, 11, 15). We were intrigued by the possibility that fractions containing the putative granule proteins (the low mol wt proteins in Fig. 1, C and E) might have peroxidase activity. A solution assay for peroxidase indicated that these fractions contained very low activity compared to crude extensin (Table II). Non-denaturing gel electrophoresis of crude extensin showed it to contain at least 6 bands with peroxidase activity (Fig. 4). The proteins in the “42 kD” fraction, if they are indeed part of the central granule, probably have some other structural or enzymatic function. We are nonetheless interested in whether the synthesis of any of the peroxidase bands is specifically enhanced by wounding in parallel with the extensins.

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