Cross-Linking Patterns in Salt-Extractable Extensin from Carrot Cell Walls

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ABSTRACT

Extensins are hydroxyproline-rich glycoproteins (HRGPs) found in the primary cell walls of dicots. Extensin monomers are secreted into the wall and covalently bound to each other, presumably by isodityrosine (IDT) cross-links, to form a rigid matrix. Expression of the extensin matrix is correlated with inhibition of cell elongation during normal development and with increased resistance to virulent pathogens. We have isolated extensin from carrot root tissue (Daucus carota L.) by published techniques and have used gel filtration chromatography to purify fractions enriched in monomers and oligomers. We refer to this protein as “extensin-1” to distinguish it from “extensin-2,” a second extensin-like HRGP from carrot which we will describe later. We prepared extensin-1 for electron microscopy by shadowing it with platinum. Monomers are highly elongated (≈64 nanometers) and kinked at several sites. Kinks occur at all sites on molecules with nearly equal probability, but do not appear to occur at their ends. The distribution of kinks is similar to that of tyrosine-lysine-tyrosine sequences, which have been shown to be capable of forming intramolecular IDT cross-links, so we suggest that kinks are visible manifestations of intramolecular IDTs. Oligomers likely result from IDT cross-links between monomers, and may be regarded as transient precursors of the fully cross-linked matrix. Nearly 60% of cross-links involve the ends of molecules while the rest are scattered among internal sites. We discuss how the relative positions and proportions of intra- and intermolecular cross-links in extensin-1 may affect the structure, and in turn the function, of the extensin matrix.

Extensins are HRGPs which are major structural components of the primary cell walls of dicotyledonous plants (23, 28). Extensins from carrot and tomato have been studied most intensively, and in addition to hydroxyproline (up to about 45%), they contain considerable amounts of tyrosine, lysine, serine, histidine, valine, and threonine (7, 33, 36, 38). The sequences Ser-Hyp-Hyp-Hyp-Hyp, Tyr-Lys-Tyr-Lys, and Thr-Hyp-Val occur repeatedly in extensin and are likely important to its structure and function (7, 12, 21). Carbohydrate constitutes about two-thirds of the glycoprotein mass, most of which is arabinose linked to hydroxyproline as tetra-arabinosides (20, 38). Single residues of galactose are linked to some serine residues (25). Several other types of HRGPs are found in the cell walls of higher plants and green algae: arabinogalactan proteins (14); solanaceous lectins and agglutinins (for review, see Refs. 23, 28); Chlamydomonas cell wall proteins (31) and sexual agglutinins (9); and the recently described HRGP from soybean seed coats (5). One feature that distinguishes extensins from other HRGPs is that they alone appear to be covalently cross-linked or ‘insolubilized’ in the cell wall: all others are freely soluble or held in the wall by ionic or other weaker interactions. Extensin molecules are thought to be secreted into the wall as monomers which then become covalently bound to each other by peroxidase-catalyzed IDT residues (15). Cooper and Varner have shown that extensin insolubilization and IDT formation are both inhibited by peroxidase inhibitors and free radical scavengers (10) and that insolubilization in isolated cell walls occurs concomitantly with IDT formation (11). The hypothesis that IDT is the intramolecular cross-link has not been proven, however. For example, much of the IDT associated with wall-bound extensin may occur in intramolecular cross-links (12).

The name ‘extensin’ was first applied to a highly insoluble, HRGP residue from cell walls (19). It now appears that a family of related extensin-like HRGPs may be precursors to the cross-linked extensin matrix. Two distinct extensin precursors from tomato suspension cultures have been purified and characterized (33). We present preliminary evidence for two extensin-like HRGPs from carrot. This report will pertain to only Ext-1, which appears to be identical to the extensin characterized by Varner and his colleagues at the protein (36, 38) and nucleic acid levels (6, 7). Ext-2, like Ext-1, is rich in hydroxyproline and arabinose, is soluble in 10% TCA and has a net positive charge (34).

The cross-linked extensin matrix is rigid, inextensible, and resistant to extraction or degradation. Extensin expression has been correlated with the ability of cells to resist lysis in hypotonic medium (19), inhibition of further cell elongation during normal development (32), and increased resistance to attack by virulent pathogens (13). Extensin-like HRGPs also may play an important role in morphogenesis (1). Our goal was to understand how the structure of the cross-linked extensin matrix might relate to these functions. To this end, we have isolated salt-extractable Ext-1 monomers and oligomers, which we regard as precursors of the matrix as a whole (10, 33), and have studied their structure and composition by EM and various biochemical methods.

MATERIALS AND METHODS

Extensin Purification. Aerated carrot root slices provide a convenient source for isolating large quantities of extensin (8). Organically grown carrots with tops (Daucus carota L.) were purchased at a local market. Slices of phloem parenchyma 1 to 2 mm thick were incubated in 20 mM K-phosphate (pH 6.0) plus 50 μg/ml chloramphenicol at 25°C for 3 d with vigorous shaking. The buffer was changed daily after the slices had been rinsed three times with distilled H2O. Extensin was purified by a method
that combined aspects of published techniques (33, 36). Carrot slices (200–400 g) were homogenized in a Virtis homogenizer in 80% (v/w) incubation buffer without chloramphenicol. All steps except chromatography were performed at 0 to 4°C. The cell wall residue was washed 6 to 8 times with distilled water and collected on two layers of Miracloth. The washed wall residue was extracted three times with 150 ml of 0.2 M CaCl2. The extract was concentrated by ultrafiltration to about 3 ml using a PM-10 or PM-30 filter (Amicon, Danvers, MA), made 10% (w/v) in TCA, and allowed to form a precipitate overnight. The TCA extract was centrifuged for 15 min at 15,000g, and the supernatant was dialyzed for 3 d against several changes of distilled H2O and then lyophilized. The ‘crude extenin’ which resulted was chromatographed on a CM-cellulose column (microgranular; Sigma) equilibrated with 25 mM Tris-HCl (pH 8.0), and eluted with a linear gradient of NaCl in the same buffer. Ext-1 eluted at about 25 mM NaCl and Ext-2 eluted at about 90 mM NaCl. Salt concentration was determined by conductivity. For some chemical analyses, samples from several experiments were pooled and chromatographed a second time on the same column.

**Gel Electrophoresis.** SDS-PAGE was performed using 7.5% acrylamide according to Laemmli (18). Acid-urea PAGE was performed in 6% acrylamide according to Stuart and Varner (36), except that the molarities of the gel buffers were reversed. K-acetate was used at 0.75 M (pH 3.0) for the resolving gel and at 0.15 M (pH 5.2) for the stacking gel. Better resolution was obtained in this gel system if dilute samples (in water) were lyophilized and taken up in a small volume of sample buffer. All gels were stained with silver (29).

**Electron Microscopy.** Extensin in 50% glycerol at a final concentration of 1.0 μg/ml was sprayed onto freshly cleaved mica and dried in vacuo in a Balzer’s freeze-etch machine. Samples were rotary shadowed with platinum/carbon at room temperature at an angle of 5 to 6° using the same device (37). Replicas were floated on distilled H2O, mounted on 300 mesh grids, and examined and photographed with a JEOL 100C or Hitachi H-600 electron microscope. Grid squares containing small holes or spots of dirt of a particular morphology (which form when glycerol droplets dry) could be detected at low magnification and were found to always contain extensin molecules (37). Fields of molecules were enlarged to a total magnification of 150,000 diameters for analysis. A Hewlett-Packard 9874A digitizer was used to measure the lengths of extensin monomers and the positions of kinks in monomers and crosslinks in oligomers. All positional data is given in relation to the nearest end of a molecule, since the ends cannot be distinguished morphologically.

**Gel Filtration Chromatography.** Preparations of pure Ext-1 were chromatographed on Sephacryl S-400 (Pharmacia) to separate monomers from oligomers. Protein was eluted at 9.0 ml/h in a column (1.1 × 48 cm) which was equilibrated in 150 mM Tris-HCl (pH 8.0).

**Amino Acid Analysis.** Amino acid analysis was performed for us by G. Cassab and J. Varner (Washington University, St. Louis) as described (5). In order to quantitate IDT, we (together with R. Harris, Chemistry Department, University of Colorado, Boulder) utilized a second method which is sensitive to this amino acid. Protein was hydrolyzed in evacuated tubes containing constant boiling 6 N HCl for 18 h at 110°C. Amino acids were eluted from an AA511 column (Interaction Chemicals, Mountain View, CA) using Buffelle buffers (Pierce), and detected by post column derivitization with o-phthalaldehyde (‘Fluorodrhyde,’ Pierce) (2). Pure IDT was generously provided by D. Lamport (Michigan State University, East Lansing) and quantitated as described by Fry (16). To cleave IDT linkages, samples were treated with acidified NaClO2 (0.3% w/v in 0.12% v/v acetic acid) for 15 min at 60°C prior to hydrolysis (15, 30).

**RESULTS**

**Purification and Characterization of Extensin-1.** Extensin and other proteins were released from the cell wall residue of aerated carrot slices with 0.2 M CaCl2. A silver-stained SDS polyacrylamide gel demonstrates that 10% TCA precipitates a characteristic set of proteins and that the same proteins are found in the supernatant (Fig. 1, A and B). In addition, the TCA supernatant contains a large amount of Ext-1, which remains in the stacking gel or just barely enters the resolving gel, so we refer to this fraction as ‘crude extenin.’ Ext-1, which has a monomer mol wt of about 86 kD, may migrate anomalously in this gel system due to its high level of glycosylation and/or its very basic isoelec-
tric point (36). Ext-2, a second TCA-soluble protein, has an apparent mol wt of about 180 kD in this gel system (Fig. 1B).

Chromatography of crude extensin on CM-cellulose (Fig. 2) yields a single major peak which is highly enriched in Ext-1 (the smaller second peak contains Ext-2 and other proteins). No other proteins are seen on a silver-stained SDS polyacrylamide gel of this fraction, even when the gel is overloaded (Fig. 1, C and D). This criterion is not sufficient to establish purity, however, since the smear of stained material in the stacking gel could contain a number of related proteins. Alditol acetate analysis of the neutral sugar composition of the Ext-1 fraction indicates that it contains 95% arabinose and 5% galactose, which is consistent with previous work (38).

Pure salt extractable Ext-1 is highly enriched in monomers, but as can be seen by acid-urea PAGE, it also contains detectable amounts of higher mol wt proteins which we believe to be covalent oligomers of Ext-1 (Fig. 3). In support of this idea is the fact that the relative migration of the putative dimer and trimer bands suggest that their mol wt are exactly two and three times that of the monomer band. Chromatography of Ext-1 on a Sephacryl S-400 gel filtration column (Fig. 4) yields a single peak which contains only monomers (Fig. 3B) and a faster eluting shoulder which is enriched in oligomers (Fig. 3C). Ext-2 migrates as a distinct species in this gel system (Fig. 3D) with an apparent mol wt of about 43 kD, assuming a mol wt of 86 kD for Ext-1 monomers (36).

The amino acid composition of Ext-1 purified by ion-exchange and gel filtration chromatography is shown in Table I. As expected, it is especially rich in hydroxyproline and contains significant amounts of serine, valine, tyrosine, histidine, and lysine. Also shown are amino acid profiles for carrot extensin purified by equilibrium density centrifugation in CsCl (38) and one derived from a carrot genomic clone thought to encode extensin (7). The three profiles are quite similar, and despite minor differences, they probably represent the same protein. A different amino acid analyzer was calibrated to detect and quantify IDT. Ext-1 (which had not been subjected to gel filtration chromatography to separate monomers from oligomers) was found to contain 5.3 half-IDT residues per molecule, which is equivalent to about 1.9 mol % (Table I).

Electron Microscopy. Proteins that contain elongated domains can be visualized readily in the electron microscope after they have been shadowed with platinum at a low angle (37). A preparation of Ext-1 purified by ion exchange chromatography contains predominantly monomers and a few oligomers (Fig. 5a). In contrast, material taken from the faster-eluting shoulder of the gel filtration column is enriched in oligomers (Fig. 5b).

**Fig. 2.** Crude extensin was chromatographed on a CM-cellulose column (1.0 x 10 cm) equilibrated with 25 mM Tris-HCl (pH 8.0) and eluted with a linear gradient of NaCl in the same buffer. Ext-1 eluted at 25 mM NaCl and Ext-2 eluted at 90 mM NaCl.

**Fig. 3.** Analysis of extensin purification by acid-urea PAGE. A, Pure Ext-1 from a CM-cellulose column contains predominantly monomers (1X), and much smaller amounts of dimers (2X), trimers (3X), and higher order oligomers (not resolved on this gel); B, the major peak from a Sephacryl S-400 column contains only monomers of Ext-1; C, the faster eluting shoulder from the S-400 column is enriched in Ext-1 oligomers; D, the Ext-2 peak from a CM-cellulose column contains a distinct protein species with an apparent mol wt about half that of Ext-1 monomers. This sample is contaminated with a small amount of Ext-1. Each lane contained about 15 μg of protein. The gel was stained with silver.

**Fig. 4.** Pure Ext-1 was chromatographed on a Sephacryl S-400 gel filtration column (1.1 x 48 cm) in 150 mM Tris-HCl (pH 8.0) at 9.0 ml/h. The major peak (M) contained only monomers and the faster eluting shoulder (O) was enriched in oligomers.
Monomers are highly elongated and measure 84.4 ± 7.5 nm (n = 68). Treatment of extensin molecules with acidified sodium chlorite fragments them into pieces of various size (Fig. 5c).

Examination of Ext-1 molecules at higher magnification reveals several features about their structure. Monomers show distinct bends or 'kinks' at several sites along their lengths (Fig. 6, a–c). Loops in monomers (Fig. 6d) are relatively rare and probably form from intramolecular IDT cross-links between widely separated sites on these molecules. Oligomers most likely result from IDT cross-link formation between molecules and may be assembled from monomers in a variety of ways, including: side to side (Fig. 6, e–f); end to end (Fig. 6g); both ends of one molecule to different sites on the side of another one (Fig. 6h); and one end of each of two different molecules to the side of the other to form a loop (Fig. 6i). Trimmers (Fig. 6j), tetramers (Fig. 6k), and indecipherable higher order polymers (Fig. 6l) also are observed. This last structure may represent the complexity of the insoluble extensin matrix as a whole.

Measurement of kink position with a digitizer shows that they occur at nearly all sites on Ext-1 molecules with equal frequency, but are absent at their ends (Fig. 7a). The average number of kinks per molecule from two separate preparations was 2.11 ± 0.93 (n = 108) and 2.47 ± 1.23 (n = 60). Cross-links occur predominantly at the ends of molecules, but can occur at any site (Fig. 7b). Figure 7c shows the relative positions of tyrosine residues and Tyr-Lys-Tyr sequences, which were deduced from a genomic clone (7). All tyrosine residues are potential sites for intermolecular cross-link formation, and the two tyrosines in the sequence Tyr-Lys-Tyr may form an intramolecular IDT cross-link (12).

DISCUSSION

Structure and Function of Extensin. Brysk and Chrispeels (3) suggested that extensin was an elongated or asymmetric molecule based on its relatively small apparent mol wt and its exclusion from a G-200 Sephadex column. Our electron micrographs show
monomers to be elongated rods with an average length of 84.4
nm, which is close to a previous estimate of 80 nm (38). The
protein backbone of extensin has been shown by circular dichro-
ism spectroscopy to be entirely in the extended polyproline II
helical conformation (38). A molecule containing 274 residues
in this conformation (7) is predicted to measure 85.5 nm (0.312
nm/residue; 22). In the following report (35), we show that the
glycoprotein has a Stokes' radius of 89 Å and discuss the impor-
tance of carbohydrate moieties in maintaining it in a rigid, rod-
like form.

The results of a large number of studies on extensin-like
HRGPs from both higher and lower plants suggest a single
unified function for these proteins, that of strengthening cell
walls through the formation of a rigid and highly insoluble

Fig. 6. Electron micrographs of individual Ext-1 molecules at higher magnification. Accompanying diagrams highlight kinks in monomers and the manner in which cross-links form within or between molecules. a to c, monomers showing a variable number and location of kinks; d, looped monomer with an intramolecular cross-link; e to f, side-to-side dimers; g, end-to-side dimer; h, both ends of one molecule linked to different sites on the side of another; i, one end of each of two different molecules linked to the side of the other; j, trimer; k, tetramer; l, indecipherable polymer (×250,000).
matrix. Repercussions of increased wall strength appear to include protection from lysis (19), inhibition of cell elongation (32), protection from pathogens (13), and regulation of morphogenesis (1). There is considerable evidence that salt-extractable extensin is a precursor of the insoluble extensin matrix of plant cell walls (10, 33) and strong circumstantial evidence that insolubilization involves the formation of covalent isodityrosine cross-links between extensin monomers (10, 11, 15). We set out to understand how the structure of the wall-bound extensin matrix might give rise to its many functions.

Cross-Linking Patterns in Salt-Extractable Extensin. It is evident from micrographs of large extensin polymers (Fig. 6l) that little useful information about matrix structure can be discerned from such tangled aggregates. A more tractable approach to this problem was to study oligomers, which may be regarded as transient intermediates between secreted monomers and the cross-linked matrix as a whole, and determine at which sites on these molecules cross-link formation occurs most commonly. Having a fraction enriched in oligomers (Figs. 3, 4, and 5b) greatly aided this task. Cross-links can form at all sites on extensin molecules (Fig. 7b), but show a strong preference for their ends. A matrix formed by such molecules would be relatively open, which suggests that the extensin matrix could wrap around cellulose microfibrils (24). The existence of two interconnected polymer systems in the cell wall raises the question of whether newly synthesized extensin is intercalated among the extant wall polymers (intussusception) or deposited near the plasma membrane-like cellulose (apposition). The degree to which Ext-I molecules may be able to penetrate the wall is unclear since they have a hydrodynamic (Stokes') radius of 89 Å (35) and the measured limiting cell wall pore diameter is about 40 Å (4). We are currently using immunoelectron microscopy to address these questions (34).

The amino acid sequence for Ext-I derived from a genomic clone (7) includes 33 tyrosine residues which are evenly distributed along the entire molecule (Fig. 7c). Since all of these tyrosines are candidates for IDT formation, it is surprising that intermolecular cross-links occur with so much greater frequency at the ends of molecules than within molecules (Fig. 7b). A possible explanation for this result comes from the observation that salt-extractable Ext-I contains 5.3 half-residues of IDT per molecule (Table I). Since this material is highly enriched in monomers, nearly all of the IDT must be due to intramolecular cross-links. It follows that the presence of IDT linkages within molecules could prevent their formation between molecules. We cannot explain why we are able to detect significant amounts of IDT in salt-extractable extensin when others were not (10, 11, 33). Fry (15) has shown that insoluble cell wall protein (presumably extensin) from a wide variety of species contains IDT (half-residues) in a ratio of 1:15 with hydroxyproline. If this relationship holds for carrot Ext-I (which contains 116 hydroxyprolines);

![Graph](https://via.placeholder.com/150)

**Table 1. Amino Acid Composition of Ext-I**

<table>
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<th>Carrot Extensin/ Genomic Clone (7)</th>
<th>Carrot Extensin/ CsCl Isolation (38)</th>
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<td>HYP</td>
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<tr>
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</table>

* Amino acids found in less than 0.05 mol %.  
† Amino acids found in less than 0.05 mol %.

Not determined.  
* Determined independently of other values (see 'Materials and Methods').  
† Half-residues of IDT per 274 total residues, or 1.93 mol %, based on 30 residues of serine per molecule (7). The IDT peak could be eliminated by pretreatment with NaClO₃.
7), then insoluble extensin would contain about 7.75 half-IDTs per monomer. Thus, if 5.3 of these residues were involved in intramolecular cross-links (Table 1), then each monomer would be able to form only 2.5 IDT cross-links with other monomers, which again suggests a relatively open extensin matrix.

**Relationship between Kinks and Intra- and Intermolecular Cross-Links.** Epstein and Lamport (12) have shown that tyrosines in the sequence Tyr-Lys-Tyr can form an intramolecular cross-link. Most of the tyrosine residues in the central regions of Ext-1 molecules occur in this sequence whereas those at the ends do not (7). Figure 7 shows that the distribution of kinks in monomers is similar to that of Tyr-Lys-Tyr sequences, and from the relative positions of kinks and cross-links are complementary. From these data, we suggest that kinks are visible manifestations of intramolecular IDT cross-links. Consistent with this idea is the fact that each monomer contains about 2.65 IDT residues (5.3 half-residues) and a similar number of kinks (2.11 – 2.47). In addition to preventing the formation of intramolecular cross-links, intramolecular IDT could put bends into otherwise linear molecules, which would effect the extensin molecules interact with other components of the wall. Such covalent cross-links also could make extensin molecules more rigid by preventing rotation at these sites.

The suggested relationship between kinks and intramolecular IDT has not been proven. We expected that molecules treated with acidified sodium chloride would be straight and un-kinked when examined in the electron microscope, but instead, they were fragmented into short rods (Fig. 5c). This result was surprising since O'Neill and Selvendran (30) suggested that this treatment had little effect on protein or carbohydrate moieties of cell wall glycoconjugates. We tried to demonstrate that extensin which contains less IDT also contains proportionally fewer kinks. For one experiment, carrot slices were treated with ascorbate to inhibit IDT formation (10), and for another, extensin was isolated from microsomal vesicles, under the presumption that IDT is formed only in the wall (11, 15). Unfortunately, both of these experiments gave equivocal results (not shown). Epstein and Lamport (12) suggested that intramolecular IDT does not put bends into a protein backbone which is in the polyproline II conformation. Although we have not proven that kinks are due to IDT, we do feel confident that they are real structures and not the results of interactions with the mica substrate.

**Control of Extensin Matrix Structure.** The relative positions and proportions of intra- and intermolecular IDT cross-links could have profound effects on the rigidity and porosity of the extensin matrix, which could in turn modulate the suggested wall strengthening properties of the matrix. IDT formation is presumed to be catalyzed by wall-bound peroxidases (10, 11, 15). Since each type of IDT linkage appears to be formed from tyrosine residues which occur in different amino acid sequences (and perhaps in different carbohydrate milieus as well), it is not unreasonable to assume that a different peroxidase could catalyze each type of reaction. The differential expression of a cell of each putative peroxidase could regulate the degree of cross-linking of the extensin matrix in its walls. Although salt-extractable extensins from wounded and unwounded carrot tissues appear to be identical chemically (38), the matrix formed by each could be different. Likewise, although pea epidermis capable of elongation growth contain some insoluble extensin, it is possible that the noted correlation between their inability to grow further and extensin accumulation (32) could involve qualitative (i.e., different proportions of intra- and intermolecular cross-links) as well as quantitative changes in the extensin matrix. The exact properties of the matrix also might be controlled by the differential expression of multiple precursors, such as Ext-1 and Ext-2.

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