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Visualization of the Action of Ligninolytic Enzymes on High yield Pulp Fibers

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High-yield pulps from wheat straw and from poplar wood were treated with manganese-peroxidase (MnP) or with laccase (Lac), before and after a second refining stage. The micromorphology of the degradation performed by the enzymes on the fibers was examined using electron microscopy. The preferential site of attack and the diffusability of the enzymes in the fiber walls were evaluated. It appears that MnP and Lac produce typical defibrillation patterns. In addition, Lac shows a particular ability to degrade lignin from the middle lamella. A correlation between the observed patterns, the type of enzyme and the nature of lignins present in the different parts of the fiber wall, as demonstrated with immunocytochemical probes, is discussed.

The purpose of the pulping process is the separation of wood fibers from each other. Pulping can be achieved either by chemical treatments intended to degrade and remove the material that glues together the fibers, or by mechanical treatments which physically tear apart the fibers from the wood tissue, or also by a combination of chemical and physical action. Obviously, the nature of the treatments influences the resulting pulp properties, and also the pulp yield. A characteristic difference between mechanical and chemical pulps is the higher lignin content of the former. This gives mechanical fibers their stiffness and opacity, but enhances their tendency to yellow and their poor flexibility. In search of an alternative to chemical treatments to improve mechanical pulp properties, biological treatment with ligninolytic fungi has been envisaged (*1-3*). Several reports described the application of lignin-degrading fungi at different stages of refining, and demonstrated the effectiveness of biomechanical pulping with white-rot fungi in saving energy and in increasing pulp strength (*3-7*). Fungal treatment with white-rot fungi by stationary solid state cultivation before soda pulping on graminaceous material resulted in a higher yield of pulps with decrease in kappa number and less fine formation(*6*).

Although the residual lignin in unbleached chemical pulps is substantially modified , mostly by alkaline condensation occuring during the kraft pulping process, some fungi can nevertheless delignify it (*8,9*). It was suggested that manganese peroxidase (MnP) plays an important role in the bleaching of hardwood kraft pulp by white-rot

fungi (*10*). Other enzymes known to take part in lignin oxidative breakdown are also potential candidates for improving pulp properties (*10,11*). Thus, several attempts to apply ligninolytic enzymes to pulps have been made, most of them on chemical pulps with the aim of developing a chlorine-free biobleaching process. Lignin peroxidase (*12*), manganese peroxidase (*13,14*) and laccase (*15*) all increased pulp brightness, and demonstrated therefore their ability to degrade or modify lignin. In the enzyme action there is contact between the pulp and the enzymes (*16*) which adsorb on the fibers during solid state fermentation (*17*).

In this work, we studied the action of MnP and laccase (Lac) treatments on the structure of fibers of high-yield pulps. Micromorphological changes in the cell walls of mechanical and chemimechanical pulps (CMP) were examined by transmission electron microscopy (TEM). The relationship between the enzymes and lignin distribution was investigated using immunocytochemical methods.

Materials and Methods

Pulps and Enzymes.

Wheat straw CMP (yield: 85%) and refined CMP were from the SAICA mill (Zaragosa, Spain). Poplar wood CMP and refined CMP pulps were from SICEM-SAGA spA (Canossa, Italy). MnP was produced from *Phanerochaete chrysosporium* I-1512 (CNCM, Institut Pasteur, Paris, France) using a new bioreactor design combining an airlift column and biofilm immobilization as previously described (18). After 5 days of incubation, the culture supernatant was harvested and concentrated by ultrafiltration using a 10 000 Da membrane (Millipore S.A., Molsheim, France). Concentrate activity was about 15 000 U/L MnP and 3 000 U/L LiP.

Lac was produced from *Pycnoporus cinnabarinus* MIC 11 (600 U/L Lac) in the presence of ferulic acid as an inducer (*19*). The cultures in the bioreactor were harvested after 8 days, corresponding to maximal enzyme activity. The biomass was removed by filtration and the filtrate was concentrated by ultrafiltration followed by diafiltration.

Treatment of Pulps with Enzymes.

For MnP treatment the pulps (40g dry weight) were resuspended at 2% weight consistency (w/v) in lactate buffer (0.1 M, pH 4.5) and the final pH adjusted to 4.5 when needed. Four volumes of enzymatic solution (300 U each) were added at 1 hour intervals. The suspension was mechanically agitated and hydrogen peroxide (0.01 M) was introduced in the suspension at a rate of 5 mL/hour. After 4 hours, the pulp was filtered in a Buchner funnel and rinsed twice with distilled water. For controls, samples were treated with lactate buffer plus hydrogen peroxide and MnP plus hydrogen peroxide in the absence of Mn^{2+}, respectively.

For Lac treatment the pulps (40g dry weight) were suspended in lactate buffer (0.1 M, pH 4.5) at 2% consistency. Lac (150 U) was added in the presence of 2,2'-azinobis (3-ethyl-benzothiazoline-6-sulfonate) (ABTS) (1 mM). The mixture was kept under gentle stirring at 40°C for 16 hours. At the end, the pulp was filtered in a Buchner funnel and rinsed with distilled water. For control, samples were treated with ABTS and laccase alone, respectively.

Fixation of Samples for Electron Microscopy.

Small samples of the pulps were fixed in a freshly prepared mixture of 0.2% glutaraldehyde, 2% paraformaldehyde in 0.1 M phosphate buffer pH 7.0. Samples dehydrated through a series of increasing ethanol concentrations up to 70% (v/v) were embedded in London Resin white (LRW) (hard mixture) and polymerized 24 h at 50°C.

Chemical Staining.

Polysaccharides were stained on thin sections by the periodic acid-thiocarbohydrazide-silver proteinate (PATAg-method of Thiery), as modified by Ruel *et al.* (*20*).

Immunocytochemical Labelings.

Labelings were performed on thin sections floating in plastic rings. The protocol was as described in Joseleau *et al.* (*21*) with a few modifications: BSA was replaced by 5% non-fat dried milk both in TBS_{500} (0.5 M NaCl in 0.01 M tris-phosphate buffer, pH 7.4) and TB (0.01 M tris-phosphate buffer, pH 7.4). The secondary marker was protein A-gold (pA_{10} or pA_5) (Amersham) diluted in tris buffer containing fish gelatin. When pA_5 was used, the technique of silver enhancement was applied (Amersham kit). After rinsing in TB, the sections were fixed in 2.5% glutaraldehyde, rinsed in water and transferred onto copper carbon coated grids. Post staining was in 2.5% aqueous uranyl acetate. Observations were performed with a Philips CM 200 cryo transmission electron microscope operating at 80 kV.

Labeling of manganese-peroxidase and laccase used an immunocytochemical method. Two polyclonal antisera raised in rabbits against the MnP complex and Lac from *Ceriporiopsis subvermispora* were used. Due to the strong homology among fungal enzymes, the antisera could be applied for labeling MnP from *P. chrysosporium*, and Lac from *P. cinnabarinus*. Immunocytochemical controls were the following: omission of the primary antibody, substitution of the primary antibody with the non-immune serum, and incubation with primary antibody that had first been saturated with the corresponding antigen. All the control tests were negative.

Results and Discussion

Pulps from wheat straw and poplar wood were used. For straw the pulping treatments were first a coarse primary defibration in the presence of 1M NaOH which gave chemimechanical pulp (CMP), then a secondary refining with soda on the preceding material, yielding refined pulp (R-CMP). From poplar wood a chemimechanical pulp was prepared by 1M NaOH impregnation of wood chips and primary defibration (CMP), followed by a second refining with soda and hydrogen peroxide to give R-CMP.

Changes in the Micromorphological Structure of Fibers Induced by Pulping Processes.

Ultrastructural description of pulps is difficult due to their heterogeneity. However general consistent trends can be observed and predominating morphological characteristics may be related to a type of pulping process, to a type of tissue and in some cases to the specificity of enzyme treatment. Thus, CMP are characterized by bundles of associated cells still showing a tissue organization. This is illustrated with CMP wheat straw pulp (Fig. 1A) showing aggregates of fibers, sclerenchyma, vessels and pluristratified parenchyma. The action of soda impregnation is visible in the slightly swollen secondary walls of fibers and on cell corners and middle lamellae exhibiting the beginnings of tissue breakdown. The effect of the second refining can be seen on R-CM pulp. The number of isolated fibers is higher, and more fibers are present in the aggregates. The alkaline cooking favors swelling of the S_2 layer and dissociation between S_1 and compound middle lamella (Fig. 1B). CMP of poplar consisted mainly of aggregates of fibers and vessels. The more fragile ray cells have been disrupted (Fig. 1C) and produced fines. The effect of secondary refining of this pulp was to produce R-CMP in which the alterations observed in CMP were accentuated. In this pulp more dissociation between fibers and vessels is visible, and the disruptions have been enhanced with,in some parts of the secondary wall, zones

where cellulose microfibrils have been dissociated (Fig. 1D). This resulted in a great heterogeneity in which almost unmodified fiber and vessel walls were found as well as highly defibrillated parts of secondary walls. In addition, numerous fines originating from parenchyma and peeled off middle lamellae can be seen.

Straw Pulps Treated with Manganese Peroxidase.

The compactness and structural heterogeneity of CMP pulps rendered difficult the identification of the effects due to the enzyme action. However, observation of a great number of images allowed us to ascribe the effects due to the enzymes. The visualization of MnP by immunolabeling (*22*) with an antiserum directed against MnP isoenzymes from *C. subvermispora* (*23*) and which cross-reacted with MnP from *P. chrysosporium* showed the interaction between the enzyme and the pulp. The labeling was most abundant on the fibers located at the periphery of the tissues (Fig. 2A). It demonstrates that in such a compact and highly aggregated pulp, the diffusion of the enzyme is low. In fact the enzyme was only seen interacting with fibers in desorganized areas, where a slight defibrillation had occured (Fig. 2B).This may be a crucial point in the use of enzyme pretreatment of mechanical pulps. Morphologically, the effects due to MnP appeared more clearly on the R-CMP. Here again the heterogeneity of the pulp prevented the enzyme from performing evenly . However, MnP-treated fibers showed definite signs of delamination and defibrillation due to the enzyme action (Fig. 2C). Such modifications were not observed in controls where Mn^{2+} or enzyme were omitted in the treatment. This confirms previous observations of the typical defibrillating effect of MnP (*22*). The labeling with the anti-MnP antiserum was restricted to these modified areas. In zones where an advanced and intense defibrillation had occured, it is interesting to note that the labeling of MnP was low when not totally absent (Fig. 2D). This suggests that when lignin has been extensively removed the enzyme either does not bind to the area or has been removed during washing of the pulp. Extensive removal of lignin is supported by the great fragility of the microfibrils of these areas under the electron beam. This is a well established observation in TEM that cellulose microfibrils which are no longer protected by lignin and hemicelluloses show a shortened life time when working at 80 kV (*24*).

Poplar Pulps Treated with Manganese Peroxidase.

CMP from poplar treated with MnP showed slight defibrillation with the action of the enzyme localized at the internal face of fibers. The defibrillated areas correspond to the most accessible zones. These ultrastructural alterations occurred within a narrow band which can be depicted as a fringe in which cellulose microfibrils became conspicuous and acquired random orientations (Fig. 3A). The modifications of fiber microstructure due to MnP on R- CMP were of the same type, but greater, probably because of the better accessibility.

Immunocytochemical visualization of the enzyme showed that, here again, the action of MnP was largely dependent on the morphology of the fibers in the pulp. On compact fibers, the labeling showed that the penetration of the enzyme was almost nil. On broken fibers showing clear traces of fracture, the enzyme penetration was limited to the surface of the fracture. Accumulation of MnP, however, could be observed wherever the treatment had resulted in an opening of the fiber ultrastructure, particularly in zones undergoing significant defibrillation (Fig. 3B). The ability of MnP to penetrate was considerably enhanced by the second refining treatment.

Straw and Poplar Pulps Treated with Laccase.

Straw pulps (CMP and R-CMP) were treated with Lac from *P. cinnabarinus* in the presence of mediator 2,2'-azinobis (3-ethyl-benzothiazoline-6-sulfonate) (ABTS) (*14*). As for MnP, fibers treated with Lac-ABTS showed ultrastructural modifications, the most distinct effect being delamination accompanied with a clear separation of cellulose microfibrils (Fig. 4A). These modifications were not observed on the reference

treatment with ABTS and laccase alone, respectively. The involvement of Lac in defibrillation is suggested by the association of the enzyme with the microfibrils in zones where defibrillation was enhanced (Fig. 4A). As was observed with MnP, the immunolabeling of Lac revealed only a weak labeling in the areas where defibrillation is the most pronounced. This again suggested that the enzyme is not retained when delignification has been extensive.

An interesting result of the action of Lac-ABTS is the degradation of cell corners and adjoining middle lamellae. Lignin degradation in these parts of the fiber walls gives rise to spherical particles (Fig. 4B). These electron-dense particles resemble those observed in steam exploded wood (*25,26*) and described as resulting from the tendency of lignin molecules to undergo "coalescence" once they have been released from their native state in the walls (*27*). Another explanation could be the formation of repolymerized compounds by the phenoxy radicals generated by Lac from the phenolic moieties of lignin. This is compatible with the enzyme's mechanism of action and has been shown to occur *in vitro* (*28*).

The effect of the Lac-ABTS system on high-yield pulps from poplar appeared to consist mainly of enhanced defibrillation. This again is supported by the presence of the enzyme in cell walls in which prior chemimechanical fragmentation facilitated a large penetration (Fig. 4C). Another feature which seemed to be associated with the action of Lac on poplar pulps was attack on middle lamellae. However, in contrast to what was observed with wheat straw pulps, no formation of spherical particles occurred. This suggests that the lignin moieties in the middle lamellae of hardwoods and grasses may be structurally different, and not undergo Lac-mediated oxidation with the formation of the same degradation intermediates.

Altogether, the action of Lac on high-yield poplar pulps was not as effective as that observed on wheat straw pulps. This difference in susceptibility to Lac oxidation between hardwood and grass lignin is consistent with hardwood lignins being extensively etherified at their phenolic groups (*29*). This should make them less susceptible to Lac oxidative cleavage by one-electron abstraction from phenolic substructures.

Relationship between the Enzyme Effects and the Nature of Lignin.

Several studies have indicated that syringyl and guaiacyl moieties in lignin have different susceptibilities to oxidation by MnP and Lac systems (*28,30*). The nature of lignin substructures, whether condensed or noncondensed, also influences lignin depolymerization by the oxidative enzymes (*28,30-32*). It was therefore interesting to investigate the action of MnP and Lac on fibers in relation to lignin structures. This was done using antibodies directed against synthetic lignins (*32*). Thus, labeling of guaiacyl-syringyl (GS) lignin with immunogold probes directed against dehydrogenation polymers (DHPs) respectively obtained by end-wise (ZT) or by bulk (ZL) polymerizations allowed us to distinguish the noncondensed lignins corresponding to the former from the more condensed ones corresponding to the latter (*33-34*). Figures 5A and 5C show the dense labeling of untreated CMP from wheat straw with both probes. This indicated that GS lignin with noncondensed and condensed interunit linkages are common in the high-yield pulps (*35*). The noncondensed GS lignin appears more abundant in the S_2 than in the S_1 layer and is virtually absent in middle lamellae (Fig. 5A). This shows that different types of lignins are present in the different morphological zones of fibers. Labeling of MnP-treated straw pulps with the same probes resulted in a less intense gold particle distribution both for noncondensed and condensed GS lignins (Fig. 5B and 5D). In fibers whose structure had been opened by the enzyme, there was still a significant amount of noncondensed lignin present, whereas the labeling was much lower in zones in which defibrillation had occured (Fig. 5B). Labeling of the more condensed type of GS lignin showed that the diminution of number of gold particles due to MnP treatment of the pulp was also greater in the more defibrillated areas of fibers. This shows that MnP

had an action both on condensed and noncondensed GS lignins. The stronger decrease in the labeling intensity provided by the anti-GSZL probe on MnP-treated pulp suggests that the enzyme removed more easily the condensed lignin units. This does not agree with the higher susceptibility of noncondensed substructures to MnP (*30*). However, the attack of lignin on the less resistant phenolic β-O-4 linkages by MnP may result in the solubilization of condensed fragments of lignin, thus explaining the reduction of the labeling with the anti-GSZL after MnP treatment.

In a similar approach, using the appropriate anti-guaiacyl antibodies (*33*) , it could be seen that guaiacyl lignin of the condensed type was largely represented in high yield wheat straw pulp (Fig. 5E), as expected from lignin analysis of wheat straw (*29,36*). The treatment with MnP did not induce significant loss of condensed guaiacyl lignin, as judged by the high intensity of the labeling (Fig. 5F) performed in parallel on the untreated and MnP-treated pulps, with the same antibody dilution. This indicates that G units and particularly the condensed type are more resistant to MnP than GS units, in agreement with studies on synthetic lignins (*30*) which demonstrated that G polymers were less rapidly oxidized than GS polymers. This was also the case when DHPs were degraded by *P. chrysosporium* (*37*).

The above results showing that guaiacyl-syringyl units, of the condensed type, were more degradable by MnP in straw high-yield pulps were confirmed in pulps from poplar wood. Here also, a substantial decrease in the labeling of GS lignins was observed after MnP treatment, whereas labeling of condensed G lignins was not significantly reduced. Interestingly, the treatment of poplar R-CMP pulp with Lac, beside inducing a clear diminution of GS units, resulted in a strong reduction in the labeling of condensed G lignins. This result is difficult to explain since *in vitro* Lac was reported as degrading GS noncondensed substructures more rapidly than G condensed linkages (*15,28*).

Another difference was observed in the labeling of G lignin after treatments with MnP and Lac-ABTS, respectively, showing that more G lignin was removed by Lac than by MnP. Both enzymes readily oxidize free phenolic units but Lac-ABTS can also oxidize nonphenolic units that are unattacked by MnP (*15,32*). This could explain the more extensive removal of guaiacyl moieties by Lac plus redox-mediator.

Conclusions

Transmission electron microscopy combined with immunocytochemical labeling of high yield pulps from wheat straw and poplar wood has shown that treatments with MnP and Lac performed local ultrastructural alterations in the fiber walls. Both enzymes were more effective after secondary refining of the pulps, the refining allowing improved penetration of the enzymes. This results in a softening and swelling of the fiber wall, and in an enhancement of defibrillation. It is important to note that the spatial delignification effects performed by the enzymes is largely oriented by the previous mechanical treatments which create the necessary openings for their penetration. This agrees with previous conclusions by Messner and Srebotnik (*38*) that biopulping with ligninolytic fungi does not remove the bulk of lignin but causes alterations in the cell wall structure.

Our observations show that both MnP and Lac affect the secondary wall structure in a similar way, although MnP does so more intensely . However, Lac showed a particular tendency to degrade lignin in middle lamellae and cell corners. This was more pronounced with wheat straw pulp, possibly because of the different nature of grass lignin in this cell wall area. The modification and removal of lignin by Lac in the middle lamellae could be a crucial factor leading to improved cell separation during biopulping. From the results of this study it seems doubtful that single isolated enzymes could perform extensive delignification of high-yield pulps at the first refining stage. However, if the enzyme treatment is placed after a second alkaline refining of CMP, the action of ligninolytic enzymes is more effective on fiber ultrastructure. The

relationship between the nature of lignins, their ultrastructural localization and their susceptibility to lignin oxidative systems, when more clearly understood, should allow better uses of ligninolytic enzymes in pulp manufacture.

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Caption for figures

Figure 1. A and B: Micromorphology of wheat straw pulp; A, CMP. Three fibers (F) are still associated a vessel (V). Cell corners have been delignified (arrow) and S_3 detached (arrowhead) by the first soda treatment. Uranyl acetate staining; B, R-CMP. Structure of an isolated fiber: the compound middle lamella is detached and a defibrillation is clearly visible in S_3 and S_2 (arrows). Uranyl acetate.

C and D: Micromorphology of poplar pulp. C, CMP. Aggregates of fibers (F) are separated from a highly loosened ray cell (RC), the isotropic layer appears in black (arrow). PATAg staining; D, R-CMP. Fibers are more dissociated and some of them have been defibrillated (arrows). Uranyl acetate.

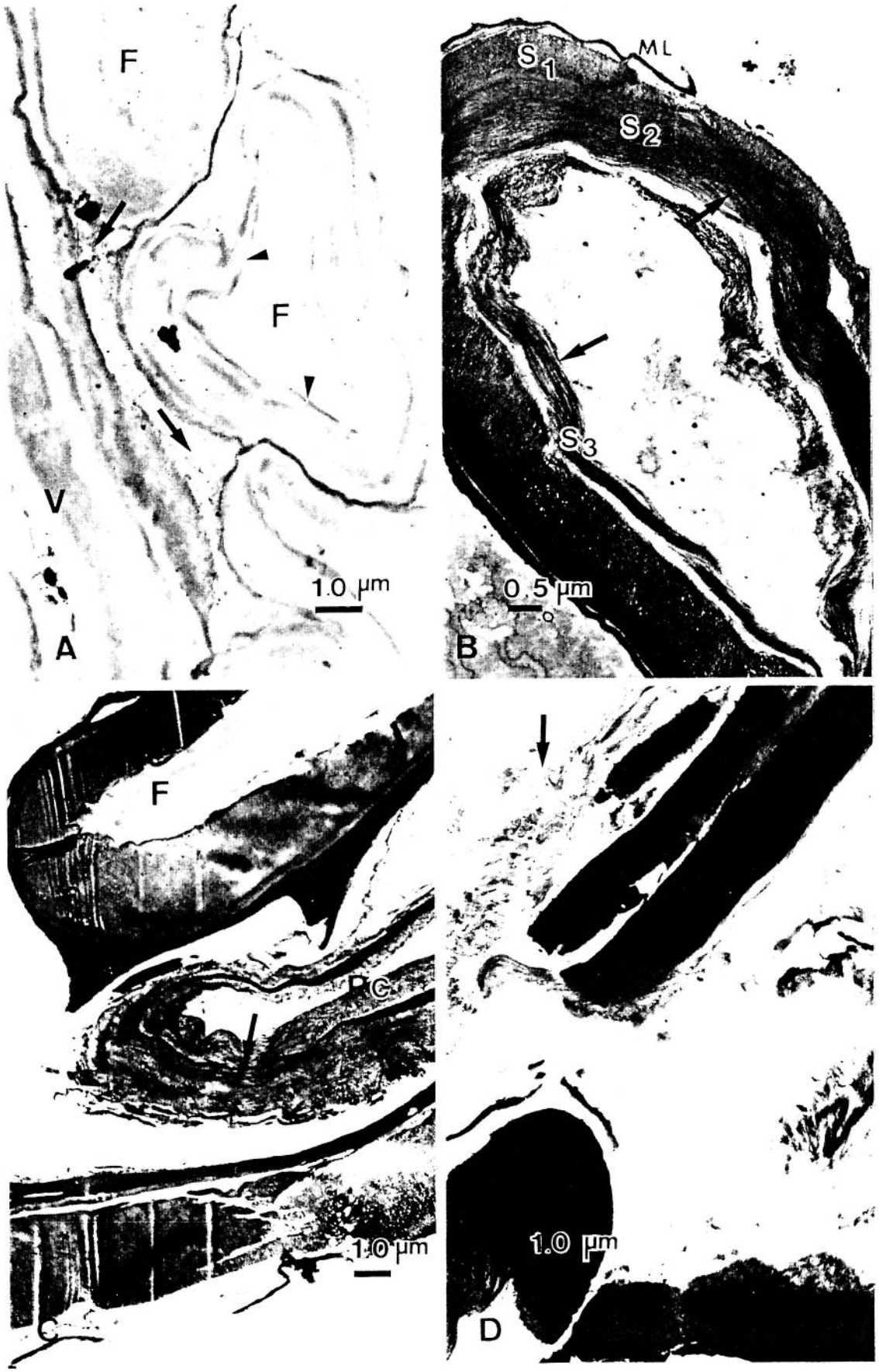
Figure 2. Wheat straw pulp treated with MnP: immunogold labeling of the enzyme with anti-MnP. A, CMP, fiber seating outside of a fiber aggregate: MnP is concentrated in the outer layer (arrows); B, CMP; part of a fiber in which MnP has penetrated inside the defibrillated areas (arrows); C, R-CMP; part of a fiber showing a strong delamination of the S_2 layer; D, R-CMP. Highly defibrillated fiber: the separation of cellulose microfibrils is clearly visible around the lumen and MnP is no longer visible.

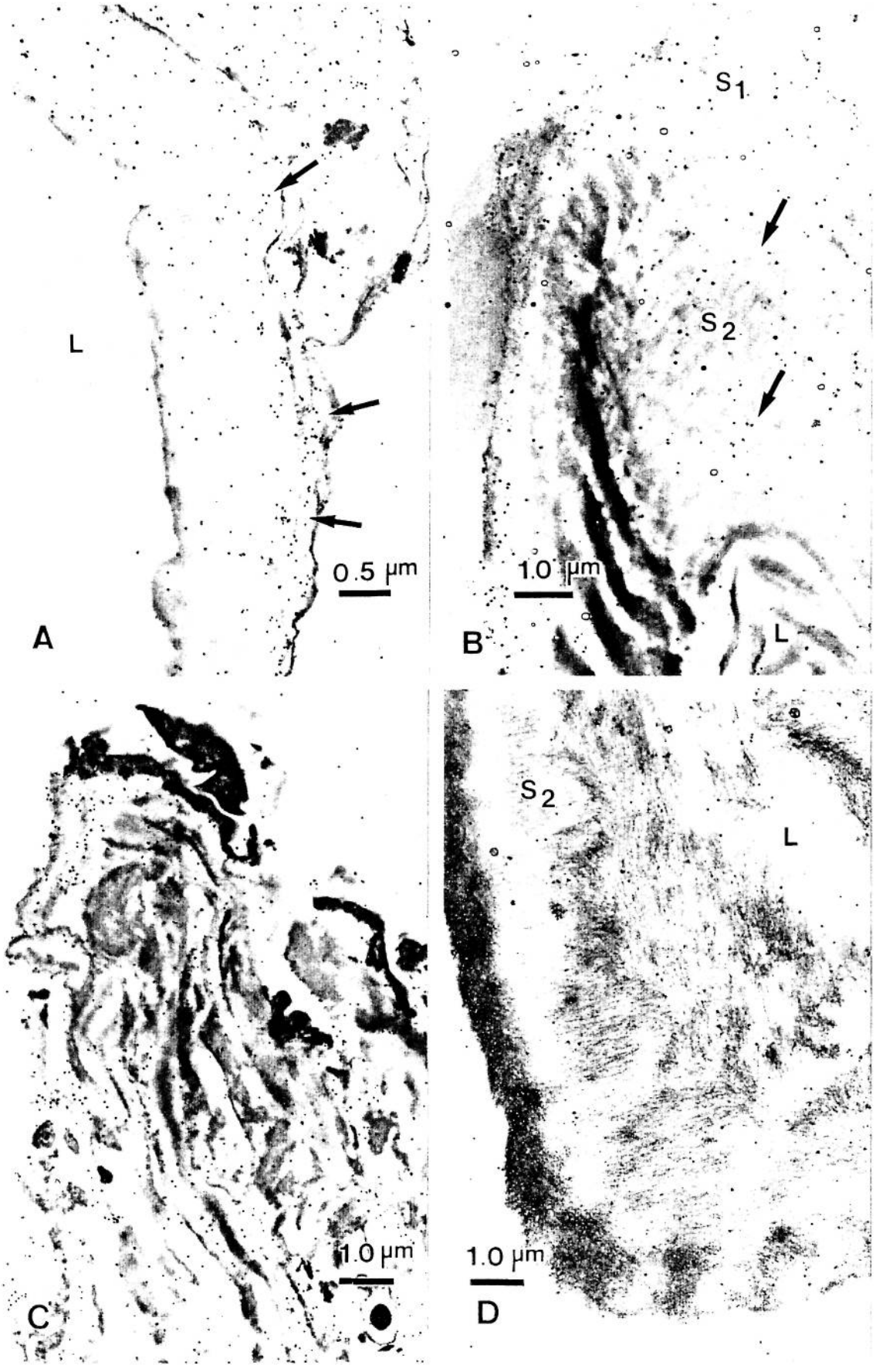
Figure 3. Poplar pulp treated with MnP. A, CMP. Inner part of a fiber showing cellulose microfibrils unmasked and randomly orientated. PATAg staining; B, immunogold labeling of MnP. The enzyme is associated with the fiber undergoing defibrillation.

Figure 4. Effects of laccase on pulps. Immunogold labeling with anti-Lac. A, wheat straw CMP. Loosening of the inner part of S_2 depicting the defibrillation effect of Lac. The enzyme is present in the less opened areas (arrowheads); B, wheat straw R-CMP. Loosening of the whole fiber. Cell corners (Cc) and middle-lamella (ML) have been degraded leaving lignin spherical particles (arrows); C, poplar CMP. The dense gold labeling shows the penetration of Lac into the loosened areas.

Figure 5. Specific immunogold labeling of lignins in wheat straw R-CMP treated with MnP. (A and B): anti-GS(ZT). A, untreated pulp. The labeling is more abundant in S_2 layer; B, MnP treated pulp. No change in the intensity of the gold labeling; (C and D): anti-GS (ZL). C, untreated pulp; D, MnP treated pulp. A strong decrease in the number of gold particles follows the action of the enzyme; (E and F): anti-G(ZL). E, untreated pulp; F, no diminution of the G(ZL) distribution after MnP treatment.

Figure 1.

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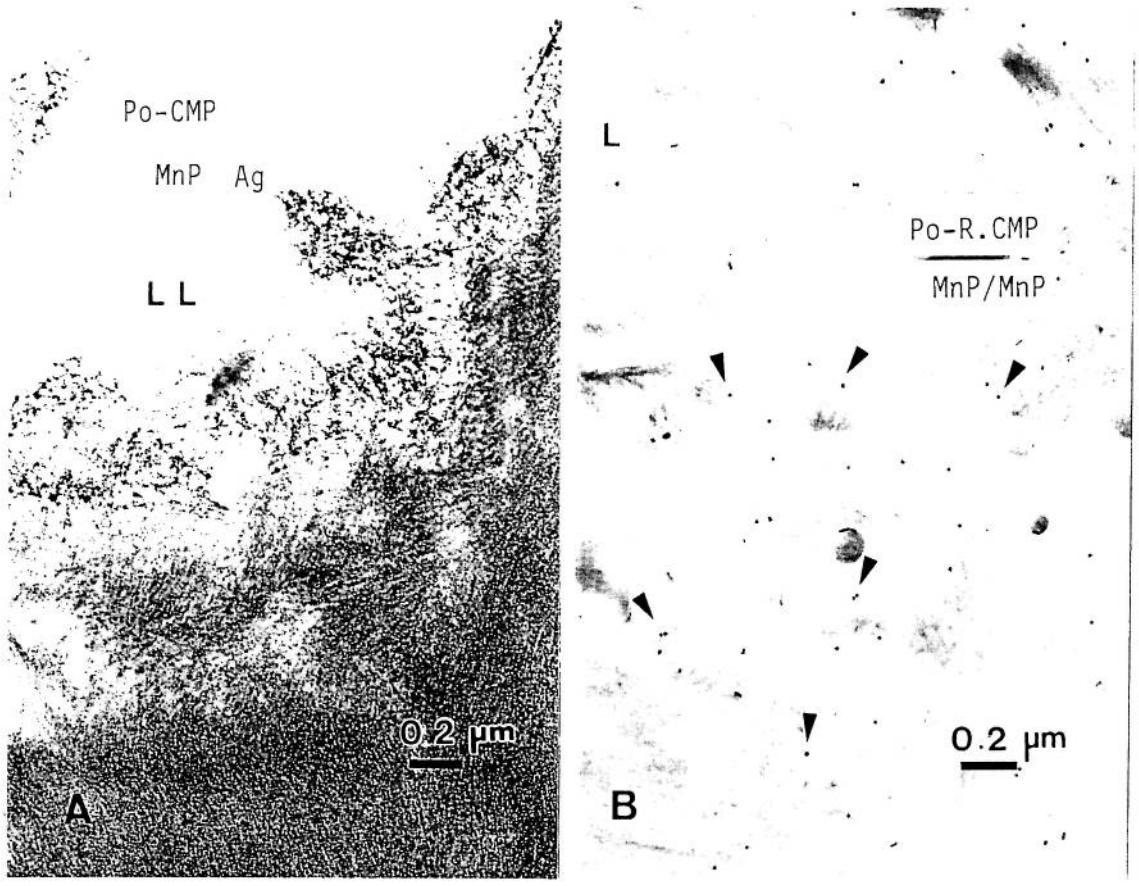
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Po-R.CMP
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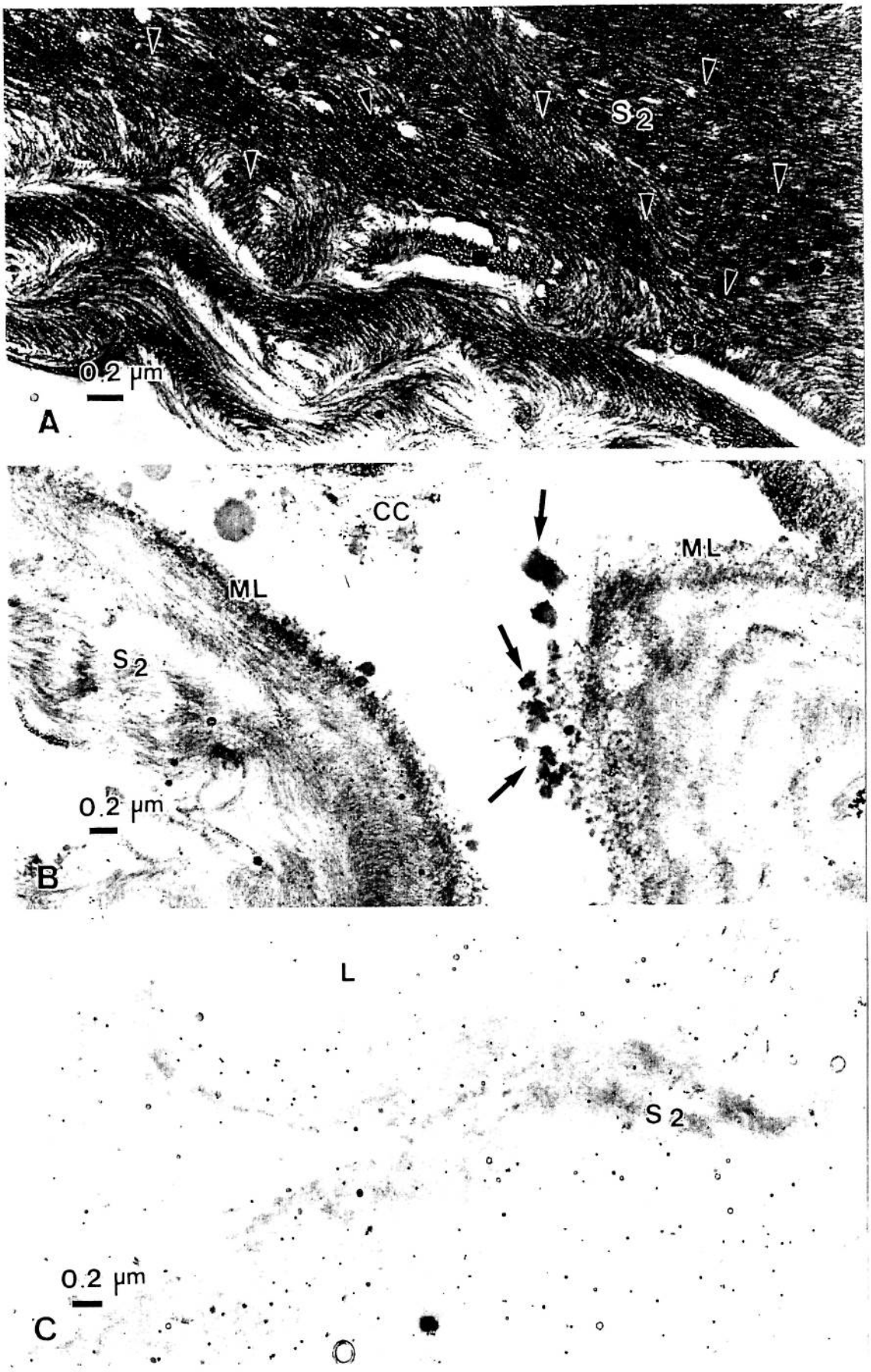
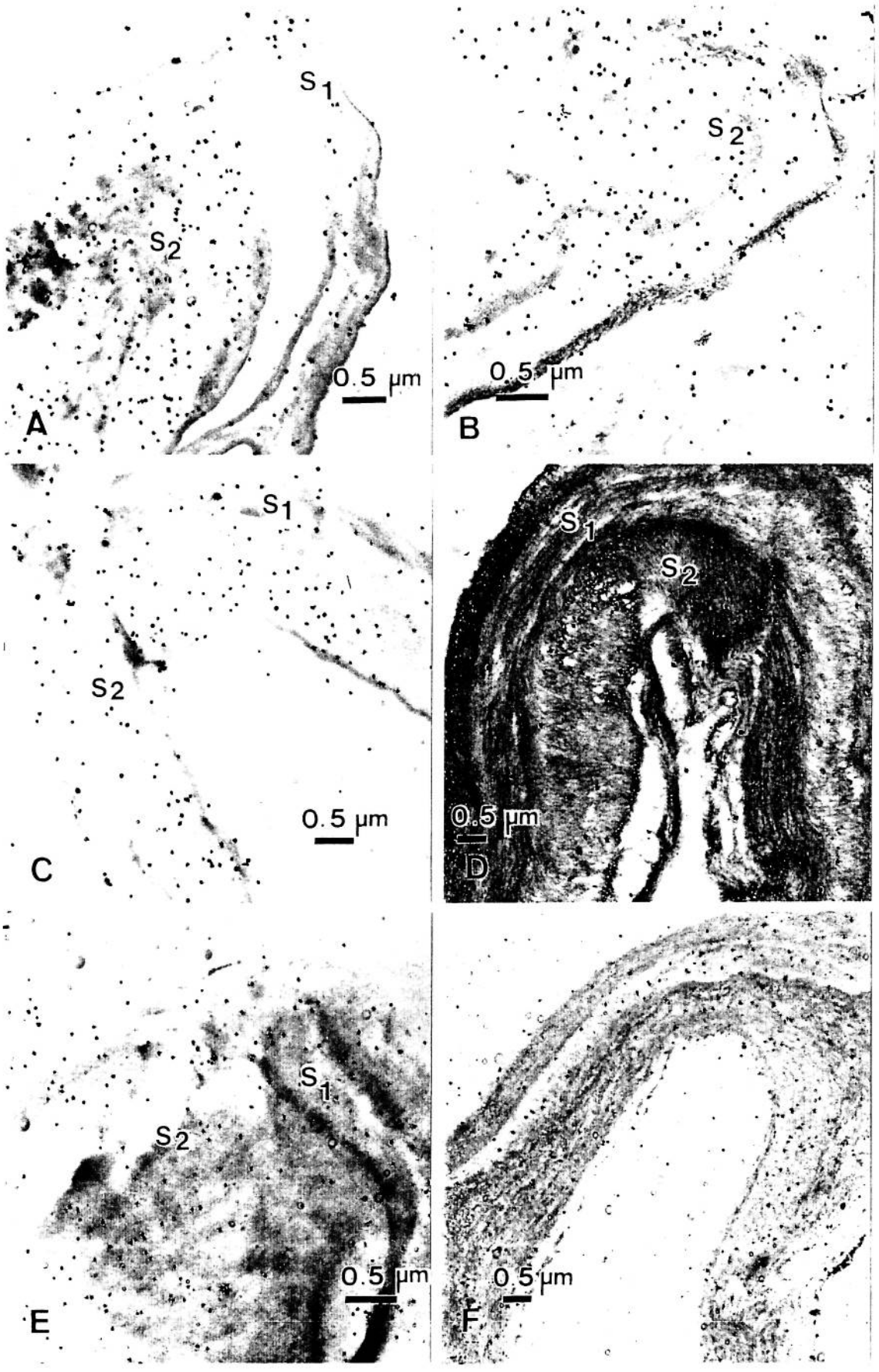
B

Figure 4.




S1
S2
0.5 μm
A
S2
0.5 μm
B
S1
S2
0.5 μm
C
S1
S2
0.5 μm
D
S1
S2
0.5 μm
E
0.5 μm
F