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Cell Wall Bound and Soluble Peroxidases in Normal and Dwarf Tomato

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Normal and dwarf tomato shoots were analyzed for their peroxidase isoenzyme content and total peroxidase activity. Both plant types were shown by electrophoresis to contain three isoenzymes that moved toward the anode and one that moved toward the cathode. Both types contained a cell wall bound peroxidase that was electrophoretically different from the four soluble isoenzymes. Cell wall bound peroxidase was separated from soluble peroxidase isoenzymes in crude extracts by using purified cell walls. With this method, accurate measurements of the total cell wall bound and soluble peroxidase activities were obtained. Cell wall bound peroxidase accounted for 30% and 56% of the activity in crude extracts of normal and dwarf plants, respectively. Total peroxidase activity in the normal plant was 74% cell wall bound and 81% in the dwarf plant. Cell wall bound peroxidase activity was 5.2 times greater in the dwarf when compared to the normal plant.

The ionic binding of peroxidases to cell walls has been studied for quite some time, and as yet, no definitive agreement can be reached as to how much or what kind of peroxidases are, in fact, bound to the cell wall. A number of review articles have summarized these studies (Lamport, 1965; Fry, 1986; Cassab and Varner, 1988). It would appear that the amounts and types of peroxidases vary depending on the phylogeny and ontogeny of the plant, and the issue is further complicated by attempts to relate the results of tissue culture model systems back to the whole plant (Mader et al., 1975; van Huystee and Chibbar, 1987).

It has long been known that dwarf plants generally contain higher peroxidase activity than their normal counterparts (Kamerbeek, 1956; McCune and Galston, 1959). However, it is not known whether this dwarfing is the result of increased or premature lignification due to increased peroxidase activity or whether increased peroxidase activity is caused by the dwarfing due to other factors. Although the peroxidase activities in dwarf plants are greater than in the normal plants, it is not known whether this increased activity is due to the cell wall ionically bound peroxidases or the nonionically bound, soluble peroxidases, or both.

This present research describes the types and amounts of total nonionically bound, soluble peroxidases and the ionically bound cell wall peroxidase present in dwarf and normal tomato plants. It further defines the peroxidase that is associated with the cell wall and its total activity in relation to the other soluble, nonionically bound peroxidase isoenzymes.

MATERIALS AND METHODS

Plant Material. Normal (d^+) and dwarf (d^x) tomato plants (*Lycopersicon esculentum* Mill.) were grown in a greenhouse from seed to a height of ca. 1 and 0.4 m, respectively, and were ca. 6 months old. The slow growth rate and morphology of the dwarf plant were described by Rick and Butler (1956), and the

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plants used in this study were descendents of Rick's stock.

Extraction of Peroxidases. Apical 100-g portions of the plants, including leaves and stems but devoid of fruit, were excised and homogenized with 200 mL of H_2O in an Omnimixer for 30 s, and the slurry was filtered through Miracloth. The filtrate was centrifuged for 20 min at 37000*g*, the pellet discarded, and the supernatant (pH 5.7) saved as the crude extract. The pulp, remaining in the Miracloth, was quantitatively removed and successively extracted with H_2O four times by the homogenization and centrifugation procedures described above. The four H_2O washes contained the soluble, nonionically bound peroxidases. The H_2O-washed pulp was then successively extracted four times with 0.2 M $CaCl_2$ by the above procedures. The $CaCl_2$ washes contained the ionically bound cell wall peroxidase. The resulting $CaCl_2$-washed pulp was rinsed with H_2O in a Buchner funnel to remove the excess $CaCl_2$ and designated the cell wall fraction. Dry matter (DM) of the cell wall fractions was determined by standard AOAC method 7.007 (AOAC, 1984).

Separation of Cell Wall Bound Peroxidase from the Crude Extract with Cell Walls. Cell walls from the dwarf and normal plants were prepared by using the H_2O and $CaCl_2$ wash procedures described above. Columns (1 cm × 8 cm) of the cell walls were prepared and equilibrated with H_2O. Aliquots (5 mL) of the crude extracts were dialyzed against H_2O and applied to the columns and the cell wall columns washed with H_2O. Three-milliliter fractions were collected at a flow rate of 1 mL/min until no peroxidase activity could be detected in the effluent. The cell wall bound peroxidase was eluted with 0.2 M $CaCl_2$. All fractions were assayed for peroxidase activity and isoenzyme content.

Peroxidase Assay and Electrophoresis. Peroxidase activity was determined spectrophotometrically in a reaction mixture consisting of 100 μmol of phosphate buffer (pH 6.7), 50 μmol of guaiacol as the hydrogen donor, and 20 μmol of H_2O_2 in a total volume of 2.4 mL. Aliquots (0.1 mL) of appropriately diluted fractions were added to initiate the reaction. The tetraguaiacol produced was quantified with an extinction coefficient of 26.6/cm per mM at 470 nm. Starch–gel electrophoresis (Evans and Alldridge, 1965) at pH 8.8 was performed on all fractions, and the gels were stained with saturated benzidine in H_2O as the hydrogen donor and 0.05% H_2O_2. This stain was also used for the histochemical studies. Polyacrylamide gel electrophoresis (PAGE) at pH 8.8 and 10% acrylamide were also used to determine whether the type of gel had any effect on the type of isoenzymes detected.

RESULTS AND DISCUSSION

The peroxidase isoenzymes that were found in the crude extract are shown in Figure 1. Peroxidases A–C moved toward the anode while peroxidase A′ was the only isoenzyme that moved toward the cathode at this pH. The D complex (D_c) appeared as a continuous streak in the gel with no appearance of discrete bands. In addition, the filter paper strip with which the sample was applied stained intensely for peroxidase activity after electrophoresis, thus indicating that much of the activity was still left on the applicator paper and did not penetrate the gel. Electrophoresis using PAGE showed the normal pattern for peroxidases A–C and A′ but did not allow this D complex to penetrate the acrylamide gel; much peroxidase activity was detected at the point of application of the sample. Apparently, the complex was too large to penetrate the acrylamide gels and would only partially penetrate the starch–gels at sufficient amounts to be detected as a continuous streak. The H_2O washes of the pulp from the tomato shoots only contained traces of isoenzymes A–C and A′; no D complex was detected in any of the H_2O washes. The $CaCl_2$ washes yielded only the D complex (Figure 1), which indicated this complex was ionically associated with the cell walls of the plant. The presence of this complex in the crude extract but not in the H_2O washes is probably due to the extraction of the cell wall bound isoenzyme by the endogenous plant salts

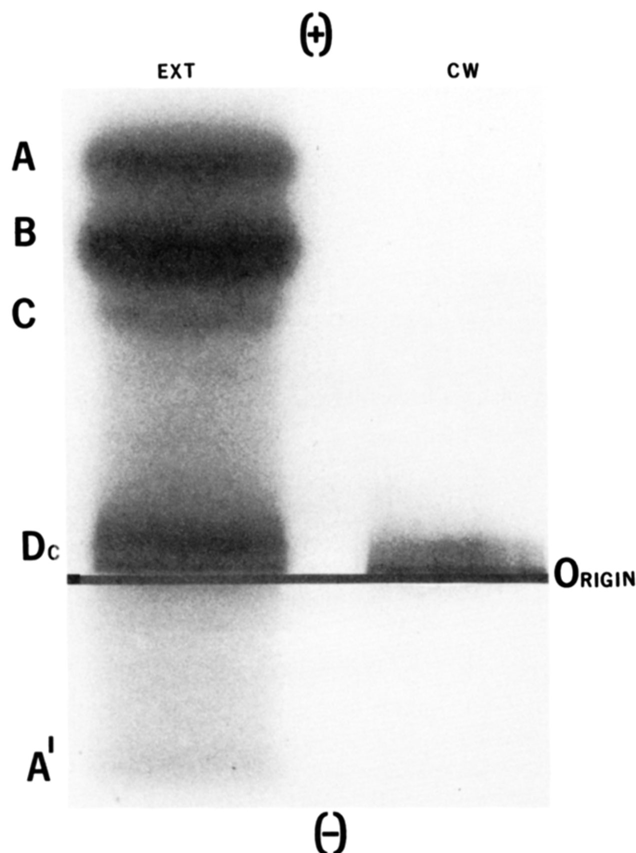

Figure 1. Zymogram showing the peroxidases present in the crude extract (EXT) and the peroxidase associated with the cell walls (CW).

solutes), which sufficiently increased the ionic strength of the extract to elute part of the complex from the walls. In a crude extract, both ionically bound and soluble (nonionically bound) peroxidase isoenzymes were extracted; therefore, to use this crude extract for a measurement of total activity for soluble, nonionically bound isoenzymes would be misleading.

To separate this bound activity from the soluble activity in the crude extract, the cell wall columns were employed. Soluble, nonionically bound peroxidases A–C and A′ in the crude extract passed through the column and were not adsorbed. Elution of the cell wall columns with 0.2 M $CaCl_2$ released only the D complex; no other isoenzymes were detected. Therefore, this method was used to quantify the amounts of ionically bound, cell wall peroxidase present in the crude extracts in relation to the soluble, nonionically bound isoenzymes.

The results of this separation and the H_2O and $CaCl_2$ extractions are in Table I. The cell wall bound D complex accounted for 30% of the total activity in the crude extract of the normal tomato shoot and approximately 92% of the total soluble activity was present in the crude extract. In contrast, of the total peroxidase activity in the crude extract of the dwarf tomato shoot, 56% was accounted for by the cell wall bound D complex. In this case, there was 1.3 times more bound activity than soluble activity in contrast to the normal tomato shoot where more was soluble than bound. In the dwarf, 86% of the soluble, nonionically bound activity was extracted in the crude extract. On a 100-g wet weight basis, the dwarf tomato shoots had 3.5 times more total soluble peroxidase activity than the normal plants. Overall, there was 5.2 times more peroxidase activity (soluble and bound) in the crude extract of the dwarf plant compared to the normal on a wet weight basis.

Table I. Total Activities[a] of Soluble and Cell Wall (CW) Bound Peroxidases in Normal (d^+) and Dwarf (d^x) Tomato Shoots

extractant	normal		dwarf	
	soluble	CW bound	soluble	CW bound
crude H_2O ext	153.0 ± 20.5	66.4 ± 9.9	499.5 ± 27.9	638.2 ± 59.6
first H_2O wash	10.6 ± 0.7		45.7 ± 14.3	
second H_2O wash	2.2 ± 0.4		20.6 ± 2.1	
third H_2O wash	1.0 ± 0.2		11.2 ± 2.1	
fourth H_2O wash	0.5 ± 0.1		6.2 ± 1.0	
first $CaCl_2$ wash[b]		349.7 ± 1.9		1501.3 ± 77.8
second $CaCl_2$ wash		38.0 ± 3.3		216.6 ± 8.0
third $CaCl_2$ wash		9.8 ± 0.5		57.8 ± 5.4
fourth $CaCl_2$ wash		6.1 ± 1.3		33.5 ± 3.5
totals	167.3 ± 21.5	470.0 ± 9.8	583.2 ± 18.1	2447.4 ± 132.4
total soluble + bound	637.3 ± 30.5		3030.6 ± 34.7	

[a] Micromoles of tetraguaiacol produced per minute ± SD from three separate experiments using 100 g of fresh material. [b] 0.2 M $CaCl_2$.

Table II. Percentage Dry Matter[a] of Whole Shoots and Cell Wall Fractions in Normal and Dwarf Tomato Plants

	whole shoot	cell wall	cell wall/whole shoot (×100)
normal (d^+)	8.4 ± 0.3	1.8 ± 0.1	21.4
dwarf (d^x)	7.9 ± 0.1	2.7 ± 0.1	34.2

[a] Averages ± SD of three determinations. Dry matter (DM) was determined by AOAC Method 7.007 (AOAC, 1984) and expressed as grams of DM/100 g fresh weight.

Extraction with 0.2 M $CaCl_2$ released considerably more peroxidase activity than in the original crude extract from both normal and dwarf tomato shoots, the D complex being the only isoenzyme present in the starch–gel (Figure 1). In the normal tomato shoot, cell wall bound activity was 2.8 times the soluble activity and the bound peroxidase activity comprised 74% of the total peroxidase activity. In contrast, the dwarf tomato shoot had cell wall bound peroxidase activity that was 4.2 times greater than the soluble activity. The cell wall bound activity accounted for 81% of the total peroxidase activity in the dwarf tomato shoot. Contrasting dwarf and normal tomato shoots as a whole, soluble, nonionically bound peroxidases A–C and A′ were 3.4 times greater in the dwarf while the cell wall bound D complex was 5.2 times greater in the dwarf tomato shoot. Overall, total soluble and bound activity in the dwarf was 4.8 times greater than in the normal tomato shoot. Of the total activity applied to the cell wall columns, 94–98% of the activities was recovered.

The dry matter data from the normal and dwarf plants are in Table II. The data were similar for the whole shoots in normal and dwarf plants, being ca. 8% DM. However, the H_2O- and $CaCl_2$-washed pulp, or cell wall fraction, was 1.5 times greater in the dwarf plant. As a percentage of the DM, the cell walls from the dwarf plant were 12.8 percentage units greater than the normal counterpart, 21.4% and 34.2%, respectively.

The specificity of this cell wall binding for one peroxidase isoenzyme is not yet known but is indicative of a certain amount since the other isoenzymes did not bind at this neutral and physiological pH. It has been shown that the cell walls can act as ion-exchange columns (Jansen et al., 1960) provided that pectin is present in the cell walls and not removed by polygalacturonase. This binding can be specific for some endogenous enzymes and nonspecific for some exogenous proteins. The binding capacity of the tomato cell walls for the D complex is great and much more than the endogenous amounts that were extracted in the crude extract and with the $CaCl_2$ washes. Extracts containing a mixture of the isoenzymes or only the D complex have been applied to the cell wall columns in excess of 10 000 μmol of tetraguaiacol equivalents with complete adsorption of the bound isoenzyme. The upper limits of the binding capacity of the cell walls for the D complex have not yet been determined, but it will be far in excess of the physiological concentrations (activities). The specificity of this binding does not extend to the genotypic difference between the normal and dwarf tomato shoots since cell walls from normal or dwarf plants will bind the D complex from its counterpart.

From the present data, the physiological concentration (activity) of the cell wall bound peroxidase is 260 μmol of tetraguaiacol equiv/g of cell wall for the normal tomato shoot and 906 μmol of tetraguaiacol equiv/g of cell wall for the dwarf shoot. On this basis, the dwarf tomato shoot had 3.5 times the activity of the normal plant on a per gram cell wall basis as opposed to the 5.2-fold increase based solely on the 100-g wet weight basis (Tables I and II).

In addition to this cell wall peroxidase and the other nonionically bound peroxidases, additional peroxidase activity was still detected histochemically in the H_2O- and $CaCl_2$-washed cell wall fractions (pulp). Exhaustive washing with 8 M urea, higher salt concentrations, and detergents would not remove this activity. It is highly probable that this peroxidase activity was incorporated into the cell walls in a covalent manner. This residual activity was not due to unbroken cells. The washed tomato cell walls were autoclaved for 30 min at 121 °C (103.5 kPa) to destroy this residual activity, and the cell walls still retained their ability to bind the D complex. In addition, tomato fruit pericarp cell walls contain no D complex (Evans and Alldridge, 1965) but did bind the D complex in the crude extract from shoots of either normal or dwarf tomato plants. Thus, the capacity to bind the D complex was present in the tomato fruit cell walls, but isoenzyme synthesis had not occurred, perhaps because these cell walls would not normally lignify.

Numerous functions have been attributed to peroxidases (Saunders et al., 1964; Gaspar et al., 1982; Paul, 1986), and the cell wall peroxidase found in the normal and dwarf tomato shoots might very well be involved in lignin biosynthesis, suberization, and/or resistance to pathogens, provided the proper precursors are synthesized and transported at the appropriate time during the ontogeny of the plant. Whether higher peroxidase activity, soluble or bound, in dwarf plants is the cause or result of this dwarfing is not yet known.

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Carbon-13 NMR Studies of Glycinin and β-Conglycinin at Neutral pH

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Carbon-13 NMR spectra have been obtained at 4.7 T for soy β-conglycinin and glycinin in pH 7.0 solutions containing 35 mM phosphate and 0.4 M NaCl. Peak assignments for amino acids in both proteins and for the carbohydrate side chain of β-conglycinin were based on published compositions and chemical shifts and the results of *J*-modulated double-spin-echo experiments. Structural differences between glycinin and β-conglycinin included (a) differences in the apparent areas of spectral envelopes for aliphatic, α-carbon, and carboxyl groups and (b) bandwidth differences in peaks for glutamine and glutamate, (c) differences in backbone and side-chain flexibility, and (d) appearance of the carbohydrate moiety of β-conglycinin. Structural similarities included (1) proline in the trans conformation and (2) a single peak for the guanidino carbon of arginine.

Nuclear magnetic resonance spectroscopy (NMR) is a powerful tool for obtaining both gross structural and microenvironmental information about proteins. NMR studies of food proteins from seeds have emphasized the alcohol-extractable proteins (prolamines) from cereal grains. The NMR characteristics of corn zein (Augustine and Baianu, 1986, 1987), wheat glutenins and gliadins (Baianu, 1981; Baianu et al., 1982), wheat gluten (Belton, et al., 1987), and C hordein from barley (Tatham et al., 1985) have been studied. None of these studies has investigated the aqueous salt-extractable proteins of cereal grains or oilseeds by NMR.

NMR studies of soy proteins have emphasized the nonstorage proteins such as soy leghemoglobin (Trewhella et al., 1986; Mabbutt and Wright, 1983), lipoxygenase (Slappendel et al., 1982), and trypsin inhibitor (Baillargeon et al., 1980) or metabolism of specific amino acids during soybean germination (Coker et al., 1987). While purified soy proteins have been examined previously by NMR (Kakalis and Baianu, 1985; Baianu, 1989), and some peaks tentatively assigned, to our knowledge no effort to fully examine glycinin and β-conglycinin by ^{13}C NMR at neutral pH has been made.

To date, the structural studies of soy storage proteins have focused on gross structural changes observable by either calorimetric (Hermansson, 1978) or spectrophotometric (Dev et al., 1988) methods, particularly CD and ORD (Ishino and Kudo, 1980; Yamauchi et al., 1979; Koshiyama and Fukushima, 1973). Recently, the conformational conclusions made with CD and ORD have been qualitatively confirmed with FT-IR (Dev et al., 1988). In this study, the structures of the soy storage proteins glycinin and β-conglycinin are examined by solution-state ^{13}C NMR, many peaks assigned, relaxation times determined, and some conclusions drawn regarding structure and conformation of these two proteins.

PROCEDURE

Protein Isolation and Purification. Crude glycinin was prepared from Nutrasoy 7B flakes (Archer Daniels Midland Co., Decatur, IL) by a modification of the procedure of Thanh et al. (1975), which is based on the differential solubility of β-conglycinin and glycinin in 0.063 M Tris buffer (pH 6.6) con-

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