A Developmentally Regulated Hydroxyproline-Rich Glycoprotein from the Cell Walls of Soybean Seed Coats

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ABSTRACT

In soybean seeds the level of hydroxyproline is regulated in a developmental and tissue-specific manner. The seed coat contains approximately 77% of the total hydroxyproline in the seed at all stages of development. We determined the ratio of hydroxyproline to dry weight in a number of tissues within the seed; however, only the seed coat shows an increase in this ratio during development. Within the many cell layers of the seed coat, hydroxyproline is most abundant in the external layer. The hydroxyproline is present as an hydroxyproline-rich cell wall glycoprotein. The protein is rich in hydroxyproline (36%), lysine (11%), proline (10%), histidine (9%), tyrosine (9%), and serine (8%). The carbohydrate portion is 90 mole % arabinose and 10 mole % galactose. The arabinose residues are attached to hydroxyproline mostly in the form of trisaccharides. The apparent molecular weight of this glycoprotein is 100,000 daltons.

Hydroxyproline-rich glycoproteins of the cell wall are present in a wide variety of plants (16). The level of the HRGPCW is usually low in plants; but, in some instances, a rise above normal levels has been observed upon wounding (3, 27) and infection (8, 11), and the level is usually higher in culture (15).

Van Etten et al. (28) reported that seed coats and pericarpers of many plant species contain high levels of the amino acid hydroxyproline. The testa is usually a hard coat whose physiological importance arises from the presence of an outer and inner cuticle, and one or more layers of thickened protective cells. These features confer upon the testa mechanical strength and some degree of impermeability to water and/or gases including O2, so as to exert a regulatory influence on the metabolism, growth, and development of the inner tissues and organs of the seed (23). Indeed, the seed coats may determine seed size (5, 26).

Here, we report that during soybean seed development the level of hydroxyproline in the testa increases dramatically, and the hydroxyproline to cell wall dry weight ratio is highest in the external layer of the seed coat. Moreover, this hydroxyproline is present as a cell wall glycoprotein which differs in its amino acid composition and arabinosylation pattern from the carrot cell wall glycoprotein previously characterized (29). This HRGPCW may play a structural role in the seed coat (28) and could be characteristic of this tissue.

1 Supported by grants from the National Science Foundation (PCM 7923550 and PCM 8104516), and the United States Department of Agriculture (83-CRCR-1-1217).
2 Abbreviation: HRGPCW, hydroxyproline-rich glycoprotein from cell wall.

MATERIALS AND METHODS

Plant Material. Seeds of Glycine max (var Provar) were obtained from six stages of development: 8, 18, 19, 20, 21, and 26 d after anthesis from plants grown in the greenhouse. At these stages of development, the palisade and hour glass cell layers were easily dissected from the rest of the seed coat. Integrity and purity of the separated layers was verified by inspection by light microscopy.

Hydroxyproline Determination. Hydroxyproline content was determined colorimetrically by the method of Drozd et al. (6) after proteins or tissues were hydrolyzed in 6N HCl at 120°C for 3 h.

Cell Wall Isolation. Fresh seed coats (about 5 g) were ground in a glass homogenizer in 0.1% K-acetate buffer (pH 5.0) with 4 mm Na2S2O5 and 1% insoluble PVP. The cell walls were washed with 0.5% Nonidet P-40 (Sigma), 2 mm Na2S2O5, and then resuspended and centrifuged 10 times with 50 ml of cold 2 mm Na2S2O5. Finally, the cell wall pellet was extracted with 10 ml of 0.2 M CaCl2, 4 M Na2S2O5 for 12 h at 5°C.

Isolation of Hydroxyproline-Rich Cell Wall Glycoprotein. The CaCl2 extract was loaded onto a CM-Sepharose CL-6B (Sigma) column (30 x 2.5 cm) previously equilibrated with 0.02 M Tris-HCl (pH 8.0). The column was washed with two volumes of 0.02 M Tris-HCl (pH 8.0) and the attached material was eluted with a linear gradient of 0.02 to 0.5 M Tris-HCl (pH 8.0). The hydroxyproline containing fractions (salt concentration, 0.3 M Tris-HCl) were pooled and solid CsCl was added to a final density of 1.4 g cm-1. Samples were centrifuged for 72 h at 250,000g in a Beckman SW-65 Ti rotor. The resulting CsCl gradient was fractionated. The hydroxyproline containing fractions (density of 1.435 g cm-3) were pooled and dialyzed against 0.1 M NaCl overnight and against deionized H2O for 2 d. The entire procedure was carried out at 5°C. In all the fractionation procedures, the salt concentration of the samples was determined using a refractometer, the protein content by UV absorption at 280 nm, and the hydroxyproline content as described above.

Chemical Analyses. Acid hydrolysis for the amino acid analyses was performed in constant boiling HCl (Pierce) with 0.05% (v/v) mercaptoethanol under N2 for 24 h at 110°C. The resulting amino acids were then analyzed on an automatic amino acid analyzer. Sugar composition was determined by GC of alditol acetate derivatives of the hydrolyzed polysaccharides (1 h at 120°C in 2 M TFA [2]) on a 1% (w/v) OV-275 Gas Chrom A Columns (180 x 0.2 cm). Hydroxyproline arabinosides were separated by gel filtration (13).

Gel Electrophoresis. SDS-PAGE was done according to Laemmli (14) with 5% and 10% acrylamide. The gels were fixed and stained with periodic acid-silver stain (7).

Precipitation with β-Galactosyl-Yariv Reagent. The precipitation of arabinogalactan-protein with β-galactosyl-Yariv reagent was carried out according to Jemmyn and Yeow (12).
FIG. 1. Pattern of total hydroxyproline distribution during soybean seed development. A, in the entire seed; B, in the seed coat. Each value represents the mean of three replicates.

Table 1. Ratios of Hydroxyproline to Dry Weight in Different Parts of the Developing Soybean Seed

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Hydroxyproline: Dry Wt Ratio (μg/mg) at Following Days after Anthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8</td>
</tr>
<tr>
<td>Seed coat</td>
<td>0.3</td>
</tr>
<tr>
<td>External layer</td>
<td>—*</td>
</tr>
<tr>
<td>Internal layer</td>
<td>—</td>
</tr>
<tr>
<td>Hilum</td>
<td>—</td>
</tr>
<tr>
<td>Cotyledon</td>
<td>0.01</td>
</tr>
<tr>
<td>Axis</td>
<td>—</td>
</tr>
</tbody>
</table>

* Not determined.

Scanning Electron Microscopy. Seeds were dissected, and then fixed for 2 h in 3% (v/v) glutaraldehyde containing 100 mM phosphate buffer (pH 7.2). After two 15-min rinses in phosphate buffer, tissue samples were slowly dehydrated in graded ethanol series, and samples in absolute ethanol were dried using the critical point dryer, fractured, attached to specimen studs, and coated with gold-palladium prior to viewing with a Hitachi S-450 scanning electron microscope.

RESULTS

Distribution of Hydroxyproline in Soybeans. The seed coat has the highest ratio of hydroxyproline to dry weight (5.2 μg Hyp/mg dry weight) of the soybean plant compared with roots (0.2 μg/mg), leaves (0.3 μg/mg), entire seed (0.9 μg/mg), stems (1.3 μg/mg), and flowers (1.5 μg/mg).

The hydroxyproline content increases in the entire seed and in all parts of the seed during its development (Fig. 1A). The seed coat has about 77% of the total hydroxyproline in the seed at all stages. The ratio of hydroxyproline to dry weight increases during development only in the seed coat and not in the cotyledons and axis (Table I). To determine the distribution of hydroxyproline in the various tissues of the seed coat, we dissected it into the external and the internal layers and the hilum region. The external layer consists of two cell layers: epidermal palisade cells and hair glass cells. The internal layer has thin-walled parenchymatous tissue, vascular cells, and compressed cells (Fig. 2). The total hydroxyproline content in the different sections of the seed coat was determined and is shown in Figure 1B. The maximum accumulation of hydroxyproline occurs in the external layer. At 26 d after anthesis, the external layer contains 73% of the total hydroxyproline of the seed coat. Moreover, the ratio of hydroxyproline to dry weight is greater than in any other part of the seed (Table I). We also determined the amount of hydroxyproline in isolated cell walls of different parts of the seed. The highest ratio of hydroxyproline to cell wall dry weight is localized in the outermost layer of the seed coat (data not shown).

Isolation of Hydroxyproline-Rich Glycoprotein from the Cell Walls of Soybean Seed Coats. The HRGPCW of soybean seed coat was isolated from seeds at stage 'N' (25 d after anthesis [21]). This particular stage was chosen because 60 to 80% of the pentidyl-hydroxyproline is solubilized with high salt concentration indicating that it is not covalently linked to the cell wall. We followed the same procedure reported by Stuart and Varner (27) with minor modifications. The density of the major protein eluted from the cation exchange chromatography was 1.435 g cc⁻¹ in a CsCl gradient. This is virtually the same density as that of the HRGPCW from carrots (27).

The HRGPCW was purified to homogeneity as indicated by the appearance of a single band in SDS-PAGE after staining with periodic acid-silver stain in 10% and 5% polyacrylamide gels. In 5% polyacrylamide gels, the HRGPCW shows an apparent mol wt of 100,000 D. With this technique, however, the mol wt of glycoproteins can be overestimated because they usually show a larger hydrodynamic radii than proteins with similar mol wt (1).

The HRGPCW is rich in Hyp, Ser, Pro, Tyr, His, and Lys, which make up 83% of the residues (Table II).

The seed coat HRGPCW has arabinose and galactose as major carbohydrates (Table II). The sugars were identified by comparison of retention times of known sugar standards as well as by co-elution of sample peaks coincident with the standards on the gas chromatograph. The arabinosylation pattern as determined by gel filtration (13) showed that arabinose is primarily attached to hydroxyproline in oligosaccharide chains of three residues (see Table II). The purified seed coat cell wall glycoprotein shows no reaction with the β-galactosyl-Yariv reagent, which precipitates the arabinogalactan proteins, another class of hydroxyproline-rich proteoglycans (9). Cell wall extracts from seed coats show little reaction with this reagent. However, in the case of cell wall extracts from soybean flowers, 33% of the total hydroxyproline reacts with the Yariv reagent.

DISCUSSION

The presence of hydroxyproline in the seed coat of several plant species has been reported earlier (28). From the present study, it is clear that the accumulation of hydroxyproline is developmentally regulated in the soybean seed and is greater at 26 d after anthesis than at the other stages analyzed. Moreover, the highest level of hydroxyproline is always found in the testa at the different stages of seed development, and interestingly,
within the many cell layers of the coat, hydroxyproline is most abundant in the outermost layer.

The peptidyl-hydroxyproline isolated from soybean seed coats is an hydroxyproline-rich glycoprotein. This glycoprotein is approximately 2% of the total dry weight of the seed coat. Six amino acids (Hyp, Ser, Pro, Tyr, His, and Lys) constitute 83% of the residues. This amino acid composition is similar to the amino acid composition of the well characterized HRGP from: (a) aerated carrot slices (29); (b) potato tubers (20); (c) tobacco callus (22); and (d) tomato cell suspension cultures (25). The relatively high content of the basic amino acids histidine and lysine and the low content of acidic residues indicate that the protein is a basic molecule, being positively charged at neutral pH values. This charge could be important for the interaction of the protein with the structural components, particularly polyuronates of the cell wall. The seed coat HRGPCW has a ratio of
Ably that the electrophoresis rather than is tested in (4:1)

Hyp to Pro (4:1) is that similar to those of the bacterial agglutinins of potato tubers and tobacco callus (20, 22), but different from the carrot cell wall glycoprotein (46:1) (29).

The carbohydrate content of the seed coat HRGPCW is similar to that from other plant HRGPCWs (18, 29, 30). However, in the seed coat HRGPCW, the arabinose is mainly bound to hydroxyproline in short side chains of three residues (HypAra), rather than HypAra4.

The seed coat HRGPCW migrates as a single broad band after electrophoresis in 5% polyacrylamide gels containing 10% SDS (data not shown), comparable to the bacterial agglutinins of potato tubers and tobacco callus (20, 22). This broad banding pattern is fairly characteristic of glycoproteins and presumably is a consequence of the heterogeneity of the carbohydrate chains (18).

Finally, one distinguishing characteristic of the seed coat HRGPCW is that it accumulates during soybean seed development. Moreover, in the seed coat, the ratios of hydroxyproline to dry weight is highest as compared to any other part of the soybean plant. This is of special interest in relation to a possible role in structure (28) and the protective function of the testa. It has been suggested that the cell wall glycoprotein is secreted as a soluble monomer which becomes insolubilized in the wall by formation of isodityrosine cross-links (4, 10). We are now interested in determining whether these isodityrosine cross-links exist in the seed coat HRGPCW and are related to its presumed structural role.

Acknowledgments—The authors are grateful to Meryl Weinstein (Fine Arts Major) for technical assistance in the first hydroxyproline assays, Terry Takehiro for technical assistance in the arabinosylation profile, and Mike Veith for excellent assistance in the scanning electron micrographs.

Table II. Composition of Hydroxyproline-Rich Glycoprotein from Cell Walls of Soybean Seed Coats

<table>
<thead>
<tr>
<th>Amino Acids</th>
<th>mol %</th>
<th>Sugars</th>
<th>mol %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyp</td>
<td>36.2</td>
<td>Ara</td>
<td>90.3</td>
</tr>
<tr>
<td>Asx</td>
<td>2.1</td>
<td>Galb</td>
<td>9.2</td>
</tr>
<tr>
<td>Thr*</td>
<td>1.3</td>
<td>Glic</td>
<td>0.5</td>
</tr>
<tr>
<td>Ser*</td>
<td>8.2</td>
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<td></td>
</tr>
<tr>
<td>Glx</td>
<td>2.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro</td>
<td>9.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gly</td>
<td>4.0</td>
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<tr>
<td>Ala</td>
<td>1.9</td>
<td></td>
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</tr>
<tr>
<td>Val</td>
<td>2.5</td>
<td>Hyp-Ara</td>
<td>10</td>
</tr>
<tr>
<td>Cys</td>
<td>1.0</td>
<td>Hyp-Ara</td>
<td>3</td>
</tr>
<tr>
<td>Met</td>
<td>2.2</td>
<td>Hyp-Ara</td>
<td>7</td>
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<tr>
<td>Ile</td>
<td>0.9</td>
<td>Hyp-Ara</td>
<td>10</td>
</tr>
<tr>
<td>Leu</td>
<td>1.3</td>
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<td>11</td>
</tr>
<tr>
<td>Tyr</td>
<td>8.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phe</td>
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<td></td>
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</tr>
<tr>
<td>His</td>
<td>8.8</td>
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</tr>
<tr>
<td>Lys</td>
<td>10.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arg</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Not corrected for losses during hydrolysis.

b Galactose is presumably linked to serine (Lamport et al., 1977).

c Glucose is thought to be a contamination.

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