

# Isolation and Characterization of Monoclonal Antibodies Directed Against Plant Plasma Membrane and Cell Wall Epitopes: Identification of a Monoclonal Antibody that Recognizes Extensin and Analysis of the Process of Epitope Biosynthesis in Plant Tissues and Cell Cultures

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**Abstract.** Membranes from tobacco cell suspension cultures were used as antigens for the preparation of monoclonal antibodies. Use of solid phase and indirect immunofluorescence assays led to the identification of hybridomas producing antibodies directed against cell surface epitopes. One of these monoclonal antibodies (11.D2) was found to recognize a molecular species which on two-dimensional analysis (using nonequilibrium pH-gradient electrophoresis and SDS-PAGE) was found to have a high and polydisperse molecular mass and a very basic isoelectric point. This component was conspicuously labeled by [<sup>3</sup>H]proline *in vivo*. The monoclonal antibody cross-reacted with authentic tomato extensin, but not with potato lectin nor larch arabinogalactan. Use of the monoclonal antibody as an immunoaffinity reagent allowed the purification of a tobacco glycoprotein which was identical in amino acid composition to extensin. Finally, immunocytological analyses revealed tissue-specific patterns of labeling by the monoclonal antibody that were identical to

those observed with a polyclonal antibody raised against purified extensin. We have concluded that monoclonal antibody 11.D2 recognizes an epitope that is carried exclusively by extensin.

Analysis of cellular homogenates through differential and isopycnic gradient centrifugation revealed that biosynthesis of the extensin epitope was found on or within the membranes of the endoplasmic reticulum, Golgi region and plasma membrane. This result is consistent with the progressive glycosylation of the newly-synthesized extensin polypeptide during its passage through a typical eukaryotic endomembrane pathway of secretion. The 11.D2 epitope was not found in protoplasts freshly isolated from leaf tissues. However, on incubation of these protoplasts in appropriate culture media, biosynthesis of the epitope was initiated. This process was not impeded by the presence of chemicals that are reported to be inhibitors of cell wall production or of proline hydroxylation.

**T**HE synthesis, modification, and targeting of proteins and glycoproteins within the endomembrane/secretory system of eukaryotic cells is at best a complex process (Garoff, 1985). This process starts at the endoplasmic reticulum/polyribosome interface, which is the site of translation and transmembrane insertion of these proteins and glycoproteins (Blobel, 1980) and, if appropriate, of core glycosylation. The next compartment comprises the Golgi region, which is primarily involved in the elaboration of glycoprotein glycosylation (Kornfeld and Kornfeld, 1985), and the sorting of molecules destined for different final cellular locations (Farquhar, 1985; Griffiths and Simons, 1986). Beyond the Golgi region, dichotomous vesicle-mediated pathways lead the molecules to these destinations (Griffiths and Simons, 1986).

Analysis of endomembrane/secretory systems in eukaryotic cells has been greatly facilitated by the identification of protein and glycoprotein markers that progress along

specifically-programmed pathways within these systems. The availability of markers that are enzymes has allowed the selection of conditional-lethal mutants defective at various stages in the endomembrane secretory pathway in yeast (Schekman, 1985). The isolation of cDNAs and genomic DNA sequences encoding specific marker proteins has led to the selective modification of putative targeting signals within these markers (Garoff, 1985). Finally, the availability of unique markers, such as the Vesicular Stomatitis Virus G-protein, that are targeted to specific cellular destinations (the plasma membrane), has allowed the molecular dissection of the controls governing this process as well as permitting (through the availability of mutant virus forms) the development of reconstituted systems *in vitro* (Balch et al., 1987).

From these studies, it is clear that a primary requirement for the successful analysis of the endomembrane/secretory pathway in plants leading to the plasma membrane would be

the identification of analogous protein or glycoprotein markers that are processed through this pathway. In higher plants, there has been little information available concerning specific markers of this type. One possible way to identify these markers is to use monoclonal antibody techniques, since these techniques provide a powerful means for the analysis of membrane systems under circumstances in which the molecular identities of specific membrane components are unknown. Monoclonal antibodies by definition are directed against single epitopes (Koeher and Milstein, 1975); therefore complex membrane systems can be resolved according to the reactivity of their epitopes against different monoclonal antibodies. This leads to epitope analysis, the purification to homogeneity of individual antigens, and ultimately to the characterization of cognate genes.

We have previously reported the production and partial characterization of a series of monoclonal antibodies directed against plant cell surface epitopes (Meyer et al., 1987). Here, we detail the biochemical, subcellular and developmental characteristics of the molecule recognized by one of these monoclonal antibodies (11.D2). We conclude from these data that the epitope resides exclusively on a basic glycoprotein indistinguishable from the class of hydroxyproline-rich glycoproteins (HGPRs)<sup>1</sup> termed extensins (Shwalter and Varner, 1988). The biosynthesis of this molecule appears to be under tissue-specific and developmental regulation within intact plants and within protoplasts. This represents the first time that it has been possible to identify the molecular nature of the antigen recognized by a monoclonal antibody that is directed against a component of the higher plant cell surface.

## Materials and Methods

### Plants and Cell Cultures

Tobacco plants (*Nicotiana tabacum* L. cv. *xanthi*) were grown under standard greenhouse conditions. A suspension culture derived from *N. tabacum* L. cv. *xanthi* root tissue was maintained in darkness at 25°C with shaking (80 rpm) as 100-ml aliquots within 500 ml erlenmeyer flasks in MS medium (Murashige and Skoog, 1962) containing 3% (wt/vol) sucrose, 100 mg/liter inositol, 0.4 mg/liter thiamine, 0.1 mg/liter 6-benzylaminopurine and 1 mg/liter naphthaleneacetic acid. The cells were subcultured at 5-d intervals.

### Preparation of [<sup>3</sup>H]Proline- and [<sup>3</sup>H]Leucine-labeled Cells

Tobacco cells were collected 3 d after subculture by centrifugation at 100  $g_{(av)}$  for 5 min. They were radiolabeled ("pulse" conditions) by resuspension of 5 ml packed volume of cells (2 g fresh weight) in 25 ml of the MS culture medium supplemented with 5  $\mu$ Ci/ml either of L-[2,3,4,5-<sup>3</sup>H]proline (sp act 110 Ci/mmol) or L-[4,5-<sup>3</sup>H]leucine (sp act 120 Ci/mmol; Amersham Corp., Arlington Heights, IL). The cells were incubated under standard growth conditions for 24 h. For "chase" conditions, labeled cultures were collected by centrifugation at 100  $g_{(av)}$  and were washed by three cycles of centrifugation and resuspension in 50 ml of MS medium containing 1 mM of the appropriate amino acid. The cells were subsequently cultured for a period of 72 h. The radiolabeled cells were harvested by vacuum filtration onto Miracloth (Calbiochem, Inc., La Jolla, CA) placed on a Millipore Type XX10-047-30 filter holder (Millipore Corp., Bedford, MA). They were washed with 500 ml of ice-cold HB medium (50 mM Tris-HCl pH 8.0, 1 mM Na<sub>2</sub>EDTA, containing 1 mM phenylmethylsulfonyl

fluoride (PMSF), 1 mM dithiothreitol (DTT), and 100 mg/liter butylated hydroxytoluene). The cells were resuspended in 5 mL HB medium supplemented with 0.3 M sucrose (HBS) and were homogenized as described under Antigen Preparation. Proteins were isolated by phenol extraction (Schuster and Davies, 1983) before gel electrophoresis.

### Protoplast Preparation and Culture

Tobacco leaf protoplasts were prepared and cultured as previously described (Galbraith and Shields, 1982), sometimes including 5 mg/L 2,6-dichlorobenzonitrile (DB) and 100  $\mu$ M 3,4-dehydro-L-proline (DHP) within the culture medium (Galbraith and Shields, 1982; Cooper and Varner, 1983). The protoplasts were collected by centrifugation at 100  $g_{(av)}$ , were frozen in liquid nitrogen and were stored at -80°C until analysis.

### Antigen Preparation and Injection Protocols

Cells (10 g fresh weight) were harvested by vacuum filtration onto Miracloth and were washed with 5 vol of ice-cold HB medium. All further procedures were carried out at 0-4°C. The cells were resuspended in 4 vol of HBS medium, and were homogenized with 20 up-and-down passages of a motor-driven pestle using a Potter/Elvehjem homogenizer. The homogenate was filtered through Miracloth and was centrifuged at 10,000  $g_{(av)}$  for 10 min. The supernatant (33 ml) was layered over 5 ml of 50% (wt/wt) sucrose dissolved in HB medium and was centrifuged for 60 min at 100,000  $g_{(max)}$ . Total membranes were collected from the interface, were resuspended in sterile PBS to a concentration of 1 mg/ml, and were emulsified with complete Freund's adjuvant. BALB/c mice were given three intra-peritoneal injections (50  $\mu$ g protein/100  $\mu$ l) at 2-wk intervals. 3 d before sacrifice, one intravenous booster injection was given (10  $\mu$ g of protein in 100  $\mu$ l of PBS).

### Hybridoma Production and Screening

Hybridomas were produced by fusion of immune spleen cells with X63-Ag8.653 myeloma cells (Kearney et al., 1979), according to the protocol of Oi and Herzenberg (1980). Culture media were aseptically collected from wells, starting 14 d after the initiation of culture.

### Antigen Deglycosylation

All procedures were performed at 4°C. Total plant membranes, collected from the sucrose step gradient, were mixed with 0.1 vol of 0.15% (wt/vol) sodium deoxycholate, followed by addition of 0.1 vol of 72% (wt/vol) trichloroacetic acid after 10 min. Precipitates were washed three times with ice-cold 5% trichloroacetic acid, once with water-saturated diethyl ether, and dried before deglycosylation on ice, as described by Edge et al. (1981). Deglycosylation was complete after 3 h, as assessed using a Concanavalin A-affinoblotting procedure as described by Faye and Chrispeels (1985).

### Hybridoma Screening and Expansion

The culture filtrates were screened by dot-analysis on nitrocellulose (Type BA85; Schleicher and Schuell, Keene, NH), using 1- $\mu$ l aliquots of deglycosylated antigens solubilized by heating to 100°C in 10% (vol/vol) glycerol, 1.5% (wt/vol) SDS, 50 mM DTT, 0.02% (wt/vol) bromophenol blue, buffered by 62.5 mM Tris-HCl, pH 6.8, (SDS-PAGE loading buffer) at a protein concentration (Peterson, 1977) of 0.5 mg/ml. Before use, the blots were air-dried, rinsed in TBS buffer (500 mM NaCl, 20 mM Tris-HCl, pH 7.5), were blocked using TBS containing 2.5% (wt/vol) BSA for 60 min, and were finally rinsed with TBS. Hybridoma culture supernatants (50-100- $\mu$ l aliquots) were applied to the nitrocellulose using a Bio-Dot filtration manifold (BioRad Laboratories, Richmond, CA) according to the manufacturer's instructions. The blots were washed and probed as described by Birkett et al. (1985), using a 1:2,000 dilution of horseradish peroxidase-conjugated goat anti-mouse antibody (BioRad). The blots were rinsed in TBS before development using the protocol recommended by the manufacturer (BioRad). Subcloning was carried out through tenfold serial dilution of the hybridomas into a medium comprising equal volumes of DME containing 10% (vol/vol) FCS, 2 mM L-glutamine and 50 mg/liter gamarycin, and the same medium conditioned by the growth of Buffalo Rat Liver (BRL 3A) cells to confluence (Giss et al., 1982). Those wells that screened positively at the highest cell dilution were subcloned again. Positive clones were stored in liquid nitrogen. Ascites was produced in isogenic mice as described by Parham (1983). Monoclonal antibody was purified from ascites fluid through ammonium sulfate precipitation and immunoaffinity chroma-

1. *Abbreviations used in this paper:* ER, endoplasmic reticulum; HRGP, hydroxyproline-rich glycoprotein; NEPHGE, nonequilibrium pH-gradient electrophoresis.

topography using anti-mouse antibody as the solid phase reagent (Hudson and Hay, 1980). Dot analyses of the epitopes recognized by mAb 11.D2 were performed as described above, with the exception of using the alkaline phosphatase detection system.

### Gel Electrophoresis

One-dimensional SDS-PAGE were run as described by Laemmli (1970). Lyophilized protoplast samples were resuspended in SDS-PAGE loading buffer to a concentration equivalent to  $10^6$  cells/ml and were heated to 100°C for 5 min; other protein samples were resuspended to a concentration of ~5 mg/ml, and were heated similarly. Two-dimensional gel electrophoresis, using nonequilibrium conditions of isoelectric focusing (nonequilibrium pH-gradient electrophoresis [NEPHGE]) in the first dimension, was performed according the methods of O'Farrell et al. (1977). Protein samples for two-dimensional gel analysis were prepared by phenol extraction (Schuster and Davies, 1983). Silver-staining was done as described by Monroy and Schwartzbach (1983), and fluorography was carried out using EN<sup>3</sup>HANCE (New England Nuclear, Boston, MA).

### Western Blotting

SDS-PAGE gels were subjected to electroblotting at 10 V/cm for 2 h using either Type BA83 or BA85 nitrocellulose (Schleicher and Schuell, Keene, NH). Transfer of proteins onto polyvinylidene difluoride (PVDF) membranes was performed according to Matsudaira (1987) at 10 V/cm for 2 h.

### Antibody Probing

Hybridoma culture supernatants were adjusted to 0.5% (vol/vol) in Tween-20 and 1.0 M in NaCl before use. Affinity-purified monoclonal antibody was diluted to 2.5 µg/ml in HST; monoclonal antibodies from ammonium sulfate-fractionated ascites fluids were diluted to 25 µg/ml in the same buffer. Incubations with primary antibodies were carried out either for 1 h or overnight, at room temperature, and washed as described for the dot-blots. Antibody localization was determined either as described for HRP or with the nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate system (Blake et al. 1984). Alkaline phosphatase-conjugated goat anti-mouse antibody was obtained from BioRad Laboratories.

### Tissue Printing

Printing of freshly cut, free-hand sections of intact plant tissues onto nitrocellulose was performed as described by Cassab and Varner (1987). Blots were probed with antibody as described for the Western blots. The distribution of total protein was found using India ink (Hancock and Tsang, 1983).

### Immunoaffinity Purification of the 11.D2 Antigen

A crude extensin-enriched cell wall fraction (Smith et al., 1986), prepared from 150 g (fresh weight) tobacco cells, was used as the starting material for immunoaffinity purification of the 11.D2 antigen. Monoclonal antibody 11.D2, from ammonium sulfate-fractionated ascites fluid, was coupled to cyanogen bromide-activated Sepharose 4B-CL (Sigma Chemical Co., St. Louis, MO) using optimized conditions (Pfeiffer et al., 1987). A column of 4 ml bed vol was prepared in a 20 ml disposable plastic syringe. The gel was then pre-washed extensively with 5 column-vol each of TBS, 0.1 M sodium bicarbonate, pH 10.5, TBS, 0.1 M sodium citrate, pH 3.0, and finally TBS. All further procedures were carried out at room temperature. The cell wall extract was dissolved in 4 ml of TBS and clarified by centrifugation for 10 min at 15,000  $g_{(max)}$ . After application of the supernatant to the affinity column, and washing with TBS, specifically-bound material was eluted with 0.1 sodium citrate, pH 3.0. This eluate (17 ml) was neutralized with 1 M Tris-HCl, pH 8.0, and was dialyzed against a solution of 1 mM Na<sub>2</sub>EDTA and 50 mM Tris-HCl, pH 8.0.

### Amino Acid Analysis of the 11.D2 Antigen

The immunoaffinity-purified 11.D2 antigen (63 µg) was concentrated using a Centriprep-30 concentrator (Amicon Corp., Danvers, MD) to a volume of 375 µl. Recrystallized urea was added to a final concentration of 6 M. The antigen was reduced under nitrogen by incubation for 3 h at 37°C with 1 mM DTT. The antigen was alkylated in the dark using an excess (2.2 mM) of iodoacetamide. Unreacted reagents were removed by dialysis against dis-

tilled water. Aliquots of purified material were hydrolyzed in vacuo for 24, 48, or 72 h in 6 N HCl (Pierce Chemical Co., Rockford, IL) at 110°C. Amino acids were converted to phenylthiocarbonyl derivatives before separation by HPLC (Bidlingmeyer et al., 1984) using a Pico-Tag column (Waters Associates, Milford, MA)

### Subcellular Fractionation

All procedures were performed on ice, in a walk-in cold room maintained at 4°C. For analysis by differential centrifugation, suspension culture cell homogenates (20 ml) were centrifuged at 1,000  $g_{(max)}$  for 10 min. The supernatant was centrifuged at 10,000  $g_{(max)}$  for 10 min. For analyses involving isopycnic sucrose gradient centrifugation, all sucrose solutions were dissolved in TE (1 mM EDTA, 10 mM Tris-HCl, pH 8.0). The membranes contained in the 10,000  $g_{(max)}$  supernatant were isolated as described previously, and were diluted to 20 mL and 18% (wt/wt) in sucrose. An aliquot (4 ml) of this was applied to a 28 ml 20-50% (wt/wt) continuous sucrose gradient, formed in a SW28 centrifuge tube above a 4.0 mL step of 50% (wt/wt) sucrose. Isopycnic separation was carried out at 100,000  $g_{(av)}$  for 180 min. Fractions (1 ml) were diluted with 4 ml of 10% (wt/vol) sucrose dissolved in TM (1 mM MgSO<sub>4</sub>, 1 mM Tris-MES, pH 7.4). The membranes were collected by centrifugation at 200,000  $g_{(max)}$  for 30 min. The membrane pellets were resuspended in 100 µl of 10% (wt/vol) sucrose in TM. Enzyme assays were performed as described by Galbraith and Northcote (1977), with the exception of the inclusion of 50 mM KCl in the Mg<sup>2+</sup> ATPase assay.

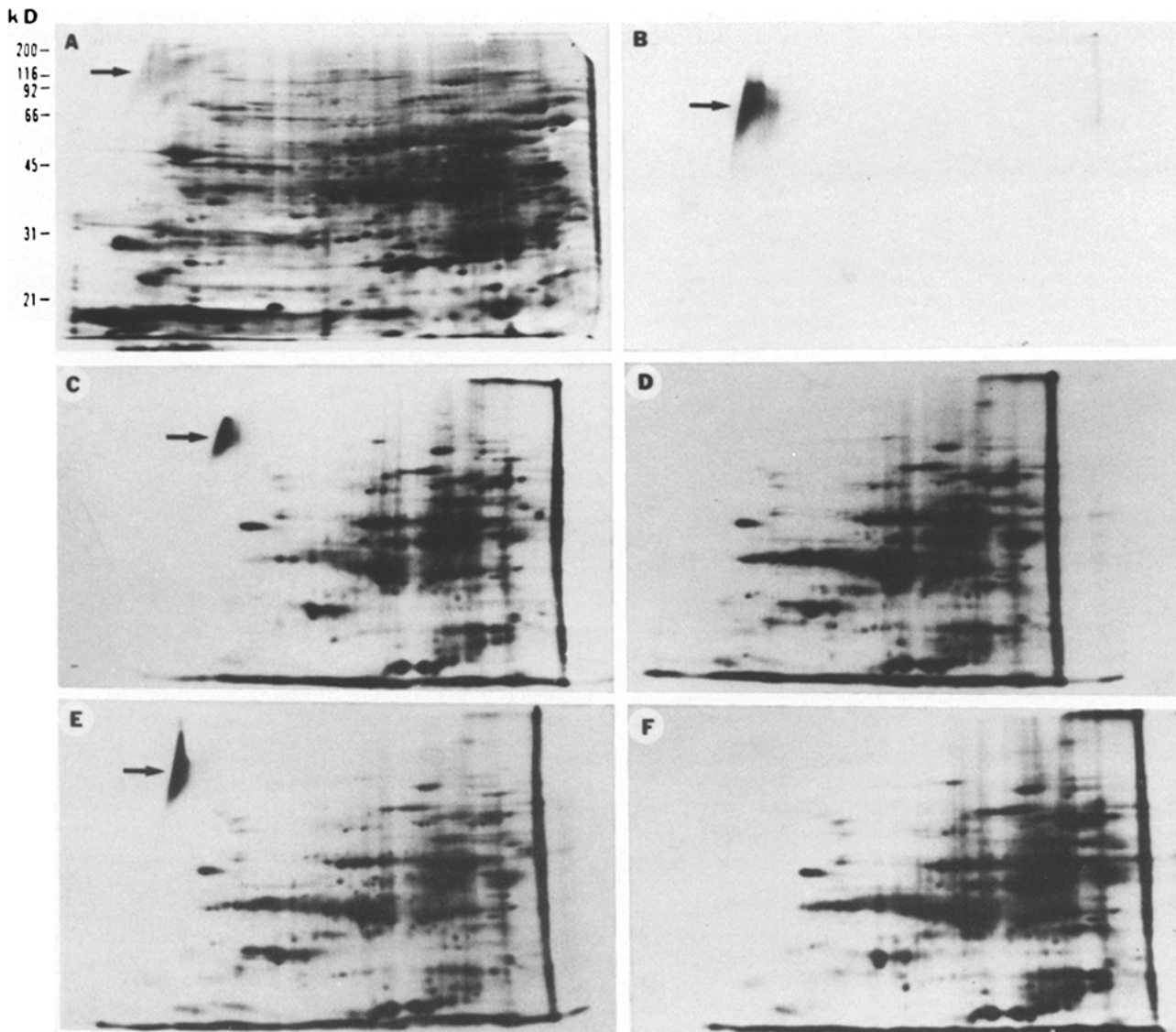
### Results

#### Characterization of the Epitope Recognized by Monoclonal Antibody 11.D2

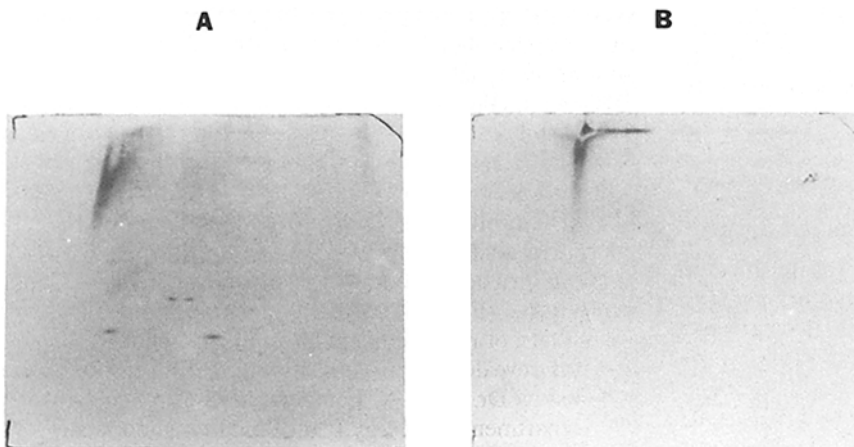
Monoclonal antibody 11.D2 was produced by a vigorously-growing subclone isolated from hybridoma line 4G5 (Meyer et al., 1987). Western blots of one-dimensional SDS-PAGE gels indicated that this mAb specifically recognizes an epitope carried by a molecular species of high and hetero-disperse molecular mass (~80-140 kD). To further characterize this species, we performed two-dimensional analyses of total tobacco cell culture protein extracts using a combination of nonequilibrium pH-gradient gel electrophoresis and SDS-PAGE. Silver staining revealed many well-resolved molecules (Fig. 1 A). Western blotting of these gels identified a single molecular species bearing the 11.D2 epitope (Fig. 1 B). This component has a NEPHGE mobility indicating a basic isoelectric point. Optimal transfer of the proteins from two-dimensional gels required the use of the electro-transfer protocol of Matsudaira (1987), although for one-dimensional separations, electrotransfer onto nitrocellulose (Towbin et al., 1979) was satisfactory.

Fluorographic analysis of two-dimensional NEPHGE/SDS-PAGE gels, using samples from cell cultures labeled *in vivo*, revealed that the epitope was one of few proteins that were intensely labeled by [<sup>3</sup>H]proline (Fig. 1 C) but not by [<sup>3</sup>H]leucine (Fig. 1 D). Growth of the labeled cells in the presence of nonradioactive amino acids for 72 h resulted in a 250% increase in the amount of protein in the cultures, with the specific activity of the total protein decreasing to 20% ([<sup>3</sup>H]proline) or 28% ([<sup>3</sup>H]leucine) of the original values. Gel analysis indicated that the specific activity of the 11.D2 antigen decreased, with a concomitant increase in molecular mass (Fig. 1 E).

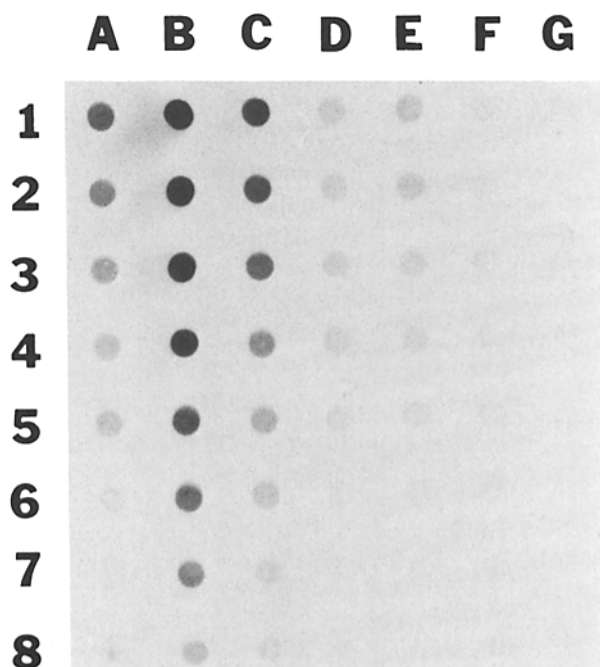
A pattern of migration similar to that of the tobacco species was revealed when authentic tomato extensin (kindly provided by Dr. D. T. A. Lampert, Michigan State University-Department of Energy Plant Research Laboratory) was



**Figure 1.** Identification of the 11.D2 epitope. Two-dimensional (NEPHGE-SDS) analysis of total proteins extracted from tobacco cell suspension cultures. All gels are oriented with the basic end of the NEPHGE dimension toward the left. Fig. 1, C-F are from the same 7-d fluorographic exposure, with 25,000 cpm loaded per gel. (A) Two-dimensional gel (50  $\mu$ g total protein), after silver-staining. (B) Western blot (50  $\mu$ g total protein), probed with mAb 11.D2. (C) Fluorographic analysis (10  $\mu$ g total protein) after "pulse" labeling for 24 h with [ $^3$ H]proline. (D) Fluorographic analysis (9.6  $\mu$ g total protein) after "pulse" labeling for 24 h with [ $^3$ H]leucine. (E) Fluorographic analysis (50  $\mu$ g total protein) after a 72-h "chase" of proline-labeled cells. (F) Fluorographic analysis (35  $\mu$ g total protein) after a 72-h "chase" of leucine-labeled cells.



**Figure 2.** Two-dimensional electrophoretic comparison of the tobacco 11.D2 antigen with purified tomato extensin, using two-dimensional gel analysis. The first dimension comprised pH 3.5-10 NEPHGE for 2,000 volt-hours. The second dimension comprised SDS-polyacrylamide gels (10% acrylamide). After transfer, the nitrocellulose blots were probed with mAb 11.D2. (A) Sample (250  $\mu$ g) of Triton X-114-insoluble antigen (Meyer et al. 1987). (B) Sample (5  $\mu$ g) of tomato P1b extensin precursor.

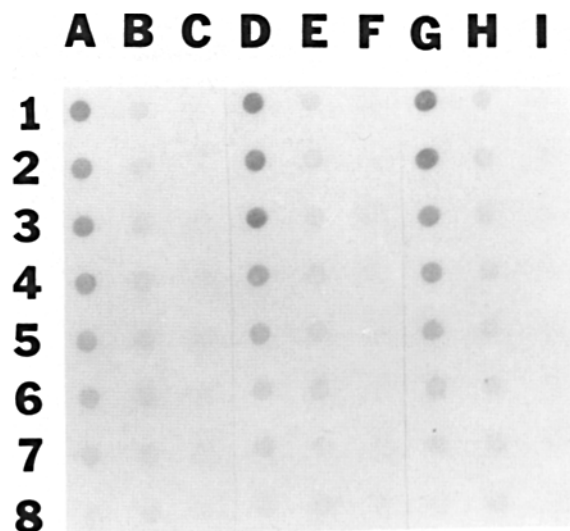


**Figure 3.** Dot-blot analysis of the cross-reactivity of mAb 11.D2 to various cell wall HRGPs. Rows 1-8 contain serial (one-half) dilutions of the indicated proteins, from 2,000 ng (row 1) to 16 ng (row 8). Columns: (A) Total proteins extracted from tobacco cell cultures. (B) Tomato extensin P1. (C) Tomato extensin P2. (D) HF-deglycosylated P1. (E) HF-deglycosylated P2. (F) Potato lectin. (G) Control (no protein).

subjected to two-dimensional Western blot analysis using mAb 11.D2 (Fig. 2). Dot-analysis showed that the limit of detection in this assay was very low ( $\sim 0.2$  pmol; Fig. 3, dot B8), confirming that mAb 11.D2 was specifically recognizing the major species within the tomato extensin preparation. Chemical deglycosylation with anhydrous hydrogen fluoride decreased the antigenicity of this extensin sample (columns D and E in Fig. 3). A second HRGP (*Solanum tuberosum* (potato) lectin; Sigma Chemical Co., St. Louis, MO) was not recognized by mAb 11.D2 at amounts up to 40 pmol (column F in Fig. 3). The interaction between mAb 11.D2 and the epitope present in tobacco protein extracts was not affected by the presence of larch arabinogalactan (Grade I; Sigma Chemical Co., St. Louis, MO) or 3-O-beta-D-galactopyranosyl-D-arabinoside (60% alpha-anomer, 40% beta-anomer; Sigma Chemical Co.) in the incubation media (Fig. 4). In this latter case, the highest concentration that we used greatly exceeded that reported to yield 50% inhibition of binding of mAbs directed against arabinogalactan-containing plant glycoproteins (Anderson et al., 1984).

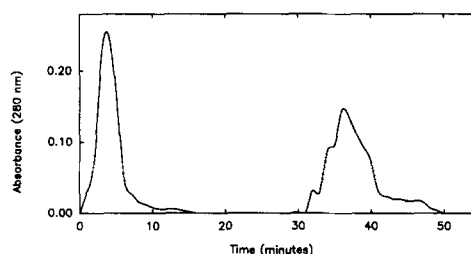
#### Purification and Analysis of the 11.D2 Antigen

The antigen recognized by mAb 11.D2 was purified to homogeneity through use of immunoaffinity chromatography. Based on our initial observations suggesting that mAb 11.D2 recognized an epitope carried by extensin, we used a cellular eluate enriched in extensin (Smith et al., 1986) as the starting material for this purification. When this eluate was subjected to chromatography on mAb 11.D2-linked



**Figure 4.** Competition analysis of the mAb 11.D2/epitope interaction. The antibody was diluted in HST buffer and was preincubated with the described amounts of the competitor species for 60 min at room temperature before being used to probe the antigens. Competing molecules: (A, B, and C) Larch arabinogalactan (1 mg/ml); (D, E, and F) 25 mM galactopyranosyl arabinose; (G, H, and I) 25 mM glucose. Rows 1-8 contain serial (one-half) dilutions of the indicated antigens (as described for Fig. 3). Columns: (A, D, and G) Total tobacco cell protein; (B, E, and H) potato lectin; (C, F, and I) control (no protein).

Sephacrose, approximately equal amounts of UV-absorbing material were recovered in the unbound and specifically-bound, citrate-eluted fractions (Fig. 5). Amino acid analysis of the specifically-bound material indicated a very high hydroxyproline content, with successively lesser proportions of lysine, serine, valine, proline, threonine, histidine, and tyrosine (Table I). An unidentified peak, eluting between the phenylthiocarbonyl derivatives of threonine and alanine in this system, increased in amount with increasing hydrolysis time, yet accounted for less than 2.5% of the UV absorbance. The composition data are consistent with the observed pattern of migration seen on two-dimensional gel electrophoresis and with the absence of labeling with [ $^3$ H]leucine (Figs. 1 and 2).



**Figure 5.** Immunoaffinity chromatography on 11.D2-Sepharose. The salt-eluted, trichloroacetic acid soluble fraction from 155 grams (fresh weight) XSR suspension-cultured cells was applied to a 4 ml column of 11.D2-Sepharose and incubated for 30 min. The column was washed with 10 bed vol of TBS, then eluted with 5 bed vol of 0.1 M sodium citrate, pH 3.0. The flow rate was  $\sim 1.25$  ml/min; the UV absorbance of the effluent was monitored at 280 nm.

**Table I. Amino Acid Composition of the 11.D2 Antigen**

Amino acid	mol
	%
Asx	0.0*
Glx	0.0
Hyp	44.6
Ser	9.1‡
Gly	0.0
His	4.3
Arg	0.0
Thr	5.1‡
Ala	0.7
Pro	7.6
Tyr	3.8
Val	8.3§
Met	0.0
Cys	0.0
Ile	0.0
Leu	0.3
Phe	0.0
Lys	16.7
Trp	ND

\* Indicates undetectable levels.

‡ Extrapolated to zero time from values at 24, 48, and 72 h of hydrolysis.

§ Value from 72 h hydrolysis. ND, not done.

### Tissue Distribution of the 11.D2 Epitope

We examined the tissue locations of the epitope recognized by mAb 11.D2 through tissue printing (Cassab and Varner, 1987). Initially, we compared the distribution of the 11.D2 epitope with that of a polyclonal antibody raised against soybean seed coat extensin (Cassab and Varner, 1987). Thus, in tissue blots of immature *Glycine max* fruit, the epitope recognized by mAb 11.D2 was restricted to the inner sclerenchymatous layer in the seed pod, to the testa and to those tis-

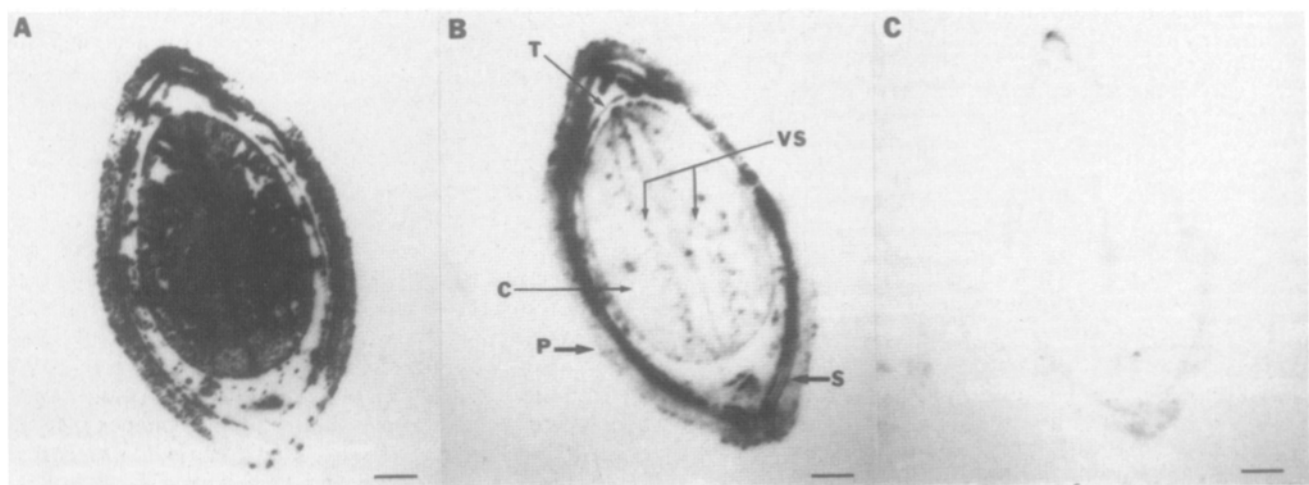
suces near the vascular system of the cotyledons (Fig. 6). This pattern is identical to that reported by Cassab and Varner (1987) and is quite different from that seen using a control mAb of identical isotype, and from the pattern of transfer of total protein as revealed by India ink (Fig. 6).

Analysis of tobacco stems sectioned at the uppermost internode (using a young plant that was ~30% of its mature height), revealed that specific antibody binding was restricted to thin layers of cells close to the epidermis and internal to the xylem (Fig. 7, A-D). In basal segments a similar pattern was observed (Fig. 7, E-H), although increased staining of the cortex and pith was apparent.

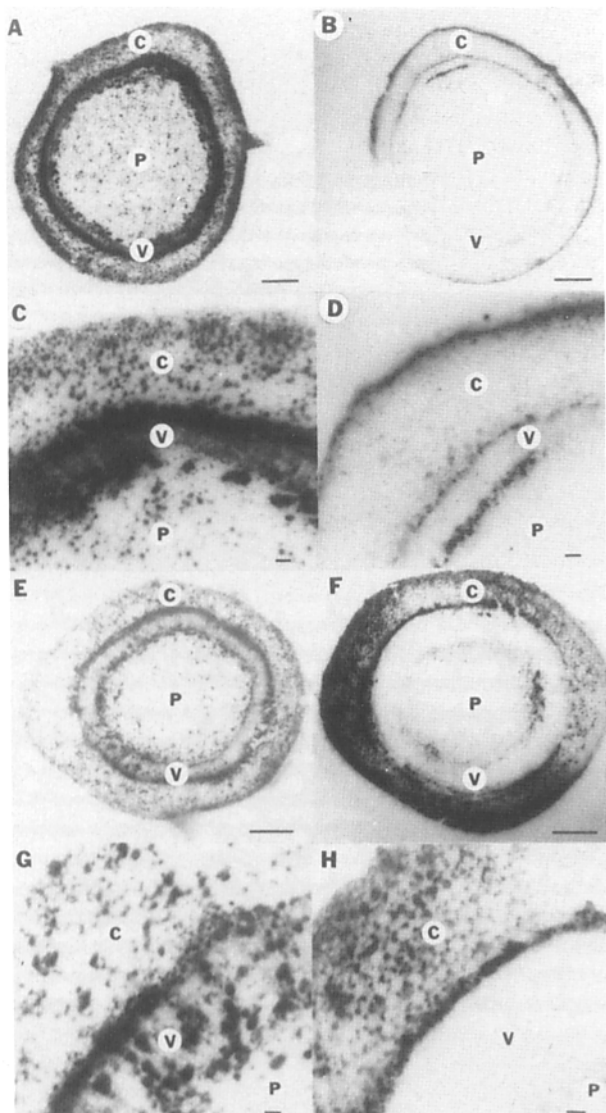
### Subcellular Localization of the 11.D2 Epitope

The subcellular distribution of the 11.D2 epitope in suspension culture cells was investigated through differential centrifugation of cellular homogenates followed by one-dimensional SDS-PAGE. Western blots were then probed using mAb 11.D2 (Fig. 8). The molecular species carrying this epitope was present at an approximately equal concentration in the subcellular membranes pelleted by centrifugation at 10,000 and 100,000  $g_{(max)}$ , but was not found in the soluble fraction of the cell nor in the 1,000  $g_{(max)}$  pellet. The absence of this cell wall protein from the 1,000  $g_{(max)}$  pellet, which should contain most of the cell wall fragments, is presumably either due to its insolubility in the low ionic-strength SDS-PAGE sample buffer or due to the formation of intermolecular crosslinks with other cell wall components, thus preventing its elution.

Based on these data, we used isopycnic sucrose gradient centrifugation to examine the cellular membranes contained within the fraction precipitated by differential centrifugation between  $10^5$  and  $6 \times 10^6$   $g/min$  (Fig. 9). Total membrane protein was broadly distributed through the gradient, the three peaks representing obvious bands of turbidity. One of these, at a density of 1.165 g/ml, corresponded to the position of most of the fumarase activity (a mitochondrial mark-



**Figure 6.** Tissue prints of immature fruits of *Glycine max*. Tissue prints were prepared as described by Cassab and Varner (1987). *A* and *B* are from opposite halves of the same cross-section. (*A*) Stained with India ink. (*B*) Probed with mAb 11.D2. (*C*) Probed with control mAb 109.3 (directed against dinitrophenol). Structures labeled: (*T*) testa; (*VS*) vascular supply of seed; (*C*) cotyledon; (*P*) parenchyma of pericarp; (*S*) sclerenchyma of pericarp. Bars, 1 mm.



**Figure 7.** Tissue prints of *Nicotiana tabacum* stems. *A-D* are prints taken from cross-sections of the uppermost internode of 30-cm tall, greenhouse-grown plants. *E-H* are prints taken from a section of the same plant at a point 5 cm above the base. *A, C, E, and G* are stained for total protein with India ink. *B, D, F, and H* were probed with mAb 11.D2. *C, D, G, and H* are enlargements of *A, B, E, and F* respectively. Structures labeled: (*C*) cortex; (*V*) vascular cylinder; (*P*) pith. Bars, (*A, B, E, and F*) 1 mm; (*C, D, G, and H*) 0.1 mm.

er). The major peak of NADH-cytochrome *c* reductase activity, a marker enzyme for the endoplasmic reticulum and the outer membrane of the mitochondrion, was found at a density of  $\sim 1.124$  g/ml, with a minor portion associated with the mitochondrial fumarase activity. Latent inosine diphosphatase (IDPase), a marker enzyme associated with the Golgi region (Ray et al., 1969), was found at a density of 1.136 g/ml. Two peaks of  $K^+$  stimulated  $Mg^{2+}$ -ATPase activity, a putative plasma membrane marker enzyme (Hodges and Leonard, 1974), were located at densities of 1.148 and 1.165 g/ml. One-dimensional SDS gel electrophoretic separation of the membranes contained in the gradient fractions, followed by silver-staining, resolved a

large number of different proteins (Fig. 10 *A*). Western blotting revealed that the high molecular-mass component recognized by monoclonal antibody 11.D2 co-localized with marker enzyme activities associated with the ER, the Golgi region and the plasma membrane (Fig. 10 *B*).

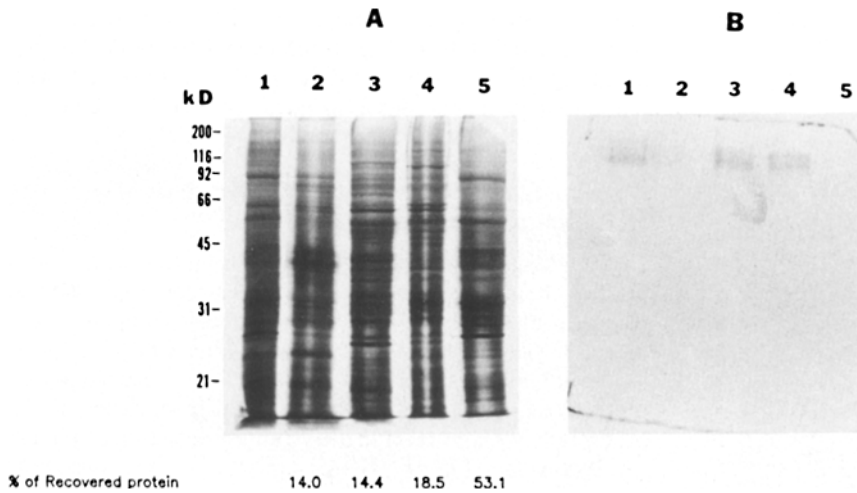
### **Developmental Controls of the Biosynthesis of 11.D2 Epitope Expression**

Leaf tissues and protoplasts freshly-isolated from leaf tissues did not contain levels of the epitope that could be detected using dot-blot analysis. However, when leaf protoplasts were placed in heterotrophic culture, biosynthesis of the epitope was readily detectable after  $\sim 48$  h (Fig. 11). After 8 d in culture, it was possible to observe a signal from as few as 30 protoplasts without reaching the limit of detection of the assay. Inclusion of DB or DHP, or a mixture of these two compounds, did not affect the total amount of epitope biosynthesis as estimated from dot-blots. However, one-dimensional SDS-PAGE analysis showed that the inhibitors caused a slight reduction in overall protein synthesis (Fig. 12 *A*). Western blotting revealed that inclusion of DHP decreased the apparent size of the molecular species carrying the epitope and increased its polydispersity (Fig. 12 *B*). The amount of reactivity on the Western blot appears to be decreased in samples treated with DHP because of the increased polydispersity. In contrast, the inclusion of DB had no effect on the apparent size and dispersity of the antigen.

### **Discussion**

The theoretical advantages of using monoclonal antibody techniques for the analysis of plant endo- and plasma-membrane systems derive primarily from the fact that clonal hybridomas secrete antibodies that are directed against single epitopes. Thus, even though plant endo- and plasma membranes obviously comprise complex mixtures of macromolecules, it should be possible to use individual monoclonal antibodies, chosen from a library raised against crude membrane preparations, for the identification and molecular dissection of the different macromolecules contained within the membranes. This approach firstly assumes that the individual macromolecules are antigenic and secondly that appropriate methods for screening the monoclonal antibodies can be developed. Previously, we have reported the preliminary characterization of a monoclonal antibody library directed against antigens derived from total cell membranes from *Nicotiana tabacum* cell cultures (Meyer et al., 1987). The initial screen, which involved the use of denatured and deglycosylated membrane proteins and glycoproteins applied as dots to nitrocellulose, led to a rapid identification of the minority of the hybridomas ( $\sim 8\%$ ) that secreted antibodies directed against epitopes present on deglycosylated membranes. Subsequent immunofluorescence analysis allowed the identification of hybridomas secreting monoclonal antibodies directed against plasma membrane epitopes (Meyer et al., 1987). A total of 34 stable hybridoma cell lines, corresponding to a minimum of 13 of the 45 original hybridomas, were successfully recovered.

Although our work, and that of others, suggests that it is relatively easy to produce monoclonal antibodies directed against plant epitopes which are located primarily at the



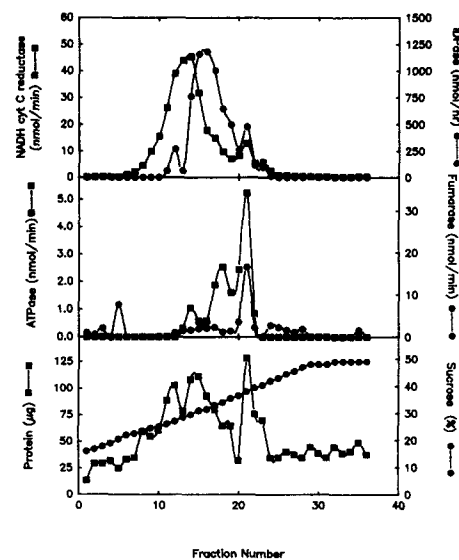
**Figure 8.** One-dimensional SDS-PAGE analysis (10% acrylamide) of the proteins contained in total cellular homogenates and in resultant membrane fractions obtained by differential centrifugation. (A) Gel after silver staining; (B) Gel probed with mAb 11.D2 after blotting onto nitrocellulose. (Lane 1) Crude homogenate; (lane 2) 1,000 g pellet; (lane 3) 10,000 g pellet; (lane 4) 100,000 g pellet; (lane 5) 100,000 g supernatant. Each lane contains 10  $\mu$ g protein. The calculated distribution of total protein between each of the fractions is presented below the individual lanes.

plant cell surface (Metcalf et al., 1986; Norman et al., 1986; Villanueva et al., 1986; Fitter et al., 1987; Hahn et al., 1987), subsequent antigen identification and characterization has proved difficult (Villanueva et al., 1986; Hahn et al., 1987). In this first case, the species recognized by a monoclonal antibody raised against soybean protoplasts comprised a molecule, or series of molecules, with an extremely high and polydisperse molecular mass (Villanueva et al., 1986). In the second case, the bulk of the molecules recognized by several independently-derived mAbs were found to have polydisperse molecular masses (60–120 kD). In neither case has a precise definition of the epitopes recognized by these mAbs been achieved.

In our work, the use of two-dimensional NEPHGE/SDS-PAGE techniques of separation was essential for the identification of the molecular species recognized by one of the monoclonal antibodies (mAb 11.D2). This single molecular species has a high, polydisperse molecular mass and displays a characteristic, curved pattern of separation on two-dimensional gels. It has an intrinsic charge that locates it well to the basic side of the range (pH 5–7) of conventional isoelectric focusing (Booz and Travis, 1980; Zurfluh and Guilfoyle, 1982; Lafayette et al., 1986). Thus the nonequilibrium aspect of the charge-based dimension of the gel separation procedure was particularly important for the identification of the 11.D2 antigen. The fact that the monoclonal antibody appears to recognize only a single molecular species argues that the epitope is not a simple glycan moiety of plant glycoproteins. Since the antibody does not recognize proteins or glycoproteins that are abundantly represented, we can be confident that non-specific interactions are not responsible for these observations.

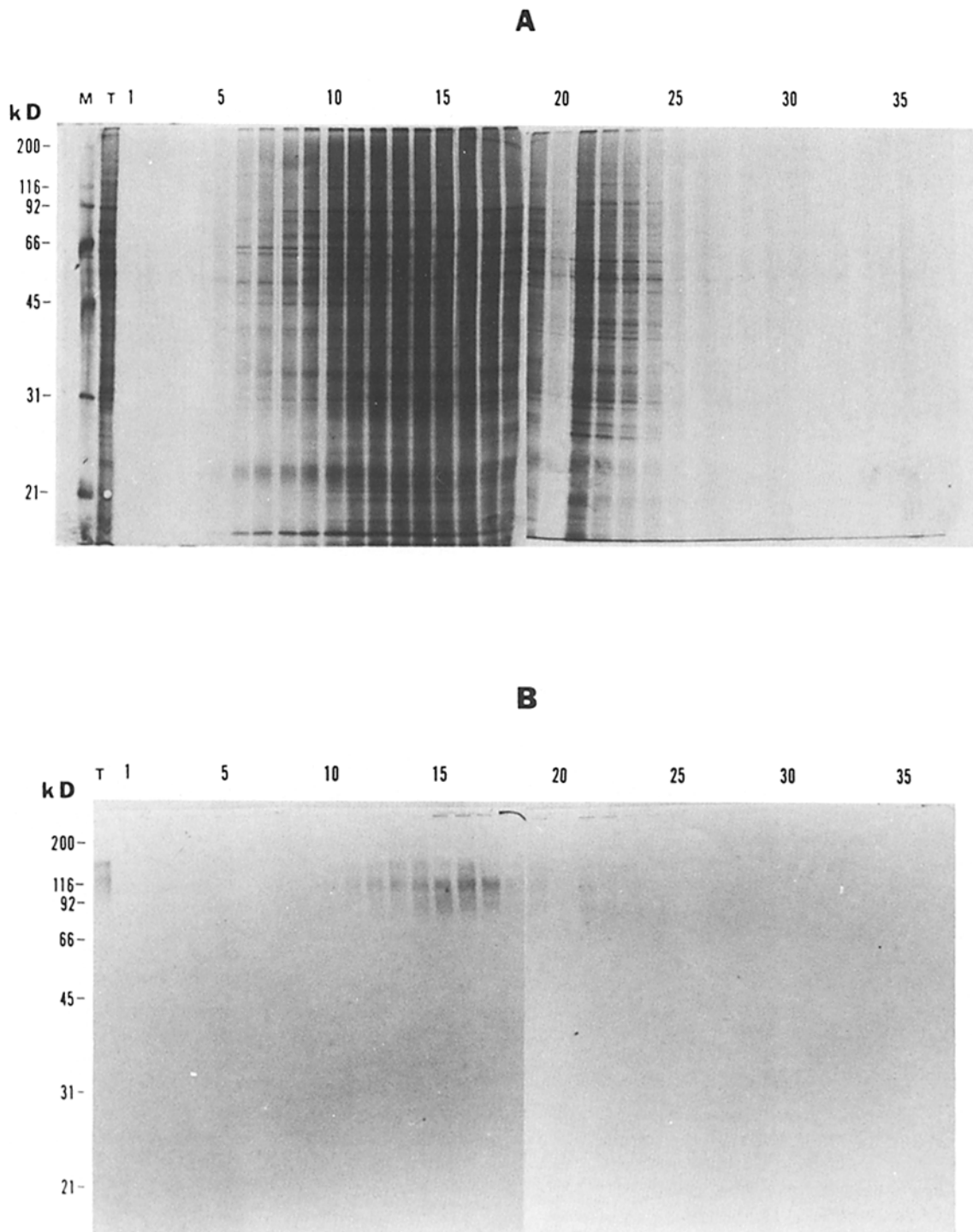
Several pieces of evidence strongly indicate that the 11.D2 epitope is carried on (one of) the hydroxyproline-rich glycoproteins termed extensins (Showalter and Varner, 1988). Firstly, it bears immunological cross-reactivity to the form of extensin that can be solubilized from tomato cell walls (Smith et al., 1986) and their patterns of mobility on two-dimensional gel analysis are almost identical. Secondly, the amino acid composition of material purified through immunoaffinity chromatography (Table I) is strikingly similar to that found for extensins purified from a wide variety of di-

cotyledonous plant species and tissues (Showalter and Varner, 1988). Further evidence linking the 11.D2 epitope with extensin was obtained through use of a tissue printing procedure specifically designed for the immunolocalization of extensins in developing soybean seeds (Cassab and Varner, 1987). Our results demonstrate that the pattern of distribution observed using mAb 11.D2 was similar to that reported using polyclonal antibodies raised against purified seed coat extensin (Cassab and Varner, 1987); the vascular supply of the cotyledons and the seed coat are specifically recognized by both antibodies, and we have also observed intense staining of the hilum in appropriate sections (data not shown). These results also imply that the epitope recognized by mAb 11.D2 is a feature of extensins in divergent dicotyledonous plant species.



**Figure 9.** Distribution of marker enzymes on sucrose gradient fractionation of cellular membranes partially purified by differential centrifugation. Enzyme activities and protein amounts are expressed as total amounts per fraction.

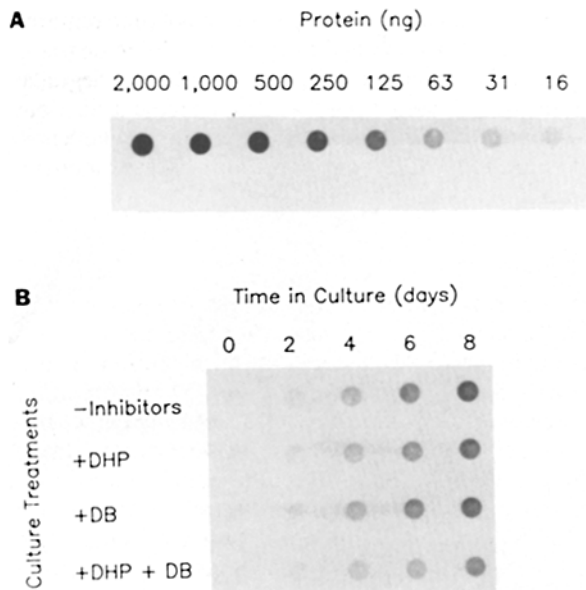




**Figure 10.** One-dimensional SDS-PAGE (10% acrylamide) analysis of the membrane fractions obtained by isopycnic sucrose gradient centrifugation. 5% of each fraction was loaded per lane. (A) Gel after silver staining. (B) Gel probed with mAb 11.D2 after blotting onto nitrocellulose. Lanes: (M) molecular weight markers; (T) crude membrane fraction (5  $\mu$ g). All remaining lanes are numbered according to the numbers given in Fig. 9.

These morphological data provide further information that may relate to the functional role of extensin in the movement of plant tissues. In particular, the intense staining of the pericarp by mAb 11.D2 is restricted to the sclerenchyma; this novel observation is consistent with the proposal that extensin may be a marker for sclerenchyma (Cassab and

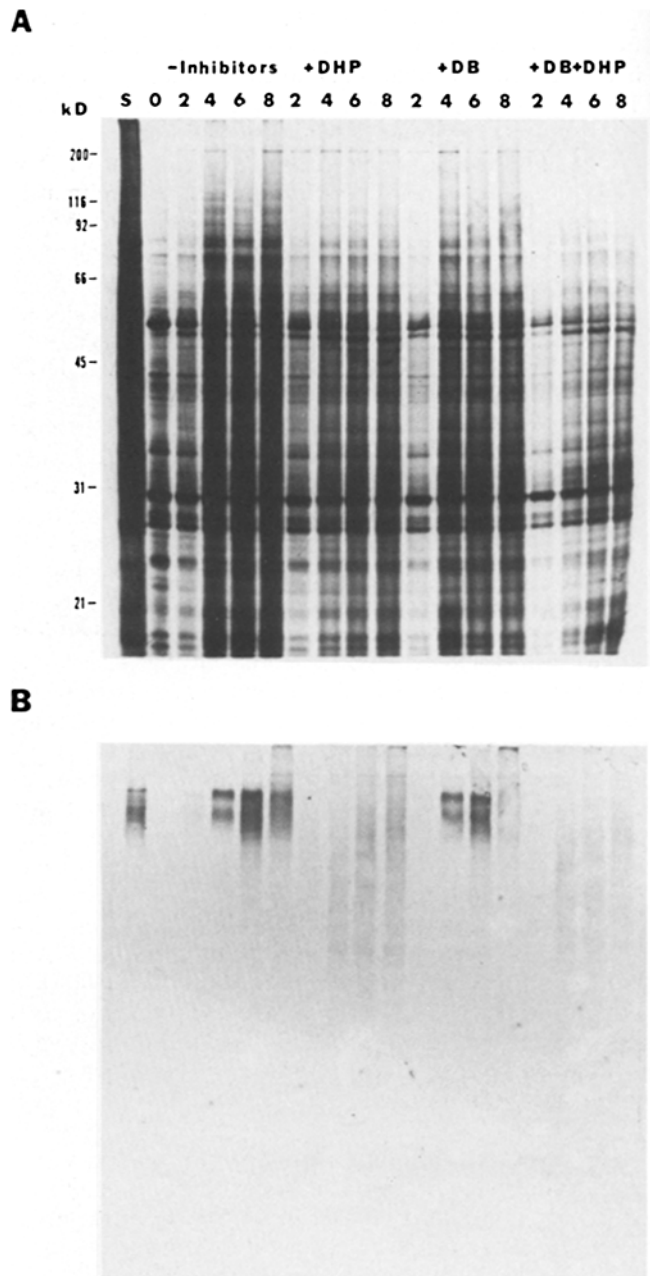
Varner, 1987). However, in *Glycine max*, the pericarp comprises two layers of sclerenchyma, separated by parenchymatous tissue. The outer layer of sclerenchyma lies just below the outer epidermis, whereas the inner layer lies proximal to the internal epidermis. It is believed that these layers are involved in the process of seed dehiscence, through the shrink-



**Figure 11.** Induction of expression of the 11.D2 epitope in leaf protoplasts. (A) Standard dilutions of total proteins extracted from cell cultures. (B) Equal numbers (250) of protoplasts that had been cultured for 0–8 d in the presence or absence of 100  $\mu$ M 3,4-dehydro-L-proline and 5 mg/liter 2,6-dichlorobenzonitrile. The protoplasts were solubilized in SDS-sample buffer before dotting on nitrocellulose. Blots A and B were probed with mAb 11.D2 and developed under identical conditions.

age of the inner layer of sclerenchyma (termed the “tissue of movement”) relative to the outer layer (the “tissue of resistance”) during desiccation of the fruit, resulting in the opening of the valves and the dehiscence of the seeds (Monsi, 1943). The cellulose microfibrils in the walls of the two sclerenchymatous tissues are differentially oriented (Monsi, 1943), and this may dictate the directions along which tissue movement can occur. Our results from tissue printing indicate that staining is restricted to the “tissue of movement”; we have not observed staining of the outer sclerenchyma of the pericarp. The differences in amounts of salt-elutable extensins between these two tissues that are destined for crucial roles in differential movement are interesting, but obviously require further analysis. Since we have only examined seeds and seed pods from  $\sim$ 21 d post-anthesis and since dehiscence occurs at a later time, a full understanding of the development of the two sclerenchymatous layers of the pericarp and of the role of extensin in dehiscence will require analysis of a series of temporal stages during seed development. It is also known that extensins can become insolubilized through cross-linking within the cell wall (Lampert and Epstein, 1983; Cooper et al., 1984); thus further experiments should also include an investigation of the distribution of both free (salt-extractable) extensin (using the tissue printing technique) and total extensin (using immunocytological analysis of tissue sections).

In *Nicotiana tabacum*, the tissue printing procedure has provided some information about the distribution of extensin within different plant organs. Our previous work demonstrated the presence of the 11.D2 antigen in extracts of plant roots and to a lesser extent in plant stems, whereas this anti-



**Figure 12.** One-dimensional SDS-PAGE (10% acrylamide) analysis of 11.D2 antigen expression in leaf protoplasts. (A) Silver-stained gel containing 10  $\mu$ g of total proteins extracted from cell suspension cultures (lane S) and 5,000 protoplasts cultured for 0–8 d in the presence or absence of DHP and DB, as indicated. (B) Duplicate gel containing 10  $\mu$ g of total cell protein and 20,000 protoplasts, as for A. The gel was blotted and probed with mAb 11.D2, as described in the text.

gen was completely absent from extracts of leaf tissues (Meyer et al., 1987). Tissue printing indicates that the extensin found within the stem tissues is differentially distributed within different cell types and, in particular, the vascular cylinder is not stained. Histochemical analysis of thin sections will be required to identify the types of cells which are stained by the antibody, before speculation about the possible functions represented by this differential distribution.

The absence of the 11.D2 antigen from leaves and from freshly-isolated leaf protoplasts contrasts with its abundance in cell suspension cultures and in cultured leaf protoplasts. This implies a form of developmental regulation of extensin biosynthesis accompanying the process of dedifferentiation that is observed in the production of cell cultures from tobacco leaf tissues and from leaf protoplasts. Preliminary experiments suggest that the initiation of extensin biosynthesis in cultured protoplasts is not controlled by the process of wounding that inevitably accompanies protoplast production. Thus, when we excised and incubated tobacco leaf discs under conditions that lead to the biosynthesis of extensin in carrot root explants (Chrispeels, 1969), no increase in levels of the 11.D2 antigen was observed (data not shown). This result parallels observations concerning changes in extensin mRNA levels in wounded leaves (Showalter and Varner, 1987). Other workers have shown that conversion of carrot suspension-cultured cells into protoplasts results in the rapid accumulation of a 1.5-kb HRGP mRNA associated with wounding (Ecker and Davis, 1987). The apparent lack of HRGP mRNA induction in wounded leaves either suggests that the signal for induction may differ between suspension-cultured cells and leaf cells, or that the biosynthesis of HRGPs occurs much more slowly in leaf cells. The absence of extensin from freshly-isolated leaf protoplasts and its appearance during heterotrophic protoplast culture are clearly consistent with the observation that extensin biosynthesis accompanies the initiation of cellular growth in plants (Showalter and Varner, 1988). The inclusion of an inhibitor of cell wall biosynthesis (DB), which prevented the appearance of a Calcofluor-positive cell wall (data not shown), did not prevent accumulation of the 11.D2 epitope. This suggests that the action of DB is not to prevent endomembrane vesicle fusion with the plasma membrane (Galbraith and Shields, 1982). The fact that the inclusion of dehydroproline had no effect on this result circumstantially argues that the epitope is not glycosidically linked to hydroxyproline, although we cannot currently exclude the possibility of minor levels of glycosylation.

The process of extensin biosynthesis in cell suspension cultures appears to occur within the cytoplasmic membranes of the secretory pathway as indicated by Western blot analysis of purified subcellular membranes subjected to one-dimensional SDS-PAGE. The polydisperse molecular mass of the molecule and its unique isoelectric point can be interpreted in terms of the addition of neutral, rather than charged sugars in the ER and Golgi region. We were unable to detect putative, non-glycosylated precursors of this main molecular species in the ER. This either suggests that the 11.D2 epitope is a glycan moiety, or that glycosylation may occur very rapidly following translation, resulting in a very low steady-state level of non-glycosylated precursors. A third possibility is that the precursors may be synthesized within a light-vesicle subset of the ER, similar to those described for animal cells by Lodish et al. (1987). This vesicle population may not carry the marker enzyme used to define the ER (NADH-cytochrome C reductase), and would probably not be pelleted under the conditions described for the fractionation of the membranes in the 10,000 g supernatant. Finally, it is possible that the smallest components are particularly susceptible to degradation, and that resuspension of the enriched membrane fractions in the absence of inhibitors of

proteolysis, coupled to the prolonged period of time required for complete fractionation and analysis, results in degradation. This possibility seems unlikely, since obvious degradation of proteins was not observed when the gradient fractions were silver-stained. We are currently examining whether in vitro translation products can be recognized by the monoclonal antibody.

### Epitope Mapping

Although our results indicate that the epitope recognized by mAb 11.D2 is exclusive to extensin, we have not identified the molecular nature of this epitope. Competition experiments imply that the epitope is not shared by other types of HRGP, including larch arabinogalactan and *Solanum tuberosum* lectin, and is not carried by an arabinogalactan disaccharide reported to be a common epitope of monoclonal antibodies raised against plant membrane proteins (Anderson et al., 1984). Since potato lectin not only has an amino acid composition similar to extensin but also contains patterns of glycosylation (hydroxyproline arabinosides and serine galactosides) that are identical in structure to those predominating within the extensin molecule (Ashford et al., 1982), it is probable that mAb 11.D2 does not recognize a simple epitope carried by these carbohydrate moieties. Alternatives include that the antibody recognizes a complex carbohydrate structure that is presented differently on extensin and potato lectin, that the antibody recognizes an as yet unidentified carbohydrate linkage or structure unique to extensin, or that it recognizes the polypeptide portion of the molecule. It has been observed that polyclonal antibodies raised against either glycosylated or deglycosylated extensins exhibit a low degree of cross-reactivity to potato lectin despite the considerable homology between the carbohydrate substituents of these molecules (Kieliszewski and Lampion, 1986; Cassab and Varner, 1987). This suggests that the carbohydrate portions of extensin may not be particularly antigenic and thus that the immunodominant epitopes found in the polyclonal antisera arise from polypeptide-dependent conformations seen only in extensin and not in potato lectin. The fact that chemically-deglycosylated extensin monomers exhibit decreased reactivity to mAb 11.D2 (which conventionally is interpreted to suggest that the epitope on the mature glycoprotein resides within the glycosyl moieties) might be explained in terms of the loss upon deglycosylation of a conformation-dependent epitope residing within the polypeptide portion of the molecule. At present there are conflicting data regarding the contribution of carbohydrate to the extended rod conformation of extensin. Van Holst and Varner (1984) and Stafstrom and Staehelin (1986) have suggested a role for carbohydrate in maintaining the overall three-dimensional structure of the extensin molecule. Alternatively, recent evidence from other workers (Heckman et al., 1988) has been interpreted to indicate that carbohydrate may not be involved in overall conformation (although this requires the assumption that succinylation of the deglycosylated extensin molecule has no effect upon this conformation). If mAb 11.D2 does not simply recognize a carbohydrate epitope removed by deglycosylation, it may prove useful as a structural probe of the extensin molecule both in vivo and in vitro.

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