Plant Physiol. (1988) 88, 1446–1453
0032-0889/88/88/1446/08/$01.00/0

Production and Characterization of Monoclonal Antibodies to Wall-Localized Peroxidases from Corn Seedlings[1]

Received for publication March 4, 1988 and in revised form July 26, 1988

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ABSTRACT

A library of 22 hybridomas, which make antibodies to soluble wall antigens from the coleoptiles and primary leaves of etiolated corn (*Zea mays* L.) seedlings, was raised and cloned three times by limit dilution to assure monoclonal growth and stability. Two of these hybridomas made immunoglobulin G antibodies, designated mWP3 and mWP19, which both effectively immunoprecipitated peroxidase activity from crude and partially purified preparations of wall peroxidases. Direct peroxidase-binding assays revealed that both antibodies bound enzymes with peroxidase activity. As judged by immunoblot analyses, mWP3 recognized a M_r 98,000 wall peroxidase with an isoelectric point near 4.2, and mWP19 recognized a M_r 58,000 wall peroxidase. Immunogold localization studies showed both peroxidases are predominately in cell walls.

Plant peroxidases (EC 1.11.1.7) are of importance in a variety of cellular functions such as lignin biosynthesis, NADH-dependent hydrogen peroxide formation, suberization, metabolism of aromatic compounds, and ethylene production (11). Peroxidases are found in both the cytoplasm and the cell wall of plant cells, and the cell wall isozymes differ from the cytoplasmic ones (9). Most of the literature on wall peroxidases concerns factors that induce changes in their activity, as measured by colorimetric assays, or studies of their localization. For example, several authors have tested the possibility that peroxidase changes are involved in the control of cell elongation and have found that there is an inverse relationship between peroxidase activity levels and growth (2). The localization studies utilize a variety of different techniques, including cytochemical staining (13), radioactive labeling (31), and immunofluorescence assays with polyclonal antibodies (8).

A number of wall peroxidase isoenzymes have been identified and partially purified from different plant species and tissues. Despite these studies, very little is known about the protein chemistry of wall peroxidases. As van Huystee reviewed (28), the most appealing approach for studying the various functions of plant peroxidases in detail is to employ immunological assays. Efforts to quantitate, purify, and biochemically characterize wall peroxidases would be greatly aided by the availability of monoclonal antibodies against them. In this paper, we describe the production of monoclonal antibodies against cell wall antigens from corn coleoptiles and the identification of two of them as being directed against two different wall-localized peroxidases.

MATERIALS AND METHODS

Chemicals. Alkaline phosphatase-labeled goat anti-mouse Ig(G+M+A)[3] was purchased from Kirkegaard & Perry Lab., Inc. (KPL). Nitrocellulose membrane (0.45 μm pore size) was purchased from Schleicher & Schuell, and Cyt *c*, *o*-dianisidine, *o*-dianisidine tetrazotized, β-naphthyl acid phosphate, protein A-Sepharose 4B, 4-chloro-1-naphthol, Antifoam A, amido black, aprotinin, thimerosol, and 4-aminoantipyrine were purchased from Sigma. Nonfat dry milk (Carnation) was purchased from a local grocery store. The other chemicals were all reagent grade.

Plant Material and Growth Conditions. Seeds of corn (*Zea mays* L., Silver Queen and Merit varieties) were surface-sterilized in 10% Clorox for 15 min and rinsed with distilled water for 6 h. Seeds were sown on moist vermiculite and grown for 7 d in the dark at 20°C. Seedlings were irradiated with white light for 15 min at 15 h before harvesting, then returned to darkness until harvesting. All further manipulations were carried out under white fluorescent room light.

Extraction of Cell Wall Antigens. Coleoptile sections (1.5 cm) with enclosed leaves were excised from 3 mm below the coleoptile tip of 7-d-old seedlings. The sections were packed into plastic syringe barrels (25), rinsed in cold distilled water for 30 min, and infiltrated with ice-cold extraction buffer, which was 10 mM NH_4HCO_3 (pH 7.7), 1 mM $Na_2S_2O_5$, and 50 mM $CaCl_2$ with 0.05% aprotinin, for 4 min under vacuum and another 3 min without vacuum, and then centrifuged at 1,000*g* for 8 min. This sequence was repeated twice. The cell wall extracts were pooled. Prior to being injected into mice, the wall proteins were lyophilized. For some enzyme assays and other purposes, the crude wall protein preparations were concentrated and desalted by centrifugation through a Centricon-10 microconcentrator (Amicon) or they were brought to 95% saturation with solid ammonium sulfate and after 30 min stirring at 4°C, centrifuged at 25,000*g* for 20 min. The protein pellets were resuspended in 10 mM NH_4HCO_3 (pH 7.7) with 0.05% aprotinin.

Immunization. Balb/c mice were initially immunized by foot pad, subcutaneous, and intraperitoneal injections of 500 μg of extracted wall proteins emulsified in complete Freund's adjuvant. The mice were given subcutaneous and intraperitoneal injections

[1] This work was supported in part by a grant, NSG 7480, from the National Aeronautics and Space Administration to S. J. R.

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[3] Abbreviations: Ig, immunoglobulin; ELISA, enzyme-linked immunoadsorbent assay; PBST, phosphate buffered saline with Tween-20; BLOTTO, bovine lacto transfer technique optimizer; 4AAP, 4-aminoantipyrine; TBS, Tris-buffered saline; TBST, Tris-buffered saline with Tween-20; NS-1, nonsecretor-1; IEF, isoelectric focusing; pI, isoelectric point.

at biweekly intervals with 250 μg wall proteins in incomplete Freund's adjuvant. A final intravenous injection of 250 μg wall proteins in PBS (50 mM Na phosphate, 150 mM NaCl, pH 7.5) was given 3 d prior to the spleenectomy.

Production and Screening of Hybridomas. Hybridomas were produced from the fusion of spleen cells from the immunized mice with P3 × 63Ag8 myeloma cells and selected by the same procedures as those described in Silberman *et al.* (22). Specific hybridomas were screened for production of antibodies to wall antigens using a solid-phase ELISA and Western blotting, employing crude wall proteins as the antigens. Those hybridomas secreting antibodies that gave strong reactions by either ELISA or Western blot assays were further cloned three times by limit dilution to assure monoclonal growth. Among these clones, the 22 that had the highest titers as estimated by both ELISA and Western blot analyses were subcloned and used to raise ascites fluids.

Production of Monoclonal Antibodies. The procedures employed were basically the same as those described in Silberman *et al.* (22). Pristane-primed mice were inoculated with 5×10^7 to 10^8 hybridoma cells. Two to 3 weeks later, the peritoneal cavities of visibly swollen mice were tapped with an 18-gauge needle three times, and the ascitic fluids were pooled, and centrifuged at 12,000*g* for 5 min to remove cells and large debris. Ascites fluids were then delipidated following the procedure of Neoh *et al.* (20). Monoclonal antibodies from delipidated ascitic fluids were purified by chromatography on CM Affi-gel Blue (Bio-Rad) (12). The purity of purified antibodies was determined by SDS-PAGE, and this permitted an estimate of antibody levels used for immunotitrations, the direct peroxidase-binding assay, and immunoinhibition. Subtyping was determined according to the Boehringer-Mannheim mouse immunoglobulin subtype identification kit (22).

Immunotitrations of Wall Peroxidases. Fractions containing about 30 μg of wall proteins that had been concentrated by ammonium sulfate were incubated at 4°C for 6 h in PBS (pH 7.5) plus the indicated amounts of immune IgG (mWP3 and mWP19) and NS-1 ascites as nonimmune mouse ascites control. The antibodies in NS-1 ascites should represent only mouse host antibodies, because cell line of NS-1.4.1 makes a κ-light chain but does not secrete it. After incubation, 10 μL of a 10% protein A-Sepharose 4B was added, and the samples were further incubated an additional 1 h at 4°C. Following centrifugation, the supernatants were assayed for peroxidase activity as described below.

Western Blots. Discontinuous SDS-PAGE was performed according to Laemmli (16) using minislab gels containing 9% acrylamide. The mol wt was determined by the method of Weber *et al.* (30). Concentrated wall proteins (12–15 μg/well) were electrophoretically separated on a 9% SDS-polyacrylamide gel (16) in a Hoefer minislab gel apparatus, and then transferred by electrophoresis to a nitrocellulose membrane (0.45 μm pore size) by the method of Towbin *et al.* (26) in a Hoefer Transfer apparatus at 80 V for 1.5 h at 4°C. All the following procedures were done at room temperature. Amido black or India ink (Pelican) staining of the control strip revealed the band pattern of the transferred proteins. The blotted nitrocellulose was blocked into BLOTTO for 2 h as described in Johnson *et al.* (15). The BLOTTO solution was 5% (w/v) nonfat dry milk, 0.01% Antifoam A, and 0.0001% Thimerosol in PBS (pH 7.5). Blotted nitrocellulose membrane was incubated in each purified monoclonal antibody in PBS containing 0.05% Tween-20 (0.05% PBST) for 3 h. Strips were then washed in 0.5% PBST for 1 h and incubated with alkaline phosphatase-labeled goat anti-mouse Ig(G+M+A) antibodies in PBS for 1 h. After washing out the remaining antibodies, the immunoblots were visualized in a solution containing 0.25 mg/mL *o*-dianisidine tetrazotized and 0.25 mg/mL β-naphthyl acid phosphate in sodium borate with 1.2 mg/mL of $MgSO_4$ (pH 9.7) (6) or KPL phosphatase substrate system (KPL).

Enzyme Assay. Peroxidase assays were carried out using 4AAP as a substrate (27) in a total volume of 1 mL of 20 mM HEPES (pH 7.2) with 0.006% of H_2O_2 at 25°C. The enzyme activity was expressed as ΔOD_{510} min^{-1} mg^{-1}. Protein content was estimated by the method of Lowry *et al.* (17) using bovine serum albumin as a standard.

Nondenaturing Gel Electrophoresis. Polyacrylamide nondenaturing gel (7.5% acrylamide and 5% *N,N'*-methylene bisacrylamide) electrophoresis was performed in an alkaline pH range using a Bio-Rad tube gel electrophoresis apparatus according to the method of David (7). The pH of the running gel was 8.5, with bromophenol blue used as the tracking dye. In the tube gel electrophoresis, peroxidase bands were visualized by placing gels in a peroxidase substrate solution of 20 mM Tris-HCl buffer (pH 7.5) with 150 mM NaCl containing 0.15 mg/mL *o*-dianisidine and 0.06% H_2O_2. For eluting these bands and thus obtaining a partial purification of wall peroxidases, 2 mm gel slices were cut from the nondenaturing tube gel and put into microfuge tubes, each containing 100 μL of elution buffer (PBS, pH 8.0). Elution proceeded at 4°C for 12 h, and after clarification by centrifugation, the supernatants were used for the peroxidase enzyme assay described as above. Positive fractions were designated as partially purified peroxidase preparations and were used for immunoprecipitation of peroxidase with antibodies.

Immunoprecipitation of Peroxidases. A preabsorbed antibody complex was prepared by adding 40 μL of each monoclonal antibody derived from partially purified antibodies or NS-1 control to 70 μL of a 10% (w/v) suspension or protein A-Sepharose 4B in PBS (pH 7.5). After incubating in an end-over-top mixer overnight at 4°C, the preabsorbed antibody complex was centrifuged and washed three times with PBS and the addition of 1% BSA in the middle wash step. Wall proteins (30 μg) concentrated by ammonium sulfate were added to the preabsorbed antibody complex. The antibody-antigen complex was incubated for 12 h at 4°C, and centrifuged. The resulting supernatant was then assayed for peroxidase activity. The pellet was washed five times with PBS, dissolved in SDS-containing sample buffer (16) with reduction by β-mercaptoethanol, boiled for 3 min, and subjected to electrophoretic separation on SDS-PAGE, followed by Western blot analysis as described above.

Direct Peroxidase-Binding Assay. The procedure was essentially as described in Suresh and Milstein (24). On a 1 × 5 cm strip of nitrocellulose membrane, 3 μL aliquots (each containing 2 μg of antibody) of partially purified antibody preparations of mWP3, mWP19, and NS-1 control were spotted at 1 cm intervals. After 5 min, the strip was washed with PBS for 10 min and then blocked in 5% BLOTTO for 20 min. The strip was then incubated with 0.5 mg of wall protein extract in 5% BLOTTO for 2 h with shaking. Unbound proteins were removed by washing in 0.5% PBST three times (10 min each wash). The strip was then developed with 0.4 mg/mL 4-chloro-1-naphthol and 0.03% H_2O_2 in PBS.

Immunoinhibition of Peroxidase Activity. Serial dilutions of purified antibodies were incubated with 4.5 μg of partially purified wall peroxidase preparation at 4°C for 2 h. The peroxidase activity in the solution was then directly measured (no centrifugation) with 4AAP as the substrate.

Isoelectric Focusing Gel Electrophoresis. From 1 g dry weight of freeze-dried coleoptile, crude wall proteins were eluted with the same extraction buffer described earlier, then clarified by centrifugation. From this preparation, 40 μg of concentrated proteins in 5 μL was subjected to analytical flat isoelectric focusing on IsoGel (FMC) plate containing ampholytes in the pH range 3.0 to 10.0. The samples were loaded 2 cm from the

negative pole of the gel, and subjected to focusing for 90 min at 500 V at 10°C. After the focusing step, the gel was briefly rinsed with transfer buffer (20 mM Tris, 500 mM NaCl, pH 7.5), and transferred to nitrocellulose membrane by contact diffusion for 25 min. After the transfer, immunostaining was carried out as described for Western blots.

Periodate Treatment of Wall Peroxidase Preparation. Aliquots of a wall protein preparation concentrated by Centricon-10 (1.5 μg of total protein per aliquot) were spotted on a strip of nitrocellulose membrane. After 5 min, the strips were briefly rinsed with 50 mM Na phosphate (pH 7.5), then some were incubated in 40 mM Na metaperiodate in the same phosphate buffer in the dark for 15 or 30 min, respectively. Others were blocked in 5% BLOTTO for 30 min. Strips treated with Na metaperiodate were washed with distilled H_2O, phosphate buffer, and PBS (each two changes for 15 min), then blocked in 5% BLOTTO. After the blocking step, the nitrocellulose membrane was treated the same as for Western blots.

Immunogold Localization. Corn coleoptiles with enclosed primary leaves were fixed in 2% formaldehyde and 0.17% glutaraldehyde in 0.1 M Na phosphate buffer (pH 7.5) for 1 h and then fixed in 2% formaldehyde for 4 additional hours at room temperature. Samples were washed in cold Na phosphate buffer and dehydrated through an ethanol series, and then embedded in Lowicryl K4M (Polysciences, Inc.) and polymerized by UV irradiation at 4°C overnight. Silver to gold sections were cut, and picked up on Parlodion-coated nickel grids, and then washed in 0.1 N HCl for 10 min followed by a wash in distilled H_2O for 5 min. They were then blocked in 5% nonfat dry milk in PBS for 30 min. They were incubated for 1 h in purified mWP3 or mWP19 diluted in 0.05% PBST (final antibody concentration ranged from 150 to 200 ng/μL), washed two times with 0.5% PBST followed by TBS (20 mM Tris, 150 mM NaCl, pH 8.2) containing 0.5% Tween-20 (0.5% TBST), and incubated with gold (15 nm)-conjugated goat anti-mouse antibody (Janssen) diluted 1:20 in 0.05% TBST for 30 min. After the sections were washed in 0.5% TBST three times and once in distilled H_2O, they were dried and poststained with 5% saturated uranyl acetate for 30 min.

RESULTS

Production of Monoclonal Antibodies. The harvested ascites fluids were delipidated, then all of them were tested by Western blot and dot blotting. Twelve of 22 antibodies clearly showed positive reactivities with crude wall protein preparations in the Western blot, but all of them showed strong reactivities in the dot blot. Two IgG antibody preparations, designated mWP3 and WP19, immunoprecipitated peroxidase activity from the wall protein extracts. As shown in Figure 1, mWP19 can immunoprecipitate the specific wall peroxidase it recognizes at an antibody concentration three times lower than that required for mWP3 to immunoprecipitate the peroxidase it recognizes under these experimental conditions. But dilution curves for these antibodies show mWP3 has a higher titer than mWP19 (data not shown).

Identification of Peroxidase in the Nondenaturing Gel. Ammonium sulfate at 95% saturation precipitated most of the proteins in the cell wall preparation, including all of the peroxidase activity. Every wall protein preparation consistently showed virtually the same protein pattern when analysed by SDS-PAGE. The range of M_r observed was from 15,000 to 100,000. Wall proteins concentrated by ammonium sulfate were loaded on the nondenaturing gel for electrophoresis at an alkaline (pH 9.5) pH. After electrophoresis, gels were either stained with substrate or eluted with appropriate buffer. Partially purified peroxidases eluted from the nondenaturing gel (see "Materials and Methods") were assayed for peroxidase activity. Analysis by SDS-PAGE of

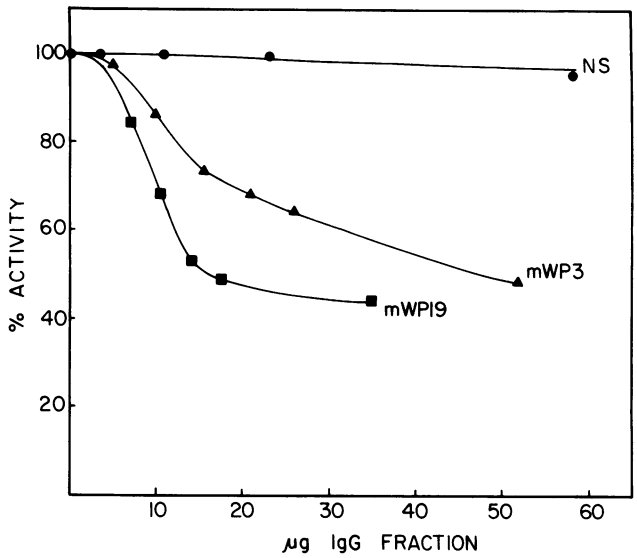

FIG. 1. Comparison of titers of mWP3 and mWP19 needed to immunoprecipitate peroxidase activity from a crude wall protein preparation concentrated by ammonium sulfate. Fractions containing about 30 μg of wall proteins were incubated at 4°C for 6 h in PBS (pH 7.5) plus the indicated amounts of IgG (mWP3 and mWP19) and NS-1 control which were preabsorbed with protein A-Sepharose 4B. After centrifugation, the resulting supernatants were assayed for peroxidase activity.

those eluted fractions showing peroxidase activity revealed some protein bands in each fraction. When positive fractions having the same protein patterns in the SDS-PAGE were pooled and assayed by dot blot for cross-reactivity with mWP3 and mWP19, one broad band near the cathodic pole of the gel showed cross-reactivity with both monoclonal antibodies (data not shown).

Immunoprecipitation Studies. The preabsorbed NS-1 control and preabsorbed ascitic fluids for mWP3 and mWP19 which were used for immunoprecipitation of peroxidase activity from corn wall protein preparation showed no endogenous peroxidase activity. When partially purified peroxidase preparations were subjected to the immunoprecipitation test, all of the activity was immunoprecipitated by both mWP3 and mWP19 (Fig. 2). Also a significant fraction of the total peroxidase activity present in crude wall protein extracts could be immunoprecipitated by preabsorbed mWP3 and mWP19, but not by the preabsorbed NS-1 control (data not shown). Peroxidase activity was recovered in the immunoprecipitate pellet after incubation both with mWP3 and with mWP19. The concentration of $MgCl_2$ (3.5 M) used to elute antigen from the immunoprecipitate pellet (18) did not interfere with peroxidase activity. Western blot analysis of the peroxidase immunoprecipitated from concentrated crude wall proteins or partially purified wall peroxidase preparation showed that the enzyme had a M_r of 98,000 (lanes 3 and 4 in Fig. 3). Because the second antibody used for the Western blot also recognized the denatured mouse immunoglobulin (both heavy and light chains of IgG), this blot also exhibited strong immunostained bands of both heavy and light chains of IgG (★). Western blot analysis of mWP3 in the absence of wall proteins showed only heavy and light chains of IgG in the immunoblot (data not shown).

In our SDS-PAGE and Western blot analysis of wall proteins, a 15 μg sample is typically loaded per lane, and loading more than 30 μg protein on a lane in the minigel distorts the banding pattern of proteins on the gel. With sample loads of 15 to 30 μg, we have not been able to detect the binding of mWP3 to a M_r 98,000 band (or to any other band) in crude wall protein preparations by direct Western blot analysis. Apparently, in crude wall protein samples of 15 to 30 μg, there is either not enough M_r

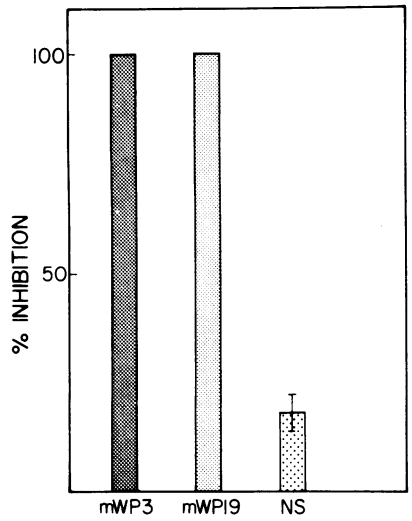

FIG. 2. Immunoprecipitation of partially purified peroxidase preparations by mWP3 and mWP19. Forty μL (about 10 μg) of protein present in an electrophoretically purified band containing peroxidase activity were added to the antibody-protein A-Sepharose complex. The antibody-antigen complex was incubated for 12 h at 4°C, and centrifuged. The resulting supernatant was then assayed for enzyme activity and the pellet was used for the experiment described in Figure 3 (lane 3).

98,000 peroxidase to be detected by direct Western blot analysis or not enough of the peroxidase that is present is transferred and bound stably enough to the nitrocellulose to be stained by the labeled second antibody. To stain this peroxidase by mWP3 in a Western blot analysis, the enzyme had to be first concentrated by being immunoprecipitated from pooled wall protein preparations so that much more of it could be loaded per lane of the SDS-polyacrylamide gel. In contrast, mWP19 could strongly stain a single M_r 58,000 protein band in a direct Western blot analysis of 15 μg of crude wall proteins (Fig. 4).

Direct Peroxidase-Binding Assay. Both mWP3 and mWP19 bound to peroxidase in crude wall protein preparations when they were tested in direct peroxidase-binding assay described in Suresh and Milstein (24), while the NS-1 control showed only minimum nonspecific binding (Fig. 5).

Immunoinhibition of Peroxidase Activity. It can be seen from Figure 6 that mWP3 inhibits a peroxidase activity when using 4AAP and H_2O_2 as substrate, but mWP19 does not. Analysis of multiple runs revealed that the small apparent stimulation of peroxidase activity by mWP19 was statistically insignificant, but the inhibition by mWP3 was significant (t test analysis, $P < 0.05$).

Immunodetection of Wall Peroxidase on IEF Blots. When reacted with an immunoblot of concentrated crude coleoptile proteins that had been separated by IEF, mWP3 showed a significant reaction only with a protein that had an apparent pI near 4.2 (Fig. 7D) and that stained positive for peroxidase activity (Fig. 7C). This designation of the peroxidase recognized by mWP3 as anionic was consistent with the same conclusion reached by analysis of mWP3 reaction with isoperoxidases separated by nondenaturing gel electrophoresis.

Periodate Treatment of Wall Peroxidase. Wall proteins modified by periodate treatment were still recognized by mWP3 and mWP19. Their cross-reactivity appeared to be the same as that of unmodified wall proteins on dot blots (data not shown).

Immunogold Localization of Wall Peroxidases. The antibodies specific for wall peroxidases labeled mainly the cell wall area of both coleoptiles and enclosed young leaves of corn. Because there was so little cytoplasm present in coleoptile cells, the mesophyll cells of leaf sections were chosen for this study. Similar staining

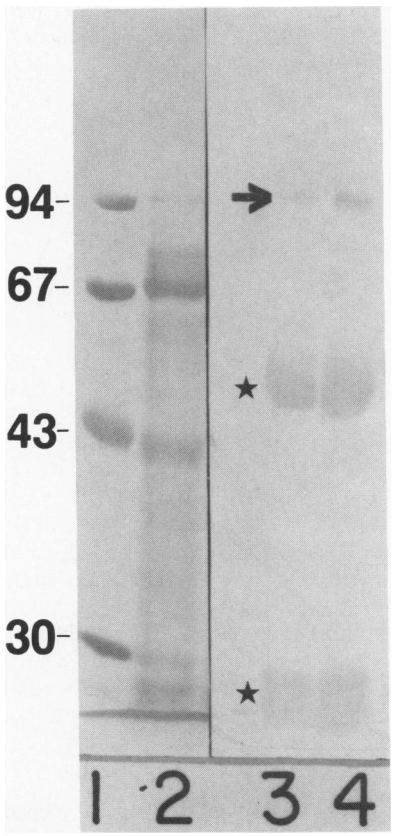

FIG. 3. Western blot analysis of immunoprecipitated pellets of mWP3. The lanes were as follows: (1) M_r markers (values in kD) stained with 0.1% amido black; (2) crude wall proteins concentrated by ammonium sulfate stained with 0.1% amido black; (3) protein immunoprecipitated by mWP3 from a partially purified peroxidase preparation that was electrophoretically separated on a nondenaturing gel—the immunoprecipitated protein was electroblotted and visualized (arrow) using mWP3 as the primary antibody; and (4) same as lane (3) except crude wall proteins concentrated by ammonium sulfate were used for immunoprecipitation. Notice that (★) in the lanes (3) and (4) showed the heavy and light chains of mouse IgG.

patterns were revealed by two different antibody preparations as shown in Figure 8. Most of labeling with both mWP3 (Fig. 8, A and C) and mWP19 (Fig. 8B) was located in the wall area. Preabsorption of mWP3 with a partially purified wall peroxidase preparation significantly reduced its staining of wall antigens (data not shown). There was no labeling of cell wall with the NS-1 control ascites (Fig. 8D). Other controls tested were P3 × 63Ag8 ascites (containing antibodies from IgG_1-secreting myeloma cells), M75 ascites (containing antibodies from IgM-secreting myeloma cells), and monoclonal antibodies raised against oat phytochrome. None of these showed any specific staining in the wall.

DISCUSSION

The method we used to select monoclonal hybridomas is similar to that used by Silberman *et al.* (22) for the production of monoclonal antibodies to oat phytochrome. It has been shown to be an effective method for ensuring that the final hybridomas are derived from single cells. All 22 monoclonals were demonstrated to be directed to some antigenic site on various cell wall antigens, including both proteins and carbohydrates. Thus our Western blot analysis, using minislab gel electrophoresis, to test either hybridoma supernatants or ascitic fluids has proven to be a rapid and useful screening method. Anderson *et al.* (1) have

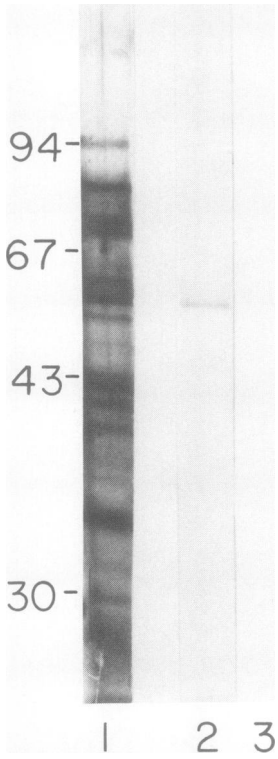

FIG. 4. Western blot analysis of mWP19. A preparation of wall proteins was subjected to SDS-PAGE and transferred to nitrocellulose as described under "Materials and Methods." The lanes were as follows: (1) total wall proteins stained with India ink; (2) same protein sample immunostained with mWP19; and (3) same as lane (2), but probed with NS-1 control. Numbers in the left margin represent mol wt in kD.

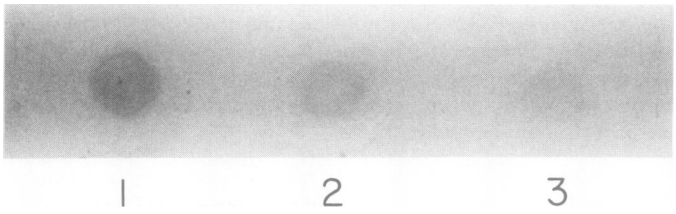

FIG. 5. Direct peroxidase-binding assay. On a strip of nitrocellulose membrane, equal amounts of antibodies in 3 μL aliquots of partially purified mWP3 (1), mWP19 (2), and NS-1 (3) were spotted at 1 cm intervals. After incubating it with crude wall proteins, the strip was developed with 0.4 mg/mL 4-chloro-1-naphthol and 0.03% H_2O_2 in PBS. Stained dots in (1) and (2) indicate that peroxidases in the crude wall protein preparation bound to the spotted mWP3 and mWP19, but a minimum nonspecific binding could be seen in NS-1 control.

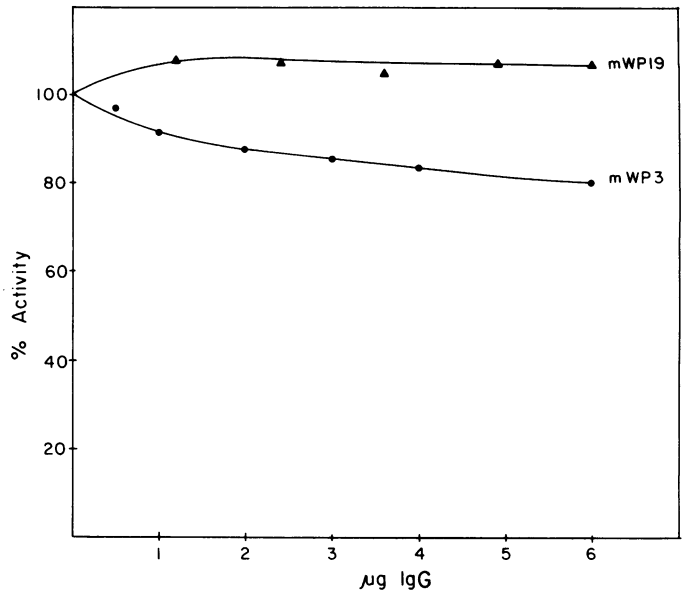

FIG. 6. Immunoinhibition of peroxidase activity. Partially purified wall peroxidase preparation (2 μg) was incubated with serial dilutions of purified monoclonal antibodies in a final volume of 20 μL of which 12 μL were assayed for peroxidase activity. Each graphed point represents the average of four experiments.

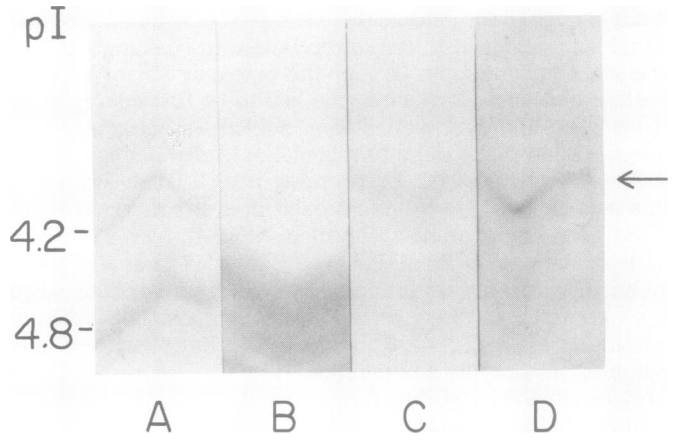

FIG. 7. Immunodetection of wall peroxidase on IEF blots. Forty μg of total crude wall proteins were subjected to IEF and transferred to nitrocellulose as described under "Materials and Methods." The lanes were as follows: A, pI markers (values in pI); B, protein samples stained with India ink; C, same as lane (B), but stained for peroxidases with *o*-dianisidine and H_2O_2 as substrate; and D, same as lane (B), but immunostained with mWP3. Staining in the range of pI of 3.0 to 5.0 is shown here. Outside this range there was essentially no significant immunostaining by mWP3.

indicated that attempts to raise monoclonal antibodies to whole plant extracts may result in hybridomas that produce antibodies to sugars associated with arabinogalactan proteins. Although several of the monoclonal antibodies in our library do react with cell wall polysaccharides, more than half of them (including mWP3 and mWP19) show no cross-reactivity with any one of several monosaccharides and polysaccharides tested, but do bind to protein bands in Western blot analysis. To directly test whether mWP3 or mWP19 reacted with carbohydrates associated with wall proteins, we treated wall protein preparations with concentrations of periodate long enough to oxidize carbohydrate moieties from glycoproteins (23). Because this treatment did not affect the cross-reactivity of wall proteins with either mWP3 or mWP19, it is unlikely that these antibodies are reacting with carbohydrates on the peroxidases they recognize.

The preparation of wall proteins was done by the centrifugation method of Terry and Bonner (25). This method involves separating the solution present in extracellular spaces from intracellular spaces by centrifugation at 1,000*g*. By microscopic examination and growth tests and assaying the extract for cytoplasmic marker proteins, they have shown that the extracted cells were not broken and less than 1.5% of the extract could be contaminated by cytoplasmic proteins, with these coming mainly from the cut surfaces of coleoptiles. So, it was highly probable that the origin of most antigens used to raise the monoclonal antibodies was the extracellular space of the coleoptile tissue.

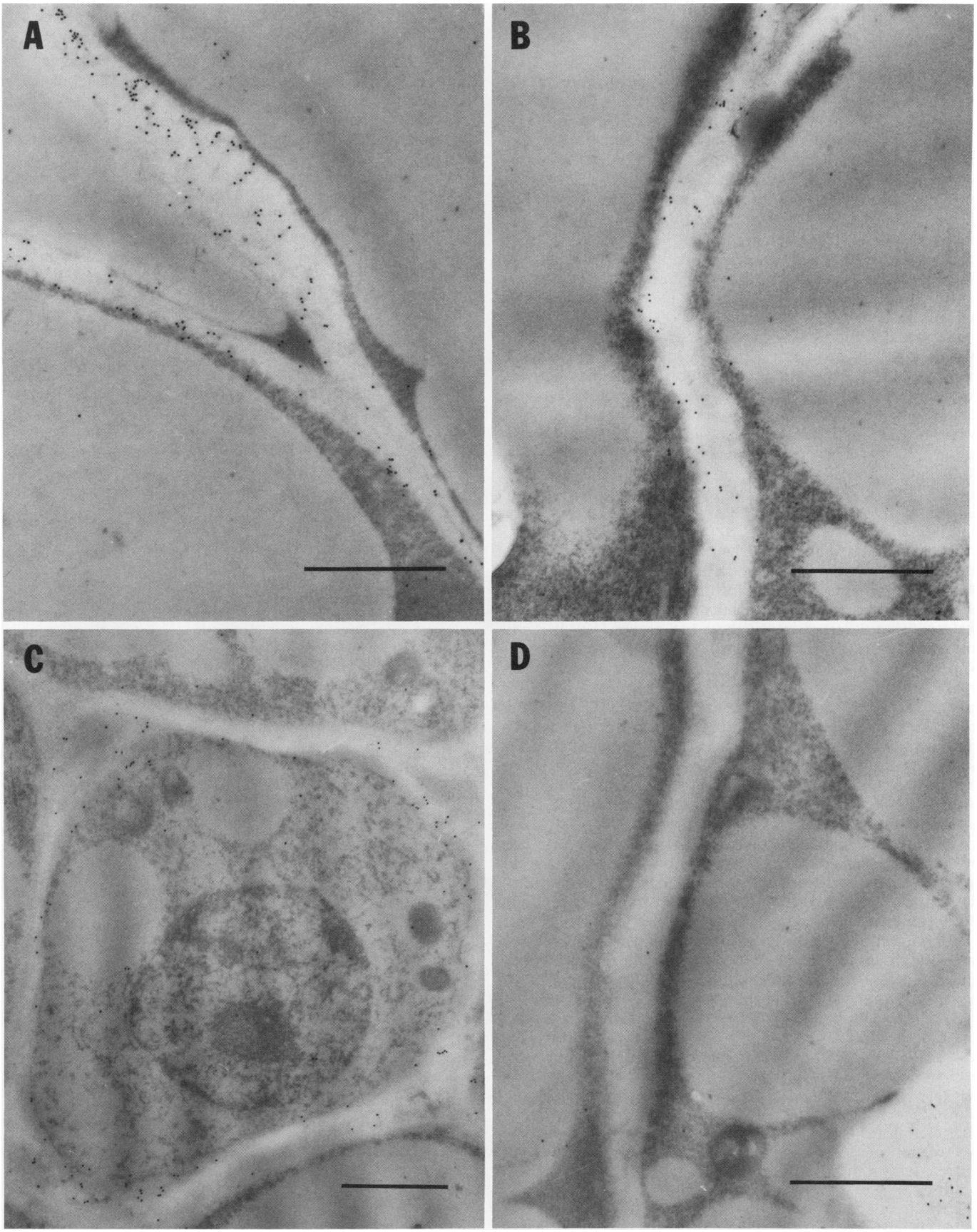

FIG. 8. Electron micrographs of enclosed young leaf cells of corn coleoptile immunolabeled for peroxidases. Shown are mWP3 labeling predominately the cell wall region of the leaf (A) and leaf vascular tissue (C), and mWP19 labeling predominately the cell wall region of the leaf (B). No labeling was seen on the cell wall treated with the NS-1 control (D). There is some nonspecific binding of gold to the starch grains (see lower right corner); a common pattern seen with immunogold labeling of plant tissues. A, B, and D, ×26,000; C, ×19,500; bar = 1 μm.

Consistent with this supposition, most of the antigens recognized by mWP3 and mWP19 were found by immunogold localization studies to be in cell walls.

The wall peroxidase that crossreacts with mWP3 has a M_r of 98,000, which is higher than most other plant peroxidases, but close to that of isoperoxidase A_2 (M_r = 89,000 in SDS-PAGE) from tobacco tissue culture (21). mWP19 reacts with a wall peroxidase of M_r 58,000, near the mol wt of many other plant peroxidases. mWP3 and mWP19 each appears to cross-react with only one peroxidase in the wall, so the epitope each recognizes must be distinctive to that peroxidase. The probability that that epitope occurs on more than one protein is low but not zero, so any other corn protein that has the same epitope as the one recognized by mWP3 or mWP19 either has the same mol wt as the peroxidase recognized by that monoclonal antibody or occurs in corn cells at a much lower concentration than that peroxidase.

Marucci (19) reported all of the horseradish peroxidase antibodies raised in the rabbit, cat, mouse, hamster and chicken showed 20 to 90% of maximum inhibition of peroxidase activity. As shown in Figure 6, mWP3 is directly inhibiting wall peroxidase activity up to 20%, depending on the amount of antibody, when measured with a partially purified wall peroxidase preparation. Whether this percentage represents the proportion of M_r 98,000 wall peroxidase in the preparation, or indicates that mWP3 does not completely inhibit peroxidase activity will be further investigated when the M_r 98,000 wall peroxidase is purified.

The main peroxidase isoenzymes can be separated into a cationic and an anionic group by electrophoresis, ion exchange chromatography, and isoelectricfocusing of enzyme extracts. The chemical constitution and the preference for various substrates are somewhat different among these two groups (10). It is still unclear which isozymes are responsible for the different functions of peroxidases in plant cells. Even the use of purified isozymes in assays for substrate specificity did not determine a definitive function for either cationic or anionic peroxidases (3). Conroy *et al.* (5) examined homology among horseradish peroxidase isoenzymes and among cationic peroxidases from root plants using antisera specific for the horseradish cationic peroxidase. Their experiments suggest that cationic peroxidase isoenzymes are strongly conserved during evolution, and that anticatalytic assays are poor indicators of homology.

van Huystee *et al.* (29) studied the cationic peroxidase release by cultured peanut cells, using an immunological method. Hu *et al.* (14) recently reported 10 monoclonal antibodies against the same cationic peroxidase from peanut suspension cell culture. By a combination of competitive ELISA, Western blot, and direct antigen-binding assay, their antibodies were subdivided into four groups depending upon different epitopes on the cationic peroxidase. All of their antibodies have shown a weak cross-reactivity with the anionic peanut peroxidase.

Based on our immunoblots of peroxidases separated by IEF and probed with mWP3 and mWP19, we expect that both the M_r 98,000 and the M_r 58,000 wall peroxidases are anionic, with pI's below 4.5. Their patterns of migration on nondenaturing gels at both alkaline and acidic pH ranges are also consistent with this conclusion. The peroxidase that reacted with mWP3 after elution from native gels migrated significantly toward the positive terminal in an alkaline gel (pH 8.5), but it did not migrate toward the negative terminal even when the gel pH was 4.5, indicating that it may have an isoelectric point below 4.5.

There have been several reports on polyclonal antibodies directed against anionic peroxidases in plants, but mWP3 and mWP19 are the first monoclonal antibodies described that are directed against wall-localized anionic peroxidases. Other immunocytolocalization (4, 13) studies have shown some peroxidases are associated with Golgi vesicles and small membraneous vesicular structures in the cytoplasm. Our immunogold localization studies show that both mWP3 and mWP19 bind to antigens present in the cell walls of enclosed young leaves. We are now determining the ultrastructural localization of the peroxidases recognized by mWP3 and mWP19 in the cell walls of both developing and differentiated tissues of corn, so as to approach questions relating to the functions and syntheses of these peroxidases.

In conclusion, we have isolated a series of monoclonal antibodies raised against two wall-localized peroxidase isozymes. These monoclonal antibodies were shown by immunoprecipitation and by immunoblot analyses to be specific for wall peroxidases by their ability to recognize them but not other proteins present in the cell wall. These antibodies should prove useful in further studies on the function and structure of the wall-localized peroxidases related to the cell wall metabolism.

Acknowledgments—The Central Hybridoma Facility of the University of Texas at Austin (Dr. P. D. Gottlieb, Director) provided essential facilities and technical assistance for the production of the monoclonal antibodies. We thank the Cell Research Institute (Dr. D. T. Brown, Director) of the University of Texas at Austin for the use of their electron microscope.

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