DOE/CH/10520--T3

Quarterly Report for

S9X021591

DOE-BCTR Program

National Renewable Energy Laboratory

A Study of Overproduction and Enhanced Secretion of Enzymes

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September, 1993

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Project Summary and 1993 Planned Activities

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Over-production of Coriolus versicolor Polyphenol Oxidase

Amplification of Genomic DNA Fragment

Substrate Induction

Enhanced Secretion of Coriolus versicolor Polyphenol Oxidase

Comparison of Ultrastructures of Hyphae Cultured on a Solid Surface and in Liquid Medium

Transmission Electron Microscopy

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PROJECT SUMMARY

Wood decay within Forests, a significant renewable photosynthetic energy resource, is caused primarily by Basidiomycetous fungi, e.g., white rot fungi. These organisms possess the ability to degrade lignin, cellulose and hemicellulose, the main organic polymers of wood. In the case of the white rot fungi, e.g., Coriolus versicolor, the capacity results from the fungus' ability to elaborate extracellular cellulolytic and ligninolytic enzymes. With regard to the latter, at least one of the enzymes, polyphenol oxidase (PPO) appears within a defined growth medium. It appears that extracellular PPO arises from intracellular PPO. Fungal PPO seems to convert the putative tree synthesized disease resistance factors, diphenols to diquinones and oligomerizes syringic acid, a lignin derivative. Because PPO appears to be inducible, it is conceivable that the C. versicolor culture system could be a model for achieving over-production of enzymes relevant to the paper-pulp industry and the agricultural community. The current proposal is concerned with the over-production and enhanced secretion of PPO, cellulase and lignin peroxidase. The proposal is divided into two segments: 1) over-production of lignocellulolytic enzymes by genetic engineering methodologies and hyper-production and enhanced secretion of these enzymes by biochemical/electron microscopical techniques. The former approach employs recombinant DNA procedures, e.g., isolation of C. versicolor genomic DNA, ligation of appropriate nuclease generated DNA fragments into a vector and the subsequent transformation of Escherichia coli to yield E. coli harboring a C. versicolor DNA insert. This approach is being carried out by Dr. Arthur L.

Williams at Howard University. The biochemical/electron microscopical method involves substrate induction and the time-dependent addition of respiration and PPO inhibitors to elevate C. versicolor's ability to synthesize and secrete lignocellulosic enzymes. In this connection, cell fractionation/kinetic analysis, TEM immunoelectron microscopic localization and TEM substrate localization of PPO are being employed to assess the route of secretion. This approach is being performed by Dr. W.V. Dashek and N.L. Moore at Clark Atlanta University. Both approaches will culminate in the batch culture of either E. coli or C. versicolor, in a fermentor with the subsequent development of rapid isolation and purification procedures to yield elevated quantities of pure lignocellulosic enzymes.

A summary of the 1993 Proposed Research Tasks is presented in Table 1.

1993 Planned Activities:

Summary of Proposed Research Tasks

Task	Approaches
Complete Purification of Intracellular and Extracellular PPOs	Employ Hydroxylapatite, Hydrophobic Interaction and Affinity Chromatographies Subsequent to Dialysis, Ammonium Sulfate Fractionation and Gel Filtration of Crude Medium.
Hyper-Production of Enzymes Establish Optimum Conditions for Substrate Induction of Cellulases and Ligninases	Supplementation of Cultures with Appropriate Substrates at Appropriate Times, Quantification of Intracellular and Extracellular PPO, Cellulase and Ligninase Spc. Acts.
Establishing the Route of Secretion	
Completion of TEM Substrate Localization Method	Continued TEM Development of Dihydroxyphenylalanine Insertion between Glutaraldehyde pre-and Osmium Post-Fixations
Completion of Identification of Cell Fractions Utilized in Time-Dependent Localization of PPO in Cell Fractions	Identify Organelles' Contents of Cell Fractions Containing PPO Derived from Homogenates of *C. versicolor* Cultured over time.
	TEM and Marker Enzyme Analysis
Completion of TEM Immuno-Electron Microscopic Localization of PPO	Repeat ELISA Quantification of Antibody Titer in Crude Serum Derived from Immunized Rabbits; Repeat Immunochromatographic Purification of PPO and Subsequent Tagging of Antibody with Colloidal Gold
Inception of Investigation on Time Dependent Appearances of Wall-Degrading Enzymes, Mechanism of Release of PPO, Cellulase and Ligninase Secretion into the Periplasmic Space	Determine the Time-Dependent Appearances of Intracellularly Synthesized Wall-Degrading Enzymes, Composition of the Wall by Following Changes in its Composition with Time of Culture
Enhance Secretion of Enzymes	
Completion of In Progress Respiration Inhibitor Studies to Separate Synthesis from Secretion	Complete the Assay of Intracellular and Extracellular PPO for Cultures Subjected to the Time-Dependent Additions of NaF & Na Azide
Development of "Batch Culture" Techniques for *C. versicolor*	Scale-up *C. versicolor* Culture by Employing a Fermentor
Isolation, Purification and Characterization of PPO, Cellulases and Ligninases from *C. versicolor* Growth Medium from non-Batch and 'Batch Cultured' *C. versicolor*	Adaptation of an Isolation and Purification Protocol for 'Non-batch Cultured' to 'Batch-Cultured' *C. versicolor*
	Determination of Amino Acid and Sugar (if any) Profiles of Purified Enzymes

TECHNICAL ACCOMPLISHMENTS

Over-Production-Polyphenol Oxidase

During the past year, a collaborative effort was initiated with Dr. David McMillin, a plant molecular geneticist at Clark Atlanta University, to over-produce polyphenol oxidase and eventually cellulase and ligninase. This effort involves restriction fragment length polymorphism (RFLP) localizations (Kochert et al, 1989; Kochert, 1991, 1992) of PPO genes (Newman et al, 1993) upon a chromosome(s) and subsequent polymerase chain reaction (PCR) amplification (Erlich, 1989; White et al, 1989, Novo, 1992) of the appropriate C. versicolor genomic DNA fragment. To this end, four different DNA isolation procedures have been screened to yield "workable" amounts of high MW DNA. Whereas application of a fungal procedure yielded a limited amount of DNA, employment of another DNA isolation procedure provided high MW DNA. Ms. C. Hutto, who received technician support, from the DOE-BCTR award participated in this research. The primers necessary for Dr. McMillin to perform the PCR amplification were ordered.

It is anticipated that the collaboration with Dr. McMillin (a supplementation of A.L. Williams' research at Howard University) will be long-term as joint proposals regarding the over-production of ligno-cellulosic enzymes by combined restriction fragment length polymorphism and polymerase chain reaction are being prepared. To this end, Clark Atlanta University and DOE-BCTR approved Dr. McMillin's possession of the DOE equipment purchased by Dashek from DOE-NASA, DOE-AICD

and DOE-BCTR funds except for those pieces of equipment transferred to Dr. A.L. Williams by Dr. Brian Davidson.

Finally, the cloning of cDNAs encoding for C. versicolor PPO should be greatly aided by the recent reports of such cloning or related gene organization/expression in higher plants (Cary et al, 1992; Shahar, 1992; Hunt et al, 1993; Newman et al, 1993). In this connection, Dashek possesses an extensive array of recombinant DNA technological literature (see publications manual).

Substrate Induction

Because preliminary investigations (Taylor et al, 1987, 1988, 1989) revealed that the time-dependent addition of one mg catechol (catecholase substrate of PPO complex) per ml liquid culture resulted in bimodal growth and differential ultrastructural responses as well as growth medium pigmentation, experiments involving catechol augmentation at day 0 followed by transfer to catechol-free medium at the seventh day of culture (presumed post synthesis - Moore et al., 1993) were performed. The experimental design consisted of supplementing certain cultures with 100 mM catechol while not administering the o-diphenol to others. Seven days later, certain mycelia from both culture types were transferred to medium lacking catechol. In addition, other mycelia from both culture types were not transferred with their culturing being continued without interruption for another 7 days. Thus, regardless of the culture manipulations, the duration of the experiment was 14 days when all cultures were harvested. Whereas the collected mycelia were rapidly frozen in liquid nitrogen and subsequently stored at -20°C, the growth media

were frozen, lyophilized and then simultaneously dialyzed (spectrofluor MW cut-off 14,000) against 2 l of 100mM pH 5.0, acetate buffer for 18 h. Although this dialysis within one container resulted in marked removal of pigmented substances from the samples to the dialyzing solution, some back diffusion of the substances into those growth medium samples which lacked catechol was observed. Thus, it will be necessary to dialyze those homogenates from mycelia which were never exposed to catechol separate from those which were. In this connection, it may be more appropriate to ultrafiltrate the samples separately through YM10 Amicon Diaflo filters (MW cut-off, 10,000). This can be accomplished with the stored mycelia possibly yielding an enhancement in PPO synthesis which can be quantified.

Enhanced Secretion

Comparison of the Ultrastructures of Hyphae Cultured on a Solid Substrate and in Liquid Culture

A comparison of the ultrastructures of hyphae cultured upon and in defined medium containing or lacking agar was concluded this quarter. The ultrastructure of hyphae from both cultures were similar (Fig. 1A,B) except that hyphal growth upon agar exhibited a hyphal sheath external to the cell wall (Fig. 1B). The occurrence of a sheath for agar grown hyphae is consistent with the reports in the literature for fungi grown upon a solid substrate (Evans et al, 1981; Palmer et al, 1983). Presumably, this sheath is fragile enough to be sloughed off during shake liquid

cultures. With regard to many speculations for the role of hyphal sheaths in wood decay, it is possible that the sheath may facilitate wood degradation by storing or concentrating degrading agents and translocating these for initial conditioning and subsequent attack on cell wall polymers (Palmer et al, 1983).

Transmission Electron Microscopic Substrate Localization of PPO

Attempts to localize intracellular hyphal PPO via a transmission electron microscopy substrate localization technique (Moore et al, 1993) were continued. In previous DOE quarterly reports, Moore and I stated that marked cytoplasmic distortions were noted in those glutaraldehyde-fixed hyphae which were treated with cacodylate-buffered dihydroxyphenylalanine (DOPA). Recent, extensive comparisons of many micrographs of aldehyde-fixed hyphae treated with cacodylate buffer only or treated with cacodylate buffered dihydroxyphenylalanine have not revealed a "clear-cut" substrate localization of PPO (Fig. 2A,B). In addition, distortions in both the cytoplasms of buffer-treated and buffered DOPA-treated hyphae were observed mandating a thorough investigation of the application of the higher plant PPO, DOPA transmission electron microscopy technique (Czaninski and Catesson, 1974) to fungal systems. However, the distortions in hyphae exposed to DOPA were more apparent.

Transmission Immunoelectron Microscopy

During the past year, progress toward achieving meaningful transmission immunoelectron localization(s) of intracellular hyphal PPO occurred. The process involved: a) purifying another "batch" of anti-PPO by immunoaffinity chromatography utilizing Pharmacia's Mab trap G (Fig. 3), b) refining the procedures for colloidal gold tagging of anti-PPO and c) preparing liquid cultured hyphae for immunoelectron microscopy for applying colloidal gold tagged anti-PPO.

With regard to purification, an identical Mab trap G elution profile to that (Fig. 4) previously reported from my laboratory (Moore et al, 1993) was obtained. Both

the purified antibody and the serum from which it was purified have been stored at -20°C. As for colloidal gold tagging of the antibody, a more in depth understanding (Fig. 5) of the procedure (Fig. 6) employed by Moore et al (1993) should result in an improvement in colloidal gold tagging of anti-PPO for use in the transmission immunoelectron microscopic localization of PPO. Finally, hyphae cultured in a defined liquid medium were fixed, dehydrated and embedded in Lowicryl K4M (Fig. 7). Thus, the possibility of localizing intracellular PPO by immunoelectron microscopy has become a reality and is being performed in conjunction with Mr. Lawrence Brako, an EMSA certified electron microscopist at Morehouse Medical School.

Regulation of Secretion

In previous DOE reports, Moore and I stated that PPO accumulated intracellularly when hyphae were exposed to respiration inhibitors. However, the data supporting this statement were not provided. Table 1 presents the intracellular PPO spc. acts. for hyphae cultured in liquid medium supplemented with NaF and NaAzide. The extracellular fractions (growth medium) remain frozen for subsequent PPO analyses. In this connection, Gilson manometric attempts were made to assess the abilities of hyphae cultured with and without respiration inhibitors to consume oxygen. However, refinements in our use of Gilson manometry are required before meaningful statements regarding hyphal respiration with and without inhibitors can be rendered.

Purification of Extracellular Polyphenol Oxidase

Progress regarding purification of Coriolus versicolor's extracellular PPO to homogeneity was summarized in Moore et al (1993b). The contents of this abstract were concerned with separating PPO from an endocellulase of commercial importance which occurs within the growth medium (see previous DOE reports submitted from my laboratory). This separation may possibly be achieved through the combined application of affinity chromatography employing tyrosine-sepharose, hydroxylapatite and phenyl sepharose-CL4B hydrophobic interaction chromatographies.

In this connection, PPO(s) have been purified from a variety of higher plants, e.g., peaches (Wong et al, 1971, Flurkey and Jen, 1980 a,b), spinach chloroplasts (Goldbeck and Cammarata, 1980), carrots (Sonderhall et al, 1985), broad bean (Genesco et al, 1972), grapes (Lamikanru et al, 1972), seeds (Chilaka et al, 1993) and potato glandular-trichomes (Kowalski et al, 1993).

Research Participants

Ms. Nina Moore was awarded a second Master of Science degree two summers ago and was employed by Emory University as a laboratory technician in molecular biology. She continues to do well. During the past year, Ms. Moore helped prepare the DOE-BCTR reports. Mr. Lawrence Brako served as an electron microscopy consultant. Ms. Moore's salary was converted into a technician position. Ms. Hutto served as the technician and was accepted into graduate school at the University of California in San Francisco. She will begin graduate work Sept., 1993. Dr. Dashek has moved to the University of Georgia where he is an adjunct associate research

botanist in the Department of Botany. He is enhancing his skills in plant molecular genetics and molecular biology as a guest of Dr. Gary Kochert. In addition, Dashek will complete the research laboratory manual (see publications) while at UGA. There he possesses daily access to the GALIN computerized literature search system. Thus, he has been able to up-date his knowledge of contemporary PPO research, enhance his information base regarding molecular biology skills and improve his literature holdings regarding the more commercially relevant cellulases and ligninases (Van der meer et al, 1987; Zodrazil and Reinger, 1988; Dodson et al, 1989; Stewart, 1989).

Publications

During this past year, an abstract by Moore and Dashek appeared in the abstract booklet for the annual meeting of the American Society of Plant Physiologists. This abstract summarized the research accomplished toward purifying Coriolus versicolor's extracellular polyphenol oxidase. In addition, a reviewed symposium paper by Moore et al was revised and will appear in Biotoxins, Biodeterioration and Biodegradation. This paper, which is concerned with mechanisms of polyphenol oxidase secretion, was scheduled to appear in 1992 but will now be published in 1993. In addition, a more thorough manuscript including recent findings, is being prepared for submission to International Biodeterioration, Mycological Research or Mycologia. It will be forwarded to the DOE-BCTR program for review prior to submission. In this connection, thorough literature searches using GALIN have been performed. Finally, a research laboratory manual, an outgrowth of the DOE-BCTR-sponsored polyphenol oxidase research, continues to be performed

for Wm. C. Brown. The manual is a collection of polyphenol oxidase experiments together with some on monoamine oxidases. The chapters (modules) which have been submitted to Wm. C. Brown Publishers are: Centrifugation/Protein Assay, Polyphenol Oxidase Assay, Polyphenol Oxidase Purification, Polyphenol Oxidase Antibody Production, Restriction Fragment Length Polymorphism and TLC/GC of Monosaccharides Derived From Detergent-Release of Membrane Bound Polyphenol Oxidase, a Glycoprotein. At the moment, a module on Polyphenol Oxidase Gene Cloning is in preparation.

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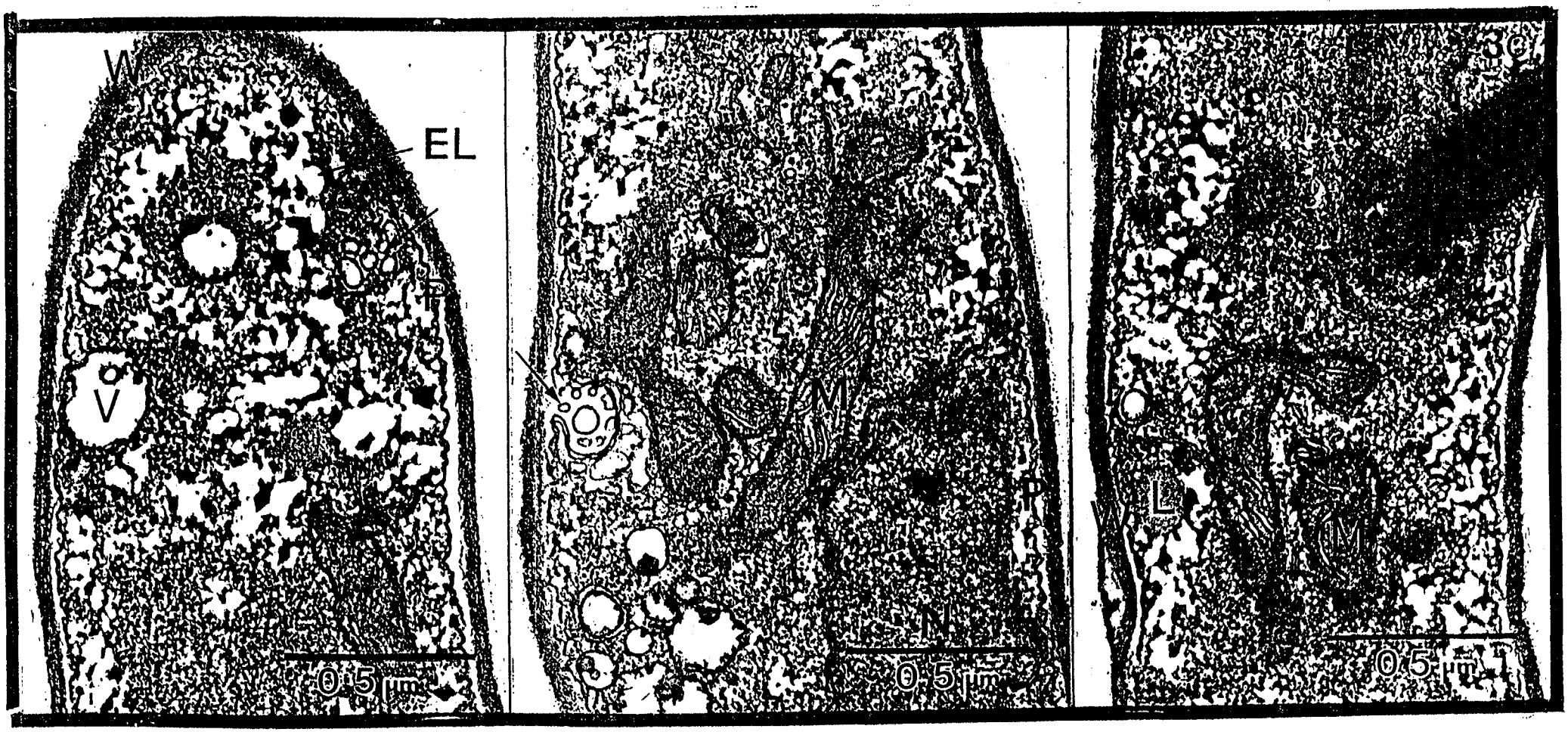
1A



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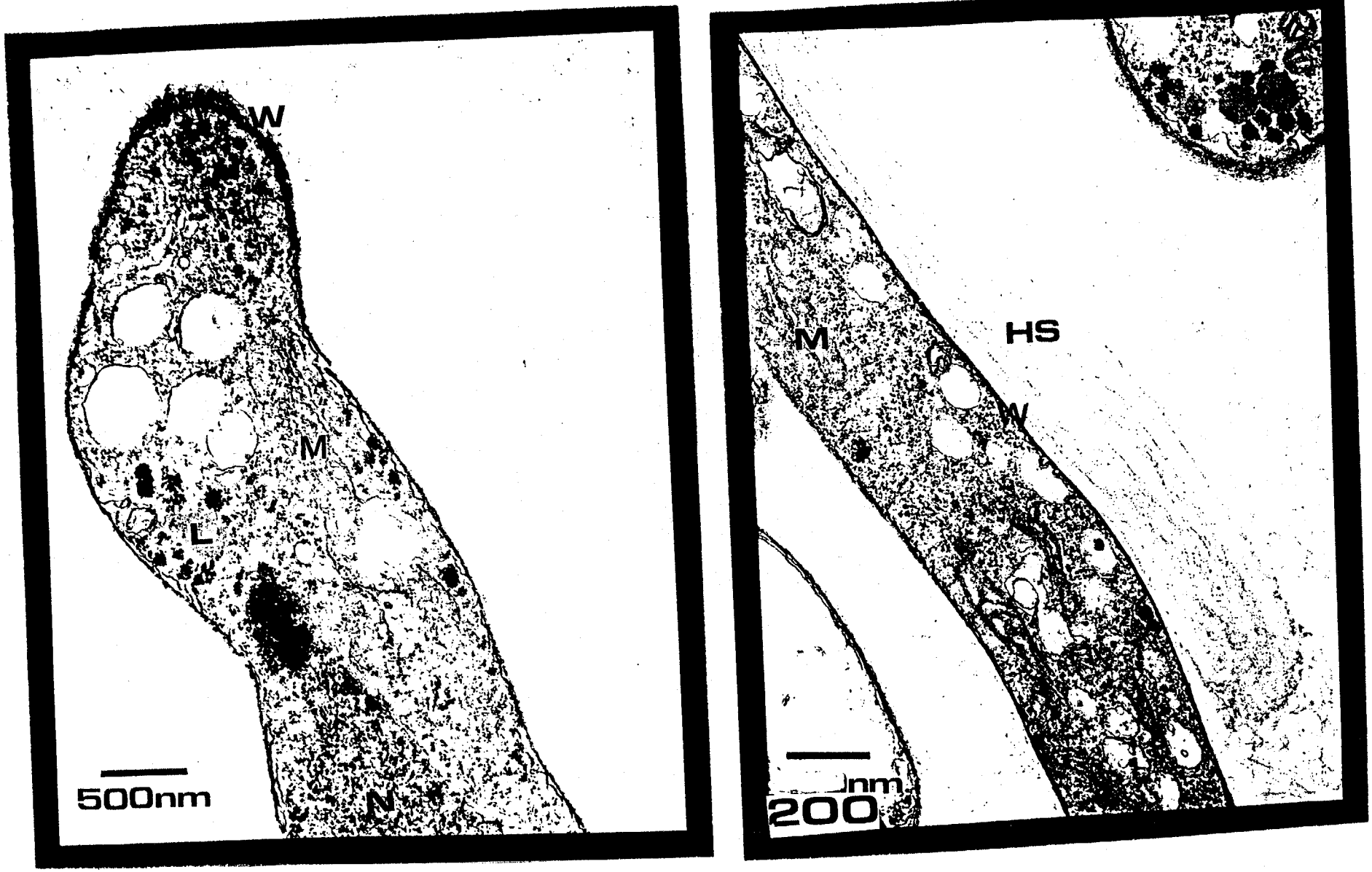

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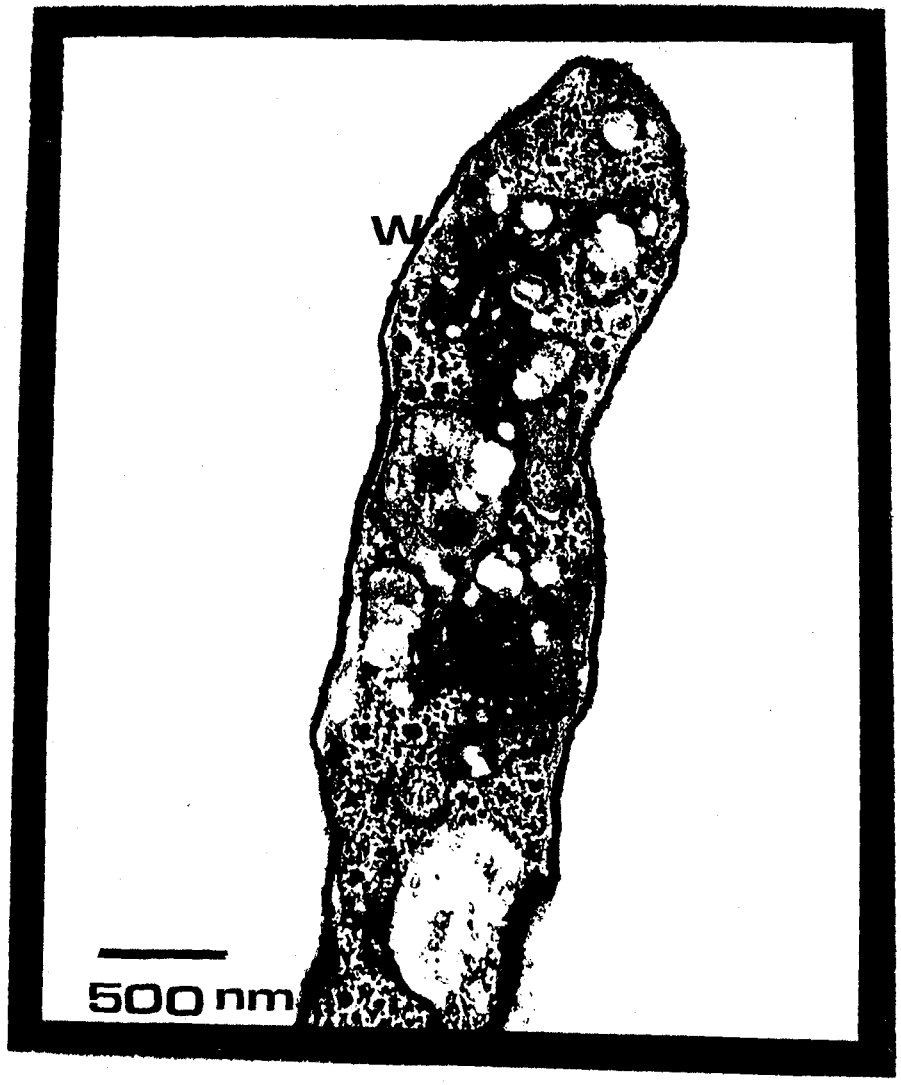

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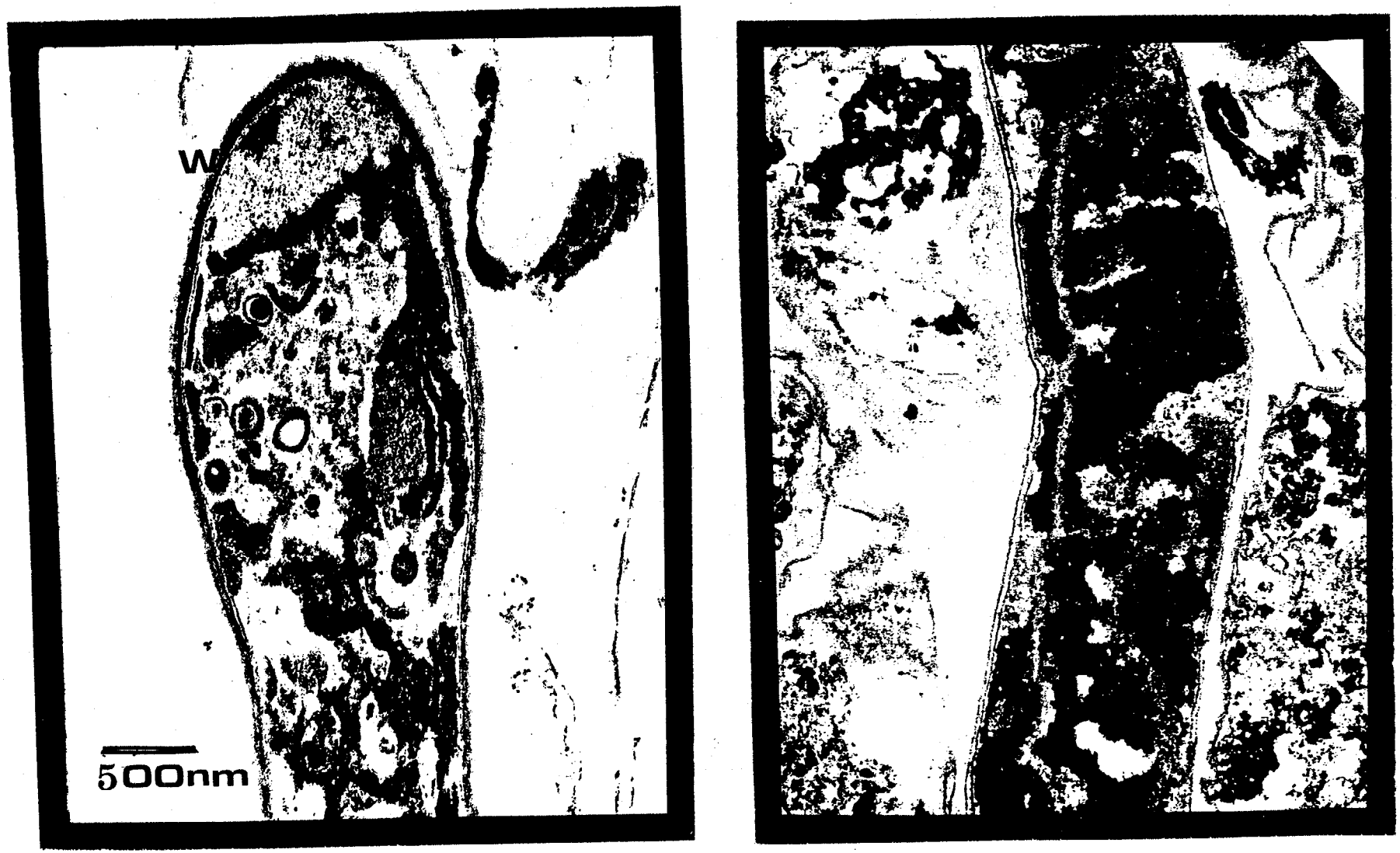


Buffer Preparation

1. Dilute the 10 X buffer concentrates **for one separation** as follows:
   a. Add 10 ml Binding Buffer concentrate to 90 ml high quality water for a total volume of 100 ml.
   b. Add 2 ml Elution Buffer concentrate to 18 ml for a total volume of 20 ml.
2. Prepare collection tubes by adding 60 to 100 µl of Neutralizing Buffer per ml of fraction to be collected. This allows for immediate renaturing of the purified sample. Neutralizing buffer should not be added once the purified fraction is collected.

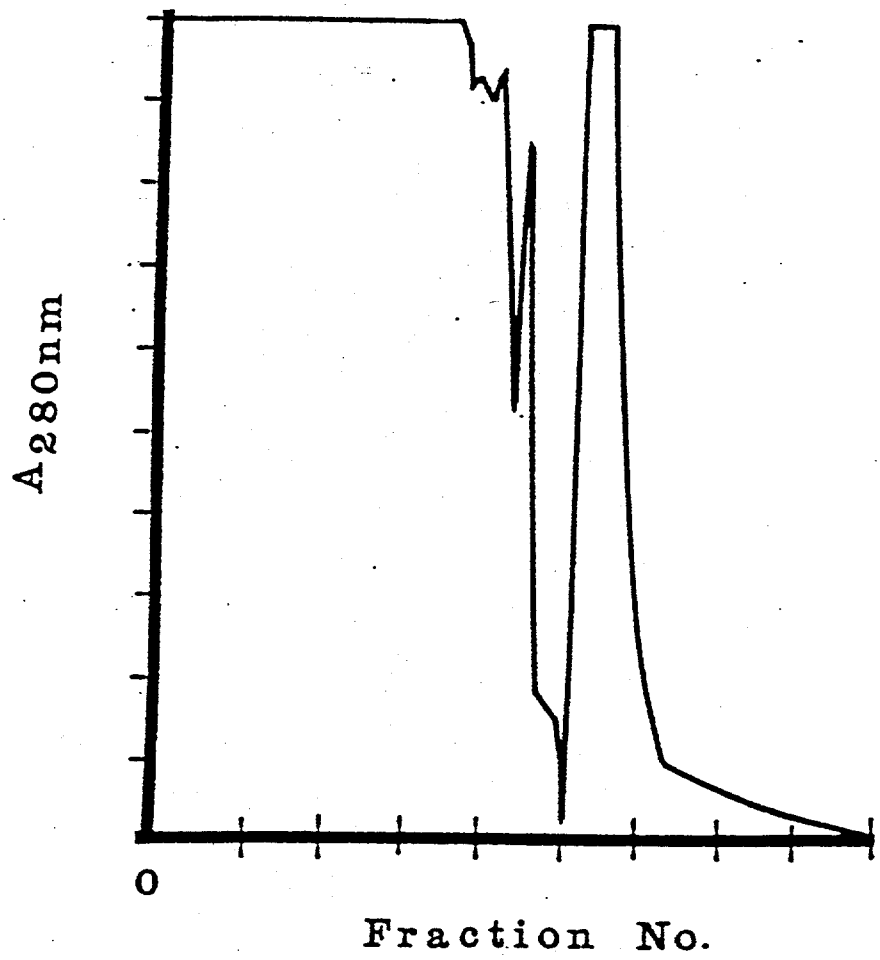
Sample Preparation

The Protein G Sepharose 4 FF column allows purification of up to 5 ml ascites fluid or 25 ml cell culture supernatant. The minimum sample volume should be 250 µl.

1. For serum, ascites fluid or cell culture supernatant samples, centrifuge (10,000 x g for 10 minutes) and filter (0.22 µm).
2. Dilute sample 1:1 with Binding Buffer to ensure proper ionic strength and pH for optimal binding. Cell culture supernatant should not be diluted with Binding Buffer.

Purification Protocol

1. Open the Protein G Sepharose 4 FF column by removing the top cap first. This will avoid air bubbles being drawn into the gel. Pour off the 20% ethanol storage solution.
2. Equilibrate the Protein G Sepharose 4 FF column by filling it to the top with Binding Buffer (~30 ml). Allow the column to drain. The column will stop flowing automatically as the meniscus reaches the top frit, preventing the column from drying out. (See Fig. 1)
3. Apply the prepared sample to the top frit, allowing it to absorb into the gel. (See Fig. 2)
4. Wash away unbound proteins by filling the column to the top with Binding Buffer (~30 ml) Allow the buffer to pass through the column, eluting unbound materials. (See Fig. 3)
5. Elute the bound IgG by filling the column with Elution Buffer to the black line (~15 ml) on the column. Collect the antibody fraction into the prepared tubes. To obtain concentrated samples, collection is best done in 1 ml fractions. If collecting 1 ml fractions, collection may be easier by reducing the flow rate (dependent upon the height of the eluent above the gel bed). This can be accomplished by dispensing three separate 5 ml aliquots of elution buffer, or by placing a syringe needle onto the column tip. Fractions can be monitored by absorbance at 280 nm. (See Fig. 4)


A_{280nm}
0
Fraction No.

Dialyze antibody (affinity purified) 1 liter against PO_4 buffer
6 h (room temperature)

↓

Dialyze against 2nd 1 liter PO_4 buffer

↓

Centrifuge 1:20 dilution 2800 rpm
25 min, 4°C

↙ ↘

pellet
(discard)

supernatant
(10 ml of gold sol)
Adjust pH to 7.4

Invert 6 times and let sit for 5 min

↓

Add fresh/filtered 1% PEG-Carbowax
20 to 10 ml gold

↓

Centrifuge 10K 30 min at 4°C

↙ ↘

Aspirate supernatant and discard

pellet

↓

resuspend in 1 ml (20mM Tris-Buffered Sol)

↓

Vortex suspension dilute 1:20 w/dH_2O

↓

Absorbance 520nm

S

Adsorption Isotherms and Colloidal Gold Procedures

GENERAL PROCEDURES:

Dialyze antibody against 2 liters of phosphate buffer prepared as follows:

2.16 g Na_2HPO_4-$7H_2O$ or 1.12 g Na_2HPO_4 anhydrous
0.266 g NaH_2PO_4
2 liters H_2O, pH 7.4

Dialyze at 4°C, overnight

Adjust pH of gold to 7.4 using 0.2N K_2CO_3

ISOTHERM:

*ADD GOLD TO THE ANTIBODY

0.1 ml antibody (Start with a low concen. of Ab)
+ 0.5 ml gold sol
Wait two minutes
+ 0.1 ml 10% NaCl
Wait five minutes

BLUE=UNSTABLE PINK=STABLE

Use the concentration of antibody to stabilize a known amount of gold in the last pink tube in series of concentrations.

CONJUGATION

1. Add protein to tube
2. Add H_2O (10% of volume), spin 2800 rpm, 25 min., 4°C, to get rid of clumps
3. Add gold to tube (enough to stabilize the antibody)
4. Invert 6 times and let sit 5 minutes
5. Add 1% PEG (Carbowax 20) and invert 6 times
6. Spin at 10K for 30 minutes at 4°C.
7. Aspirate as much supernatant as possible
8. Resuspend pellet in 1 ml 0.22u filtered buffer
   20mM Tris buffered saline, pH 8.2 + 0.1%BSA and 0.05% Na Azide
9. Standardize O.D.$_{525}$ for Au_{18}
   O.D.$_{540}$ for Au_5
   Commercial preps-O.D. at 520nm is 3.5 for 15nm and 7.0 for 30nm.

NOTE: Use only acid washed glassware, glass distilled water and 0.22u filtered reagents.

Store at 4-8°C. DO NOT FREEZE!

Fig. 6 Protocol for Colloidal Gold Tagging of PPO Antibody

From: C. Claussen, Forest Products Laboratory, Madison, WI.

POST-EMBEDDING IMMUNOLABELING IN LOWICRYL K4M

Reference: Abrahamson, D.R. 1986. Post-embedding colloidal gold immunolocalization of laminin to the lamina rara interna, lamina densa, and lamina rara externa of renal glomerular basement membranes. J. Histochem. Cytochem. 34:847-853.

A. ***Embedding in Lowicryl.*** (Modified from Altman, L.G., B.G. Schneider, and D.S. Papermaster. 1984. Rapid embedding of tissues in Lowicryl K4M for immunoelectron microscopy. J. Histochem. Cytochem. 32:1217).

1. ***Fix*** tissue in 1-4% formaldehyde (freshly prepared from paraformaldehyde) in 0.1 M phosphate buffer, 2 hrs at 0-4°C. Fixative may also contain 0.05 - 0.1% glutaraldehyde. The choice of fixative must be made empirically. Trim tissue cubes to a maximum dimension of < 1.0 mm.

2. ***Wash*** in several changes of 0.1 M phosphate plus 3.5% sucrose followed by 0.5 M ammonium chloride.

3. Dehydration/infiltration should take place on a rotating platform at room temperature as follows. Dehydrate in a graded series of dimethylformamide (DMF) in dH_2O:

   50% DMF --- 10 min.
   75% DMF --- 10 min.
   90% DMF --- 10 min.

4. Infiltrate tissue in:

   1 part Lowicryl: 2 parts DMF --- 10 min.
   1 part Lowicryl: 1 part DMF --- 10 min.
   100% Lowicryl --- 20 min.
   100% Lowicryl --- 25 min.

To prepare Lowicryl (Polysciences, Warrington, PA) (done at room temperature):

   2g Crosslinker "A"
   13g Monomer "B"
   Add 75mg Initiator "C" and gently mix with a paddle
   Makes approximately 20mls.

5. ***Embedding.*** Place tissue in GELATIN capsules that are approximately 1/2 full with fresh Lowicryl. Fill until brimming with additional Lowicryl and cover with capsule top. Blocks can be labeled after hardening - do not include labels now as the paper may affect polymerization.

6. ***Polymerization.*** Select a cardboard box large enough to hold the UV lamp and apparatus described below and line it on all six sides (including the top and bottom) with aluminum foil, shiny side facing the inside of the boc. (Our box

measures 80cm long, 45cm tall, and 27cm wide). Cut a hole at the bottom of one of the sides of the box for the lamp cord and place the UV lamp (25 Watts, General Electric #F25T8/BL, [single 18 inch tube]) inside. Place filled Lowicryl capsules in a flexible plastic ELISA or clear plexiglass plate where the wells have been cut out so that the base of the capsule protrudes beneath the bottom of the plate. The ELISA plate can then be attached to a clamp on a ring stand.

Position the bottom of the capsule directly above the lamp with a lamp-to-tissue distance of exactly 10cm. Place the box in a cold room or refrigerator. Close the top of the box and turn on the UV lamp. Polymerize for 2 hrs at 4°C. (UV polymerization at room temperature will usually occur too quickly and result in uneven polymerization).

B. *Sectioning*. Lowicryl blocks can be trimmed and sectioned using conventional procedures except that the meniscus in the knife boat should be as low as possible to minimize wetting of the block face. The first few thin sections cut should not be used for labeling. For two-sided labeling, pick up sections on uncoated 400 mesh nickel grids. **DO NOT USE** copper grids for immunogold labeling.

C. *Immunolabeling*. All solutions, except gold, should be filtered through a 0.22μm cellulose acetate (Corning) filter.

1. Treat grids with sections with 1 M ammonium chloride in PBS, 1 hr, room temperature.

2. Rinse in a gentle stream of PBS.

3. Treat with 0.1% BSA in PBS, 1.5-2 hrs. room temperature.

4. Rinse by passage through 2 - 3 drops of BSA in PBS, 30 sec/drop.

5. Wash with a gentle stream of PBS.

6. Incubate sections with primary antibody diluted with 0.1% BSA in PBS (we use affinity purified IgG at a final concentration of 15μg/ml), **OVERNIGHT-24 hrs** at 4°C.

7. Wash thoroughly with PBS.

8. Incubate with appropriate colloidal gold-antibody conjugate (10 nm, Janssen Pharmaceutica, Beerse, Belgium) diluted 1:3 with PBS, 2-3 hrs., room temperature.

9. Wash thoroughly with PBS.

10. Wash thoroughly with dH_2O.

11. Stain with 1% uranyl acetate in dH_2O, ~30 sec., wash and stain with lead citrate for 30 sec. Wash with dH_2O.

Effects of Sodium Fluoride and Sodium Azide Additions on Intracellular/ Extracellular Total Protein and Polyphenol Oxidase Activity

Treatment [1]	Intracellular		Extracellular	
	mg 280 nm Absorbing Substances	Polyphenol Oxidase Spc. Act.	mg 280 Absorbing Substances	Polyphenol Oxidase Spc. Act.
None	0.26	194.04 ± 93.68		
0.03M Sodium Fluoride	1.16±0.87	446.53 ±547.09		
0.001M Sodium Azide	0.51± 0.18	906.27± 1226.20		

[1] An inhibitor added at day 6 and mycelia harvested at day 16

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