Immunocytochemical Localization of a Wheat Germ Lysozyme in Wheat Embryo and Coleoptile Cells and Cytochemical Study of Its Interaction with the Cell Wall

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ABSTRACT
Among several wheat (Triticum aestivum L.) germ proteins able to lyse Micrococcus lysodeikticus, one lysozyme (W1A) was purified by ion-exchange chromatography, gel filtration, and preparative polyacrylamide gel electrophoresis. Polyclonal antibodies against this lysozyme were raised in rabbits. The in situ localization of W1A lysozyme was achieved by the indirect protein A-gold technique. Large amounts of W1A lysozyme were found in cell walls whereas intercellular spaces, cytoplasm, and organelles were nearly free of labeling. Specificity of labeling was assessed with several controls. In an attempt to detect the presence of binding sites, W1A lysozyme was complexed to colloidal gold. Particles were specifically distributed in large amounts over wheat embryo and coleoptile cell walls. The absence of labeling over isolated coleoptile cell walls treated with 0.1 and 0.4 molar potassium hydroxide for hemicellulose extraction indicated that W1A lysozyme binding sites were probably of hemicellulosic nature.

Ever since the first report on plant lysozyme activity in turnip (17), lysozymes (EC 3.2.1.17) have been studied in various plant tissues and latices (for review see 10, 22). In the last 20 years, several plant lysozymes have been purified from dicotyledons (10, 15, 19, 21, 29, 30). Plant lysozymes exhibit strong chitino-lytic activity in addition to hydrolysis of N-acetyl-glucosaminyl-N-acetylmuramic linkages in bacterial cell walls. Since endogenous substrates have not been found, plant lysozymes are assumed to be involved in defense or resistance against infection (11).

Several histochemical and cytochemical methods have been developed for identification of lysozymes in human and animal cell compartments (27). Most of these techniques have been based on the hydrolytic properties of the enzyme using Micrococcus lysodeikticus (syn: luteus) or chitin as substrates. Immunofluorescence and immunoenzymatic labeling have also been used (1, 23, 24). More recently, studies concerning the localization of intracellular lysozymes in human tissues involved immunoelectron microscopic techniques combining the use of specific antibodies and colloidal gold as an electron-dense marker (13).

So far, the literature on plant lysozymes has been restricted to enzyme purification procedures and determination of in vitro enzymatic activity toward various substrates (22). To our knowledge, no attempt has ever been made to study plant lysozymes in situ.

This report describes the use of an indirect immunocytochemical technique (5, 6) for localizing one lysozyme (W1A) purified from wheat germ (2). The use of polyclonal antibodies against this lysozyme in conjunction with the protein A-gold technique has allowed localization of this enzyme. Furthermore, in an attempt to detect the presence of a putative endogenous substrate, W1A lysozyme was conjugated to gold and applied to tissue sections. Results suggest that W1A lysozyme can interact with some polysaccharide(s) in the cell wall.

MATERIALS AND METHODS

Plant Material. Wheat seeds (Triticum aestivum L. cv Laval 19) were germinated in a commercial Biosta sprouter in the dark at 22°C and provided daily with tap water. Embryos were excised from mature seeds and processed for electron microscope studies. Coleoptiles were harvested when they were about 2.5 cm long.

Isolation of Wheat Coleoptile Cell Walls and Sequential Removal of Polysaccharides. Twenty-five g (fresh weight) of etiolated coleoptiles were homogenized in a mortar using 50 mL of 0.05 M Tes buffer (pH 7.2) at 4°C. Walls and cell debris were collected by vacuum filtration on a Whatman GF/C filter and successively washed with 50 mL of the following solutions: 0.5 M potassium phosphate (pH 7.0) (2x); bi-distilled water (4x); chloroform:methanol (1:1, v/v) at 45°C for 30 min (2x); acetone (1x); methanol (2x); bi-distilled water (2x). The recovered material was frozen at −80°C and lyophilized.

Removal of polysaccharides was performed according to Carpita (12) with slight modifications. For extraction of starch, 250 mg of lyophilized walls was suspended in 100 mL of DMSO and was shaken over night. Wall material was then collected by vacuum filtration on a Whatman No. 1 filter paper. For extraction of pectic substances, wall material collected by filtration was incubated with gentle shaking in 100 mL of 0.5% (w/v) ammonium oxalate/HCl (pH 4.5) at 100°C with two changes at 1 h intervals. Insoluble cellulose and hemicelluloses were recovered by filtration and were frozen and lyophilized. This remaining material was successively suspended in 100 mL of potassium hydroxide at 0.01 M, 0.1 M, and 0.4 M containing 3 mg/mL of sodium borohydride and was agitated with magnetic stirring under nitrogen for 1 h. Samples collected between each potassium hydroxide treatment were washed with bi-distilled water, frozen, and lyophilized.

Tissue Processing. Wheat embryos, coleoptiles, untreated, and treated coleoptile cell walls embedded in 1% aqueous agarose, were sliced with a double-edge razor blade into small pieces (1

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mm) in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) and incubated for 2 h at 4°C. Some samples were postfixed in 1% osmium tetroxide in sodium cacodylate buffer for 1 h at 4°C. All samples were dehydrated in a graded ethanol series and embedded in Epon 812. Ultrathin sections were collected on nickel grids (200 mesh).

**Purification of Wheat Germ Lysozyme WIA and Preparation of Polyclonal Antibodies.** Lysozyme purification was performed as described by Audy et al. (2). The purification procedure involved ammonium sulfate fractionation, ion-exchange chromatography, gel filtration, and preparative PAGE under native conditions. Antiserum against WIA lysozyme was raised in rabbits by four intramuscular injections (1 week apart) of purified WIA lysozyme (0.25 mg) emulsified in complete (first injection) or incomplete (subsequent injections) Freund's adjuvant. One week after the last intramuscular injection, rabbits were boosted with an intravenous injection. Animals were bled from the marginal ear vein 9 d after the intravenous injection. Antiserum was separated from red cells by centrifugation at 10,000g for 20 min at room temperature. Aliquots (1 mL) were stored at -20°C.

**Western Blotting.** Analytical PAGE under denaturing conditions was performed as described by Audy et al. (2). The electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose was performed using a transfer cell (Trans-blot cell, Bio-Rad) in 50 mM Tris, 384 mM glycine, and 0.1% (w/v) SDS. Proteins were transferred at room temperature for 3 h at 375 mamp. Immunodetection (28) after western blotting was performed according to Fortin et al. (16).

**Preparation of Protein A-Gold Complex.** Colloidal gold with a mean particle diameter of 15 nm was prepared according to Frens (18).

Preparation of the protein A-gold complex was adapted from Bendayan (3). The minimal amount of protein A for full stabilization of the gold solution at pH 6.9 was estimated to be 30 μg. A 10-fold excess of protein (300 μg) was dissolved in 100 μL of distilled water and mixed with 10 mL of colloidal gold at pH 6.9. After centrifugation, the red pellet was resuspended in 0.5 mL of PBS (pH 7.4) containing 0.02% PEG 20,000. This was the stock solution.

**Immunocytochemical Labeling.** Labeling was carried out at room temperature. Ultrathin tissue sections were incubated for 5 min on a drop of PBS (pH 7.4) containing 1% ovalbumin and transferred onto a drop of the diluted anti-WIA antiserum (1:40 with PBS-ovalbumin) for 60 min in a moist chamber. Sections were thoroughly rinsed with PBS and incubated with the protein A-gold complex diluted 1:30 in PBS-PEG for 30 min. Sections were washed with PBS, rinsed with distilled water and dried. Staining with uranyl acetate and lead citrate was performed before examination in a Siemens Elmiskope 102 electron microscope.

Specificity of labeling was assessed with five control experiments: (a) incubation of sections with the protein A-gold complex, without the antiserum step; (b) incubation of sections with the antiserum previously absorbed with an excess of the corresponding antigen; (c) incubation of sections with the antiserum, followed by 30 min incubations with nonlabeled protein A and then with the protein A-gold complex; (d) incubation of sections with preimmune serum; (e) incubation of sections with the colloidal gold suspension alone.

**Preparation of WIA Lysozyme-Gold Complex.** The optimal pH value for complexesing colloidal gold with purified WIA lysozyme was 9.3 (isoelectric point of this enzyme). The minimal amount of WIA lysozyme needed to stabilize the gold solution at pH 9.3 was determined by adding 1 mL of colloidal gold to 0.1 mL of serial dilutions of WIA lysozyme. After a few minutes, 100 μL of 10% NaCl solution was added, and flocculation was determined visually.

For preparation of the WIA lysozyme-gold complex, 0.10 mg of WIA lysozyme was dissolved in 100 μL of distilled water and mixed with 10 mL of colloidal gold at pH 9.3. After centrifugation, the dark-red sediment was resuspended in 0.5 mL of PBS-PEG (pH 6.0). This was the stock solution.

**Cytochemical Labeling.** Ultrathin tissue sections were floated on a drop of PBS-PEG (pH 7.2) and transferred onto a drop of the WIA lysozyme-gold complex at 1:2 dilution in PBS-PEG (pH 7.2) for 30 min at room temperature in a moist chamber. Sections were rinsed, dried, and contrasted as described above. The following controls were carried out: (a) incubation of sections with the uncomplexed WIA lysozyme followed by incubation with the WIA lysozyme-gold complex, (b) incubation of sections with a nonenzymic protein-gold complex: bovine serum albumin (BSA)-gold complex, (c) incubation of sections with stabilized or unstabilized gold suspensions.

**Reagents.** All chemicals for electrophoresis and protein assays were purchased from Bio-Rad Co, Mississauga, Ontario, Canada. Lyophilized cells of Micrococcus lysodeikticus, protein A, BSA, and sodium citrate were purchased from Sigma Chemical Co, St. Louis, MO, USA. Complete and incomplete Freund's adjuvant were obtained from Difco laboratories, Detroit, MI, USA. Tetrachloroauric acid was purchased from BDH Chemicals, Montreal, Canada, and PEG 20,000 from Fisher Scientific, Quebec, Canada.

**RESULTS**

**Immunocytochemical Localization of WIA Lysozyme.** The basic requirements for reliable immunocytochemical labeling are the high specificity of the antibodies and the preservation of antigenicity and ultrastructure (3).

Antibodies were produced against the electrophoretically purified WIA lysozyme which exhibited only one band (25,400 M) after Coomassie blue staining in SDS-PAGE (2) and Figure 1A.

Immunodetection of purified WIA lysozyme (Fig. 1B) and...
FIGS. 2–4. Immunocytochemical localization of wheat germ WIA lysozyme using the postembedding protein A-gold technique.

FIG. 2. Endosperm cell walls of wheat embryos are intensely labeled. A few gold particles, likely corresponding to nonspecific labeling, are occasionally seen over the cytoplasm. x31,000. Bar = 0.5 μm.

FIG. 3. Gold particles are present over wheat coleoptile cell walls whereas intercellular spaces are free of labeling (x48,000; bar = 0.25 μm).

FIG. 4. Control test. Gold labeling is negligible over sections incubated with the anti-WIA lysozyme antiserum, previously absorbed with purified WIA lysozyme (x48,000; bar = 0.25 μm).

IS, intercellular space; CW, cell wall; Cy, cytoplasm.

Commercial hen egg white lysozyme (HEWL) (Fig. 1C), blotted on nitrocellulose revealed that antibodies reacted with a single band (Fig. 1B) with mol wt corresponding to the purified protein (Fig. 1A). No reaction was observed with HEWL (Fig. 1C). Two other isozymes of WIA lysozyme also reacted with the anti-WIA antiserum when analyzed in PAGE under native conditions (data not shown).

Good preservation of antigenicity and cellular structure was obtained by fixation with glutaraldehyde followed by embedding in Epon. Antigenicity was not maintained when tissues were postfixed with osmium tetroxide prior to embedding. As previously reported (4, 31), osmication of tissues was found to significantly reduce the accessibility of antibodies toward antigens. Since cytoplasmic structures and antigenicity were satisfactorily preserved when tissues were fixed with glutaraldehyde only, this procedure was adopted for further immunocytochemical tests.

Pretreating sections with 1% ovalbumin in PBS was essential to avoid nonspecific labeling. Omission of this step resulted in an intense background signal.

Incubation of thin sections from wheat embryos and coleoptiles with anti-WIA lysozyme antibody (1:40 in PBS-ovalbumin), followed by protein A-gold complex (1:30 in PBS-PEG) yielded an intense and specific labeling of cell walls (Figs. 2 and 3). A few randomly distributed gold particles were occasionally seen over the cytoplasm. As these particles were not consistently found from one experiment to another, they were probably related to nonspecific labeling. When sections were incubated with the antiserum previously absorbed with purified WIA lysozyme (1 mg/mL), gold deposition was negligible (Fig. 4). Similarly, sections were nearly free of labeling when (a) preimmune rabbit immunoglobulins were used instead of antiserum, (b) antiserum was omitted, (c) incubation with antiserum was followed by
incubation with unlabeled protein A followed by protein A-gold complex, and (d) incubation was performed with the gold suspension alone.

Examination of sections from wheat embryos revealed that cell walls of the endosperm were specifically labeled (Fig. 2). Gold particles did not show any preferential localization over the wall and appeared unevenly distributed. Sections of wheat coleoptiles also exhibited an intense labeling of cell walls (Figs. 3 and 4). Areas corresponding to intercellular spaces were almost devoid of labeling whereas numerous gold particles were present over primary walls. In this case, gold deposition was also irregular.

Cytochemical Localization of Interaction between W1A Lysozyme and Cell Wall. The optimal pH value for tagging W1A lysozyme to 15 nm gold particles was 9.3 and the minimal amount of enzyme necessary to fully stabilize the gold suspension was 20 μg/mL. Fixation procedures influenced the labeling pattern obtained with the gold-complexed W1A lysozyme. Postfixation with osmium tetroxide had to be avoided since it was found to impede the accessibility of the enzyme toward the cell wall.

Treatment of thin section from wheat embryos and coleoptiles with the W1A lysozyme-gold complex resulted in specific labeling of cell walls (Figs. 5–7). Cytoplasm, nuclei, mitochondria, and endoplasmic reticulum were nearly free of labeling (Figs. 6 and 7). In contrast, a few gold particles were reproducibly observed over amyloplasts especially over starch grains (Fig. 7). The specificity of the enzyme-wall interactions was supported by the low level of labeling over sections incubated with the uncomplexed W1A lysozyme and then with the enzyme-gold complex (Fig. 8). Similarly, when sections were incubated with a BSA-
gold complex or with the gold suspension alone, labeling was negligible.

An intense and specific gold labeling was always observed over cell walls, whereas intercellular spaces appeared unlabeled (Fig. 7). Over starch grains of amyloplasts, gold particles were randomly distributed (Fig. 7). Labeling of these structures was considered significant because it was observed from one experiment to another and absent in all controls.

Following incubation with W1A lysozyme-gold complex, sections from isolated wheat coleoptile cell walls displayed an intense labeling (Fig. 9). Depending on the plane of sectioning, gold particles appeared randomly distributed or mostly localized over one side of the walls (Fig. 9, arrow). A similar labeling pattern was observed over cell walls treated with DMSO for starch removal (Fig. 10). In this case also, preferential deposition of gold particles over one part of the walls was noticeable (Fig. 10, arrows). Sections of wheat coleoptile cell walls, treated with DMSO and ammonium oxalate/HCl for pectin extraction exhibited intense and specific labeling (Fig. 11). Similarly, gold particles were present over sections of isolated cell walls treated with DMSO, ammonium oxalate/HCl, and 0.01 M potassium hydroxide for the first hemicellulosic fraction removal (Fig. 12). Further treatments of isolated walls with 0.1 or 0.4 M potassium hydroxide for extraction of the remaining hemicelluloses resulted in loss of gold labeling (Fig. 13). Control experiments performed on untreated and treated coleoptile cell walls yielded negative results.

**DISCUSSION AND CONCLUSIONS**

The present results demonstrate that the use of glutaraldehyde for fixation and of Epon for embedding provides good morpho-

Figs. 9–13. Cytochemical localization of W1A lysozyme over isolated wheat coleoptile cell walls (CW).

**Fig. 9.** Untreated wheat coleoptile cell walls exhibit an intense and specific labeling. Depending on the plane of sectioning, gold particles may appear mostly localized over one side of the walls (arrow) (x40,000; bar = 0.25 μm).

**Fig. 10.** Gold particles are present over isolated walls treated with DMSO for starch removal. A preferential deposition of gold particles over one part of the walls is observed (arrows). x30,000. Bar = 0.5 μm.

**Fig. 11.** Wheat coleoptile cell walls are still intensely labeled after treatment with DMSO and oxalate/HCl for pectin extraction (x40,000; bar = 0.25 μm).

**Fig. 12.** Gold particles are present over coleoptile cell walls treated with DMSO, oxalate/HCl, and 0.01 M potassium hydroxide for the first hemicellulosic fraction removal (x40,000; bar = 0.25 μm).

**Fig. 13.** Further treatment with 0.1 M potassium hydroxide for sequential extraction of hemicelluloses results in a loss of gold labeling (x40,000; bar = 0.25 μm).
logical integrity and satisfactory preservation of cyto- and immunocytochemical reactivity. As stated by Cramer and Breton-Gorius (13) who studied the localization of lysozyme in human neutrophils, postembedding colloidal gold techniques are highly suitable for accurate in situ localization of such macromolecules. The introduction of colloidal gold in plant biology as an alternative to markers such as peroxidase or fluorescein has greatly enhanced the resolution and specificity of labeling (7, 8).

This is the first report dealing with the ultrastructural localization of a plant lysozyme and of its interaction with the cell wall. In the past, plant lysozymes have received less attention than animal lysozymes (22). In recent years, our knowledge has been mostly concerned with their structure and catalytic properties (9–11, 29). However, the physiological role of plant lysozymes is still open to question. Plant lysozymes are generally assumed to be defensive or protective enzymes (22). Because plant polysaccharides susceptible to enzymic cleavages by lysozymes have not been characterized, the possibility that such enzymes may play a physiological role did not receive much attention (26).

Our results revealed that a wheat germ lysozyme (W1A) was associated with cell walls of wheat embryos and coleoptiles. This provides good evidence that wheat cells synthesize at least one lysozyme accumulating in the wall matrix. Cytochemical experiments conducted on isolated wheat coleoptile cell walls disclosed that the cell wall-lysozyme interaction did not involve starch or pectic substances but rather hemicellulose. Since hemicellulose interments conducted on isolated wheat coleoptile cell walls disclosed that plant lysozymes are generally assumed to be defensive or protective enzymes (22). Because plant polysaccharides susceptible to enzymic cleavages by lysozymes have not been characterized, the possibility that such enzymes may play a physiological role did not receive much attention (26).

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LITERATURE CITED

4. BENDAYAN M, E PUヴィON 1984 Ultrastructural localization of nucleic acids through several cytochemical techniques on osmium-fixed tissue. Comparative evaluation of the different labelings. J Histochem Cytochem 32: 1185–1191
7. BENHAMOU N, H CHAMBERLAND, GB OUELETTE, FJ PAUΖE 1987 Ultrastructural localization of β(1–4) glucans in two pathogenic fungi and in their host tissues by means of an exoglucanase-gold complex. Can J Microbiol 33: 403–417
17. FLEMING A 1922 On a remarkable bacteriolytic element found in tissues and secretions. Proc Roy Soc 93: 306