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Cross-Linking of Soluble Extensin in Isolated Cell Walls[1]

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ABSTRACT

The extensin component of primary cell walls has generally been considered to be an intrinsically insoluble cell wall glycoprotein. Recent data have established that cell wall extensin is in fact secreted in a soluble monomeric form which slowly becomes insolubilized in the cell wall probably through the oxidative formation of isodityrosine cross-links. We now show that isolated cell walls from aerated root slices of *Daucus carota* have the capacity to insolubilize extensin through the formation of isodityrosine. This *in vitro* cross-linking is specific for the extensin glycoprotein, as other wall proteins are not cross-linked by the isolated wall system. Although extensin can be cross-linked in solution by peroxidase and H_2O_2, dityrosine and not isodityrosine is the phenolic cross-link formed. Wall-catalyzed cross-linking of soluble extensin is inhibited by L-ascorbate, and both the initial rate and total extent of cross-linking are inhibited by acidic pH in the physiological range (pH 4 to 6). We suggest several mechanisms by which acid might inhibit cross-linking and propose that cytoplasmic factors (ascorbate and/or hydrogen ions) may regulate the solubility of extensin *in vivo*.

Cell walls of dicotyledonous plants contain an insoluble glycoprotein component which is distinguished by its content of 4-hydroxyproline and has been given the name extensin (for review, see 7, 19). Despite suggestions that this integral wall polymer is important in growth control (4, 17, 25), disease resistance (10), and morphogenesis (1), the role of extensin in cell wall structure and function remains speculative and thus controversial (12, 19).

The biochemical characterization of extensin has proved especially difficult. For two decades it was generally believed that wall extensin is an intrinsically insoluble HRGP[3] which could only be solubilized by proteolysis (17, 18). Recently, we have shown, using aerated carrot root slices, that insoluble wall extensin is secreted into the cell wall as a soluble monomeric HRGP (5) which has been well characterized (28, 29). This soluble extensin[4] has a polycationic rod-like structure resembling bacterial agglutinins isolated from potato (20) and tobacco (21). The purified glycoprotein differs significantly from the other classes of soluble HRGPs (arabinogalactan proteins and *Solanacae* lectins). Extensin monomers are slowly insolubilized in the wall to form insoluble wall extensin (5, 27). This insolubilization is inhibited *in vivo* by free radical scavengers and antioxidants (especially ascorbic acid), and is accompanied by the derivatization of tyrosine to IDT (5), a diphenyl ether-linked bityrosine recently characterized from plant cell wall hydrolysates and proposed to be responsible for covalently cross-linking extensin in the wall (5, 13).

Understanding the manner in which extensin rods are assembled into insoluble wall extensin should provide new insight into the molelcular organization of plant cell walls. Because of the complexities associated with biochemical studies using *in vivo* systems, we have developed an *in vitro* system to study extensin cross-linking. Here we report the results of our studies.

MATERIALS AND METHODS

Tissue Preparation. Large tap roots of *Daucus carota* were obtained from local merchants and stored at 4°C until use. Sterile discs (7 mm diameter by 1.5 mm thick) were washed extensively with sterile H_2O and aerated at 25°C (less than 5 g tissue/50 ml/500 ml flask shaken at 120 cycles/min) for at least 30 h to induce synthesis of the wall HRGP (3). Aerated discs (2 discs/ml) were labeled in K-phosphate (50 mM, pH 6) with either [2,3,4,5-^{3}H] proline (110 Ci/mmol) or with [3,4-^{14}C]tyrosine (480 mCi/mmol) obtained from New England Nuclear.

Cell Wall Isolation and HRGP Extraction and Analysis. A cell wall fraction was prepared as follows: discs were chilled to 4°C and homogenized in 50 mM K-phosphate (pH 6), 1 mM proline, 1 mM DTT, and 0.1 mM phenylmethylsulfonylfluoride (Sigma) using a Ten-Broek homogenizer. Homogenates were centrifuged at 1,000*g* for 3 min, and the resulting cell wall pellet was washed rapidly and extensively with homogenization buffer at 4°C (about 500 ml/g fresh weight in a 25-min period). Labeled proteins were extracted from cell walls with 0.6 M NH_4HCO_3 and analyzed by isopycnic density gradient centrifugation in CsCl as previously described (6).

Cross-Linking Reactions. Suspensions of cell wall material, either labeled *in vivo* with [^{3}H]proline or mixed with [^{14}C] tyrosine-labeled soluble extensin, were buffered with 100 mM K-phosphate (pH 6 or 7) or K-acetate (pH 4 or 5) and incubated in a water bath at 0°C or 25°C. Ascorbate (Sigma) was added from a freshly prepared 100 mM stock. For each time point in the assays, three aliquots of the wall suspension were removed and independently extracted with 0.5 M $CaCl_2$, and the extracts were counted in a liquid scintillation spectrometer. Extensin was cross-linked in solution by incubating [^{14}C]tyrosine-labeled HRGP with 1 mg/ml horseradish peroxidase (type III, Sigma) in 50 mM $(NH_4)_2CO_3$ (pH 9.0) at 25°C for 18 h (16). Reaction substrate and products were analyzed by acidic-urea polyacrylamide gel electrophoresis as described (28).

Analysis of Tyrosine Derivatives Generated *In Vitro*. Labeled material was hydrolyzed under N_2 with constant boiling HCl (Pierce) at 110°C for 22 h in the presence of 10 mM phenol (less

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[3] Abbreviations: HRGP, hydroxyproline; IDT, isodityrosine.

[4] We use the term soluble extensin because the term extensin precursor (27) contains the implicit assumption that these soluble HRGPs are always destined to become completely cross-linked in the cell wall by all cell types at all stages of development (unproven), and because a strict precursor-product relationship is impossible to test in many biological systems.

than 4 mg dry wall/ml HCl [13]). Hydrolysates were dried *in vacuo*, taken into a small volume of 30% ethanol, and spotted onto Silica Gel G TLC plates (Analtech) along with authentic tyrosine, dityrosine, and IDT standards. Dityrosine and IDT were prepared by oxidation of tyrosine with H_2O_2/peroxidase (16) and ferricyanide (15), respectively. Chromatograms were developed in *n*-propanol:NH_3(7:3) and the standards were visualized with 1 N Folin-Ciocalteau reagent destained with NH_3 as described (15). Radioactive lanes were scraped into scintillation vials and counted.

RESULTS AND DISCUSSION

Cross-Linking of Extensin by Isolated Cell Walls. Salt-extractable extensin, pulse-labeled *in vivo* with [^{3}H]proline, is insolubilized by isolated cell walls incubated with neutral buffer at 25°C (Fig. 1). The rate, but not the extent, of insolubilization is decreased when the walls are incubated at 0°C. The Q_{10} for the initial rate of cross-linking (calculated from Figs. 1 and 3) is about 2.4, indicating that the cross-linking reaction could be catalyzed by a cell wall enzyme. Analysis of the salt-soluble proteins (on equilibrium density gradients) before and after an *in vitro* incubation demonstrates that the insolubilization is specific for soluble extensin (Fig. 2). This salt-extractable HRGP, which bands with a buoyant density of 1.45 g/ml (6, 28), is selectively lost from wall extracts, while other wall proteins, with lower buoyant densities, remain soluble during this long (42 h) experiment.

Isolated cell walls also insolublize exogenously supplied soluble extensin with similar kinetics (not shown). Insolubilization of [^{14}C]tyrosine-labeled monomeric extensin is accompanied by the formation of IDT cross-links in the same proportion as formed *in vivo* (Table I). It is not known whether the IDTs formed *in vitro* serve as intramolecular or intermolecular cross-links. (Nor is it known what proportion of the IDT formed *in vivo* serve as intra- and intermolecular cross-links.) Carrot extensin is also cross-linked in solution (with horseradish peroxidase and H_2O_2) into high mol wt polymers which do not enter 5% acidic-urea polyacrylamide gels (not shown). In contrast to wall-catalyzed extensin cross-linking, dityrosine and not IDT is the phenolic cross-link formed by peroxidase oxidation. These results indicate either that extensin monomers must be positioned correctly in the wall by other wall components (acidic pectins) for IDT cross-linking to occur, or that commercial peroxidase preparations lack the specific isoperoxidase responsible for IDT formation and extensin cross-linking. (It should be noted that peroxidase and H_2O_2 can nonspecifically cross-link many proteins in solution [26].)

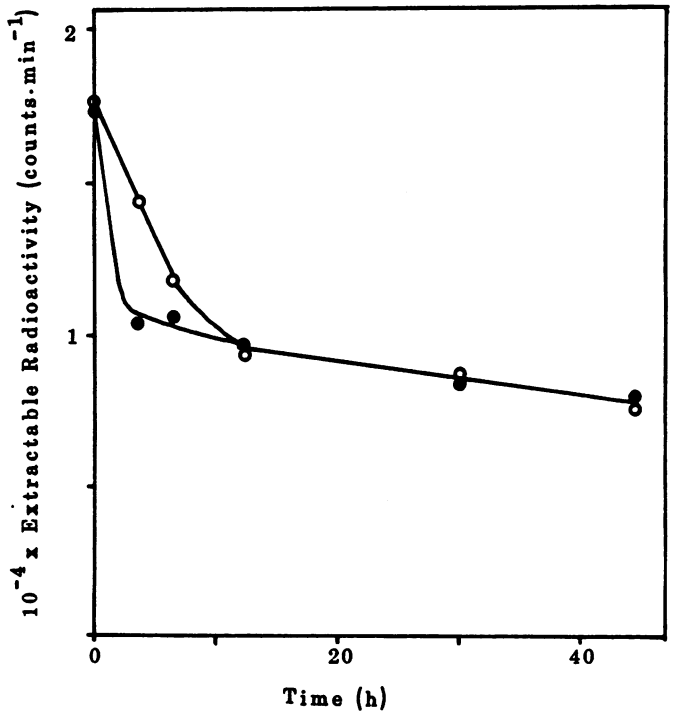

FIG. 1. Insolubilization of extensin *in vitro*. Labeled cell walls were buffered with 100 mM K-phosphate (pH 7) and incubated at 25°C (●) or 0°C (○). Aliquots of the wall suspensions were extracted with 0.5 M $CaCl_2$ at the indicated times and the extracts were counted.

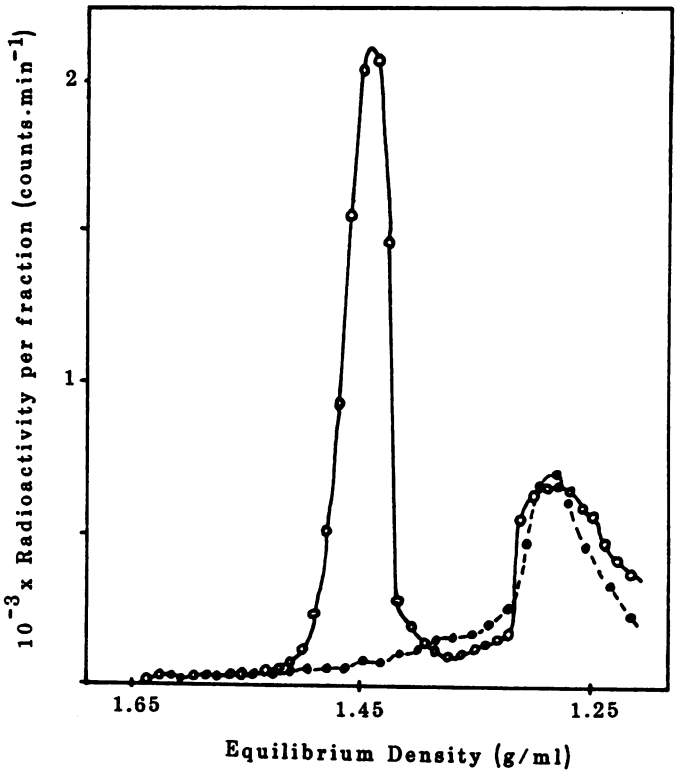

FIG. 2. Salt-extractable cell wall proteins before and after *in vitro* incubation. Proteins were salt-extracted from [^{3}H]proline-labeled cell walls immediately after wall isolation (○) or following a 42-h incubation (●) (25°C, pH 7), and analyzed on isopycnic CsCl density gradients.

Table I. *Derivitization of Tyrosine during Extensin Cross-linking*

Soluble [^{14}C]tyrosine-labeled extensin was incubated with isolated cell walls at 25°C (pH 6) for 18 h, or with horseradish peroxidase and H_2O_2. Cell wall hydrolysates (containing the insoluble extensin) were analyzed by TLC on silica gel plates (15).

Preparation	^{14}C Recovered as			
	Tyr	IDT	diTyr	Unknown[a]
			%	
Soluble extensin	100	0	0	0
Cross-linked *in vivo*[b]	65	35	0	0
Cross-linked *in vitro*, wall-catalyzed	60	30	0	10
Cross-linked *in vitro*, peroxidase/H_2O_2	14	0	60	25

[a] Unknown which remained at the origin in this TLC solvent system.
[b] From Ref. 5.

Potential Control of Extensin Solubility. Insolubilization of extensin *in vivo* is inhibited by a number of free radical scavengers/antioxidants, the most effective of which is L-ascorbate (5).

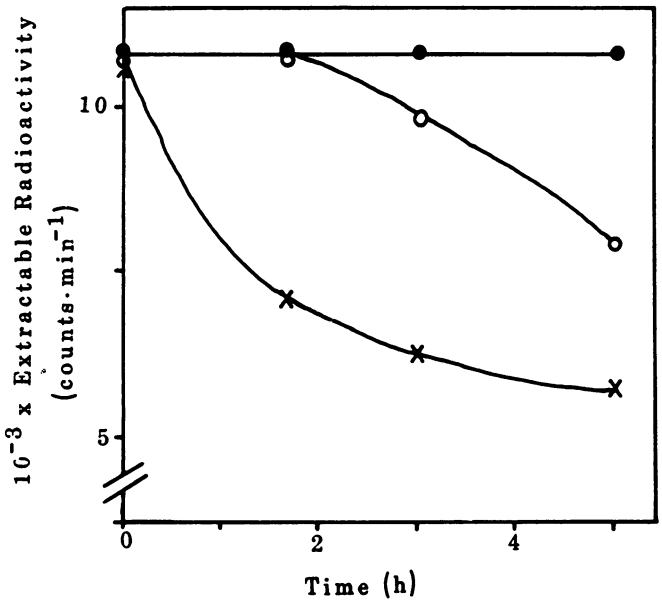

FIG. 3. Effect of L-ascorbate on *in vitro* cross-linking. Labeled cell walls were buffered with 100 mM K-phosphate (pH 7) and incubated at 25°C alone (×) or with ascorbate at 1 mM (○) or 2 mM (●), and assayed as in Figure 1.

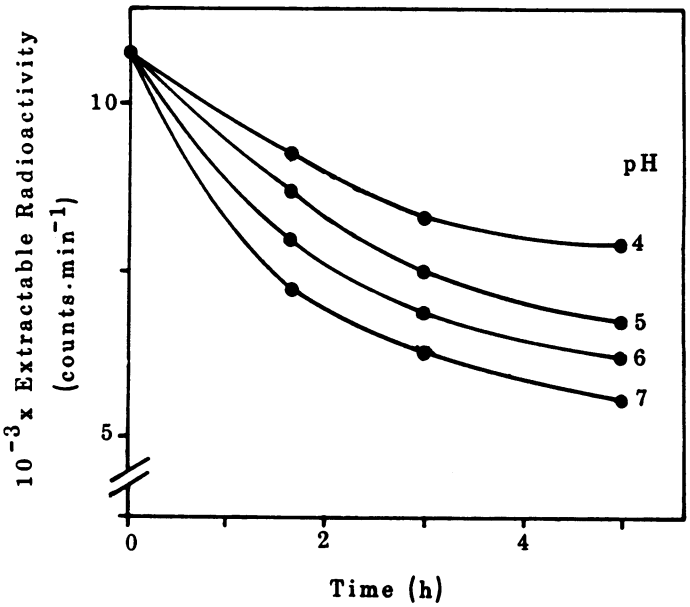

FIG. 4. Effect of pH on *in vitro* cross-linking. Labeled cell walls were incubated at 25°C with 100 mM K-phosphate (pH 6 and 7) or K-acetate (pH 4 and 5), and assayed as in Figure 1.

Wall-catalyzed cross-linking is also inhibited by ascorbic acid indicating that this *in vitro* cross-linking reaction occurs by an oxidative mechanism (Fig. 3). A lower concentration of ascorbate inhibits cross-linking completely for a short time and then appears to be oxidized by the cell wall system, indicating that isolated cell walls maintain an oxidation potential. Ascorbate is normally synthesized by plant cells, and a wall-bound ascorbate oxidase has been reported with a possible role in cell growth control (17, 22, 23). It is attractive to speculate that a cell wall-bound peroxidase-ascorbate oxidase system controls the redox potential of the wall and thus the rate of free radical phenolic cross-linking of wall polymers. The wall-bound malate dehydrogenase which has been reported (19) might also be involved in this enzyme system. Phenolic cross-linking of monomeric extensin rods would serve to 'harden' the cell wall with a hydrophilic polymer, thus decreasing wall extensibility without limiting the availability of apoplastic water.

Extensin cross-linking *in vitro* is highly dependent on the pH of the cell wall suspension. Surprisingly, both the initial rate and the total extent of cross-linking are inhibited by acid within the physiological range of pH 7 to pH 4 (Fig. 4). Thus, the solubility of extensin could be regulated by auxin-induced hydrogen ion secretion (24). A decreased wall hardening induced by acid is operationally equivalent to an increased wall loosening in a dynamic growth process. It is interesting that the data used to support the acid-induced 'release' of wall polysaccharides from isolated cell walls (2) can also be interpreted as acid-inhibition of wall-catalyzed polysaccharide insolubilization, perhaps through the formation of diferulic acid cross-links (14).

We can suggest two mechanisms by which wall pH might regulate extensin solubility. First, wall pH could control the enzyme activity catalyzing extensin cross-linking. Control of cell wall enzyme activity by wall pH has been proposed for wall-bound acid phosphatase (8) and for xyloglucan turnover (2), and some peroxidases have sharp pH optima in the physiological pH range (11). Second, wall pH should affect the structure of soluble extensin directly. Purified carrot extensin contains 11 mol% histidine (28) which could be titrated by proton secretion *in vivo*. The consequences of histidyl protonation would be 2-fold. Histidine occurs repeatedly preceding tyrosine residues in extensin peptide sequences deduced by cDNA sequencing (J. A. Chen, unpublished). Thus, acid could change the local environment of the tyrosine side chains, altering the affinity of the peroxidase cross-linking catalyst or changing the stability of tyrosyl-free radical intermediates. Histidine protonation should also increase the affinity of polycationic extensin rods for polyanionic pectins, and protein-pectin interactions might regulate the availability of tyrosine residues for oxidation, as well as the mobility of the extensin rods within the wall and thus the rate at which intermolecular IDT cross-links are formed.

In summary, our *in vitro* experiments demonstrate that plant cell walls contain all of the machinery necessary to cross-link soluble monomeric extensin through the formation of IDT. The soluble half-life of extensin *in vitro* is about 1 h, compared to about 12 h *in vivo* (5), indicating that cytoplasmic factors might exert negative control over extracellular extensin cross-linking; and ascorbic acid and/or hydrogen ions might mediate such cytoplasmic control. Finally, this *in vitro* system should be useful for studying the molecular mechanism of extensin cross-linking, and for finding inhibitors of extensin cross-linking which could be used to understand the physiological and developmental significance of cross-linked extensin and which might be active as exogenous plant growth regulators.

LITERATURE CITED

1. BASILE DV 1980 A possible mode of action for morphoregulatory hydroxyproline proteins. Bull Torrey Bot Club 107: 325–338
2. BATES GW, PM RAY 1981 pH-dependent interactions between pea cell wall polymers possibly involved in wall deposition and growth. Plant Physiol 68: 158–164
3. CHRISPEELS MJ, D SADAVA, YP CHO 1974 Enhancement of extensin biosynthesis in aging discs of carrot storage tissue. J Exp Bot 25: 1157–1166
4. CLELAND R 1968 Hydroxyproline formation and its relation to auxin-induced cell elongation in the *Avena* coleoptile. Plant Physiol 43: 1625–1630
5. COOPER JB, JE VARNER 1983 Insolubilization of hydroxyproline-rich glycoprotein in aerated carrot root slices. Biochem Biophys Res Commun 112: 161–167
6. COOPER JB, JE VARNER 1983 Selective inhibition of proline hydroxylation by 3,4-dehydroproline. Plant Physiol 73: 324–328
7. COOPER JB, JA CHEN, JE VARNER 1984 The glycoprotein component of plant cell walls. *In* WM Dugger, S Bartnicki-Garcia, eds, Structure, Function, and Biosynthesis of Plant Cell Walls. American Society of Plant Physiologists, Rockville, MD, pp 75–88
8. CRASNIER M, J RICHARDS, G NONT 1982 pH-regulation of acid phosphatase

of plant cell walls. FEBS Lett 144: 309–312
9. ELSTNER EF, A HEUPEL 1976 Formation of hydrogen peroxide by isolated cell walls from horseradish (*Armoracia lapathifolia* Gilib). Planta 130: 175–180
10. ESQUERRE-TUGAYE MT, C LAFITTE, D MAZAU, A TOPPAN, A TOUZE 1979 Cell surfaces in plant-microorganism interactions. II. Evidence for the accumulation of hydroxyproline-rich glycoproteins in the cell wall of diseased plants as a defense mechanism. Plant Physiol 64: 320–326
11. FIELDING JL, JL HALL 1978 A biochemical and cytochemical study of peroxidase activity in roots of *Pisum sativum*. I. A comparison of DAB-peroxidase and guiacol-peroxidase with particular emphasis on the properties of cell wall activity. J Exp Bot 29: 969–981
12. FREY-WYSSLING A 1976 The plant cell wall. Encyclopedia of Plant Anatomy III, 4. Gebrüder Borntraeger, Berlin
13. FRY SC 1982 Isodityrosine, a new crosslinking amino acid from plant cell-wall glycoprotein. Biochem J 204: 449–455
14. FRY SC 1983 Feruloylated pectins from the primary cell wall: their structure and possible function. Planta 157: 111–123
15. FRY SC 1984 Isodityrosine, a diphenyl-ether from plant cell-wall protein: Identification, assay and chemical synthesis. Methods Enzymol. In press
16. GROSS AJ, IW SIZER 1959 The oxidation of tyramine, tyrosine and related compounds by peroxidase. J Biol Chem 234: 1611–1612
17. LAMPORT DTA 1965 The protein component of primary cell walls. Adv Bot Res 2: 151–218
18. LAMPORT DTA 1969 The isolation and partial characterization of hydroxyproline-rich glycopeptides obtained by enzymatic degradation of primary cell walls. Biochemistry 8: 1155–1163
19. LAMPORT DTA, JW CATT 1981 Glycoproteins and enzymes of the cell wall. *In* A Pirsch, M Zimmerman, eds, Encyclopedia of Plant Physiology, New Series, Vol 13B, W Tanner, F Loewus, eds, Plant Carbohydrates II; Extracellular Carbohydrates. Springer-Verlag, Berlin, pp 133–165
20. LEACH JE, MA CANTRELL, L SEQUEIRA 1982 Hydroxyproline-rich bacterial agglutinin from potato. Extraction, purification and characterization. Plant Physiol 70: 1353–1358
21. MELLON JE, JP HELGESON 1982 Interactions of a hydroxyproline-rich glycoprotein from tobacco callus with potential pathogens. Plant Physiol 70: 401–405
22. MERTZ D 1961 The distribution and cellular location of ascorbic acid oxidase in the maize root tip. Am J Bot 48: 405–413
23. MERTZ D 1964 Ascorbic acid oxidase in cell growth. Plant Physiol 39: 398–401
24. RAYLE DL, R CLELAND 1977 Control of plant cell enlargement by hydrogen ions. Curr Top Dev Biol 11: 187–214
25. SADAVA D, F WALKER, MJ CHRISPEELS 1973 Hydroxyproline-rich cell wall protein (extensin): biosynthesis and accumulation in growing pea epicotyls. Dev Biol 30: 42–48
26. STAHMANN MA, AK SPENCER, GR HONOLD 1977 Cross-linking of proteins *in vitro* by peroxidase. Biopolymers 16: 1307–1318
27. SMITH JJ, DTA LAMPORT 1983 Isolation of putative extensin precursors from the cell surface of tomato suspension cultures. Plant Physiol 72: s-73
28. STUART DA, JE VARNER 1980 Purification and characterization of a salt-extractable hydroxyproline-rich glycoprotein from aerated carrot discs. Plant Physiol 66: 787–792
29. VAN HOLST GJ, JE VARNER 1984 Reinforced polyproline II conformation in an hydroxyproline-rich cell wall glycoprotein form carrot root. Plant Physiol 74: 247–251