Plant Physiol. (1981) 68, 956–963
0032-0889/81/68/0956/08/$00.50/0

Characterization of Carrot Cell Wall Protein[1]

I. EFFECT OF α, α'-DIPYRIDYL ON CELL WALL PROTEIN SYNTHESIS AND SECRETION IN INCUBATED CARROT DISCS

Received for publication November 15, 1979 and in revised form February 5, 1981

MACKY A. SMITH[2]
Plant Biology Program, Washington University, St. Louis, Missouri 63130

ABSTRACT

A single glycoprotein accounts for the majority of radioactivity secreted to the cell wall when incubated carrot (*Daucus carota*) discs are labeled with radioactive proline or arabinose. The ferrous chelator α,α'-dipyridyl prevents the synthesis of this protein. A new proline-labeled protein is made in the presence of α,α'-dipyridyl and is secreted to the cell wall. The protein has little, if any, carbohydrate attached to it and has a molecular weight of 55,000 daltons. This protein appears to be the nonhydroxylated, nonglycosylated form of the major cell wall glycoprotein. α,α'-Dipyridyl does not prevent proline label from becoming tightly (presumably covalently) bound to the cell wall, providing further evidence that hydroxylation and arabinosylation are not required for the covalent attachment of proteins to the cell wall. Messenger RNA extracted from incubated carrot discs produces a product which electrophoreses similarly to the protein made in the presence of α,α'-dipyridyl. The possible use of the carrot disc system to study gene structure and regulation is discussed.

A drawback of studying cell wall proteins by traditional methods is that the treatments needed to solubilize the proteins from the cell wall most often require the partial degradation of the peptide linkages of the protein (14). The approach taken in this paper is to identify precursors of cell wall proteins which have not become covalently bound to the cell wall (8, 10, 19, 20). The abundance of hydroxyproline in the cell wall proteins is very helpful in this approach, since in labeling experiments with radioactive proline, the precursor proteins are likely to be well represented (9, 15). The transient presence of precursor proteins in the cytoplasm is also useful as a criterion for identification of the precursors. Another approach to studying cell wall protein precursors is to inhibit proline hydroxylation with α,α'-dipyridyl which offers a further criterion for recognizing the glycosylated precursor proteins; the fully glycosylated species should not be present when labeling experiments are carried out in the presence of α,α'-dipyridyl (2). A final method used to study cell wall precursor proteins is *in vitro* translation of RNA isolated from tissues actively making cell wall protein, followed by identification of the protein products which electrophorese similarly to previously recognized precursor proteins.

Incubated carrot discs are well suited to the study of cell wall protein precursors. Following overnight incubation the discs begin synthesizing large amounts of hydroxyproline-rich cell wall protein (6). A hydroxyproline-rich glycoprotein (CWS-Hyp protein[3]) has been isolated from both the cytoplasm and from the cell walls of incubated carrot discs. This protein is also rich in serine and lysine, and contains arabinose as its only detectable sugar (4). In addition to salt extractable cell wall proteins, the incubated carrot discs produce hydroxyproline-rich proteins that appear to be covalently bound to the cell wall (6). These proteins contain galactose attached to many of their serine residues (5). The hydroxyproline-rich proteins from carrot discs are transiently present in Golgi bodies, suggesting that they are secreted via a membranous organelle route (10).

By studying the precursors of cell wall proteins in carrot discs, it is possible to begin answering questions concerning the diversity and physical properties of cell wall proteins, the structural requirements for secretion and attachment to the cell wall, the relationships between covalently and noncovalently bound cell wall proteins, and control mechanisms regulating cell wall protein synthesis and secretion.

MATERIALS AND METHODS

Source of Chemicals. Unless otherwise stated, all biochemicals used were the highest grade available from Sigma. Scintillation grade Triton X-100 from New England Nuclear was used in making scintillation fluid, while Triton X-100 from Sigma was used in preparing buffers. Cesium chloride (99.5% purity) was purchased from the Varlacoid Chemical Corporation. Acrylamide and N,N'-methylene-bisacrylamide were from Eastman. Radiochemicals were purchased from New England Nuclear, Amersham/Searle, and ICN Pharmaceuticals. Specific radioactivities of the radioactive materials were 261 mCi/mmol for [^{14}C]proline, 26.7 Ci/mmol for [4-^{3}H]proline, 23 Ci/mmol for [5-^{3}H]proline, 2.8 Ci/mmol for [1-^{3}H]galactose, 60 mCi/mmol for [^{14}C]arabinose, and 17 Ci/mg for Na[^{125}I] in 0.1 N NaOH.

Preparation and Incubation of Carrot Discs. *Daucus carota* discs were prepared and incubated following the procedure of Chrispeels (6).

***In Vivo* Labeling of Carrot Discs.** All labeling experiments were done without the addition of nonradioactive carrier material. In labeling experiments using α,α'-dipyridyl to inhibit proline hydroxylation, a 0.1 volume of 10^{-3} M α,α'-dipyridyl solution was added to the aged discs 10 min before the labeled compound was added.

[1] This work was supported in part by a National Science Foundation predoctoral fellowship to the author and an American Cancer Society Institutional Grant to Washington University (ACS IN36).

[2] Present Address: Warren Wilson College, Swannanoa, NC 28778.

[3] Abbreviations: CWS-Hyp protein, cell wall soluble, hydroxyproline-rich protein produced in the absence of α,α'-dipyridyl; TEMED, N,N,N',N'-tetramethylethylene diamine; FMN, flavin mononucleotide; SDS-PAGE, SDS polyacrylamide gel electrophoresis; CWS-Pro protein, cell wall soluble, proline-rich protein produced in the presence of α,α'-dipyridyl; Urea-PAGE, polyacrylamide gel electrophoresis using urea-containing buffer.

Isolation of Radioactive Cytoplasmic and Cell Wall Proteins from Incubated Carrot Discs. The procedures of Chrispeels (6) were used to isolate the salt-extractable cell wall protein and the residual cell wall material. When cytoplasmic proteins were studied, 0.4 M sucrose with 10 mM K-phosphate (pH 6.0) was used as the grinding buffer. The extract was centrifuged at 1000g at 4 C and the supernatant was used as the total cytoplasmic extract.

Determination of Radioactivity in Cytoplasmic and Cell Wall Proteins. Cytoplasmic proteins were precipitated with 10% trichloroacetic acid, trapped on a Millipore filter which was washed first with 10% trichloroacetic acid and then with acetone. The scintillation fluor contained 5 g PPO and 0.2 g POPOP/liter of toluene.

A small volume (10–40 μl) of the salt extractable fraction was counted with 10 ml of a toluene:TX-100 scintillation fluid (8 g PPO, 0.2 g POPOP, 2 liters toluene, 1 liters TX-100).

Radioactivity in the cell wall fraction which remained following salt extraction was counted by first making a slurry of the cell wall fraction with dH_2O. An aliquot (20–50 μl) of wall material was removed with a Pipetman which had its tip cut off to provide a wide orifice. Radioactivity was then counted in 10 ml of the toluene:TX-100 fluid.

Cesium Chloride Density Gradient Centrifugation. CsCl density gradient centrifugation was used to determine the density of radioactive proteins isolated from carrot discs (7). Samples of radioactive cell wall protein or cytoplasmic extracts were prepared for centrifugation by dilution with a stock solution of CsCl (usually 6.3 M). For experiments in which proteins and glycoproteins in the density region of 1.30 to 1.45 g/cm^3 were examined, the starting density of the sample was adjusted to about 1.40 g/cm^3. Centrifugation was performed in Beckman SW-65 or SW-50.1 rotors at 45,000 rpm at 4 C for 48 to 72 h. Fractions of 100 to 150 μl were collected following centrifugation. Radioactivity in each fraction was determined by counting 10 to 40 μl of each fraction with an equal volume of dH_2O in 10 ml of the toluene:TX-100 scintillation fluid. The density gradient of each tube was determined by measuring the refractive index of every fourth sample in a Bausch and Lomb refractometer (11).

Table I. *Incorporation of Radioactive Amino Acids into Cytoplasmic Proteins and Salt-Extractable Cell Wall Proteins*

Carrot discs were incubated 2 h in the presence of radioactive amino acids. Isolation and determination of radioactivity in the cytoplasmic and salt-extractable cell wall fractions was done as described under "Materials and Methods." Labeling regimens used in the individual experiments are described below. Experiment 1, 5 μCi of [^{14}C]proline with 6 discs in 10 ml incubation medium. Experiment 2, 12.5 μCi of [^{35}S]methionine or 2 μCi of [^{14}C]proline per 6 disks in 8 ml medium. Experiment 3, 5 μCi of [^{14}C]proline per 5 disks in 7 ml of medium. Experiment 4, 100 μCi of [^{3}H]proline per 6 discs in 8 ml of medium. Data are given as cpm incorporated by all discs.

Expt.	Label	Dipyridyl	Cytoplasm	Cell Wall	Radioactivity % in Cell Wall
			cpm		%
3	Proline	No	4.3×10^6	1.0×10^6	19
2	Proline	No	3.9×10^6	1.7×10^5	30
2	Proline	Yes	1.7×10^5	3.9×10^4	19
1	Proline	Yes	2.3×10^6	0.4×10^6	15
3	Proline	Yes	3.2×10^6	0.5×10^6	14
4	Proline	Yes	20.4×10^6	3.4×10^6	14
2	Methionine	No	3.4×10^6	1.6×10^5	4
2	Methionine	Yes	2.7×10^6	1.3×10^5	5

Preparation of Samples for Electrophoresis. Salt-extractable cell wall proteins and cytoplasmic proteins were precipitated by adding five volumes of acetone. In cases where small amounts of proteins were present, 50 μg BSA/200 μl sample (5 μl of a 10 mg/ml BSA solution) were added prior to acetone addition. Acetone precipitation, rather than trichloroacetic acid precipitation, was used since the main hydroxyproline-rich protein is soluble in 5% trichloroacetic acid (6).

The loading buffer for SDS polyacrylamide gels contained 62.5 mM Tris, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.002% bromphenol blue, with the pH adjusted to 6.8. The loading buffer for the urea polyacrylamide gels contained 8 M urea with 1% β-mercaptoethanol and 0.0005% basic fuchsin. Precipitated samples and added loading buffer were boiled for 5 min in both cases.

Electrophoresis of Proteins in Polyacrylamide Gels. The procedure of Laemmli (12) was used with the modification that acrylamide and bisacrylamide concentrations in the resolving gel were 15 and 0.087%, respectively.

Best resolution of cell wall proteins was obtained on a gradient polyacrylamide gel with a urea buffer. The stacking gel was prepared by mixing 1 part of Solution B (24 ml 1.0 N KOH, 1.44 ml acetic acid, 0.23 ml TEMED, 24 g urea with dH_2O to 50 ml) with 4 parts of Solution D (2.5 g acrylamide, 0.625 g bisacrylamide, 24 g urea, with dH_2O to 50 ml), 2 parts 8 M urea, and 1 part of Solution E (40 μl of 5×10^{-3} M FMN solution added to 5 ml of 8 M urea). The stacking gel was photopolymerized under a fluorescent lamp. The basic resolving gel was made with 1 part Solution A (24 ml 1.0 N KOH, 8.6 ml acetic acid, 2.0 ml TEMED, 24 g urea, and dH_2O to 50 ml), 6 parts Solution C (6.65 g acrylamide, 0.1 g bisacrylamide, 24 g urea, and dH_2O to 50 ml), and 1 part of 56 mg ammonium persulfate dissolved in 5 ml 8 M urea. When gradient-resolving gels were used, Solution C was replaced by Solution C-H (9 g acrylamide, 0.13 g bisacrylamide, 24 g urea, 10 ml 50% glycerol, with dH_2O to 50 ml) and Solution C-L (2.5 g acrylamide, 35 mg bisacrylamide, 24 g urea, 1 ml 50% glycerol, with dH_2O to 50 ml). The acrylamide gradient ran from 5 to 18%. The reservoir buffer for the urea gel contained 7.8 g β-alanine, 2.0

Table II. *Total Accounting of Radioactivity in the Cell Wall Following Labeling with [^{14}C]Proline*

Carrot discs (5 in 7 ml of medium) were labeled for 2.5 h with 7 μCi of [^{14}C]proline. Radioactivity in each fraction was determined as described under "Materials and Methods." Data are given as cpm incorporated by all five discs.

Dipyridyl	Cytoplasm	SECW[a]	RCW[b]	RCW:SECW	Radioactivity in Cell Wall
		cpm		*ratio*	%
No	4.32×10^6	1.0×10^6	0.51×10^6	0.51	26
Yes	3.18×10^6	0.5×10^6	0.36×10^6	0.68	22

[a] Salt-extractable cell wall protein.

[b] Residual cell wall protein.

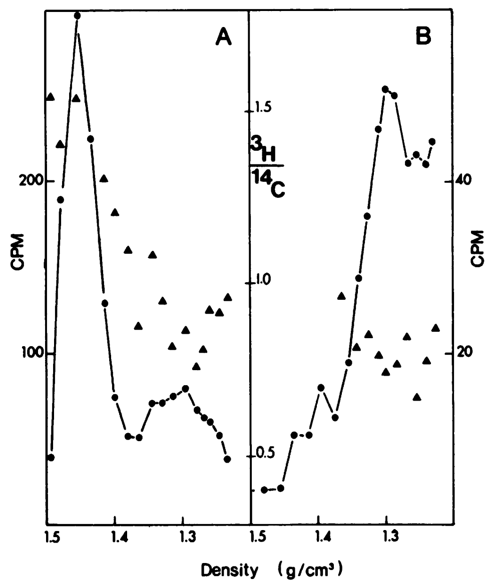

FIG. 1. Cesium chloride density gradient centrifugation of salt extractable cell wall proteins labeled with radioactive proline. Incubated carrot discs were labeled 2.5 h with 4 μCi of [^{3}H]proline and 2 μCi [^{14}C]proline. Salt-extractable cell wall proteins were isolated and 1 ml of the sample submitted to CsCl density gradient centrifugation. Starting density was 1.367 g/cm^3. In B, 1 ml of 10^{-3} M α,α'-dipyridyl was added to the incubated carrot discs 10 min before the addition of radioactive proline. The solid line indicates the [^{14}C] radioactivity profile; (▲), the [^{14}C]:[^{3}H] ratio.

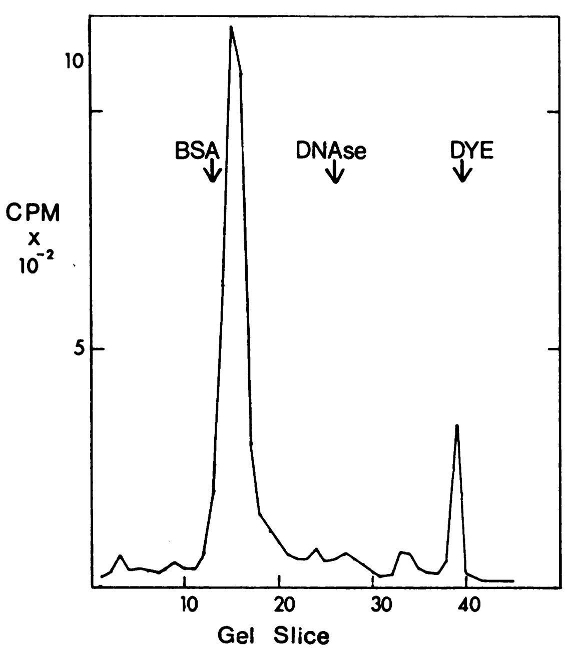

FIG. 2. SDS electrophoresis of [^{14}C]proline-labeled salt extractable cell wall proteins labeled in the presence of α,α'-dipyridyl. The acrylamide concentration was 12%. Gel slices (1-mm) were counted with toluene scintillation fluid following solubilization with NCS tissue solubilizer.

ml acetic acid with dH_2O to 1,000 ml (pH 4.5). Electrophoresis of gradient gels was at 150 v at 4 C for 12 to 14 h.

Iodination of Proteins with ^{125}I. Proteins were iodinated by the lactoperoxidase method of Thorell and Johansson (22).

Isolation of RNA from Carrot Discs. RNA was isolated by a procedure similar to that of Berridge and Lane (1). Isolation was usually done with 10 g of carrot discs with the initial homogenization in a Polytron homogenizer with 50 ml grinding buffer (0.2

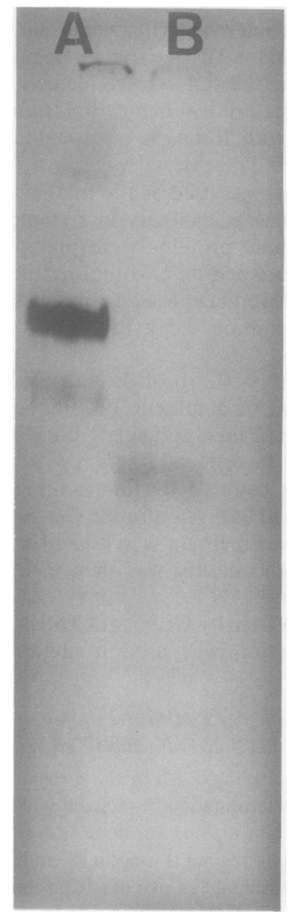

FIG. 3. Electrophoresis of salt-extractable cell wall proteins in 8 M urea polyacrylamide gels. Slot A shows cell wall proteins labeled in the absence of α,α'-dipyridyl, while Slot B shows cell wall proteins labeled in the presence of α,α'-dipyridyl.

Table III. *Radioactivity in the Cytoplasmic and Cell Wall Fraction Following a [^{14}C]Proline Pulse and Chase*

Carrot discs in 2 flasks (6 slices in 8 ml of medium) were preincubated for 10 min with 10^{-4} M α,α'-dipyridyl. Ten μCi of [^{3}H]proline were added to each flask. After 15 min, the radioactive medium was removed from both flasks. Discs in one flask were ground and the cytoplasmic and cell wall fractions separated. Radioactivity in these fractions is shown in the upper line. The other flask was incubated 2 h longer at 30 C in the presence of 0.5 mM proline and 0.1 mM α,α'-dipyridyl. The salt-extractable cell wall and cytoplasmic fractions of these carrots were isolated, and the radioactivity in each determined (bottom line of table). The grinding buffer contained 0.4 M sucrose and 10 mM K-phosphate (pH 6.0) to keep membranous vesicles intact. Data are given as radioactivity in all discs.

State	Cytoplasm	Cell Wall	Radioactivity in Cell Wall
	cpm		%
Pulse, 15 min	343,140	7,650	2
Chase, 2 h	284,060	52,200	15

M Tris [pH 8.5], 50 mM KCl, 50 mM $MgCl_2$, 50 μg/ml heparin, 5 μg/ml cycloheximide, 10 μg/ml polyvinyl sulfate). Following phenol-$CHCl_3$ extractions of the initial supernatant and precipitation with 2.5 volumes of ethanol, the pellet was treated with 3 M sodium acetate (pH 6.2) to solubilize DNA and small RNA molecules. The RNA insoluble in 3 M sodium acetate was suspended in 10 mM Hepes (pH 7.5) and stored at −20 C.

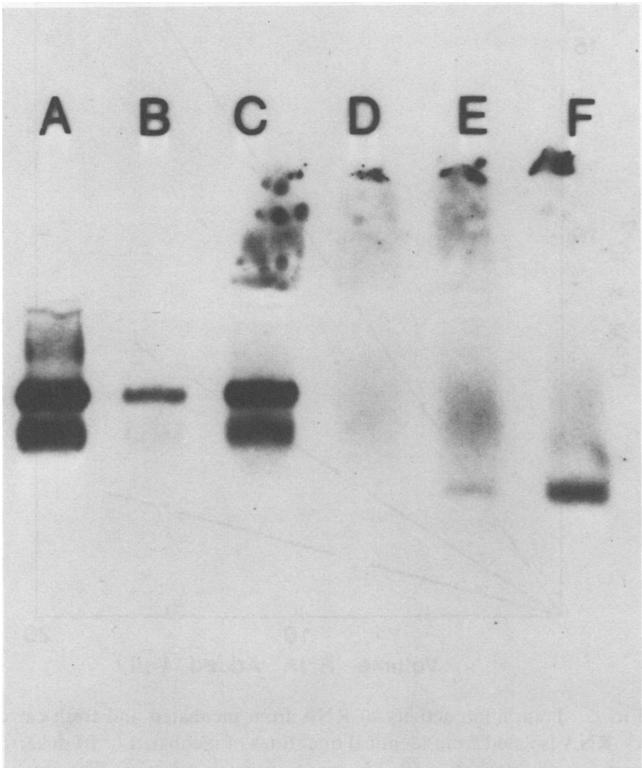

FIG. 4. Electrophoresis of radioactive cytoplasmic and cell wall proteins from a pulse-chase experiment. Four flasks of incubated carrot discs were labeled with 45 μCi 5-[^{3}H]proline for 25 min. Two flasks had been pretreated with α,α'-dipyridyl for 10 min; the others had not. One flask of each type was harvested at the end of the labeling period for the isolation of cytoplasmic proteins. The other flasks were subjected to a 3-h chase with nonradioactive proline (0.5 mM) and then harvested for isolation of cytoplasmic proteins and the salt-extractable cell wall fraction. Slot A, cell wall fraction after chase, no dipyridyl; Slot B, cytoplasmic fraction after chase, no dipyridyl; Slot C, cytoplasmic fraction at end of 25 min pulse, no dipyridyl; Slot D, cytoplasmic fraction after chase, dipyridyl present; Slot E, cytoplasmic fraction at end of 25 min pulse, dipyridyl present; and Slot F, cell wall fraction after chase, dipyridyl present.

Translation of Isolated RNA in a Wheat Germ Extract. The procedure of Boime, *et al.* (3) was used for translating RNA isolated from carrot discs.

RESULTS

Incorporation of Proline into Cell Wall Protein and the Effect of an Inhibitor of Prolyl Hydroxylase on Proline Incorporation. When carrot discs which have been aged for 20 h are incubated with radioactive proline for an additional 3 h, a large proportion of the proline incorporated into protein is secreted into the cell wall. Table I (lines 1 and 2) verifies previous results showing that the percentage of radioactive label present in the wall varies between 20 and 30% of the total incorporated label (6). The addition of α,α'-dipyridyl to the incubation medium during the labeling period does not stop the secretion of proline-containing proteins to the cell wall (Table I, lines 3 to 6), indicating that hydroxylation and arabinosyl addition may not be important in the secretion process. When methionine is used as the labeled amino acid, a much smaller percentage of the incorporated radioactivity is present in the wall (Table I, lines 7 and 8), indicating that more proline than methionine may be present in the cell wall proteins.

Not all radioactive protein present in the cell wall can be removed by salt extraction. The material remaining, called residual cell wall protein, is presumably covalently bound to the wall. Washing the walls in 8 M urea does not remove significant amounts of this radioactivity. In agreement with Chrispeels' data (6), radioactivity in the residual cell wall fraction was from 50 to 70% of the radioactivity which was salt extractable from the wall (Table II). Furthermore, α,α'-dipyridyl does not prevent the incorporation of radioactivity into the residual cell wall fraction (Table II).

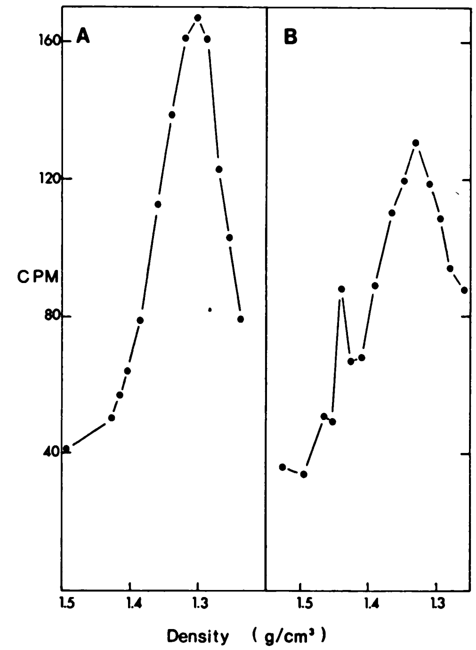

FIG. 5. Cesium chloride density gradient centrifugation of [^{35}S]methionine-labeled cell wall proteins. Salt extractable cell wall proteins isolated from discs labeled with [^{35}S]methionine in either the presence or absence of α,α'-dipyridyl were centrifuged to equilibrium in CsCl gradients. (A) with α,α'-dipyridyl; (B) without α,α'-dipyridyl.

Properties of Salt Extractable Cell Wall Proteins. The extent of glycosylation of the salt-extractable cell wall proteins and the presence of hydroxyproline in these proteins was determined by CsCl density gradient centrifugation. Most of the incorporated radioactive proline extractable from the cell wall with salt is present as a narrow peak with a density of 1.45 g/cm^3, indicative of a carbohydrate content of about 45% (Fig. 1). This glycoprotein is the CWS-Hyp protein studied by Brysk and Chrispeels (4) and accounts for over 60% of the radioactivity present, with little radioactivity being present in the region of 1.30 g/cm^3 which is typical of nonglycosylated proteins. When cell wall proteins labeled in the presence of α,α'-dipyridyl are submitted to CsCl centrifugation, the CWS-Hyp protein is totally missing, and the majority of the radioactivity is present in the region of nonglycosylated proteins (Fig. 1B). About 30% of the cytoplasmic proteins labeled with a short pulse of [^{3}H]proline have a buoyant density of 1.45 g/cm^3, in good agreement with results discussed above.

Double labeling with [^{14}C]proline and [4-^{3}H]proline can be used to determine the extent of proline hydroxylation, since hydroxylation causes loss of the *trans* hydrogen at the four position (13; Varner and Ho, personal communication). Thus, proteins containing hydroxyproline will have higher [^{14}C]:[^{3}H] ratios, as a result of the loss of ^{3}H. The peak at 1.45 g/cm^3 has a [^{14}C]:[^{3}H] ratio of 1.5 which is much higher than for the nonglycosylated protein peak and the ratio obtained when proline hydroxylation is prevented with α,α'-dipyridyl (Fig. 1). This confirms that the CWS-Hyp protein contains considerable hydroxyproline, while the hydroxyproline content in the nonglycosylated proteins is low.

Electrophoresis of Cell Wall Proteins. When the salt-extractable cell wall proteins labeled in the presence of α,α'-dipyridyl were

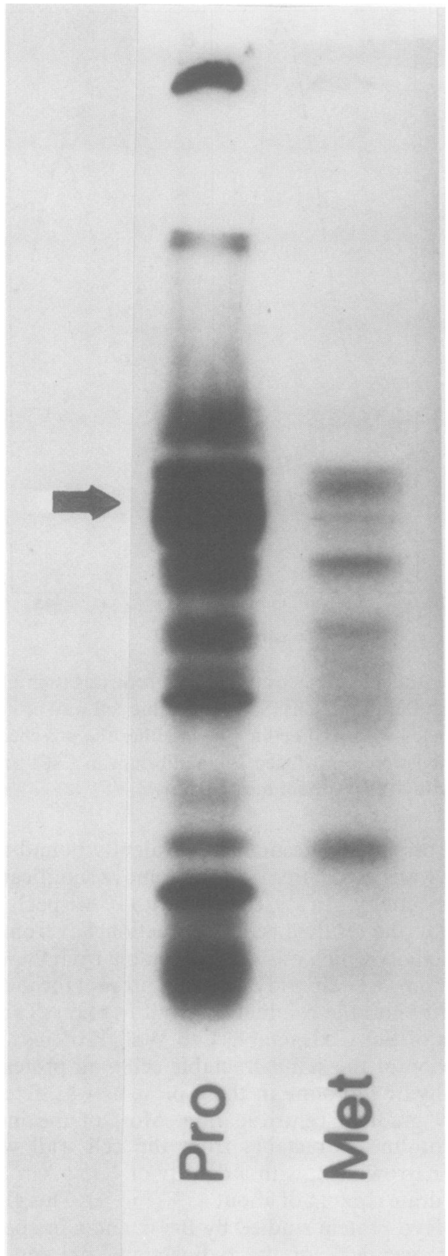

FIG. 6. SDS polyacrylamide gel electrophoresis of [^{35}S]methionine and [^{14}C]proline-labeled cell wall proteins labeled in the presence of α,α'-dipyridyl. Slot A, proline label. Slot B, methionine label. The arrow adjacent to Slot A indicates the position of the CWS-Pro band. Slot A is over-exposed, making minor protein species appear prominent.

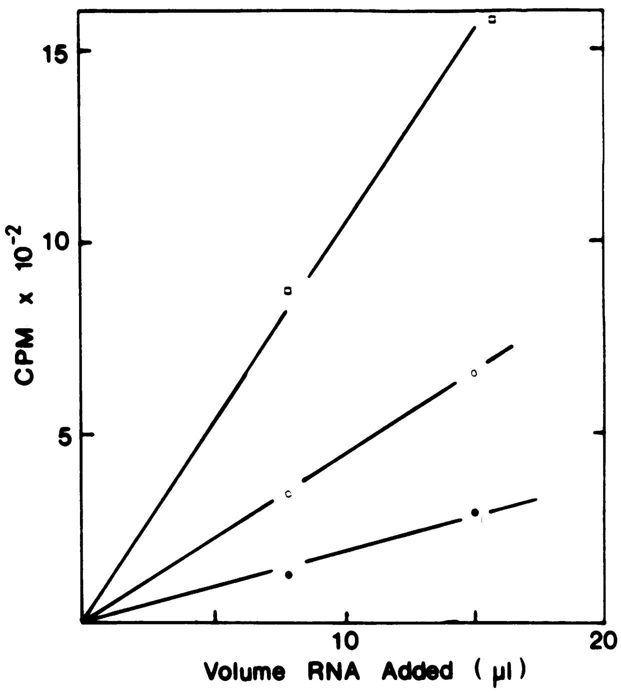

FIG. 7. Translation activity of RNA from incubated and fresh carrot discs. RNA isolated from identical quantities of incubated or fresh carrot discs was used to program 50 µl *in vitro* translation reactions. The amount of [^{3}H]proline incorporated into protein was determined by using 10-µl aliquots of each sample. (□) Incorporation obtained by using RNA from incubated carrot discs; (●) results obtained with RNA from fresh carrot discs; (○) incorporation obtained with RNA isolated from an equal mixture of fresh and incubated carrot discs.

examined by SDS-PAGE, only one major radioactive band was present (Fig. 2). The mol wt of this cell wall protein was 55,000 daltons as determined by comparison with molecular weight standards. This protein, which will be called the CWS-Pro protein, was present only when labeling was done in the presence of α,α'-dipyridyl. When the salt-extractable cell wall proteins labeled in the absence of α,α'-dipyridyl were subjected to SDS-PAGE (even in the presence of 8 M urea), too little radioactivity entered the resolving gel to obtain a molecular weight estimate for the CWS-Hyp protein.

A polyacrylamide gel system which was successful in resolving the salt-extractable cell wall proteins labeled in the absence of α,α'-dipyridyl was an acidic buffer, 8 M urea system. In this system the majority of the radioactivity migrated as one sharp band (Fig. 3, Slot A). Cell wall proteins labeled in the presence of α,α'-dipyridyl also produced only one major band in the urea gels (Fig. 3, Slot B) which migrated more rapidly than the major band present in the absence of the inhibitor. The urea-PAGE system effectively resolved the major cell wall proteins; however, estimates of molecular weights can not be obtained with these gels. For unknown reasons, some other carrot proteins were not clearly resolved by the urea gels (compare Figs. 3 and 6).

To determine the relationship between the CWS-Hyp protein and the major protein species present in urea gels, the former was purified according to the procedure of Brysk and Chrispeels (4). After being reacted with ^{125}I, the CWS-Hyp protein showed a single peak of radioactivity at a density of 1.45 g/cm^3; upon electrophoresis it produced a single band which migrated identically to the major proline-labeled species from salt extracts of carrot cell walls.

Demonstration of Secretion of CWS-Pro and CWS-Hyp. To demonstrate that the CWS-Pro protein made in the presence of α,α'-dipyridyl is actually secreted into the wall and does not merely bind to the wall during tissue grinding and wall isolation, a pulse-chase experiment was performed. Table III shows that very little radioactive proline was present in the cell wall following 20 min of incubation in [^{3}H]proline. After the 2-h chase period, the typical 15% of incorporated radioactivity was found in the cell wall.

In a similar experiment, cytoplasmic and cell wall proteins were examined in the urea-PAGE system (Fig. 4). Slot C shows that the CWS-Hyp protein was a major cytoplasmic protein following a 20-min labeling period in the absence of α,α'-dipyridyl. Its concentration in the cytoplasm was greatly reduced following a 2-h chase (Slot B) with the concomitant appearance of radioactivity at the cell wall (Slot A). Similarly, the CWS-Pro protein labeled in the presence of α,α'-dipyridyl was present in the cytoplasm

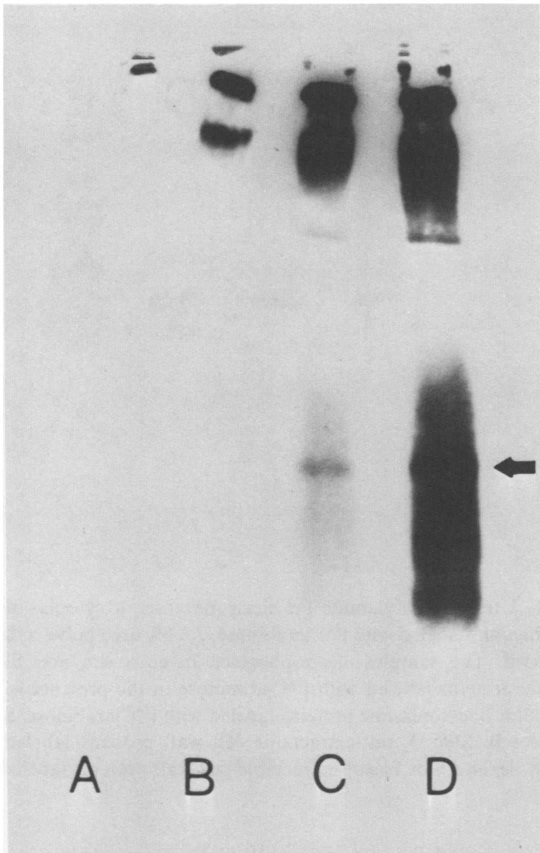

FIG. 8. Urea polyacrylamide gel electrophoresis of *in vitro* translation products produced with RNA from incubated and fresh carrot discs. An 8 M urea polyacrylamide gel with a 5 to 18% acrylamide gradient was used. 50 μl *in vitro* translation reactions were performed by using 15 μl RNA solution to drive the reaction. The source of RNA used for each gel slot is as follows: Slot A, 10 mM Hepes control; Slot B, fresh carrot discs; Slot C, mixture of fresh and incubated carrot discs; and Slot D, incubated carrot discs. The arrow beside Slot D indicates the position of the CWS-Pro protein.

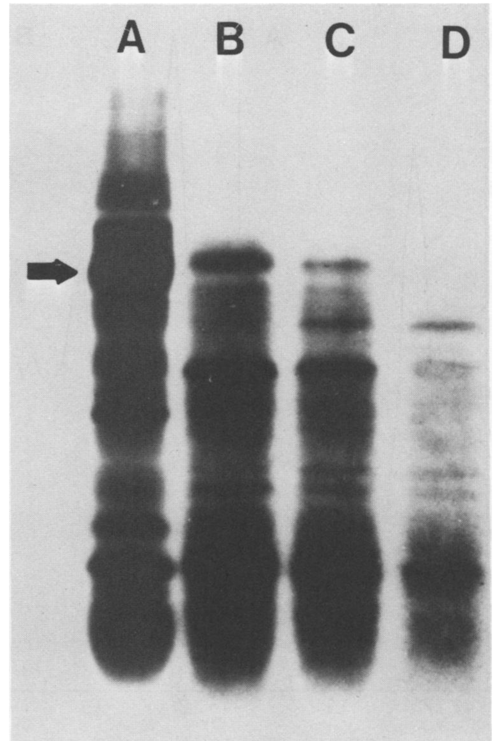

FIG. 9. SDS gel electrophoresis of *in vitro* translation products. An SDS polyacrylamide gel with an acrylamide concentration of 14% was used. Slot A, salt-extractable cell wall protein labeled with [^{14}C]proline in the presence of α,α'-dipyridyl; Slot B, products obtained with RNA from incubated carrot discs; Slot C, products obtained with RNA from a mixture of incubated and fresh carrot discs; and Slot D, products obtained with RNA from fresh carrot discs.

after the 20-min labeling period (Slot E) and was found in the cell wall following a 2-h chase (Slot F).

Labeling of Cell Wall Proteins with Methionine. Figure 5B shows a CsCl gradient of the salt-extractable cell wall proteins labeled with [^{35}S]methionine. The profile of radioactivity is very different from that seen when proline is used as label (Fig. 1A), since very little radioactivity is present in the density region of 1.45 g/cm^3. CsCl density gradient centrifugation of cell wall proteins labeled with methionine in the presence of α,α'-dipyridyl (Fig. 5A) provides no evidence on the methionine concentration in the CWS-Pro proteins, inasmuch as these proteins all band at a density of 1.30 g/cm^3. However, SDS gel electrophoresis of cell wall proteins labeled with [^{35}S]methionine in the presence of α,α'-dipyridyl showed no major methionine-labeled bands that migrated like CWS-Pro (Fig. 6). Thus, both CWS-Pro and CWS-Hyp can be classified as proline-(hydroxyproline-)rich and methionine-poor proteins.

***In Vitro* Translation of Carrot Disc RNA.** RNA was isolated from incubated carrot discs and translated in a wheat germ *in vitro* system. Translation activity was much greater in incubated discs than in freshly cut discs; an inhibitor of translation in the fresh discs probably cannot account for this difference since a mixture of incubated and fresh discs produced RNA of intermediate activity (Fig. 7).

The *in vitro* translation products were electrophoresed in polyacrylamide gels. The urea gel of Figure 8 shows that a band migrating in the same position as the CWS-Pro protein is present in the translation products of the incubated carrot disc RNA (Slot D), but is missing or greatly reduced in the products of the fresh carrot disc RNA (Slot B). Similar results were obtained by using a SDS-PAGE system (Fig. 9). A protein migrating slightly slower than the CWS-Pro protein was present when RNA from incubated carrot discs was used to program the system. This band was not present, though many other bands were detectable, when RNA from fresh carrot discs was used.

[^{14}C]Arabinose Labeling of Cell Wall Proteins. As expected from the chemical analysis of the CWS-Hyp protein by Brysk and Chrispeels (4), [^{14}C]arabinose is effectively incorporated into this cell wall protein. The incorporation of arabinose into cytoplasmic macromolecules was also examined. α,α'-Dipyridyl caused only a 35% decrease in the incorporation of arabinose into the cytoplasmic, acetone-precipitable fraction, suggesting that much of the arabinose was part of a polymer not containing hydroxyproline. A CsCl density gradient of an arabinose-labeled cytoplasmic extract showed a major peak at 1.64 g/cm^3 even when the discs were labeled in the presence of α,α'-dipyridyl (Fig. 10). The only difference between the α,α'-dipyridyl treated and nontreated samples was that the nontreated sample had a peak of radioactivity near 1.45 g/cm^3. The cytoplasmic extracts used in the CsCl gradient of Figure 10 were also electrophoresed in a urea polyacrylamide gel. The sample labeled in the presence of α,α'-dipyridyl and electrophoresed in Slot A of Figure 11 produced no detectable bands. However, the sample from the nontreated carrot discs produced one-labeled species, which co-migrated with CWS-Hyp (Fig. 11, Slots B and C).

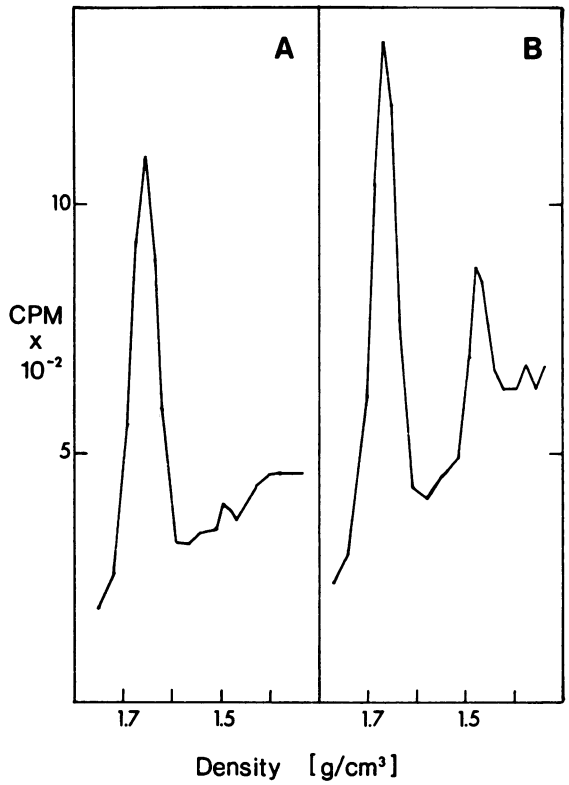

FIG. 10. Cesium chloride density gradient centrifugation of the cytoplasmic fraction labeled with [^{14}C]arabinose in the presence and absence of α,α'-dipyridyl. Carrot discs were labeled with [^{14}C]arabinose for 30 min, with one group of discs being pretreated with 10^{-4} M α,α'-dipyridyl for 10 min. The cytoplasmic fraction was isolated after homogenizing the discs in 0.4 M sucrose, 10 mM K-phosphate (pH 6.0). The starting density of the gradient was 1.55 g/cm^3. Aliquots (25 μl) of each fraction were counted with 10 ml of toluene:TX-100 scintillation fluor.

FIG. 11. Urea polyacrylamide gel electrophoresis of cytoplasmic and cell wall fractions labeled with [^{14}C]arabinose. An 8% urea polyacrylamide gel was used. The samples electrophoresed in each slot are: Slot A, cytoplasmic proteins labeled with [^{14}C]arabinose in the presence of α,α'-dipyridyl; Slot B, cytoplasmic proteins labeled with [^{14}C]arabinose; Slot C, same as slot B; Slot D, salt-extractable cell wall proteins labeled with [^{14}C]arabinose; and Slot E, salt-extractable cell wall proteins labeled with [^{14}C]proline.

DISCUSSION

A major goal of this study was to determine the number of major cell wall proteins in carrot discs. The results obtained show that one hydroxyproline-rich protein species accounts for a large fraction of the protein incorporated into the cell wall. The CWS-Hyp protein is the major species present as shown by CsCl centrifugation (Fig. 1) and by urea gel electrophoresis (Fig. 3). A second, minor band appearing in the urea gels which migrates faster than CWS-Hyp and which is not present when labeling is done in the presence of α,α'-dipyridyl appears as a major band only when the gel slot is overexposed and the CWS-Hyp grain density is saturated (Figs. 4 and 11). Labeling with proline in the presence of α,α'-dipyridyl also results in one major protein band, as determined by either urea or SDS gels (Figs. 2 and 3). When gel slots are overexposed, numerous cell wall proteins present in lesser quantities are visualized, particularly when using the SDS-PAGE system with its higher resolving power (Fig. 6).

The major protein (CWS-Hyp) extractable from the cell wall is a glycoprotein rich in proline and hydroxyproline, poor in methionine, and about 45% carbohydrate. These results are in agreement with the characterization of this protein performed by Brysk and Chrispeels (4). The results of the [^{14}C]- and [^{3}H]proline double label and CsCl density gradient centrifugation experiments indicate that hydroxyproline is only slightly represented in the nonglycosylated cell wall proteins. Most of the salt-extractable hydroxyproline is associated with the CWS-Hyp protein.

It was important to determine that the underglycosylated cell wall protein precursor produced in the presence of α,α'-dipyridyl actually is secreted into the cell wall. Collagen secretion in chick embryo and mouse cells occurs only if a minimum of 30% of the hydroxyproline residues are hydroxylated, suggesting that secretion of collagen requires triple helix formation which does not occur unless proline residues are hydroxylated (2, 18). In contrast, the CWS-Pro protein is clearly being secreted to the cell wall in the presence of α,α'-dipyridyl (Table III and Fig. 4). Thus, the structure required for the secretion of CWS-Pro does not depend upon significant hydroxylation and arabinose addition.

Although there is no direct evidence showing that the CWS-Hyp and CWS-Pro proteins have the same amino acid sequence (except for hydroxyproline substituted for proline), there are several lines of evidence suggesting that this is the case. Both proteins are the major salt-extractable cell wall proteins labeled with proline. CWS-Hyp accounts for over 60% of the radioactivity removed from the cell wall by salt, as determined by CsCl centrifugation. CWS-Pro also accounts for about 60% of the salt-extractable, proline-labeled cell wall protein as determined by antibody recognition of 60% of the input radioactivity of the cell wall proteins (21). Second, both CWS-Pro and CWS-Hyp are rich in amino acids but very poor in methionine. Third, the migration of the proteins in 8 M urea polyacrylamide gels is consistent with the proteins having similar amino acid sequences. Assuming similar sequences in both proteins, CWS-Pro should migrate faster in the urea gels since it would have the same electrical charge but have a lower molecular weight than the glycosylated CWS-Hyp. Furthermore, removal of the sugars from CWS-Hyp (by mild acid hydrolysis) results in the deglycosylated protein migrating almost as fast as CWS-Pro (data not shown).

Labeling with arabinose is a particularly useful way to study cell wall proteins, since only cell wall proteins and carbohydrates are likely to be labeled with this sugar. Electrophoresis of the salt-extractable labeled products showed only one major band on urea polyacrylamide gels. This confirms the result that only a small number of proteins are present in the cell wall.

Labeling with arabinose was also used to investigate the properties of the protein which becomes covalently bound to the cell wall. Results from experiments with radioactive proline indicated that one-half to two-thirds as much radioactive proline was associated with the residual cell wall fraction as with the salt-extractable fraction (Table II). Incorporation of radioactivity into the residual cell wall fraction was not inhibited by the presence of α,α'-dipyridyl, indicating that neither hydroxyproline nor arabinose play a critical role in the covalent attachment of protein to the cell wall.

An important question is whether or not the protein covalently bound to the cell wall is identical or at least similar to the protein which is noncovalently bound to the cell wall. Hydroxyproline-rich covalently bound cell wall proteins from many dicotyledonous plants have been shown to contain hydroxyproline-arabinose linkages (16). This suggested that studying cytoplasmic proteins labeled with arabinose would be a way to determine whether or not the covalently and noncovalently bound cell wall proteins have similar structures. Cytoplasmic proteins labeled with arabinose show only one band, which migrates like CWS-Hyp on 8 M urea polyacrylamide gels. CsCl density gradient centrifugation of these proteins shows that the only pronounced difference between the pattern obtained in the presence and absence of α,α'-dipyridyl is the peak near 1.45 g/cm^3 which is characteristic of CWS-Hyp (Fig. 10). The same figure also shows that a large fraction of the arabinose-labeled material (with or without α,α'-dipyridyl treatment) is present in a peak at 1.64 g/cm^3, a density that implies a total carbohydrate composition.

The finding of only one major arabinose-labeled protein in the cytoplasm suggests that the covalently and noncovalently bound cell wall proteins have an identical or similar cytoplasmic precursor. Cho and Chrispeels (5) found that CWS-Hyp protein contains no galactose, while peptides isolated from salt-extracted cell walls by proteolysis did contain galactose attached to serine residues. The present data, combined with Cho and Chrispeels' data, suggest a model for how cell wall protein becomes covalently attached to the cell wall in carrot discs. In this model a single cytoplasmic precursor is secreted via two alternate pathways. In one pathway the protein has arabinose added to it and enters into the cell wall as the CWS-Hyp protein. In the second pathway, the protein has galactose in addition to arabinose bound to it, enabling the latter glycoprotein to become covalently bound to a carbohydrate component of the cell wall.

The presence of proteins migrating like CWS-Pro among the *in vitro* translation products of RNA isolated from incubated carrot discs indicates that the disc system may be useful in studying gene organization and expression in plants.

It is likely that the cell wall protein mRNA accounts for 5 to 10% of the total mRNA of the discs. It should be feasible to isolate this mRNA and use it for isolating the gene for the cell wall protein. Many questions concerning the molecular biology of plants could then be studied with this system. The apparent lack of mRNA for the cell wall protein in fresh carrot discs also suggests that the carrot system may be valuable in studying changes in gene activity which occur in wounded plant tissue.

Acknowledgments—The author especially thanks Dr. Virginia Walbot who provided laboratory space, assistance, and advice contributing to the success of this work. Dr. Joseph Varner also provided valuable criticism and advice.

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