Chapter 6

Antibody-Mediated Immunochemistry and Immunoassay in Plant-Related Diseases*

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6.1 Introduction

Immunology, the scientific study of the immune response in vertebrates, has contributed greatly to contemporary biological ideas and practice. This is due to the uniqueness of concepts as well as the usefulness and wide application of its techniques in the analysis of plant macromolecules. Immunology has as its fundamental basis the response of animals to immunization with foreign cells, tissues, microorganisms, and proteins. Immunochemical methods have become widely employed because they permit the analysis of both unknown antigen mixtures (substance capable of inducing antibody formation in an animal) and highly purified antigens in terms of their cellular and tissue activity, sites of localization, and significance in chemotaxonomy and evolution. In the absence of other measurable activity (i.e., enzyme activity), immunochemical methods may provide the sole means for detecting, quantifying, and localizing a particular antigen. Immunochemistry represents a powerful research tool in the study of plant physiology/biochemistry and plant pathology. Although plants do not produce antibodies in response to foreign antigens, plants do exhibit some of the features of the immune response exhibited by vertebrates. Nevertheless, plant components, proteins, glycoproteins, and polysaccharides are usually excellent antigens.

The capacity of the mammalian immune system to produce serum glycoproteins (antibodies) in direct response to immunization with foreign proteins or polysaccharides (antigens) has been exploited in many creative ways in plant pathology. Once harvested, these antibodies possess the capacity to recognize, bind to, and neutralize the immunizing agent (antigen). Additional uses for specific antibodies include localization of enzymes or microbial antigens in plant cells, affinity chromatography, and quantitative determination of antigen concentration.

The advantages of immunological methods are as follows: (1) immunizing microbes or cells do not need to be viable; (2) confidence in sensitivity and specificity of antigen–antibody distribution is high; (3) most techniques are straightforward and simple, often using commercial kits; (4) antigen purification usually employs conventional protein methodology; (5) microgram quantities of antigen are amplified into milligrams of antibody which can be preserved for years; (6) many samples can be tested simultaneously with minute amounts of antigen and antibody; and (7) costs for production of antibody — commercial antibody production is readily available. Important concerns include: (1) availability of animal housing facilities required for polyclonal antibody (Pab) production; (2) expertise in the art of tissue culture required for monoclonal antibody (Mab) production; and (3) antigen selection and preparation of required amounts of antigen for immunization.

The purpose of this chapter is to outline the significance of immunological theory and methods and their applications to plant biochemistry and pathology.
6.2 Theories of Antibody Formation and Basic Principles of Immunization

The majority of simple and effective immunochemical techniques accessible to researchers in plant physiology/biochemistry and plant pathology entail the production of either polyclonal (Pab) or monoclonal (Mab) antibodies. In general, antibodies are produced in response to challenge or immunization by a foreign antigen which elicits a chain reaction that results in extracellular antibody production by specific cell types. Proteins and glycoproteins (including antibody molecules) make effective challenge antigens. In general, lipids make poor antigens. Most of these immune responses stem directly from the action of vertebrates responding to invading microbes (bacteria, viruses, protozoans, and fungi) or mutated cells. The cell-damaging effects of antibody-mediated and cell-mediated immunity are exquisitely designed to avoid self-recognition. Therefore, foreignness is the primary requirement needed for antibody production.

The primary mechanisms by which an antigen elicits a specific immune response are generally divided between instructive and selective theories. Instructive theories hypothesize that the antigen serves as a template upon which antibody molecules are folded to mimic an antigen combining site. This theory has generally been discarded in favor of Burnett’s clonal selection and expansion theory which assumes that an immunologically responsive cell acquired this capacity prior to encountering antigen.

The principal antibody-producing cell is the plasma cell, formed from a subpopulation of bursal-derived lymphocytes (β lymphocytes) which have the capacity to differentiate in response to antigen. Other small lymphocytes, e.g., thymus-derived (T lymphocytes), acquire a helper function of processing and presenting antigen to the β lymphocyte. The primary lymphatic organs of vertebrates coordinating efforts in a polyclonal (Pab) immune response include spleen, thymus, lymphatic system, and circulating small lymphocytes. The majority of circulating small lymphocytes are T cells, which in part helps to describe the cell population most reduced during HIV infection. Production of Mab in vitro is generally accomplished with splenic lymphocytes.

The best immunization protocol for a particular immunogen and species of animal can only be established by trial and error. Nevertheless, certain rules apply to the antigen preparations most commonly used for antibody production, such as (1) particulate antigens (i.e., whole cells) elicit stronger immune responses than soluble antigens (i.e., enzymes); (2) polymeric antigens (i.e., capsular polysaccharides) elicit stronger immune responses than monomeric antigens (i.e., proteins); (3) specific amounts of antigen per immunization range from 10 µg to 10 mg irrespective of animal size; (4) the conformation of the antigen molecule is of obvious importance in the generation of an immune response, i.e., it may be important to denature highly conserved mammalian antigens such as tubulin or actin with detergent (sodium dodecyl sulfate [SDS]) in order to challenge the immune system with an antigen significantly altered from the normal configuration of these molecules to impart “foreignness”; (5) immunogens should exceed 10,000 kDa; smaller antigens should be conjugated to larger proteins, such as bovine serum albumin (BSA); and (6) emulsification of antigen with an adjuvant increases the likelihood of stimulating an effective immune response. Freund’s complete adjuvant is a mixture of killed tubercule bacilli in oil.

Various injection routes can be explored, including intramuscular (IM), intravenous (IV), subcutaneous (SC), intradermal (ID), and intraperitoneal (IP). One key to successful immunization appears to be a multiple-route, multiple-site injection schedule. IV routes should be used cautiously to avoid anaphylaxis. Initial injection doses can range from 10 to 1000 µg, depending upon the availability and purity of the antigen (30 to 50 µg are sufficient for booster doses).

Another key to antibody production is the difference between the primary and secondary antibody response. Secondary or booster responses differ in the type of predominating antibody (IgG vs. IgM), have a much shorter lag phase, yield increased amounts of antibody, and are capable of longer sustained antibody production. The only caution is that antigen–antibody specificity tends to decrease over time as additional clones proliferate.
A typical Pab immunization schedule for an electrophoretically pure protein would include:
1. preimmunization serum control;
2. initial intramuscular injection of 1.0 mg protein in 1 mL phosphate-buffered saline (PBS) emulsified in complete Freund’s adjuvant and injected at multiple sites;
3. boost at multiple sites (ID) with 2 mg protein at 28, 42, 56, and 70 d (emulsified in incomplete Freund’s adjuvant); and
4. bleed for antibody 7 to 10 d after each booster until sufficient antibody titer is achieved.

6.3 Immunoglobulins — Production and Purification

6.3.1 Identification and Purification of Suitable Antigens

The complex ultrastructure of fungi may cause difficulties when choosing an antigen for immunological studies. A large number of fungal products have been used successfully as antigens, including whole and disrupted cells, extracellular and intracellular components, and crude and purified preparations. Fungal antigens may be either soluble metabolic products or particulates such as conidia or mycelium.

6.3.1.1 Antigen Preparation for a Decay Fungus

Sweetgum (Liquidambar styraciflua) wood blocks inoculated with Postia placenta are incubated at 27°C for 9 weeks. An extract is prepared by homogenizing the decayed wood blocks in 50 mM Tris, 0.85% NaCl, and 0.1 mM EDTA, pH 7.0, followed by ultracentrifugation at 128,000 xg for 2 h. Hemicellulases are fractionated by passing the extract sequentially through a 60- by 2-cm Sepharose® 6B (Pharmacia, Piscataway, NJ) column equilibrated with 50 mM Tris-HCl buffer and a 90- by 26-cm Fractogel® TSK HW-55 (F) (EM Science, Gibbstown, NJ) column equilibrated with 50 mM Tris-HCl buffer, pH 7.0, and 0.5 M NaCl. Chromatographic fractions are eluted in Tris, and those eluants containing xylanase activity as determined by the microadaptation of the Nelson–Somogyi reducing sugar assay serve as antigen.

6.3.2 Production of Polyclonal Antibodies

Rabbits are the ideal choice for production of Pab against fungi. New Zealand white rabbits are prebled and checked for nonspecific background reactivity to the antigen. Whole blood is clotted overnight at 4°C and centrifuged at 1200 xg for 10 min. Clarified serum is removed, filter sterilized, and frozen in 1-mL aliquots.

Fractionated xylanase is emulsified with Freund’s complete adjuvant (1:1) (Difco, Detroit, MI) and injected subcutaneously at multiple sites on the back of each rabbit. Rabbits are boosted intramuscularly with xylanase Freund’s incomplete adjuvant (Difco) 14 d postinoculation, and bled 10 d later. Monthly boosters with Freund’s incomplete adjuvant are followed by 7 to 14 d postboost bleedings until the desired antibody titer is achieved.

6.3.3 Production of Monoclonal Antibodies

Because of their high specificity, Mab to fungal antigens minimize the risk of cross reactions in antigen detection. Several advantages of Mab over Pab include the ability to obtain unlimited quantities of antibody, defined or selected specificity, and defined affinity for a single epitope.

6.3.3.1 Hybridoma Generation

Female BALB/c mice, 6 weeks old, are immunized intraperitoneally (IP) with 10 µg protein per 0.5 mL in 50% Freund’s complete adjuvant, and boosted IP at 2-week intervals with the same
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antigen in PBS. Mice are bled at 4 weeks and their serum is tested by enzyme-linked immunosor- 

bent assay (ELISA). The mouse with the highest titer is inoculated IV with 0.1 mL, and IP with 0.5 

mL antigen on two successive days, 5 d prior to the fusion. The spleen is surgically removed, and 

collected splenocytes are fused with 1 × 10^7 NS-1 myeloma cells. Fused cells are suspended in 

Dulbecco’s Modified Eagle’s Minimal Essential Medium (DME) (Gibco, Grant Island, NY) sup-

plemented with penicillin, streptomycin, 20% fetal calf serum (FCS) (Gibco), hypoxanthine, ami- 

noterin, and thymidine and plated in sterile 96-well cell culture plates. Hybridomas are selected by 

ELISA and western blot. Dilution cloning of the hybridomas yields clones from different colonies. 

Mab are isotyped using a commercially available kit employing an Ouchterlony assay (Binding 

Site, San Diego, CA). Cell lines are frozen at 4 × 10^6 cells mL^-1 in DME, 20% FCS, penicillin, 
streptomycin, and 10% dimethyl sulfoxide and maintained in liquid N_2.

6.3.3.2 Ascites Generation

Mice, 10 weeks old, are primed with 0.5 mL Pristane® (2,6,10,14-tetramethylpentadecane) (Sigma) 

IP, 10 d before injecting 2 × 10^6 hybridoma cells per 0.5 mL IP for each mouse. Ascites fluid is 

collected by tapping the lower abdominal area 10 to 18 d later. Centrifuge ascites at 5000 xg for 

5 min and freeze at –20°C.

6.3.4 Purification of Antibodies

Before purification, serum or ascites must be free of lipid contamination. Lipid clearing solutions, 

which are available commercially, are mixed with an equal volume of serum or ascites in a glass 

test tube that can withstand low-speed centrifugation. Vortex this mixture 90 s or until homogenized. 

Centrifuge at 1500 xg for 5 min and remove the clarified serum or ascites (top layer) from the 

lower layer, comprised of the solid lipid matrix. Mix the clarified sample with an equal volume of 

0.02 M sodium phosphate, pH 7.0, binding buffer.

Deliver the sample to a prepared Protein A or Protein G Sepharose® column, and allow the 

sample to drain into the gel bed. A 1-mL gel bed will immobilize 25 mg of human IgG. Columns 

of 1 or 5 mL gel bed are available commercially from either Bio-Rad or Pharmacia. Wash the 

column with a minimum of 10 mL binding buffer at a maximum flow rate of 1.0 mL/min. Continue 

to wash the column with binding buffer until the eluant is free of nonspecific protein and a 280-

nm spectrophotometric tracing returns to baseline. 

Add 5 mL 0.1 M glycine-HCl, pH 2.7, to the column and begin collecting 0.5- to 1.0-mL 

fractions in tubes containing approximately 100 µL 1.0 M Tris-HCl, pH 9.0, neutralizing buffer 

per milliliter of eluant. Alternatively, the IgG peak may immediately be passed through a buffer 

exchange column to remove acid and stored in a buffer of choice.

The column should be washed with binding buffer for regeneration followed by distilled water 

containing 20% EtOH or 0.02% thimersol for storage at 4°C. 

Total IgG can be determined with the Pierce® BCA protein assay. IgG should be stored at 

–20°C until needed.

6.4 Basic Methods

Many types of organisms deteriorate wood, but the most prevalent economic loss is caused by 

g fungi. Brown-rot, the most destructive type of decay, can rapidly cause structural failure. As little 

as 1% weight loss in wood can result in a 50% loss in bending strength.

Detecting incipient stages of decay in wood structures has been such a problem that research 

efforts in several disciplines have been directed towards the development of a successful field test. 

Visual and microscopic inspection of borings from wood, sounding of wood, radiography, sonics,
and electronic resistance have been used for detecting advanced decay, but initial stages of decay are difficult to detect with these methods. Results of direct staining of core samples with chemical indicators are subject to interpretation. The Shigometer, an electronic-type detector, has been used for the detection of early internal decay in trees and utility poles. This instrument yields unreliable results when used on wood products with a moisture content between 38 and 45%. Visual inspection and culturing still traditionally serve to evaluate wood samples for signs of fungal decay, but the shortcomings of these methods can result in misdiagnosis. Misdiagnosis can lead to unnecessary replacement of either uninfected wood or inappropriate remedial treatments in an effort to halt the spread of decay.

The role of an immunoassay is to reveal the presence of specific complexes between the antibody and an antigen that are unique to the pathogen. Recently, immunological methods have been so successful in detecting early fungal infection in agricultural crops that commercialized field test kits are available for several economically important plant pathogens. Various types of immunoassays, such as ELISA and dot blot, have been successful in detecting brown-rot decay in vitro. However, a diagnostic field test method is needed by timber inspectors who lack scientific expertise and laboratory facilities. To this end, our laboratory has studied several systems including ELISA, dot blot, and agglutination assays. Agglutination with latex beads was adequately sensitive for a presumptive test, although ELISA was necessary to verify and quantify decay. Neither of these methods was easily transferable to a field test kit, and both lacked the sensitivity for incipient decay that could be incorporated in a more recent technology known as particle capture immunoassay (PCI). The PCI is a significant advance in technology for all fields of diagnostics. This method incorporates the increased sensitivity and specificity of a double antibody sandwich ELISA with the simplicity of a test strip that can be easily transported to the field for use by the layperson. Further simplicity was added by utilizing dyed latex particles and eliminating colored enzyme/substrate reagents. These methods are described.

6.4.1 Precipitation

6.4.1.1 Immunodiffusion

The Ouchterlony gel diffusion test is one of a variety of gel diffusion techniques involving precipitation reactions between antigen and antibody. Optimal concentrations of antibody and antigen form visible bands of precipitation that provide identification of components in mixtures. The number of bands indicates the number of antigen–antibody systems present.

The gel is prepared with 0.05 M barbital buffer, pH 8.4, composed of 2.06 g Na barbital, 197.00 mL distilled H2O, and 3.0 mL HCl (1.0 N). Then 100 mL of barbital buffer are mixed with 1.00 g purified agar and 0.10 g Na azide. This mixture is boiled until fully dissolved and 4.0 mL are dispensed into 15- × 60-mm plastic petri dishes. Cool the plates 5 min uncovered and an additional 30 min covered. Plates may be stored for 3 weeks at 4°C. Punch 4-mm-diameter wells and remove the agar by aspiration. Fill the center well with 25 µL antibody and the outer wells with 25 µL of serum dilutions. Incubate at 37°C overnight in a humidified chamber and an additional 24 h at 25°C. Highest resolution is obtained at 4°C for 3 d. Precipitation lines may be viewed with background lighting. Alternatively, gels may be pressed and stained.

6.4.1.2 Immunofluorescence for Antigen Localization

Methods for the detection and localization of specific antigens in sera or in tissue structures, cells, or microorganisms visible by ordinary light microscopy can be easily accomplished using antibodies labeled with fluorescent dye. When irradiated with short wavelengths (ultraviolet, blue, or green excitation) of light, fluorescent substances emit electromagnetic radiation in the visible region. This property is known as primary or autofluorescence. Nonfluorescent objects, such as tissue background, remain dark. Light emission from a fluorochrome is called secondary fluorescence. The highest intensity of fluorescence is achieved by incident or reflected light (epillumination); however, light microscopes equipped for transmitted light are much less expensive.
Suitable antibody markers for immunofluorescent microscopy include: fluorescein isothiocyanate (FITC), lissamine rhodamine B, tetramethyl rhodamine isothiocyanate, and stilbene isothiocyanate. For fluorescence microscopy, it is essential that the best filter combinations be used. FITC exhibits an intense green fluorescence with either blue or violet excitation (365 to 405 nm), but autofluorescence of tissue components may be minimized by using short-pass filters with emission between 485 and 500 nm.

Immunofluorescence methods have greater sensitivity in antigen detection than diffusion-in-gel methods because they involve direct immunoprecipitation, visualized by the fluorescent tracer, rather than extensive precipitates in a gel matrix. On animal cell surfaces, the limit of detection has been estimated at between 100 and 1000 FITC molecules per cell surface by immunofluorescence, and the amount of binding has been shown to be quantitatively related to the number of antigen molecules or their concentration and also to that of the antibody in the incubation medium.

This immunofluorescent technique was first described by A. H. Coons (Harvard Medical School), who published the first account of fluorescein-conjugated antibody in 1942. Coons et al. showed that fluorochrome labeling allows both direct observation and precise localization of the sites of reaction antibodies. Recent developments in this field include demonstration of cell populations by means of immunofluorescence of cell surface antigens and flow cytometry in a fluorescent-activated cell sorter.

Both Pabs and Mabs are suitable for immunofluorescent labeling. Detecting antibodies can be accomplished by several methods. Using the indirect method, the primary antibody–antigen reaction is detected using a conjugated antiserum of species specificity directed against the primary antibody. In the direct method, the fluorescent dye is conjugated directly to the primary antibody; however, this is the least common usage due to inefficient use of the primary antibody.

In specific applications of this technology, fluorescent antibody immunochemistry has allowed the detection and localization of fungal metabolizes within host substrates for both Pab and Mab. Fluorescent detection of two white-rot fungi causing severe root rot, *Armillaria mellea* and *Heterobasidium annosum*, has been demonstrated using polyclonal antisera to homogenized mycelia. Lignin-degrading enzymes have been localized on the surface of intact fungal mycelium and conidiospores of the white-rot fungus *Phanerochaete chrysosporium* (Figure 6.1).

### Indirect staining method:

1. Apply blocking serum (normal goat serum 1:100) in a moist chamber for 30 min.
2. Apply unconjugated primary antibody (diluted 1:20) for 30 min.
3. Wash slide in PBS (pH 7.4) 10 min.
4. Apply FITC-conjugated goat anti-rabbit antibody for 30 min.
5. Wash (PBS pH 7.4) for 2 h; dry and coverslip.
6.4.2 Particle Agglutination

Particle agglutination tests are rapid qualitative tests based on the very specific interaction of antigen and antibody. Submicron-sized polystyrene particles are the solid supports to which antigen or antibody are adsorbed. Labeled polystyrene particles act to magnify an antibody–antigen reaction when placed in a sample containing the opposite reactant.\(^a\)

6.4.2.1 Antibody-Labeled Polystyrene Beads

Polystyrene beads, 1-mL solution (10% w/v), 0.785-µm diameter, washed in PBS to remove surfactant, are resuspended with sonification and passively adsorbed to 2 mL purified polyclonal IgG (1.4 mg protein) for 24 h at 4°C. Bound beads are centrifuged 5000 xg for 20 min to remove unbound antibody and blocked with 0.1% BSA in PBS for 1 h. The blocking solution is removed by centrifugation and pelleted beads are resuspended to 3% solids in PBS with 0.1% Triton\(^a\) X-100 and 0.02% Na azide. The adsorbed beads are stored at 4°C and vortexed thoroughly before each use.\(^a\)

6.4.2.2 Agglutination Assay

Wood extract, prepared by soaking 50 to 100 mg sawdust in 0.1% Triton\(^a\) X-100 for 2 h, is pipetted (60 µL) onto an 18-mm circle of a glass agglutination slide and spread to the edges of the well. A positive control consisting of a known positive antigen and negative controls consisting of PBS, undecayed wood block extract, and nonbrown-rot isolates are included with each test. The agglutination reagent is vortexed vigorously just before adding 10 µL to each sample. The slide is placed in a humidified chamber and mechanically rotated for 15 min at 100 rpm before macroscopic examination. A negative agglutination result shows a smooth, homologous appearance, while a positive test exhibits visible, grainy aggregates of reagent. The strength of agglutination is ranked as negative or 1 to 4, with 4 being the strongest reaction\(^a\) (Figure 6.2).

6.4.3 Crossed Immunoelectrophoresis

Crossed immunoelectrophoresis is a two-dimensional method which provides both qualitative and quantitative information about an antigen–antibody system. Several excellent articles describe this technique and a number of variations on immunoelectrophoresis.\(^{27-29}\) The method described here is that of Clarke and Freeman \(^{30}\)
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6.4.3.1 First Dimension
A 1% agarose solution is prepared in Tris-barbiturate buffer, pH 8.6. The solution is heated to 90°C until completely dissolved and dispensed in 12-mL aliquots. Aliquots are stored at 4°C for future use. On a level surface, 12 mL of agarose are carefully poured onto an 84- × 94-mm glass plate. After cooling, a sample well is punched and the gel is aspirated from the well. The well is filled with protein sample and 1 µL bromophenol blue as a dye indicator. First-dimension electrophoresis is performed with barbiturate buffer as the electrolyte using prewetted paper buffer wicks for 1.5 h at 10°C and 10 V/cm or until the dye indicator migrates across the gel. Some proteins may migrate ahead of the dye front.

6.4.3.2 Second Dimension
After electrophoretic separation of the antigen, a slice of gel is transferred to a new glass plate. Onto the remainder of the plate are cast 8 mL of agarose containing 0.9 mL antibody. Second-dimension electrophoresis is performed perpendicular to the first dimension at 10°C and 2 V/cm overnight.

Crossed immunoelectrophoresis is performed at pH 8.6 where the electrophoretic mobility of antibodies is very low. Antigens move into the antibody-containing gel. Initially, the antibody concentration is greater than the antigen concentration, so the precipitates are soluble and will migrate toward the anode. Eventually, as the concentrations of antibody and antigen equilibrate, precipitates become insoluble and form a continuous rocket (Figure 6.3). The area of each rocket is proportional to the concentration of antigen. Likewise, the area of each rocket is inversely proportional to the concentration of antibodies in the antibody-containing gel.1

After electrophoresis the gel is wetted with distilled water and covered with one wet and five dry filter papers. A glass plate and 1-kg weight are placed on top of the filter papers. The pressing is repeated twice at 3-min intervals. The gel is washed in 0.1 M sodium chloride (2 × 15 min) and the pressing repeated twice more. After pressing, the gel is dried to a fine film using hot air from a hair dryer.

The plate is stained with 1 g Coomassie Brilliant Blue R-250 in 90 mL EtOH, 20 mL acetic acid, and 90 mL H₂O for 5 min. The plate is rinsed briefly with water, then placed in destain which contains the same components as the staining solution without the Coomassie Brilliant Blue. The destained gel is a permanent record of the antigen/antibody precipitates.

6.4.4 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISAs give reliable and reproducible quantitative measurements of antibody or antigen. There are basically two forms of ELISA. In the direct method, the specific antibody is immobilized on
the solid phase and incubated successively with antigen and a second specific antibody that is conjugated with an enzyme. In the indirect method, either antigen or antibody are immobilized to the solid phase, usually polystyrene. Enzyme-labeled antoglobulin is linked to the immobilized antigen or antibody. A chromogenic substrate is added to give a visible colored reaction which yields a precise result when read photometrically. ELISAs are capable of detecting nanogram amounts of antigen or antibody.

Coat 96-well microtiter plates with dilutions of wood or plant extracts in 0.1 M Tris plate-coating buffer with 0.15 M NaCl, pH 9.0. Incubate plates at 25°C overnight, and wash five times with binding buffer. Add 0.1 mL rabbit IgG (2 µg mL⁻¹) to each well and incubate for 2 h. Wells are washed five times with PBS–Tween® 20 before adding goat anti-rabbit conjugated peroxidase. The plate is incubated for 2 h, rinsed thoroughly to remove any unbound conjugate, and each well is filled with 0.1 mL o-phenylenediamine. Color development is detected after 20 min at 490 nm with a microplate reader. Absorbance that are three times greater than the negative control value are considered positive reactions.

### 6.4.5 Dot Blot Immunoassay

Blotting membranes offer high protein binding capacity and sensitivity for immunochemical protocols. Plant tissue may be placed directly on nitrocellulose paper (NCP) for a tissue blot. Otherwise, wood extracts or plant extracts spotted onto NCP are allowed to dry before the paper is blocked overnight in Tris-buffered saline (TBS) with 3% gelatin (w/v). The NCP is incubated in rabbit IgG (2 µg mL⁻¹ PBS) for 2 h and washed three times each for 10 min in TTBS (TBS plus 0.05% Tween® 20). The blot is then probed with horseradish peroxidase-conjugated goat anti-rabbit antiserum for 2 h, washed three times, 10 min each, with TTBS, and developed with 4-chloro-1-naphthol (Figure 6.4).

### 6.4.6 Western Blotting

Electrophoretic transfer of protein from polyacrylamide gels to blotting membranes was first described by Towbin et al. in 1979. Probing membrane-bound antigens with a specific antibody solution is especially useful for detecting antibodies against antigens in complex mixtures. Less than 100 pg specific protein can be detected using horseradish peroxidase and 4-chloro- 1-naphthol. Gel chromatographic fractions described in Section 6.3.1 are separated on SDS polyacrylamide gels consisting of a 4% stacking gel and a 12% resolving gel according to the method of Laemmli. Gels are cast using a Bio-Rad Protean II apparatus (Rockville Centre, NY). Prestained molecular weight markers (Bio-Rad) are run for molecular weight estimations on the immunoblots and duplicate gels are stained with Coomassie blue. Proteins are transferred to NCP using a Bio-Rad Transblot apparatus with 25 m M Tris-HCl, 192 m M glycine, 20% methanol, pH 8.3 running buffer, precooled to −20°C. Transfers are conducted for 2 h at 100 V. Blotted NCP is blocked for 1 h in
Figure 6.5

TBS (0.02 M Tris, 0.5 M NaCl, pH 7.5) with 3% gelatin, rinsed briefly with distilled water, and placed in a Miniblotter II (Immunetics, Cambridge, MA). Supernatants from single colony clones are added to each channel and allowed to bind for 2 h. Blots are washed with TTBS (TBS plus 0.05% Tween® 20) before adding 1 µg mL⁻¹ horseradish peroxidase-conjugated goat anti-mouse IgG (H + L) in TBS with 1% gelatin. Similarly, blots may be probed with rabbit antibodies provided the appropriate conjugated anti-rabbit IgG is subsequently used. After a 2-h incubation at 25°C, the blot is washed again in TTBS and developed with 60 mg 4-chloro-l-naphthol (Sigma) in 20 mL methanol and 60 µL hydrogen peroxide in 100 mL TBS (Figure 6.5).

6.5 Specific Applications

6.5.1 Immunogold Labeling

Electron microscopic techniques permit the visualization of structure and function relationships at the macromolecular level. The ability to couple the technology of immunocytochemistry with electron microscopy offers a tremendous opportunity for expanding the useful information to be obtained from ultrastructural studies. Numerous electron-dense marker systems (horseradish peroxidase, ferritin, ruthenium red, polystyrene latex, osmium, and uranyl acetate) were developed for localization of proteins and other enzymes for transmission electron microscopy (TEM). Recent developments in immunocytochemical marker systems offer significant improvements to the practice of ultrastructural labeling — most notably colloidal gold. Colloidal gold, through minute sizing, may be conjugated to a wide variety of macromolecules, including plant lectins and hormones, bacterial proteins, and enzymes. In addition, colloidal gold labeling is contributing to a whole new field of immunocytochemistry-immunoscanning electron microscopy. Compared to TEM, scanning
electron microscopy (SEM) has had limited impact upon immunocytochemistry. However, introduction of the colloidal gold marker system along with improved sensitivity of backscattered electron detector systems offer significant opportunities in the practice of SEM immunocytochemistry.

Bioapplications of colloidal gold and SEM include: (1) localization and surface distribution of enzymes, (2) expression of cell surface lectin-binding sites, (3) surface distribution of extracellular matrix components, and (4) localization of surface antigens (immunocytochemistry). Although certain gold-labeling methods are suitable for visualization with light microscopy, colloidal gold probes are most commonly examined either by surface labeling in SEM or marking of ultrathin resin or plastic embedded tissues and cells by TEM.

The use of Pab and Mab with immunocytochemical labeling has allowed detection and localization of microbial metabolizes or enzymes on and within cells and substrates. Many factors may affect the cytochemical localization of specific enzymes resulting in negative labeling with gold complexes. One critical element is the effect of tissue processing (i.e., washing, fixation, type of embedding medium, and embedding conditions) which can induce changes in tissue components.

Numerous advantages of colloidal gold as a marker for SEM immunocytochemistry have been summarized by Hodges et al. in a recent review:

1. Monodispersed gold sols of uniform diameter (2 to 150 nm) can be easily prepared.
2. The particulate nature of colloidal gold allows precise localization of labeled sites.
3. Negatively charged gold particles can be noncovalently complexed to various macromolecules including immunoglobulins.
4. High electron density correlates with the high atomic number of gold for overcoming the metal coating of specimens.
5. A variety of sizes permits double labeling of the same sample.
6. Direct counting permits quantification of gold particles.
7. Backscattered electron imaging of colloidal gold can be used to overcome complex topography which may mask small or sparse particles.

6.5.1.1 Application of Colloidal Gold Labeling to Enzyme Localization in SEM

Extracellular wood-degrading enzymes of the brown-rot fungus, Postia placenta, are localized using colloidal gold-labeled Mab to the β -1,4-xylanase (32 to 36 kDa) fraction of P. placenta. P. placenta is grown from agar onto glass coverslips, immunolabeled with or without prior fixation, and examined by SEM. Enzymes are localized on the hyphal surface and on the clumped fibrillar elements (mycofibrils) of the hyphal sheath following fixation with glutaraldehyde. If fixation is omitted, labeling is diffuse and not localized on individual or clumped mycofibrils. We conclude that extracellular decay enzymes are weakly bound (noncovalently) to, but not identical with, the linear mycofibrillar elements of the hyphal sheath. The linear structural elements of the hyphal sheath may play an important role in transport and presentation of wood-degrading enzymes during the decay process (Figure 6.6).

Figure 6.6
6.5.1.2 Preparation, Coupling, and Labeling with Colloidal Gold

Colloidal gold beads (~30 nm) are prepared by the reduction of 4% H\text{AuCl}_4 using the method of Park et al. Gold particles, 32 nm (particle size is increased by decreasing the amount of sodium citrate), are prepared by the following method:

1. Acid wash all glassware and rinse with glass-distilled H\text{O}.
2. Add 0.5 mL of a 4% H\text{AuCl}_4 to 200 mL H\text{O} that has been filtered through a 0.2-\text{µm filter}.
3. Heat. When the solution begins to boil, quickly add 3 mL of a 1% solution of sodium citrate. Continually stir and maintain at 100°C for 30 min. Solution will turn light blue immediately; after reduction is complete, color will change to reddish-orange.
4. Cool solution and stabilize with two drops of 1% polyethylene glycol (PEG) (mol wt 20,000) to 10 mL sol.
5. Adjust pH to 7.4 with 0.2 \text{M} potassium carbonate; the size of the particles is estimated from absorbance at 520 nm.
6. Filter sterilize the gold prep and store at 4°C in a dark glass container.

Coupling of Mab to colloidal gold requires predetermination of the adsorption isotherm for each antibody. Prior to conjugation of the colloidal gold to the Mab, it is necessary to dialyze the antibody against PBS (pH 7.4) at 4°C overnight, and test an adsorption isotherm in order to determine the minimal concentration of antibody necessary to stabilize a known amount of gold sol.

1. Make 1:2 dilutions of Mab in microtiter plate wells, beginning with a low concentration of Mab.
2. Add 0.5 mL gold sol to 0.1 mL of each dilution of Mab (always add gold sol to dilutions of antibody).
3. After 2 min, add 0.1 mL NaCl to each well.
4. After an additional 5 min, determine the lowest amount of Mab to stabilize (pink) a known amount of gold. Unstable mixtures will be blue.

Conjugation of Mab to gold sol:

1. Add Mab protein to an acid-washed glass tube.
2. Add glass-distilled water (10% of volume); centrifuge at 2800 xg for 5 min at 4°C to get rid of clumps.
3. Add enough gold to each tube to stabilize the antibody.
4. Invert six times and let sit 5 min.
5. Add 1% PEG (Carbowax 20) and invert six times.
6. Spin at 10,000 xg for 30 min at 4°C.
7. Aspirate as much supernatant as possible.
8. Resuspend pellet in 1 mL 0.22-\text{µm-filtered buffer composed of 20 m M TBS, pH 8.2 + 0.1% BSA, and 0.05% Na azide.}
9. Standardize $A_{525nm}$ for Au$_{15}$ or $A_{540nm}$ for Au. Commercial preparation absorbance at 520 nm is 3.5 for 15 nm and 7.0 for 30 nm.
10. Store at 4 to 8°C. Do not freeze.

Labeling of fungal hyphae:

The brown-rot fungus, \textit{P. placenta}, is grown onto sterile 12-mm glass coverslips placed on the surface of 2% (w/v) malt agar plates. Coverslips are removed when about 50% covered (about 1 to 2 weeks) and are processed for SEM. Coverslips are washed in 0.1 \text{M} sodium acetate buffer (pH 4.5) or Triton® X-100 for 15 min to remove excess glucan and fixed in 1% glutaraldehyde and 4% paraformaldehyde in 0.1 \text{M} cacodylate buffer (pH 7.2) supplemented with either 0.5% CaCl$_2$ + 0.05% digitonin or aqueous ruthenium red (500 parts per million) for 15 min and washed in PBS (pH 7.0) as previously
described. Colloidal gold-labeled Mabs are applied directly to the washed surface of coverslips and incubated in a moist chamber for 1 to 2 h at room temperature. Coverslips are washed by dipping in distilled water to remove unattached beads, frozen in liquid nitrogen slush (LN), dehydrated by lyophilization, and air dried. Specimens are sputtercoated with gold and examined in a Hitachi S530 SEM at accelerating voltage of 10 to 25 kV. Localization of colloidal gold label is enhanced by decreasing the working distance to 5 mm and by adjustment of illumination to maximum contrast and minimum brightness.

6.5.2 Dyed-Particle Capture Immunoassay

Antibody-coated polystyrene beads form the basis for particle capture and sandwich tests. There is a variety of formats, but in all cases, the antigen links the sandwich of primary antibody-coated particles with secondary antibody. Dyed particles eliminate the need for enzyme/substrate reactions and allow for precise placement of antibody-coated particles on membranes. In this type of assay, antibody-coated particles move through the membrane. The dyed beads bring enough color to the antibody-Ag-antibody sandwich to preclude the use of enzymes. The speed and ease of particle capture assays create a new avenue for field tests for incipient wood decay.

Antixylanase monoclonal antibody (Ab₁) is passively bound to a 1- by 6-cm strip of polyester cloth (E. I. DuPont Nemours & Co., Wilmington, DE) 4 cm from one end of the strip and dried at 40°C. The entire cloth strip is saturated with 10% sucrose and 0.2% BSA in water for 1 h at 25°C, dried at 40°C, recoated with the sucrose/BSA mixture, then completely dried at 40°C. Pab-labeled colored polystyrene beads (Ab₂) are applied to the coated cloth strip in a line 1 cm from the bottom of the test strip.

The dyed-particle capture test strip is shown schematically in Figure 6.7. In step 1, the prepared test strip is dipped into a fungal extract that is wicked upward through the cloth. Step 2 shows that antigen from the fungal extract, which is specific for Ab₂, is captured by the labeled particles and further carried to the immobilized Ab₁. In positive tests, the antigen is sandwiched between the Pab-labeled beads and the Mab, and an easily visible colored line results 4 cm from the bottom of the test strip in the reaction zone. In a negative test, the Pab-labeled beads passed through the zone of capture, resulting in an absence of a colored line.

![Figure 6.7](image)

Figure 6.7

Particle capture Immunoassay (PCI): step 1, test strip is dipped into fungal extract and extract (antigen) is wicked upward; step 2, fungal antigens captured by Ab₁-labeled beads are sandwiched by a second immobilized antibody (Ab₂) to form colored line for positive result. (Clausen and Green, U.S. patent no. 5,563,040.)
References