SUBCELLULAR LOCALIZATION OF CELLULASES IN AUXIN-TREATED PEA

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ABSTRACT

Two forms of cellulase, buffer soluble (BS) and buffer insoluble (BI), are induced as a result of auxin treatment of dark-grown pea epicotyls. These two cellulases have been purified to homogeneity. Antibodies raised against the purified cellulases were conjugated with ferritin and were used to localize the two cellulases. Tissue sections were fixed in cold paraformaldehyde-glutaraldehyde and incubated for 1 h in the ferritin conjugates. The sections were washed with continuous shaking for 18 h and subsequently postfixed in osmium tetroxide. Tissue incubated in unconjugated ferritin was used as a control. A major part of BI cellulase is localized at the inner surface of the cell wall in close association with microfibrils. BS cellulase is localized mainly within the distended endoplasmic reticulum. Golgi complex and plasma membrane appear to be completely devoid of any cellulase activity. These observations are consistent with cytochemical localization and biochemical data on the distribution of these two cellulases among various cell and membrane fractions.

Cellulolytic enzymes (β-1,4-glucan, 4-glucanohydrolase, EC 3.2.1.4) occur in specific locations in tissues of higher plants at particular stages of development. Their possible regulatory roles in cell growth and differentiation (1, 7–10, 12, 14–18), and the hormonal control of their induction (5–8, 21), have been emphasized in earlier work. Although cellulases have been purified and partly characterized (5, 6), their actual localization at the cellular level has not been demonstrated. In elucidating the physiological role of these enzymes, it is imperative that their exact subcellular loci be defined. Attempts made to localize cellulase in higher plants (3, 4) and snail (20) have not met the refinements necessary for precise ultrastructural localization.

Auxin-induced growth in etiolated pea epicotyls offers a unique system for localization of cellulases. Two different species of cellulases, one buffer insoluble (BI) and the other buffer soluble (BS), have been purified and characterized from this system (5, 6). These two proteins have proved to be immunologically different. With the use of ferritin-conjugated antibodies to these two cellulases, an immunocytochemical method was developed to localize the enzymes at subcellular levels. The results were supplemented by cytochemical (4) and cell fractionation procedures.

MATERIALS AND METHODS

Plant Material and Hormone Treatment

Seeds of *Pisum sativum* L. var. Alaska were soaked for 20 min in 0.5% sodium hypochlorite. After 8 h of imbibition in tap water, seeds were planted in moistened vermiculite and grown in darkness at room temperature.

When the third internode was more than 1 cm long (7–8 days), intact seedlings were sprayed with 0.1% 2,4-dichlorophenoxyacetic acid (2,4-D), with 0.1% Tween-80 and 0.1 M NaCl (pH adjusted to 7.0) (5, 6).

Preparation of Antibody-Ferritin Conjugates

Buffer-insoluble (BI) and buffer-soluble (BS) cellulases were purified and antisera were raised as described previously (6). The specificities of the antisera to the purified proteins were tested by Ouchterlony double diffusion and immunoelectrophoresis (5, 6, 21). Serum was made to 50% saturation with $(NH_4)_2SO_4$, pH 6.5, and the resulting precipitate was washed twice with 50% $(NH_4)_2SO_4$ and dissolved in 0.1 M borate-phosphate buffer, pH 9.0 (2). Cadmium-free ferritin (Polysciences Inc., Warrington, Pa.) was centrifuged for 2 h at 100,000 *g* and the top 75% of the supernate was discarded. The pellet was resuspended in the remaining solution. It was conjugated by *m*-xylylene diisocyanate in a two-step reaction (2). First, *m*-xylylene diisocyanate was added slowly to ferritin at 4°C and stirred for 45 min. The mixture was centrifuged for 10 min at 500 *g* and the pellet was discarded. The clean brown supernate was then mixed with the gamma globulin fraction of the serum, prepared as above, and stirred for 24 h at 4°C. The mixture was centrifuged for 10 min at 1,000 *g* to remove any denatured proteins and ferritin-ferritin conjugates. The clear supernate was centrifuged at 10,000 *g* for 2 h and the top 75% of the supernate was discarded. The remaining fraction was incubated for 24 h at 4°C and centrifuged once again at 100,000 *g* to remove any unconjugated material. The final pellet was resuspended in phosphate-buffered saline and used for localization of the enzymes.

Preparations for Electron Microscopy

Slices (0.5 mm) of 2,4-D-induced swollen tissue were fixed in a high osmolarity fixative (1,970 mOsmol) containing a mixture of glutaraldehyde and paraformaldehyde in 0.1 M phosphate buffer, pH 7.2 (11), for 1 h at 0°C. The slices were subsequently washed in cold buffer for 18 h with at least 10 changes with large volumes of liquid. For cytochemical localization, slices were placed in an incubation medium containing 0.1 M phosphate buffer, pH 6.0, and 0.02% carboxymethyl-cellulose (CMC) for 10 min at 25°C. Control slices were either heat inactivated by first boiling for 10 min or incubated in only buffer without the substrate. After incubation, slices were transferred to hot Benedict's solution at 80°C for 10 min and washed thoroughly in distilled water. They were treated with 1% osmium tetroxide in phosphate buffer, pH 7.0, and dehydrated in alcohol-propylene oxide.

For immunocytochemical localization, after washing in buffer for 18 h the slices were incubated in ferritin-conjugated antibodies for 1 h at 25°C. Tissue incubated in unconjugated ferritin was used as a control. This was followed by washing in buffer for 18 h at 35°C with continuous shaking. Slices were then treated with osmium tetroxide and embedded in Epon after standard dehydration procedures. Ultrathin sections were made from the cut surface of the tissue slices as well as from deeper layers. Sections were stained with uranyl acetate and lead citrate and viewed with a Zeiss 9S electron microscope.

Fractionation of Subcellular Components

Apical segments (10 mm) of dark-grown pea epicotyls were harvested at zero time or 2 days after 2,4-D treatment and chopped with razor blades (13, 19) in 0.3 vol of extraction medium containing 0.4 M sucrose (ribonuclease free), 100 mM Tris-HCl (pH 8.0 at 2°C), and 5 mM dithiothreitol with or without $MgCl_2$. The slurry was squeezed through nylon cloth, and the filtrate was centrifuged at 500 *g* for 20 min. A sample (2 ml) of the clarified homogenate was layered on 10 ml of a 25%–55% linear sucrose gradient containing either no Mg^{++} or 5 mM Mg^{++} and centrifuged for 2 h at 45,000 *g*. After centrifugation, gradients were fractionated and aliquots removed from each fraction for determining NADH-cytochrome *c* reductase activity. The density of each fraction was determined from its refractive index. The remainder of each fraction was diluted with 20 mM sodium phosphate buffer, pH 6.2, and pelleted at 145,000 *g* for 1 h. Pellets were resuspended in 0.5 ml of 20 mM sodium phosphate buffer, pH 6.2, containing 1 M NaCl and cellulase activity was assayed (5). The amount of each enzyme activity found in the fractions was calculated as a percent of the total activity recovered in the gradient.

RESULTS

Cytochemical Localization

The distribution of reducing groups, as visualized by the presence of cuprous oxide precipitate, is shown in Fig. 1 (*a* and *b*). With this procedure, fine structure is not well preserved because of the drastic treatments, but most of the organelles are recognizable. The cuprous oxide precipitate is clearly concentrated between the plasma membrane and the cell wall (Fig. 1 *a*). Some reaction can be observed in the endoplasmic reticulum and smooth vesicles. Controls which had not been preincubated with substrate (CMC) also revealed some reaction product, presumably due to the large quantities of endogenous substrate. However, in heat-inactivated samples no reaction product could be found, indicating that the reaction is enzyme dependent. Whether these reaction products are entirely due to cellulases or some other hydrolases is not clear. However, in tissues treated

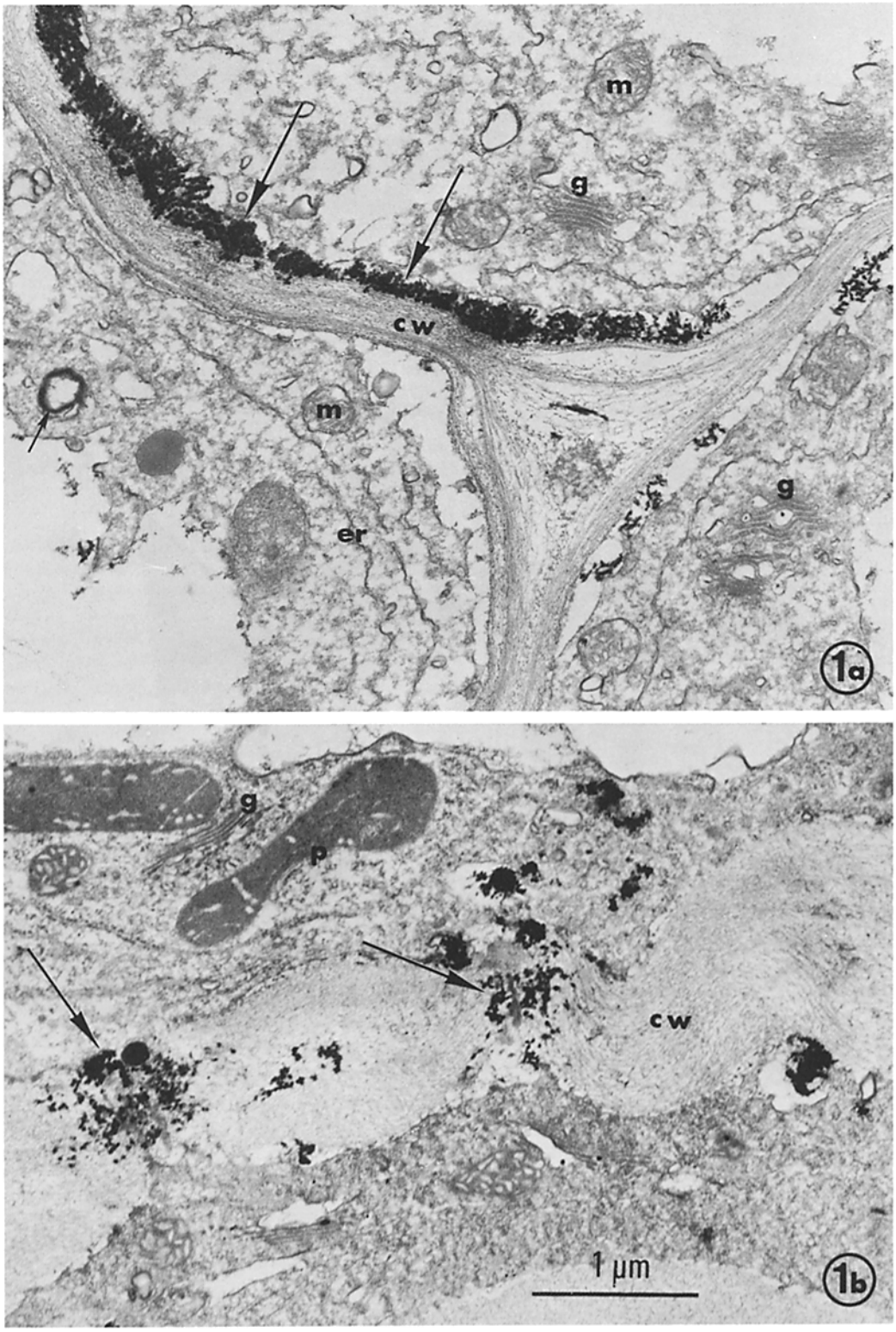

FIGURE 1 Localization of cellulolytic activity in pea epicotyl sections by formation of cuprous oxide using carboxymethyl-cellulose as a substrate. Arrows indicate reaction products; *cw*, cell wall; *g*, Golgi; *m*, mitochondria; *er*, endoplasmic reticulum; *p*, proplastid. Epicotyls were treated with 2,4-D for 2 days (*a*) or 4 days (*b*). × 25,000.

with auxin for a longer period (4 days), wall degradation is evident (Fig. 1 *b*), and cuprous oxide precipitation is particularly intense at these loci.

Immunocytochemical Localization

Since ferritin-antibody conjugates do not penetrate intact cells, localization was only possible in cells on the cut surface of the tissue. The ultrastructural organization of many of the cut cells was impaired, especially in terms of preservation of the cytoplasmic matrix.

Fig. 2 shows ferritin-antibody conjugates of BI cellulase which are primarily located in close proximity to cell-wall fibrils along the inner surface of the cell wall. Some ferritin particles are also seen attached to the endoplasmic reticulum (Fig. 2 *a*). In preparations in which the plasma membrane is pulled away from the cell wall, the conjugates are clearly attached to the wall fibrils (Fig. 3). At higher magnification (Fig. 3 *a*), ferritin can be seen to be intimately associated with microfibrils in clusters or along the fibrils. Profiles of microtubules are often seen close to the plasma membrane, but no correlation could be traced between their presence and the ferritin sites.

Fig. 4 shows ferritin-antibody conjugates of BS cellulase which are found mainly in swollen regions of the endoplasmic reticulum. Cell-wall surface, the Golgi complex, and other organelles are devoid of any BS-ferritin conjugates. Slices treated with unconjugated ferritin alone (controls) showed no trace of ferritin at any loci in the cell. Since most of the cellulase is synthesized *de novo* (21), no ferritin was found in tissue not treated with 2,4-D.

The results of both cytochemical and immunocytochemical observations suggest that auxin-induced cellulolytic activities are confined to the inner surface of the cell wall and to endoplasmic reticulum vesicles. The two cellulases, BI and BS, appear to be responsible for these two distinct loci of activities, respectively.

Localization of Cellulases in Subcellular Fractions

Apical segments of auxin-treated pea epicotyls were homogenized and fractionated into wall, total particulate, and supernate fractions and analyzed for the presence of the two cellulases. Table I shows that the two enzymes were differentially distributed between these fractions. BS was concentrated in the soluble phase, while BI was primarily bound to the cell-wall material.

Fig. 5 shows the distribution of cellulase activity in the particulate fraction after isopycnic centrifugation in linear sucrose gradients. In the absence of added $MgCl_2$, ribosomes dissociate from the ER, and all ER and marker enzymes are found in a single locus at a density of approximately 1.11 (19). Cellulase activity is found in this region of the gradient. However, in the presence of added $MgCl_2$, the integrity of the rough ER is maintained and it separates into smooth ER (remaining at density 1.11) and rough ER, which sediments at a new density of 1.18, (19). The location of cellulase activity does not alter under these conditions, indicating that it is confined to a region containing smooth ER vesicles.

DISCUSSION

The different methods used for subcellular localization of cellulase activities in 2,4-D-treated pea epicotyls are in agreement. The cytochemical technique, using copper reduction as an indicator of cellulolytic activity, is less specific since the presence of any hydrolase which releases reducing power would result in such reaction product. Most such hydrolytic activity was clearly associated with the inner surface of the cell wall. Reaction products were particularly concentrated in areas where obvious cell-wall degradation occurs (Fig. 1 *b*).

The use of ferritin-antibody conjugates for enzymic localization has not been reported with plant tissues. Frozen sections are difficult to obtain with plant tissues, particularly those such as auxin-treated pea epicotyl which contains very large vacuoles. Therefore, thin, freehand slices were cut and immediately fixed in aldehyde fixative. Ferritin conjugates reacted only with cut cells on the surfaces of the slices since ferritin does not penetrate cell walls.

The BI cellulase is clearly localized on the cell-wall fibrils on the inner surface of the cell wall, with a small amount associated with the endoplasmic reticulum (Figs. 2 and 3). The BS cellulase, in contrast, was entirely localized in the distended parts of the endoplasmic reticulum (Fig. 4). These regions of the ER vesicles have few ribosomes attached to them ("smooth ER"). It is possible that the cellulase close to the ER is nascent protein or recently formed protein being transported to the cell wall. We have recently observed (21) that BS cellulase is specifically synthesized by membrane-bound polysomes. How enzyme transport takes

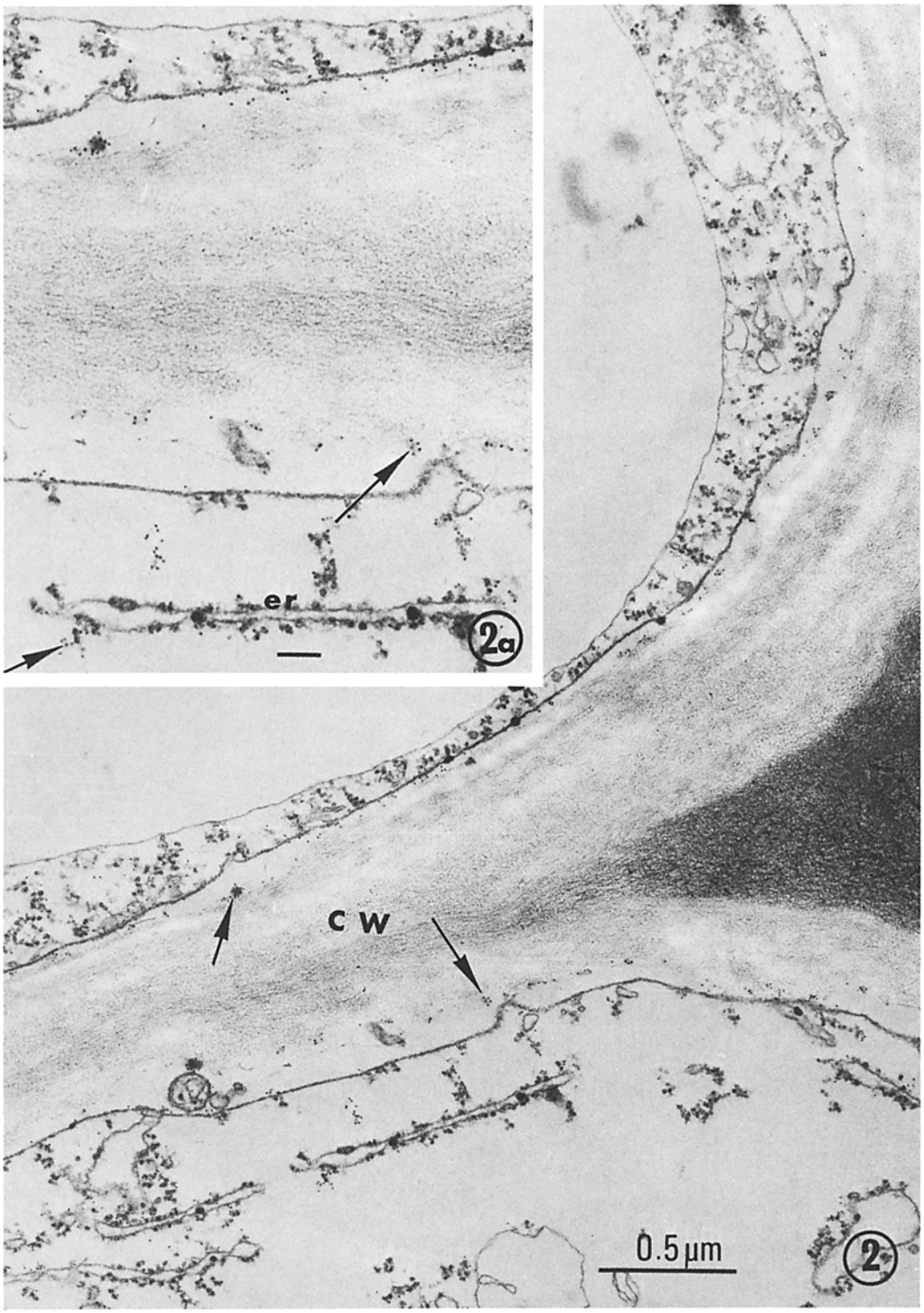

FIGURE 2 Immunocytochemical localization of buffer-insoluble (BI) cellulase, using ferritin-conjugated antibodies. Ferritin (arrows) is associated with the surface of the cell wall (*cw*) and the endoplasmic reticulum (*er*). × 41,000. The inset (2*a*) is a magnified view. Bar = 0.1 nm. × 65,600.

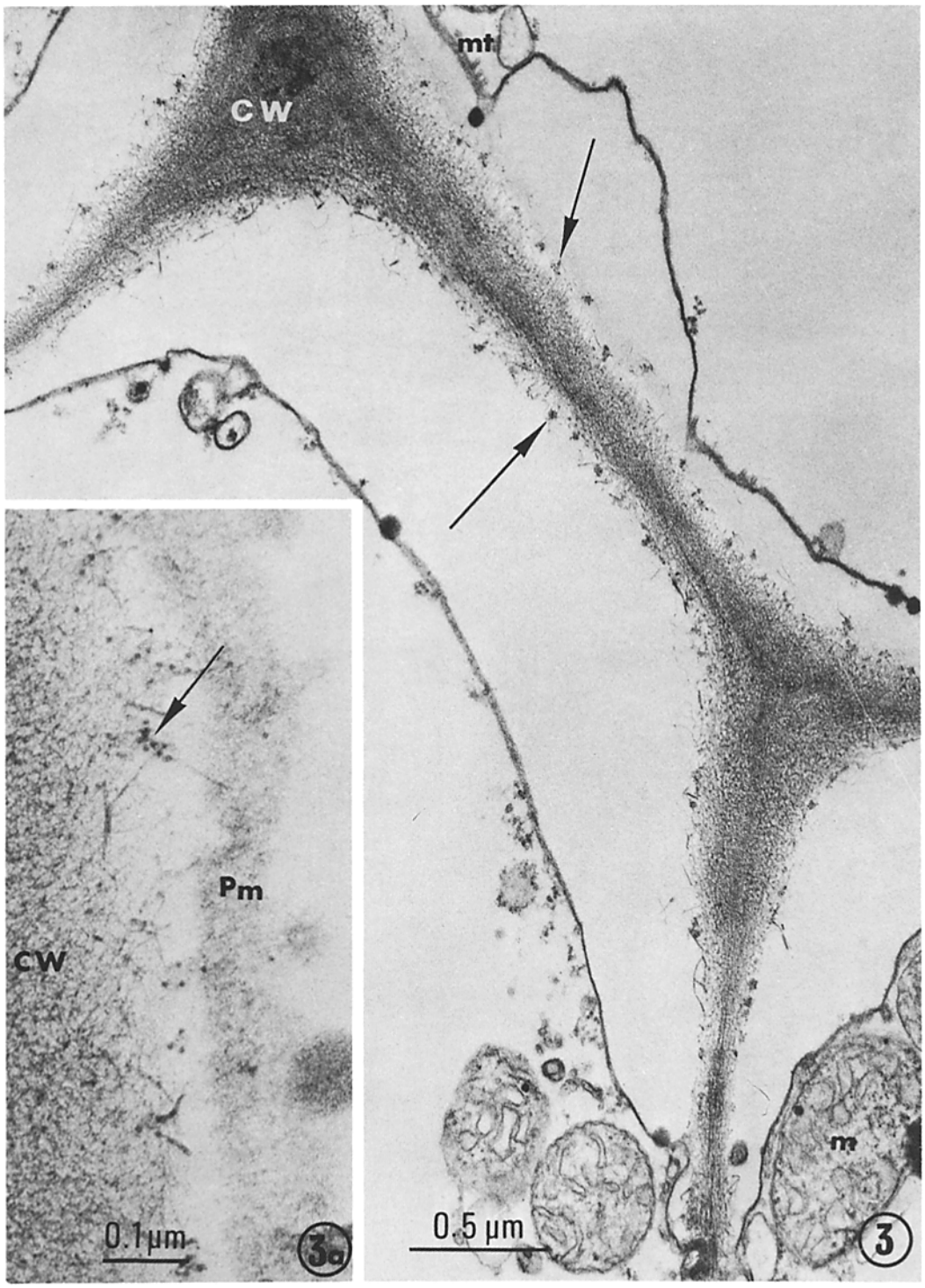

FIGURE 3 Immunocytochemical localization of buffer-insoluble (BI) cellulase, using ferritin-conjugated antibodies. Ferritin is associated with the inner surface of the cell wall (*cw*), not with the plasma membrane. Microtubules (*mt*) can be seen close to the plasma membrane. × 41,000. Inset (3 *a*) shows a magnified view of a tangential section through the cell wall and plasma membrane (*Pm*). × 135,000.

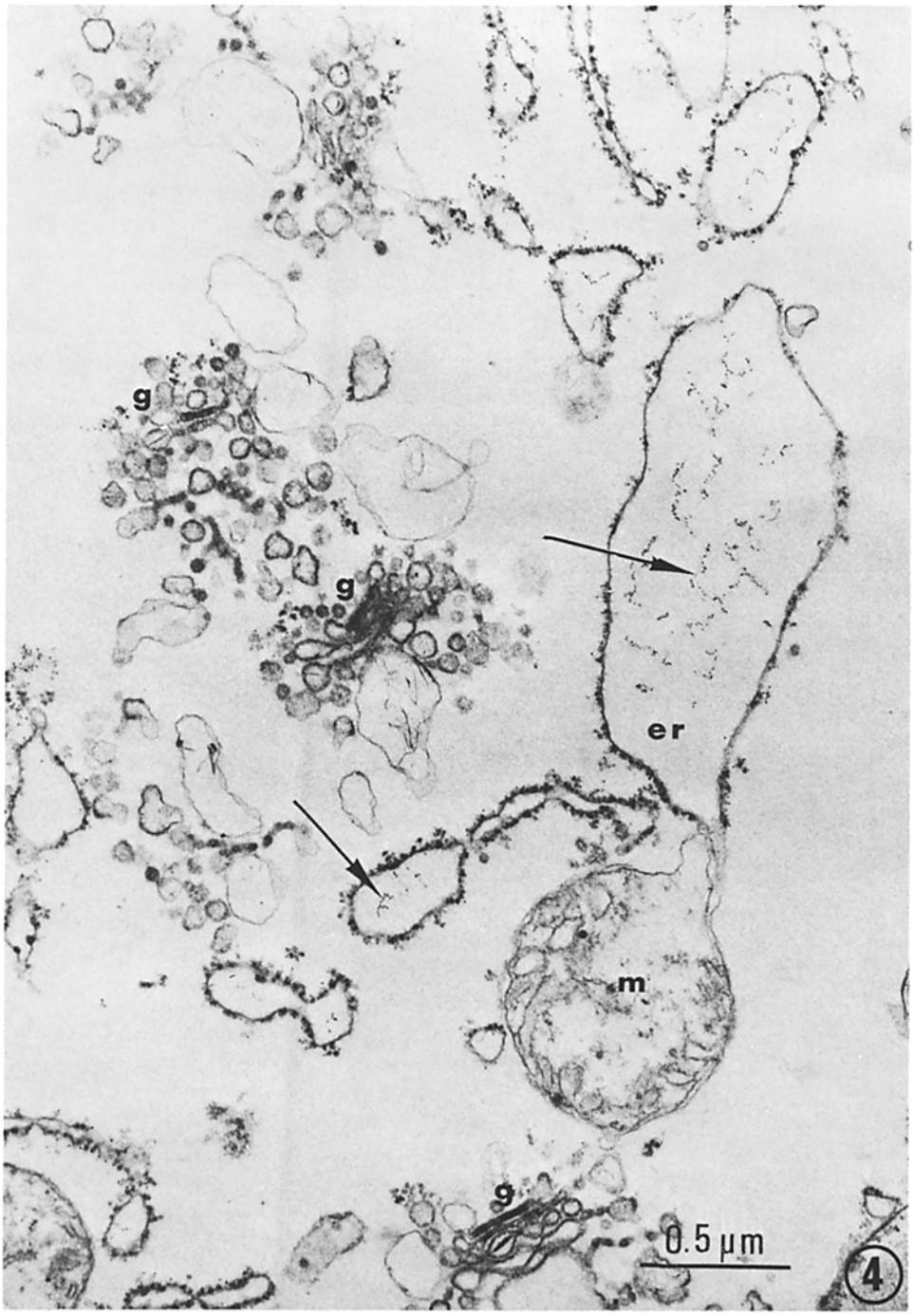

FIGURE 4 Immunocytochemical localization of buffer-insoluble (BS) cellulase using ferritin-conjugated antibodies. Ferritin (arrows) is present mainly within and around distended endoplasmic reticulum (*er*) configurations. × 41,000.

TABLE I

Distribution of Cellulase Activities in Various Cell Fractions

Fraction	Protein/ segment	Cellulase units	
		BS	BI
	μg		
Supernate	560	93	0
Particulate (500–100,000 *g*)	222	4	23
Cell wall	110	20	112

Auxin-treated tissue was homogenized in 0.02 M phosphate buffer, pH 6.2, and centrifuged for 10 min at 500 *g* to remove the cell-wall fragments. The supernate was further centrifuged for 1 h at 100,000 *g*. The particulate material and 500 *g* pellet were washed once with buffer and extracted with 0.02 M phosphate, pH 6.2, 1 M NaCl. All fractions were assayed for buffer-soluble (BS) and buffer-insoluble (BI) cellulase activities (6). The presence of NaCl in extraction of particulate and cell wall fractions does not interfere with assay.

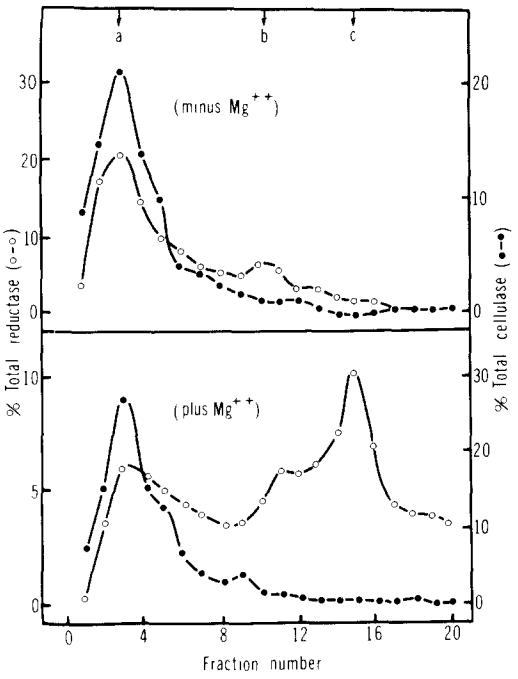

FIGURE 5 The distribution of cellulase and NADH-cytochrome *c* reductase (er marker) activities on isopycnic sucrose density gradients. Centrifugation of particulate fractions from 2,4-D-treated pea epicotyl (see Materials and Methods) was carried out with or without $MgCl_2$ (5 mM) added to the gradient in order to distinguish between smooth and rough endoplasmic reticulum. *a* = density 1.11; *b* = density 1.15; *c* = density 1.18. Golgi complex sediments at a density of 1.15 (19).

place to the cell surface is not clear, but no involvement of the Golgi complexes or microtubules could be detected in this study.

Biochemical assays of the distribution of the two cellulases among cell fractions are consistent with the cytological observations (Table I), i.e. the BI cellulase is primarily associated with wall fraction while BS cellulase is in the cytoplasm. Isopycnic sucrose density gradient profiles of cellulase (Fig. 5) also confirm that most particle-bound cellulase activity is confined to smooth vesicles coinciding with the position of the smooth ER. Other organelles, e.g., Golgi complex and plasma membrane, are present at completely different loci in such gradients (19) and do not contain appreciable cellulase activity.

These observations support the general contention that cellulases have a functional role in cell-wall metabolism in growing cells, with their biosynthesis being under hormonal control.

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