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Peptides isolated from cell walls of *Medicago truncatula* nodules and uninfected root

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Abstract

The hydroxyproline-rich root nodules of legumes provide a microaerobic niche for symbiotic nitrogen-fixing Rhizobacteria. The contributions of the cell wall and associated structural proteins, particularly the hydroxyproline-rich glycoproteins (HRGPs), are therefore of interest. Our approach involved identification of the protein components by direct chemical analysis of the insoluble wall. Chymotryptic peptide mapping showed a "P3-type" extensin containing the highly arabinosylated Ser-Hyp_4-Ser-Hyp-Ser-Hyp_4-Tyr_3-Lys motif as a major component. Cell wall amino acid analyses and quantitative hydroxyproline arabinoside profiles, predominantly of tri- and tetraarabinosides, confirmed this extensin as the major structural protein in the cell walls of both root nodules and uninfected roots. On the other hand, judging from the Pro, Glu and non-glycosylated Hyp content, the nodule-specific proline-rich glycoproteins, such as the early nodulins (ENOD-PRPs), are present in much lesser amounts. Although we isolated no PRP peptides from nodule cell walls, a single PRP peptide from root cell walls confirmed the presence of a PRP in roots and represented the first direct evidence for a crosslinked PRP in muro. Compared with root cell walls (~7% protein dry weight) nodule cell walls contained significantly more protein (~13% dry weight) with an overall amino acid and peptide composition indicating the presence of structural protein unrelated to the HRGPs. © 2000 Published by Elsevier Science Ltd.

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1. Introduction

Root nodules, nitrogen-fixing organs characteristic of the *Leguminosae* (Brown and Walsh, 1996) sequester rhizobial symbionts in a way that facilitates nutrient exchange while protecting the oxygen-sensitive nitrogenase. The ability to maintain a microaerobic compartment in an actively respiring tissue (Long, 1989; Brewin, 1991; Dalton et al., 1998) is reflected in nodule morphology that restricts groups of bacteroids to a central medulla (Cassab, 1986). Precisely how nodule structure determines function is not clear, although it is undoubtedly complex and comprised of several metabolic and structural components that determine the oxygen diffusion path and oxygen availability.

As prime structural components, there is particular interest in understanding how cell walls contribute to the physiological function of the nodule. Nodule walls exhibit a range of physical and chemical properties that can limit oxygen diffusion through the walls of the endodermis for example, with gaseous exchange restricted to certain "choke points" (Jacobsen et al., 1998). On the other hand cell wall plasticity allows cells to expand. The ensuing cell separation results in a network of intercellular spaces that facilitate oxygen diffusion throughout the nodule cortex. However, the precise molecular details that determine the cell wall properties responsible for regulating oxygen diffusion through the nodule

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are still speculative (Skot et al., 1996; Weisbach et al., 1999). It is therefore important to identify the major structural proteins and their relative contributions to the nodule extracellular matrix.

Generally three related groups of Hyp/Pro-containing proteins (Kieliszewski and Lamport, 1994) contribute to cell walls: moderately glycosylated extensins typified by repetitive Ser-Hyp_4 motifs; heavily glycosylated arabinogalactan-proteins (AGPs) characterized by clustered non-contiguous Hyp-polysaccharide addition sites (Shpak et al., 1999); and the lightly glycosylated, highly repetitive proline/hydroxyproline-rich proteins (PRPs). Although their relative proportions may vary greatly, extensins occur as covalently crosslinked networks, while AGPs, initially membrane-bound by glycosylphosphatidyl inositol anchors, probably occur generally as freely soluble macromolecules (Nothnagel, 1997; Youl et al., 1998; Svetek et al., 1999). Finally, the less abundant and less well-characterized PRPs also occur as soluble (Averyhart-Fullard et al., 1988; Tierney et al., 1988; Kieliszewski et al., 1992) and presumably as crosslinked forms (Bradley et al., 1992).

Judging from the abundance of firmly bound Hyp in root nodules (Cassab, 1986) and immunocytochemical detection (Benhamou et al., 1991), an extensin network is a component of nodule cell walls. Nodules also contain soluble Hyp-rich macromolecules in the form of AGPs (Cassab, 1986). However, HRGPs have yet to be isolated from nodules and chemically characterized. This includes the more recently discovered nodulin-PRPs encoded by nodule-specific genes whose mRNA transcripts are a prominent and intriguing feature of early nodule development and therefore implicated in nodule function (van de Wiel et al., 1990; De Bruijn et al., 1994). Thus two general questions arise: what are the relative structural contributions of each HRGP to the nodule and how can we relate their structure to the physiological and biochemical function of nodules?

In the work reported here, we analyzed the insoluble (presumably crosslinked) cell wall protein components isolated from root nodules. As a control we analyzed the walls of uninfected roots. Peptide mapping yielded major peptides characteristic of the repetitive P3-type extensin motif, SO_4SOSO_4YYYK (Smith et al., 1986) (single letter code where O = Hyp) indicating that extensin was the predominant structural protein of both nodule and root cell walls. PRPs were evident only as minor peptides in enzymic digests of root cell walls and AGP peptides not at all. Surprisingly we observed no PRP peptides in enzymic digests of nodule cell walls, although other peptides completely lacking Hyp were present, possibly corresponding to other structural proteins. Hydroxyproline glycoside profiles confirmed the conclusion that AGPs are not part of the crosslinked wall matrix and that extensin is the major structural protein in both nodules and roots.

2. Results and discussion

2.1. HRGP identification

We classify HRGPs into three classes based on their amino acid composition, peptide periodicity and Hyp glycosylation. Therefore our approach involved: (1) Isolation of the cell walls; (2) Quantitative amino acid analysis; (3) Characterization of the Hyp-glycoside profiles; (4) HF-deglycosylation of the cell walls to facilitate proteolytic digestion; and (5) Chymotryptic degradation of the HF-treated cell walls followed by peptide mapping and identification of the major peptides released.

2.2. Amino acid composition of cell walls

There was an almost two-fold difference between the protein content of nodule walls and the walls isolated from uninfected roots (Fig. 1, Tables 1 and 2). However, the low Hyp content, much less than the 30–42 mol% Hyp typical of extensins (Table 1) (Smith et al., 1984), indicates the presence of other protein lacking Hyp.

This non-HRGP protein component(s) is presumably structural as it is abundant, and like extensin, remains firmly bound to the wall during HF-solvolysis. Indeed, structural wall proteins lacking Hyp are widespread, as evidenced by earlier amino acid analyses of walls isolated from maize coleoptile and rice meristem as well as tomato, sugar beet, maize, asparagus and Douglas fir suspension culture cells (Kieliszewski et al., 1992). For example, the Douglas fir cell wall containing ~20% protein has very little Hyp (Table 2) and a relatively low glycine content; clearly neither extensins nor glycine-rich proteins, typically 67 mol% glycine (Condit and Meagher, 1986) are major contributors.

2.3. Amino acid composition after HF-solvolysis and proteolysis

For both nodule and root cell walls, anhydrous HF solvolysis of the wall polysaccharides removed about two thirds of the cell wall weight but essentially no Hyp judging by recoveries of Hyp and protein in the remaining HF-insoluble residue (~97% of total Hyp) (Tables 1 and 2). This was corroborated by amino acid analyses of the HF-soluble non-dialyzable wall fractions (large molecular weight material retained by the dialysis membrane) which contained no Hyp (not shown). However, a considerable portion of the root protein (~29% dry wt) was lost during dialysis of the HF-treated wall, although not as precipitate. In contrast, only 10% of the nodule total wall protein was lost during dialysis of the HF-treated wall. This dialyzable protein component was presumably small molecular weight material as the dialysis membrane molecular weight cutoff was 3.5 kDa.

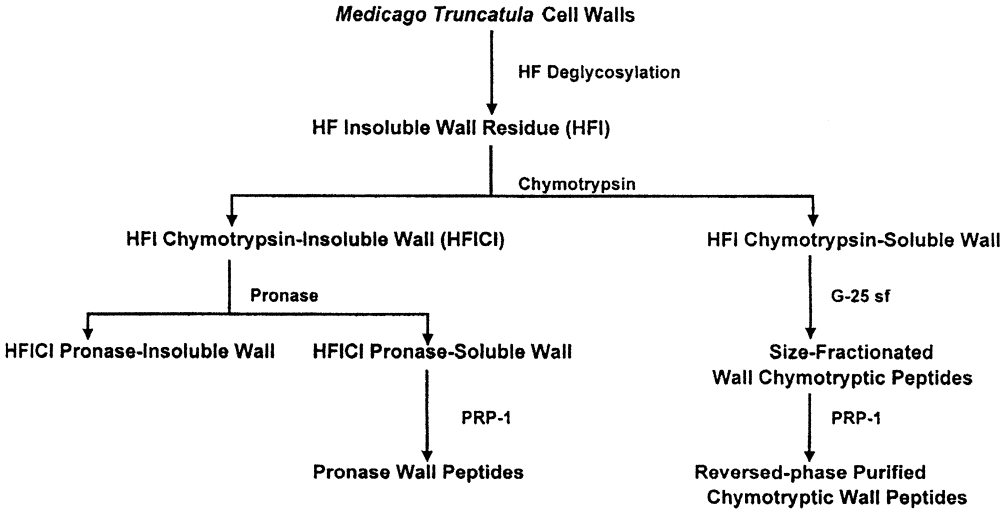

Fig. 1. Flow chart outlining the isolation of *M. truncatula* root and nodule wall peptides. Wall preparations of the root and nodule were deglycosylated with anhydrous HF before chymotryptic digestion of the remaining HF-insoluble residues (HFI). The solubilized chymotryptic peptides were isolated by gel permeation (G-25sf) and polymeric reversed-phase liquid chromatography (designated PRP-1), then sequenced. The chymotrypsin-insoluble wall residues (HFICI) were digested further with pronase.

Table 1
Amino acid compositions of *Medicago truncatula* root cell walls before and after treatment with anhydrous HF and chymotrypsin. The compositions of tomato extensins are given for comparison

				Tomato extensins[d]	
Amino acid[a]	Wall	HFI[b]	HFCI[c]	P1	P2
Asx	8.1	8.5	6.8	1.4	0.7
Glx	6.4	7.7	5.7	1.5	0.3
Hyp	13.3	12.3	14.0	32.7	41.8
Ser	9.1	7.8	8.6	9.8	12.1
Gly	7.4	6.9	7.6	1.7	0.3
His	2.5	2.5	1.9	6.1	1.0
Arg	3.1	3.0	2.4	0.7	0.1
Thr	5.1	4.9	5.0	6.2	1.0
Ala	6.6	6.5	7.2	2.9	0.5
Pro	5.4	5.8	6.4	9.6	0.8
Tyr	3.5	3.6	3.0	7.7	14.9
Val	7.1	6.9	8.0	8.3	5.1
Met	1.2	0.9	1.0	0.0	0.0
Ile	4.0	4.6	4.8	1.0	0.9
Leu	6.8	7.4	7.3	1.0	0.2
Phe	3.1	3.6	3.9	0.0	0.2
Lys	6.9	6.9	5.7	9.5	20.1
Percent protein	7	15	23	n.d.[e]	n.d.[e]

[a] mol%.
[b] HFI: HF-insoluble wall.
[c] HFCI: HF- & chymotrypsin-insoluble wall residue.
[d] Tomato extensin precursors P1 & P2 (Smith et al., 1984).
[e] n.d. not determined.

The bulk of the wall protein in both walls is HF-insoluble, suggesting that it participates in a crosslinked network. However, amino acids accounted for only 15% of the root HF-insoluble residue and for 31% of the nodule residue, evidence for the presence of an unknown wall component that is not protein. Nor is the component carbohydrate: anhydrous HF at 0°C rapidly cleaves the glycosidic linkages of neutral sugars and uronic acid residues, thereby removing wall polysaccharides, and although HF at 0°C does not cleave the bonds of amino sugars, wall polysaccharides contain no amino sugars (Mort and Lamport, 1977). This unknown bulk component has been previously noted for walls of tomato cell suspension cultures (Mort and Lamport, 1977), legume root hairs (Mort and Grover, 1988), and maize cell suspension cultures (Kieliszewski, 1989), and may correspond to suberin (Brown and Walsh, 1996), or "juvenile lignin" (Musel et al., 1997). Both contain ester linkages and therefore might be substrates for the esterase activity of chymotrypsin. Chemical analyses of wall phenolics and aliphatic components that remain after HF-deglycosylation will resolve the issue.

2.4. Proteolysis of the HF-insoluble cell wall residue

Chymotrypsin solubilized about two thirds of the insoluble residue remaining after HF-solvolysis of root or nodule walls. This chymotrypsin-soluble fraction accounted for about a half of the original wall protein and Hyp of root cell walls (Table 1) and about two thirds of the original wall protein and Hyp of nodule cell walls (Table 2). Further digestion of the chymotrypsin-insoluble root and nodule residues by pronase solubilized little more material (e.g. 40 μg containing 2 μg Hyp from 1 mg) leaving a Hyp-rich insoluble residue. The low yield and wide size range of the pronase-soluble material precluded further characterization. In summary, a significant amount of wall protein and Hyp remained completely insoluble after protease digestion, along with a non-protein, non-carbohydrate component. This suggests the wall protein is either covalently

Table 2
Amino acid composition of the *Medicago truncatula* nodule wall before and after treatment with HF and chymotrypsin. Compositions of *Medicago* PRP4, ENOD12, and the Douglas fir cell wall are presented for comparison

Amino acid[a]	*Medicago* nodule			PRPs		
	Wall	HFI[b]	HFCI[c]	PRP4[d]	ENOD12[e]	Douglas Fir wall[f]
Asx	8.3	8.5	6.8	0.4	7.6	9.4
Glx	7.6	6.8	7.7	10.9	10.1	11.8
Hyp	6.4	8.0	11.6	–	–	0.8
Ser	8.2	8.4	7.9	0.6	3.8	7.5
Gly	12.0	9.2	6.9	0.0	0.0	9.5
His	2.3	2.5	2.0	6.8	10.1	1.6
Arg	4.1	3.8	3.0	0.0	3.8	4.6
Thr	4.7	5.4	4.8	0.6	2.5	5.3
Ala	6.3	7.9	7.7	0.4	2.5	9.0
Pro	5.6	5.6	7.0	35.0	30.4	5.4
Tyr	4.0	3.0	3.3	4.9	5.1	1.9
Val	7.0	7.7	8.4	16.3	6.3	6.7
Met	1.6	1.9	1.3	0.2	0.0	n.d.
Ile	4.3	4.8	4.4	1.0	1.3	5.0
Leu	6.8	7.9	7.0	3.0	1.3	10.6
Phe	3.6	3.8	3.4	0.6	1.3	3.5
Lys	5.8	5.5	6.0	19.2	13.9	6.6
Percent protein	13	31	35	–	–	20

[a] mol%.
[b] HFI: HF-insoluble wall.
[c] HFCI: HF- & chymotrypsin-insoluble wall.
[d] Deduced from Medicago cDNA (Wilson et al., 1994).
[e] Deduced from alfalfa cDNA (Pichon et al., 1992).
[f] From Kieliszewski et al. (1992).

bound to the non-protein component or that the two comprise independent but entangled networks.

2.5. Peptide mapping

Peptide mapping of the soluble nodule and root chymotryptic digests on Sephadex G-25 (Fig. 2) gave unresolved, overlapping peaks. On reversed-phase chromatography, the G25 peaks resolved into a relatively small number of major peptides that were sequenced as well as numerous unresolved minor UV-absorbing peaks (Figs. 3 and 4; Tables 3 and 4).

HRGP peptides related to the P3-type extensin made the largest contribution to the peptide maps; i.e. from uninfected root,G5:P50 (Fig. 3b), and nodule peptides G2:P30, G4:P47 and G5:P47 (Fig. 4; Tables 3 and 4, respectively). Two unsequenced nodule peptides with elution times of 47 and 50 min respectively (see Fig. 4d) also corresponded to P3 extensin peptides judging by amino acid analyses. The predominance of P3 extensin peptides is significant as P3-type extensins seem to be the constitutive extensins of many species including tomato (Smith et al., 1986; Showalter et al., 1991), potato (Bown et al., 1993), tobacco (Chen et al., 1992; Schwacke and Hager, 1992), petunia (Showalter and Varner, 1989), soybean (Hong et al., 1994), common bean (Reynolds et al., 1995), parsley (Kawalleck et al., 1995), cotton (Qi et al., 1995), cow pea (Arsenijevic-Maksimovic et al., 1997) and even monocots like maize (Rubinstein et al., 1995). Root peptides G5:P37 and G5:P38 (Table 3) are related to P1-type extensins (possessing the VYK putative crosslink motif) while the nodules contained G2:P21 (Table 4), another extensin-like peptide.

2.6. Evidence for PRP nodulins

None of the major peptides sequenced from the enzymic digestions of root or nodule cell walls contained the penta- or hexapeptide motifs POXYZ or POOXYZ (O = hydroxyproline). Such motifs are typical of PRPs, which presumably include the early nodulins ENOD2 (typically (P)PPHEK and (P)PPEYQ pentamers and hexamers, which have been identified at the DNA level) (Franssen et al., 1987; van de Wiel et al., 1990; Dehio and De Bruijn, 1992; Perlick and Puhler, 1993) and ENOD12 (typically PPVXK, PPQXE pentamers) (Scheres et al., 1990; Govers et al., 1991; Pichon et al., 1992; Allison et al., 1993). The absence of these motifs from the major peptides was somewhat surprising considering the following:

First, PRP nodulin mRNAs are abundant and widely distributed throughout nodule tissues; i.e. cortex, PRP1; inner cortex/nodule parenchyma, PRP4 (Wilson et al., 1994); medulla, ENOD12 (Pichon et al., 1992); and meristem, PRP4 (Wilson et al., 1994).

Secondly, earlier immunohistochemical evidence localizes these proteins in the wall matrix as well as intercellular spaces (Sherrier and VandenBosch, 1994).

Thirdly, some PRPs possess XYK motifs related to the putative extensin crosslink motif VYK (Schnabelrauch et al., 1996).

Finally, *Rhizobium* induces the early appearance of a possible crosslinking peroxidase (Cook et al., 1995).

However, little direct evidence for PRP crosslinking exists. For example, PRP crosslinking in bean cells treated with a fungal elicitor (Bradley et al., 1992) involved an indirect assay based on a decrease of soluble PRPs (Bradley et al., 1992) and therefore did not exclude the possibility that PRP monomers were rapidly degraded rather than rendered insoluble by crosslinking. Furthermore, extensin peroxidase rapidly crosslinked extensin monomers in vitro but did not crosslink PRPs from soybean (Schnabelrauch et al., 1996). Nevertheless, the minor peptide G5:P44 (Table 4) isolated from the root cell shares extensive sequence identity with the repetitive motif KPPVEKPPVY of MtPRP2 and MtPRP4 (Wilson et al., 1994), with only a single A for P substitution at residue 2. Thus G5:P44 is the first PRP fragment to be isolated from the cell wall and therefore the first direct evidence for a crosslinked PRP in muro. The wall Hyp arabinoside profiles also indicate

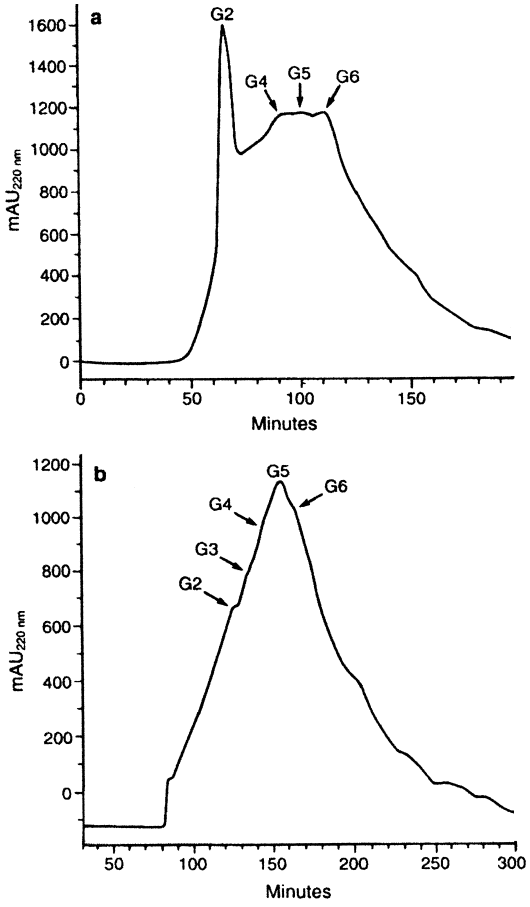

Fig. 2. Fractionation of the root (a) and nodule (b) chymotryptic peptides by G25 superfine gel permeation chromatography. The elution times vary from panel (a) to panel (b) because a longer G25 column was used to fractionate the nodule peptides (see Section 3). (a) The root cell wall yielded several chymotryptic fractions designated G2 and G4–G6. (b) The nodule cell wall yielded a major broad peak which we collected in parts (G2–G6) for further fractionation.

the presence of PRPs, as 17–18% of the wall Hyp occurred as non-glycosylated Hyp (Table 5). Unlike extensin Hyp residues (Smith et al. 1984), PRP-Hyp residues are arabinosylated to a lesser degree (Kieliszewski et al., 1995, 1992). However, PRPs, which generally contain approximately equimolar Pro and Hyp, and often substantial Glx, were not obviously retained with the insoluble wall, as the amount of wall-bound Glx and Pro did not increase after removal of the extensin-rich wall peptides by chymotrypsin (Tables 1 and 2). Thus the amino acid and peptide compositions of root and nodule walls indicate that some PRPs are probably relatively minor components of the crosslinked wall, i.e. they are freely soluble and removed during preparation of the wall fraction.

2.7. Nodule peptides lacking hydroxyproline

Our analyses of isolated nodule walls also emphasized the likely existence of major non-HRGP protein components represented by peptide G4:P22, Ala-Lys-Ala-Glu-Ile-Val-Arg-Lys-, (Table 4). Possibly the peptides lacking Hyp (G4:P22, G4:P32a and b, G4:P47b, and G6:P33 a and b) simply arise from extensin "insertion sequence" motifs (Kieliszewski and Lamport, 1994; Smith et al., 1986) which characteristically lack Hyp. Indeed, the non-HRGP peptides collectively have a biased amino acid composition rich in Ala, Lys, Pro, Val, Tyr and His, which, except for Ala, are common amino acids in extensins. On the other hand Hyp accounts for only ~6 mol% of the total amino acids contained in the nodule wall indicating abundant non-HRGP structural protein as well.

In summary, this work demonstrates extensin as the major HRGP component of the crosslinked nodule and root cell walls and supports the suggestion that "Extensins might play a special role in cell walls of legume nodules" (Perlick and Puhler, 1993). The levels of crosslinked Hyp-rich nodulins were substantially less than expected. Although discrepancies between mRNA levels and levels of expressed protein products have been noted in other systems (Shaul et al., 1999), it seems more likely that the abundance of ENOD2 and ENOD12 cognate mRNAs simply reflects a group of freely soluble PRPs whose roles in the wall do not necessarily include crosslinking. Thus it remains for future work to isolate

nodulin PRPs for further biochemical characterization. We also need to characterize the non-protein, non-carbohydrate component of the wall as it contributes substantially to the network architecture. Finally, we need to identify the hypothetical non-HRGP structural protein that contributes to the root and nodule cell walls of *M. truncatula* and to determine whether relatively protein-rich cell walls constitute a general feature of root nodules in other species.

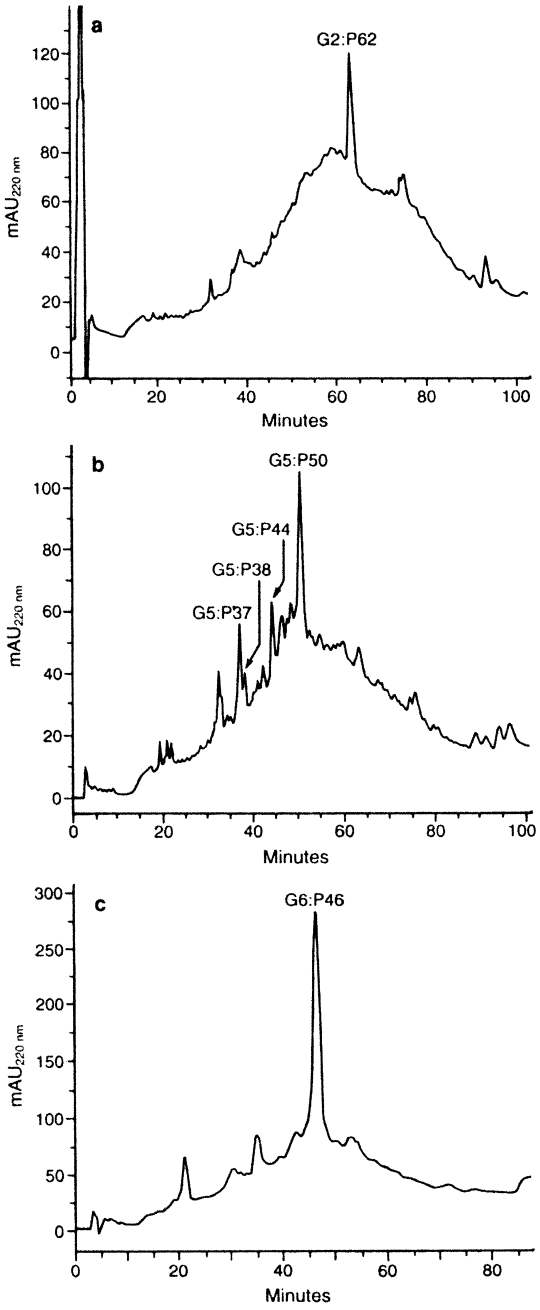

Fig. 3. Reversed-phase peptide maps of *M. truncatula* root wall chymotryptic peptides. (a) G25 peak G2 yielded one major peak, G2:P62, when fractionated on a reversed-phase column. G2:P62, gave no sequence on Edman degradation. (b) Fractionation of G25 peak G5 gave several peptides, four of which we sequenced: G5:P37, G5:P38, G5:P44, and G5:P50. (c) Fractionation of G25 peak G6 gave one major peptide, G6:P46. Note: Not shown are the reversed-phase fractionations of root G25 peak G4 and the shoulder before G4 (see Fig. 2a). Both yielded only broad UV-absorbing profiles similar to that in panel (a) above, but without any discrete peaks.

3. Experimental

3.1. Medicago truncatula nodule/root tissue preparation

We grew *Medicago truncatula* (A17), either uninoculated or after inoculation with *R. meliloti* (ABS7 m), hydroponically for 14 days as previously described (Gallusci et al., 1991; Cook et al., 1995). Roots or nodulated roots were kept frozen at −70°C. The tweezer-picked nodules were free of root tissue as determined by inspection under a magnifying glass.

3.2. Cell wall isolation

Roots or nodules were frozen in liquid nitrogen, with the tissue then ground to powder in a mortar which was suspended in 100 mM $AlCl_3$ (Smith et al., 1984) at 4°C overnight. We collected the walls by centrifugation then washed them repeatedly with dH_2O to free the walls of large cellular debris. Next we removed any small debris by layering the walls on a 20% sucrose cushion allowing the walls to settle overnight at 4°C; cell debris was retained in the water layer above the sucrose cushion. After removing the layer of cell debris, we collected the walls by centrifugation, then removed residual sucrose via alternating dH_2O-washes and centrifugation.

3.3. Anhydrous hydrogen fluoride deglycosylation

We deglycosylated cell wall preparations in anhydrous HF containing 10% (v/v) anhydrous methanol as scavenger (20 mg wall in 1 ml HF/MeOH for 1 h at 4°C), quenching the reaction in ice-cold ddH_2O (final concentration 10% (v/v) HF) (Mort and Lamport, 1977; Sanger and Lamport, 1983; Kieliszewski et al., 1995). Walls were dialyzed against ddH_2O for 2 days at 4°C, then freeze-dried.

3.4. Proteolytic degradation of HF-deglycosylated wall

We boiled HF-deglycosylated wall residue (suspended at 10 mg/ml in ddH_2O) for 5 min, then cooled the walls to room temperature before adding an equal volume of freshly prepared 3% (w/v) ammonium bicarbonate (aq) containing 5 mM $CaCl_2$ and chymotrypsin (enzyme to substrate ratio 1 to 100, w/w). After stirring the deglycosylated walls with enzyme for 24 h at 25°C, we added more chymotrypsin or pronase (final enzyme to substrate ratio 1 to 50) for another 24 h incubation. The

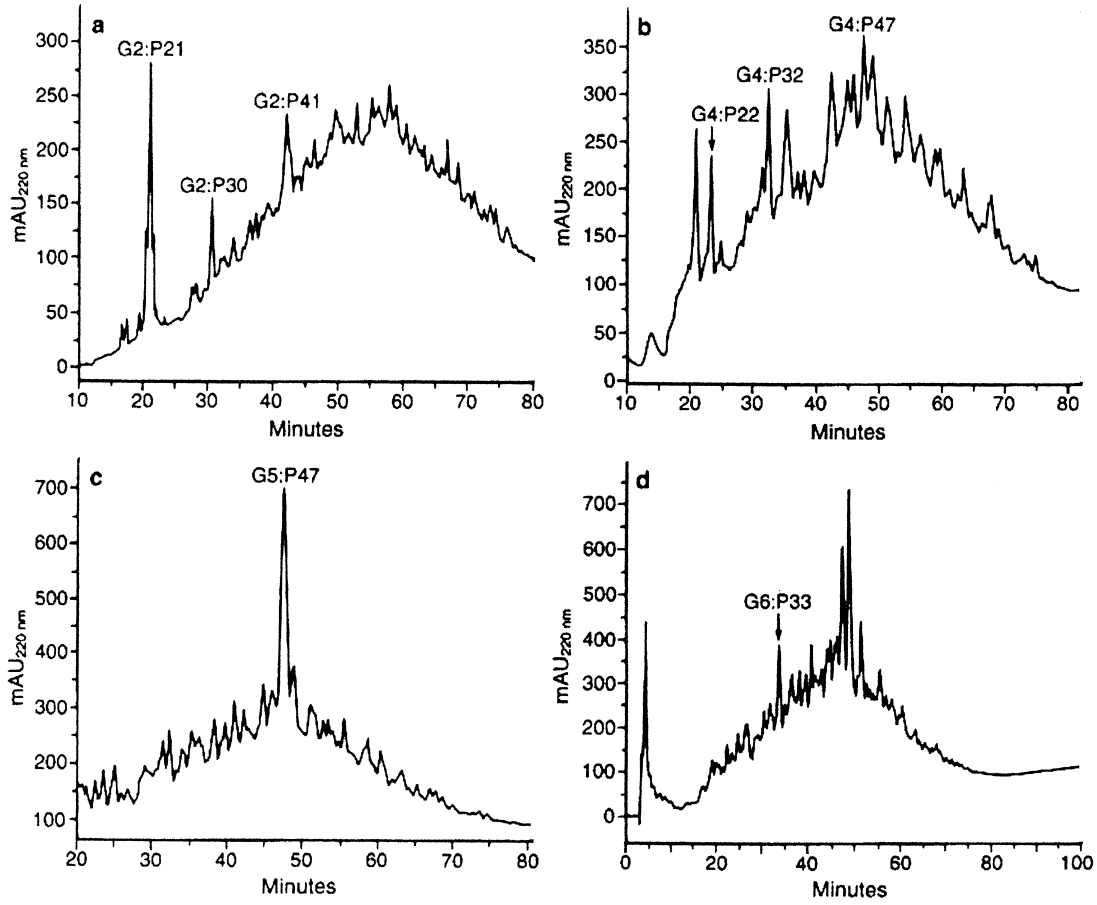

Fig. 4. Reversed-phase peptide maps of *M. truncatula* nodule wall chymotryptic peptides. (a) Reversed-phase fractionation of G25 peak G2 yielded one major peak and several minor peaks which co-chromatographed with UV-absorbing material. Only peaks G2:P21 and G2:P30 yielded peptide sequences on Edman degradation. (b) Nodule G25 peak G4 further fractionated on the reversed phase column produced a few major peaks three of which gave sequences: G4:P22, G4:P32, and G4:P47. (c) G25 peak G5 contained one major peptide fraction, G5:P47, the same peptide as G4:P47 (Table 4). (d) G25 peak G6 yielded one peptide fraction which we sequenced, G6:P33. The peak at 47 min co-chromatographed with nodule peptides G5:P47 and G4:P47a and had a P3-type extensin amino acid composition (Hyp, 65 mol%, Ser, 20%, Tyr, 6%, Lys, 8%), therefore we did not sequence it. Likewise, the peak at 50 min also had a P3-type composition except it contained more Ala (18 mol%, and less Ser, 10%), therefore we did not sequence the peptide.

chymotrypsin-insoluble residue was separated from the chymotrypsin-soluble fraction by pelleting the residue in a microfuge; the supernatant was freeze-dried and the insoluble residue was washed several times with water, then digested with pronase in 1.5% (w/v) ammonium bicarbonate as described above for chymotryptic digests.

3.5. Gel permeation chromatography of chymotryptic digests

We size-fractionated the chymotrypsin solublized fraction of the deglycosylated nodule wall or root wall on a G-25 superfine Sephadex column (10 mm i.d.×30 cm for the root peptides; 10 mm i.d.×45 cm for the nodule peptides; the use of G25 columns having different lengths was incidental and did not affect the quality of the separations) eluted at 0.2 ml/min with 100 mM ammonium acetate. Column eluates were monitored at 220 nm; G-25 fractions were collected and freeze-dried.

3.6. Reversed-phase fractionation of pronase and chymotryptic peptides

The G-25-fractionated chymotryptic peptides were further separated on a Hamilton polymeric reversed-phase column (PRP-1; 5 μm, 150 mm×4.1 mm) equilibrated with 0.1% (v/v) TFA (Buffer A), eluted with a linearly increasing gradient of 0.1% TFA in 80% acetonitrile aq. (Buffer B) at 0.5 ml/min, and monitored at 220 nm. After an initial 5 min of 100% A and 0% B, the gradient increased to 50% B by 100 min. Pronase-soluble peptides released from the chymotrypsin-insoluble residue were also fractionated on the reversed-phase column using the same flow rate and gradient conditions.

Table 3
Chymotryptic peptides isolated from the HF-insoluble residue of *Medicago truncatula* root cell walls

Peptide type	G-25/PRP-1[a] Peak	Sequence
Extensin P1-type[b]	G5:P37	Lys/Ala-Ser-Hyp-Hyp-Hyp-Hyp-Ala-Hyp-Val-Tyr
Extensin P1-type	G5:P38	Lys-Ala/Ser-Hyp-Hyp-Hyp-Hyp-Thr-Hyp-Val-Tyr
Extensin P3-type[b]	G5:P50	Lys-Ala/Ser-Hyp-Hyp-Hyp-X-Ala/Ser-Hyp-Ser-Hyp-Hyp-Hyp-Hyp-X-Tyr[d]
Extensin-like	G6:P46	Lys-Ser-Hyp-Hyp-Hyp-Ser-Ser-
PRP4-type[c]	G5:P44	Lys-Ala-Hyp-Val-Glu-Lys-Pro-Hyp-Val-Tyr

[a] G denotes a G25 peak, P denotes a polymeric reversed-phase peptide peak.
[b] Tomato P1 extensin and Tomato P3 extensin (Smith et al., 1986).
[c] *Medicago* proline-rich protein, PRP4 (Wilson et al., 1994).
[d] X denotes a blank cycle which probably corresponds to one-half isodityrosine, the crosslink amino acid which occurs in P3-type extensins (Epstein and Lamport, 1984).

Table 4
Chymotryptic peptides from the HF-insoluble residue of *Medicago truncatula* nodule cell walls

Peptide type	G-25/Reversed-phase[a] Peak	Sequence
Extensin	G2:P21	His/Asp-Ser-Hyp-Hyp-Hyp-Hyp-Val-His
Extensin P3-type[b]	G2:P30	Lys-Ser-Hyp-Hyp-Hyp-Hyp-Ser-Hyp-Ser-Hyp-Hyp-Hyp-Hyp-Hyp-Tyr
Extensin P3-type	G4:P47a[3]	Lys-Ser-Hyp-Hyp-Hyp-Hyp-Ser-Hyp-Ser-Hyp-Hyp-Hyp-Hyp-X-Tyr[d]
Extensin P3-type	G5:P47	Lys-Ser-Hyp-Hyp-Hyp-Hyp-Ser-Hyp-Ser-Hyp-Hyp-Hyp-Hyp-X-Tyr[d]
Unidentified	G4:P22	Ala-Lys-Ala-Glu-Ile-Val-Arg-Lys
Unidentified	G4:P32a[c]	Val-Ile-Ala-Pro
Unidentified	G4:P32b[c]	Ala-Lys-Pro-Asp-His-His
Unidentified	G4:P47b[c]	Lys-Tyr-Pro-His-Pro-Lys-Pro-Val-Tyr-Tyr
Unidentified	G6:P33a[c]	Lys-Val-Gly-Tyr
Unidentified	G6:P33b[c]	Glu-Asp-Pro-Asn-Ala-Arg

[a] G denotes a G25 peak, P denotes a polymeric reverse phase peptide peak.
[b] Tomato P1 extensin and Tomato P3 extensin (Smith et al., 1986).
[c] Denotes that the G25, reversed-phase peptide peak split into two peptide components on further fractionation via microbore HPLC (carried out at the Michigan State University Macromolecular Facility).
[d] X: blank cycle, which probably corresponds to one-half an isodityrosine residue (Epstein and Lamport, 1984).

Table 5
Hydroxyproline glycoside profiles of *Medicago truncatula* root and nodule walls

Hyp-Glycoside	Root wall	Nodule wall
	Percent of total Hyp	
Hyp Polysaccharide	None	None
Hyp-Ara_4	34	33
Hyp-Ara_3	37	37
Hyp-Ara_2	6	6
Hyp-Ara	6	6
Non-glycosylated Hyp	17	18

3.7. Microbore reversed-phase fractionation of chymotryptic peptides

Peptides G4:P47, G4:P32, and G6:P33 (See Table 4) from the Hamilton polymeric reverse phase column were further purified on an Applied Biosystems Model 130 microbore HPLC system (Foster City, CA) eluted at a flow rate of 40 μl/min (data not shown). The C18 Vydec column (5 μm, 0.8 mm×250 mm; San Francisco, CA) was equilibrated in 95% Start Buffer/5% End Buffer (Start Buffer was 0.05% aqueous heptafluorobutyric acid [HFBA]; End Buffer was 0.0467% HFBA containing 95% aqueous acetonitrile) and eluted with a linearly increasing gradient. The gradient increased to 45% end buffer over 140 min, then increased to 80% end buffer by 160 min.

3.8. Hydroxyproline assays

We determined the Hyp content of samples after acid hydrolysis (6 N HCl, 110oC, 18 h) as detailed earlier (Kivirikko and Liesmaa, 1959), involving alkaline hypobromite oxidation and subsequent coupling with acidic Ehrlich's reagent, monitoring at A_{560nm}.

3.9. Amino acid analysis

We determined amino acids in acid hydrolysates of walls and peptides as 6-aminoquinolyl-*N*-hydroxysuccinimidyl (AQC) derivatives by the use of a Waters AccQ.Tag™ column and reagent kit (Crimmins and Cherian, 1997; van Wandelen and Cohen, 1997).

3.10. Hydroxyproline glycoside profile

We determined Hyp arabinoside profiles for root and nodule walls after alkaline hydrolysis (0.22 M $Ba(OH)_2$ 18 h, 105°C), followed by neutralization with concentrated H_2SO_4, pelleting the precipitate and immediate application of the supernatant, containing 100–300 μg Hyp, to a 75-×0.6-cm column (H^+ form) of Technicon Chromobeads C washed with water and eluted with a linear 0 to 0.6 N HCl gradient. The post-column Hyp assay was continuously monitored at A_{560} nm as previously described (Lamport and Miller, 1971).

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