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Subcellular Fractionation of a Hypercellulolytic Mutant, *Trichoderma reesei* Rut-C30: Localization of Endoglucanase in Microsomal Fraction

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The growing mycelia of *Trichoderma reesei* Rut-C30 are richly endowed with endoplasmic reticula and a variety of pleomorphic subcellular bodies. Mycelia of the culture growing in presence of avicel pH101 was fractionated in sucrose density gradients, and several morphologically and biochemically distinct fractions were isolated. Mycelia were homogenized in a Bead Beater, and the homogenate was freed of nucleus and wall fragments by low-speed centrifugation before fractionation. Organelle-free cytosol, which did not penetrate the gradient, contained (of the total) 72% of the vanadate-sensitive ATPase, 26% of carboxymethyl cellulase (CMCase), 2% of cytochrome *c* reductase, and 13% of the protein. Significant fractions separated on a gradient were light vesicles containing heavily stained material inside and ribosomes attached to the outside surface, intact vesicles resembling condensing vacuoles, large vesicles derived from the plasma membrane, and heavy vesicles containing crystalline material. The light-vesicle fraction contained a large portion of the cell-bound CMCase activity. The particle-bound ATPase and cytochrome *c* reductase activities were concentrated in heavy fractions. The fractionation in the presence of $MgCl_2$ improved the preservation of subcellular bodies derived from the endoplasmic reticula. Although the CMCase activity of the light-vesicle fraction was 4 times higher than the activity in the heavy-vesicle fraction, the CMCase antibody-binding capacities of both fractions were about the same. This discrepancy between the catalytic activity and the antibody-binding capacity suggests that the heavy vesicles might have contained considerable amount of inactive CMCase compared with that present in the light vesicles.

Secretion of protein molecules is a general term used to denote a chain of subcellular activities that begins at the site of synthesis and culminates in passage of the molecule to the cell exterior. Secretion requires a series of membrane-bound organelles (the endomembrane system), which function in the synthesis, packaging, and externalization of the secreted materials (24). The endomembrane system is well characterized in specialized secretory cells of higher eucaryotes (21, 24), but secretory processes in nonspecialized cells such as microorganisms are relatively unexplored and difficult to study, since they account for only a minor portion of the cells' activity. It is possible that the complex array of secretory organelles found in the higher organisms may not have evolved or the physiological regulation of secretory activity may be coupled to the repression-derepression of the secretory organelle formation in microorganisms (e.g., filamentous fungi). The study of a mutant showing enhanced secretion may provide significant information on the interrelationship between the secretory organelle and the enhancement of a secretory enzyme.

The wild-type fungus *Trichoderma reesei* QM6a secretes only a small amount of cellulase, and this cellulase activity declines at late stages of growth; however, the mutant Rut-C30, when grown on crystalline cellulose (avicel pH101), synthesizes and secretes a significantly higher amount of cellulase than the wild-type cells. Cellulase is a complex of three enzymes: endoglucanase or carboxymethyl cellulase (CMCase), cellobiohydrolase, and cellobiase (9). In Rut-C30, the enhancement of CMCase is much higher than that of the net cellulolytic (disk activity) or cellobiase activity (9). The proportional decrease or increase of cellobiohydrolase has not been determined because of the lack of a specific assay based on the catalytic activity of the enzyme.

Studies on the ultrastructure of these cells demonstrate an enhancement of rough endoplasmic reticulum (rER) during the time of maximal cell-bound endoglucanase activity and the production of a variety of pleomorphic bodies from the rER. In addition, there are a large number of vesicles, many of which resemble the condensing vacuoles of animal cells (9). These cells lack the characteristic stacked appearance of Golgi bodies and contain instead many individual endoplasmic reticulum (ER)-associated saccules typical of some fungi (8, 9, 13, 14). Golgi bodies play a significant role in the packaging, processing, and distribution of secretory material within a cell (21, 24). Hence, the absence of typical Golgi bodies in *T. reesei* may indicate the presence of either morphologically uncharacteristic but functional Golgi bodies or a Golgi-independent secretory pathway which may remain repressed in the wild type QM6a, but becomes functional in the mutant Rut-C30 by a specific deregulation of the secretory activity.

The following facts suggest that the mutant fulfills the criteria necessary to study the secretory pathway using subcellular fractionation. (i) Rut-C30 synthesizes substantial amounts of a secretory protein. (ii) There is no autolytic degradation of subcellular organelles during the phase of high rate of synthesis. (iii) The rER is extensive and is differentiated into a large number of presumptive secretion-related organelles (A. Ghosh, M. Glenn, and B. K. Ghosh, Abstr. Am. Soc. Cell Biol. **97**:305a, 1983). (iv) A recently described method (2) can be used to rupture Rut-C30

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mycelia and release subcellular organelles. (v) Several marker enzymes that characterize the membrane fractions in *Neurospora crassa* can be used to characterize *T. reesei* organelles (2, 9).

In this paper we report a method of fractionation of microsome and a variety of vesicular bodies from *T. reesei* Rut-C30 mycelia. The microsome (i.e., light-vesicle) fraction contained a significant proportion of the cell-bound CMCase activity; the heavy-vesicle fraction, which contained amorphous or crystalline electron dense material, showed low CMCase activity. However, anti-CMCase antibody binding, determined by direct radioimmunoassay, was high in the heavy-vesicle fraction. In contrast to the cytochrome *c* reductase, the majority of the ATPase was concentrated in the organelle-free cytosol. However, the residual particle-bound ATPase was concentrated in the heavy-vesicle fraction.

MATERIALS AND METHODS

Cultures of *T. reesei*. Spores of *T. reesei* Rut-C30 (6) were inoculated into 500 ml of Vogel (27) medium containing 1% avicel pH101 (FMC Corp, Marcus Hook, Pa.), 1% lactose, and 0.1% proteose peptone in 2-liter conical flasks and incubated on a rotary shaker (250 rpm; New Brunswick Scientific Co., Inc.) at 28°C for 2 to 3 days. The mycelia were harvested by centrifugation at 16,000 × *g* for 20 min and washed by filtration on a coarse sintered-glass filter with 0.01 M Tris hydrochloride (pH 7.0)–0.15 M NaCl.

Cell breakage and fractionation. The washed mycelial mat was used for fractionation by the methods used by Borgeson and Bowman (2) and Bowman and Slayman (3) to isolate ER and plasma membrane from *N. crassa*. The mycelial mat was dispersed by mixing with an equal volume of glass beads (diameter 0.5 mm) and 0.01 M Tris hydrochloride–NaCl buffer (pH 7.0) with or without $MgCl_2$ (25), and homogenized for 5 min in a 60-ml chamber (Bead Beater, Biospec Products). The homogenate was centrifuged at 5,000 × g for 10 min to remove the beads, nuclei and mitochondria, and 7 ml of the supernatant was layered on a 30-ml linear sucrose gradient of 14 to 42% (wt/wt). The gradients were centrifuged for 2 h at 80,000 × *g* at 4°C and fractionated from the top into 13 fractions. The lightest fraction (8.1 ml) contained material which did not penetrate the gradient. The next 11 fractions were 2.7 ml each, and the final fraction was the pellet. One portion of each fraction was fixed for electron microscopy in a mixture of 1% tannic acid and 1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 18 h at 0°C. The washed, prefixed material was enrobed in 2% Noble agar, further fixed overnight (12 to 14 h) in 1% OsO_4, and finally treated with 0.5% uranyl acetate, dehydrated, and embedded in Spurr low-viscosity resin (10). The other portion was washed to remove sucrose by centrifugation and resuspended in 0.01 M Tris hydrochloride–NaCl buffer for biochemical assays. Fractions were sonicated for 5 s to rupture vesicles before sampling for biochemical assays. The lightest fraction was washed in an Amicon apparatus with a PM10 filter to retain the soluble proteins.

Biochemical assays. The protein content of each fraction was precipitated with 26% trichloroacetic acid, and the protein was determined by the Lowry et al. method (17). Endoglucanase was determined by reducing sugar released from carboxymethyl cellulose (Hercules Powder Co., Wilmington, Del.) (18). One unit of CMCase was defined as 1 μmol of glucose produced per min. Vanadate-sensitive ATPase (Mg dependent) was measured by P_i release. One unit was equivalent to 1 μg of P_i released per h; this was used as a plasma membrane marker (3, 4). The ATPase assay solution of 0.5 ml contained 50 μmol of Tris hydrochloride (pH 7.2) with 15 μmol of $MgCl_2$; 15 mM potassium fluoride was added to inhibit acid phosphatase, and sodium azide was added to inhibit mitochondrial ATPase (11, 19). The substrate ATP (1.38 mg) was added, and tubes were incubated for 15 min at 37°C. The reaction was stopped with 0.1 ml 50% trichloroacetic acid, tubes were centrifuged, and 0.2 to 0.3 ml of supernatant was removed and assayed for P_i by ascorbate reduction of phosphomolybdic acid (5). The cytochrome *c* reductase (NADH dependent), a putative marker for the endoplasmic reticulum, was measured spectrophotometrically (26); 1 U was 1 pmol of cytochrome *c* reduced per min.

Radiommunoassay. (i) Iodination. CMCase was purified, and a polyclonal antibody against this pure enzyme was prepared in rabbit. The immunoglobulin G (IgG) fraction was purified from the sera by ion-exchange chromatography (A. Ghosh and B. K. Ghosh, unpublished work). The goat anti-rabbit IgG was obtained from Cappel Laboratories Inc. (Cochranville, Pa.). ^{125}Iodine (carrier free as sodium iodide; specific activity, 400 mCi/ml) was used for iodination of the goat anti-rabbit IgG. The iodination was done in presence of cloramine T, and the reaction was stopped by the addition of sodium metabisulfite (12, 16). The iodinated goat IgG was purified from free iodine by Sephadex-G75 column chromatography. This column was equilibrated with 0.01 M borate buffer (pH 8.6) at room temperature, coated with 1 mg of bovine serum albumin per ml, and finally washed with borate buffer. The first radioactive peak containing iodinated IgG was collected for labeling the subcellular fractions.

(ii) Labeling. To compare the CMCase antigen contents, two subcellular fractions were iodinated: the light-vesicle fraction (microsome rich) and the heavy-vesicle fraction, frequently containing crystalline material (see Results). These two fractions were precipitated with antibody against CMCase for 24 h at 4°C; the precipitates were collected and washed with saline by centrifugation at 4°C. The saline suspensions of the precipitates, in 200-μl volumes, were iodinated with 100-μl volumes of the mixtures of radioactive and cold goat IgG. The amount of the cold IgG needed to prepare the above mixture was initially calculated from the CMCase (catalytic) activities of the fractions. However, differing concentrations of the cold goat IgG were used to determine the amount needed for maximum precipitation from cell fractions. The reaction was continued overnight at 4°C, followed by a 2-h mixing with 200 μl of 12% polyethylene glycol. Finally, the precipitates were washed with 0.1% gelatin in 0.05 M phosphate-buffered saline (pH 7.6). The radioactivities of iodinated samples were counted in a gamma counter for 10 min.

RESULTS

Low-speed centrifugation of the Bead Beater homogenate removed avicel, glass beads, cell walls, and partially fragmented mycelia. The supernatant, which will be called the cytoplasmic homogenate, was separated into 13 fractions: the organelle-free cytosol, which remained at a density less than 1.07 g/ml; 11 fractions with densitites of 1.07 to 1.19 g/ml; and a pellet containing material denser than 1.19 g/ml. The presence of Mg salt during cell homogenization and fractionation influenced the morphology and the distribution of enzyme activities of the fractions, and the results with and without Mg salt are presented separately.

Biochemical characteristics of the fractions. Protein con-

tents and enzyme activities of the subcellular fractions are presented in Fig. 1A (with $MgCl_2$) and 1B (without $MgCl_2$). Thirteen percent of the protein of the homogenate, prepared in absence of magnesium salt, did not penetrate the gradient. This protein, which is unassociated with any particulate material, is referred to as soluble protein. This soluble protein accounted for 72% of the ATPase activity but only 2% of the cytochrome *c* reductase activity, indicating that the latter was almost entirely particle bound. The majority of the activities of the particle-bound proteins, ATPase and cytochrome *c* reductase, migrated into the high-density regions of the gradient. The density range of 1.14 to 1.19 g/ml contained 84% of particle-bound ATPase, 79% of cytochrome *c* reductase, and 79% of the particulate protein. About 75% of the CMCase activity layered atop the gradient penetrated it, but unlike the marker enzymes, particle-bound CMCase activity was highest in the fractions of density 1.09 to 1.12 g/ml. Specific activities of the three proteins showed distinct peaks, i.e., CMCase 1.10 to 1.11 g/ml; cytochrome *c* reductase at 1.12 to 1.15 g/ml; ATPase at 1.16 to 1.18 g/ml. When the fractionation was performed with 3 mM $MgCl_2$, the majority of protein and marker enzyme activities sedimented at densities similar to that without $MgCl_2$. However, the CMCase activity was distributed evenly from fractions with densities of 1.12 to 1.18 g/ml and showed a peak at a density range of 1.12 to 1.14 g/ml, which is slightly denser than the fraction without $MgCl_2$, which peaked at 1.10 g/ml.

Ultrastructural morphology of fractions. The electron micrograph of a thin section of a secreting hypha is shown in Fig. 2A. This micrograph shows many rER profiles; the cisternae of these rER contain nongranular material, and the terminal regions of these cisternae are swollen (arrows). There are many vacuoles containing lightly stained material; these are generally large. In addition, small uniform-sized condensing vacuoles are present. These are delimited by bilamellar membranes and their staining densities are high but show variation. Small size, fine granularity, and the absence of any internal membrane are distinctive features of condensing vacuoles relative to the other vacuoles. Hyphae in the Bead Beater were ruptured by shear forces and by the impact of the mycelial surface with the glass beads. The suspension of ruptured hyphae contained a heterogeneous mixture of subcellular materials consisting of fragmented and reorganized ER, mitochondria, secretion granules, condensing vacuoles, vacuolar membrane, wall fragments, plasma membrane, and nuclei. Intact nuclei and large wall fragments were sedimented by centrifugation at 5,000 × *g* for 10 min. The supernatant from this low-speed centrifugation contained membranes derived from ER, plasma membrane, vacuole; in addition, there were secretion granules and condensing vacuoles that were largely intact. This supernatant was loaded onto the sucrose gradients. When the fractionation was performed in the presence of $MgCl_2$, subcellular structures with comparable morphology were found at slightly higher density compared with their location in fractions without $MgCl_2$. The structure of the fractions in the presence of $MgCl_2$ is described below.

The lightest particulate fraction (density of 1.09 to 1.10 g/ml) contained free ribosomes and a small number of ribosome-studded vesicles (Fig. 2B and C). This fraction could not be demonstrated in the absence of $MgCl_2$. The next fraction (density of 1.11 to 1.12 g/ml) consisted entirely of free ribosomes (Fig. 2D). A density fraction at 1.13 to 1.14 g/ml contained two types of vesicles: some were ribosome studded and empty, and some lacked ribosomes but contained nongranular electron-dense material (Fig. 2E). The

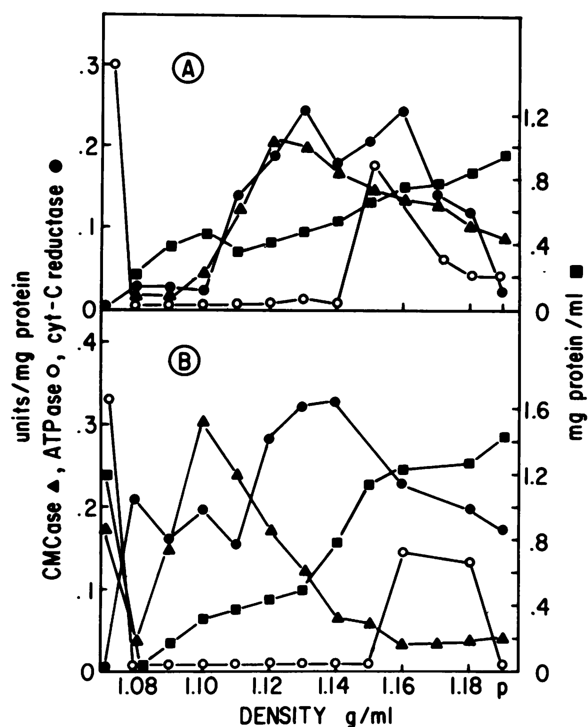

FIG. 1. Profiles of total trichloroacetic acid-precipitable protein and specific activities of CMCase, ATPase (vanadate sensitive, Mg dependent), and cytochrome *c* reductase (NADH dependent and sodium azide insensitive) determined in fractions of sucrose gradient prepared in the presence of 3 mM $MgCl_2$ (A) and in the absence of $MgCl_2$ (B).

morphology of this fraction was comparable to the morphology of fractions in the absence of $MgCl_2$ at densities of 1.10 to 1.11 g/ml (Fig. 3A and B). This fraction contained the highest specific activity of cell-bound CMCase. At a density 1.14 g/ml, multivesicular bodies were seen. Plasma-membrane derived vesicles with a variety of sizes were seen at densities higher than 1.14 g/ml; many of these vesicles showed ribosomes enclosed within the vesicular space (Fig. 3C). These enclosed ribosomes were not membrane bound and may have been free ribosomes trapped by vesiculation of the plasma membrane. The densest layer (1.18 g/ml and pellet) also contained vesicles similar in size to the large vesicles found in the lighter fractions (Fig. 3D). These vesicles, however, contained a crystalline substance with a 5-nm repeat (Fig. 3E).

Distribution of CMCase catalytic and immunological activity. The data presented above show that the cell-bound CMCase activity was concentrated in a light subcellular fraction which was largely populated with vesicles having ribosomes attached to the outside surface of the membrane. The heavy-vesicle fraction, in contrast, had a low CMCase activity and contained a highly electron dense material which was frequently crystalline. A direct radioimmunoassay for the CMCase content was performed with both the light-vesicle fraction and the heavy-vesicle fraction. The goal of this experiment was to compare the distributions of the catalytic activity and the antibody-binding capacity of CMCase in the above two fractions. The catalytic activity of the light-vesicle fraction was 4.4 times higher than that of the heavy-vesicle fraction (Table 1). The CMCase antibody binding of the light-vesicle fraction on the other hand, was

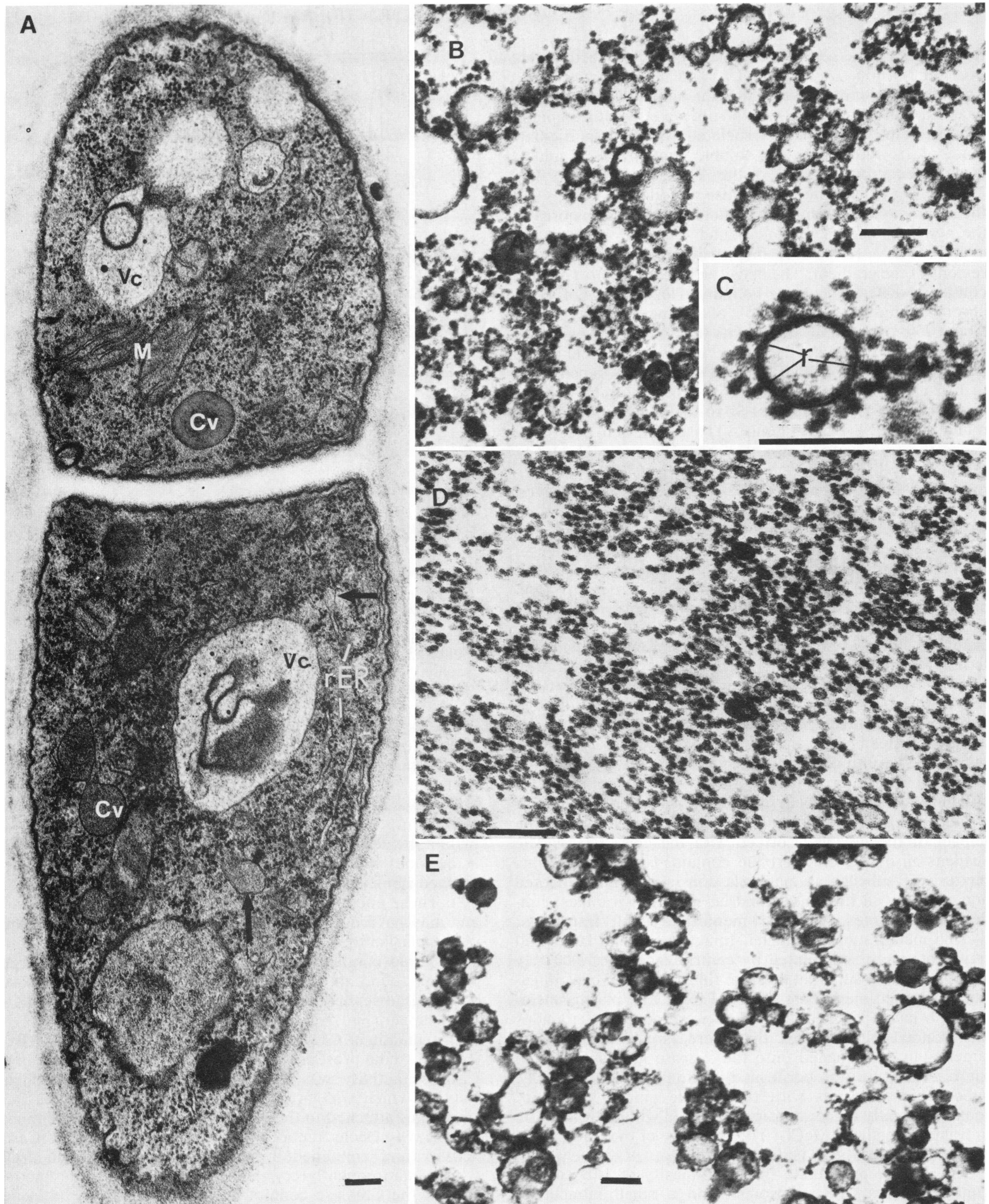

FIG. 2. (A) Ultrastructure of a mycelium from a rapidly growing culture (2 days) of *T. reesei* Rut-C30. The arrows show swollen terminals of the rER. Abbreviations: Vc, lightly stained vacuoles containing membrane material; M, mitochondria; Cv, condensing vacuole (note that staining density of the contents is higher than that present in Vc). Bar, 0.2 μm. (B) Light fraction in the presence of 3 mM $MgCl_2$ (density, 1.09 to 1.10 g/ml); small vesicles show no stained material and have ribosomes (r) attached to the outer surface of the bilayered membrane (C). Bar, 0.2 μm. (D) Free ribosomes present in fraction of density 1.11 to 1.12 g/ml. Bar, 0.2 μm. (E) Vesicle fraction mostly filled with highly stained material; however, many vesicles contained no stained material. These vesicles unlike those in B, have very few ribosomes attached to the outer surface of the membrane. Density, 1.13 to 1.14 g/ml. Bar, 0.2 μm.

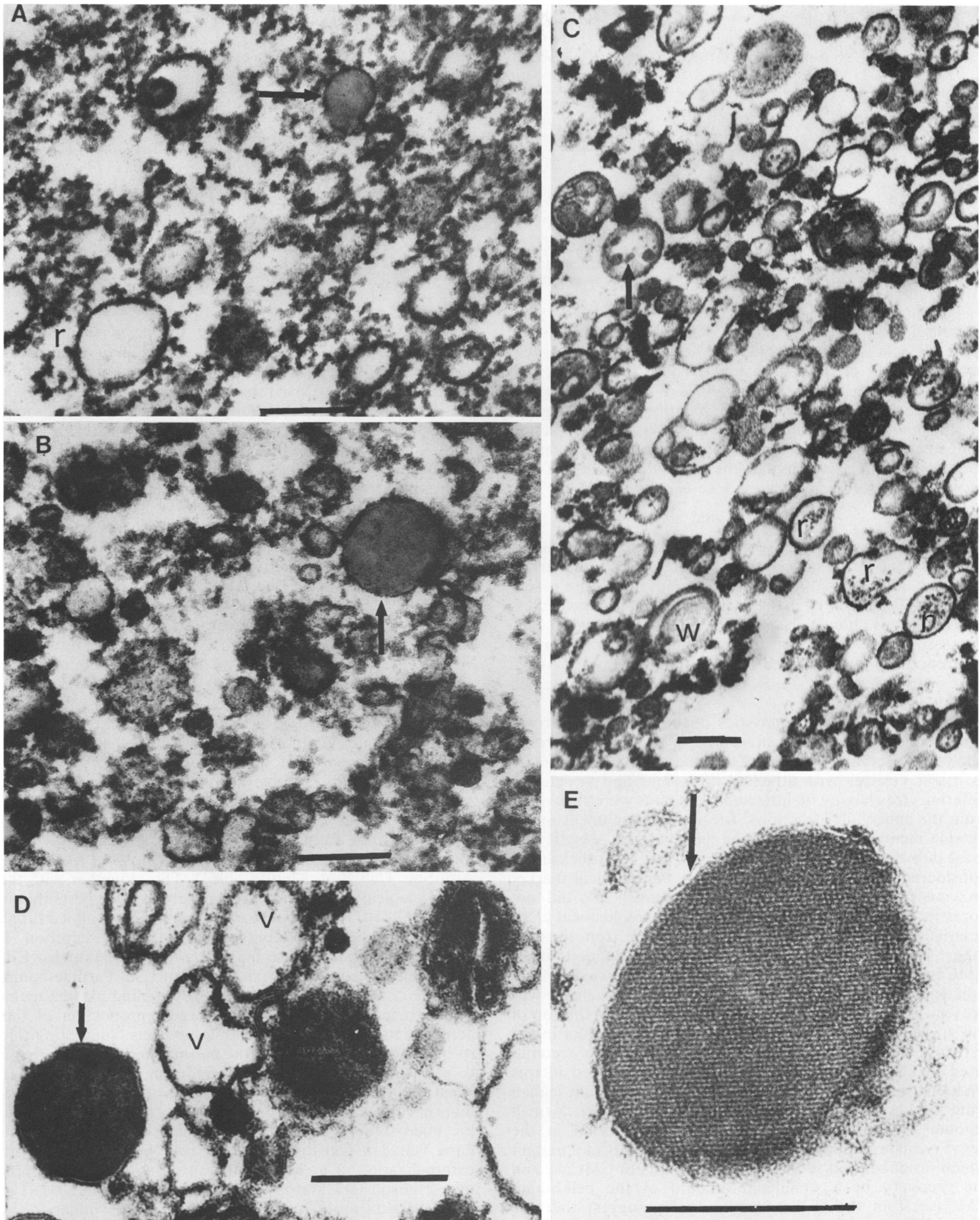

FIG. 3. (A and B) Some vesicles contain highly stained material (arrows); there are few vesicles with ribosomes attached to the outer surface of the delimiting membrane; the densities of these fractions prepared in absence of $MgCl_2$ were 1.10 (A) and 1.11 (B) g/ml. Bar, 0.2 μm. (C) Plasma membrane-derived vesicles; these vesicles generally have no stained material but many of these trap ribosomes (r); microvesicles (arrows) and whorls (W) of membrane material also are also present inside; the vesicle surface shows no associated ribosomes (density, 1.16 g/ml). Bar, 0.2 μm. (D and E) Vesicles containing crystalline material (arrows) were recovered from a heavy fraction (density, 1.18 g/ml) and the pellet; a crystalline nature is suggested from 5-nm periodicity of dense and light bands (E). The material is tightly delimited by a bilayered membrane (arrows). This fraction also contains large vesicles (v) with no stained material inside (D). Bars, 0.2 μm.

TABLE 1. Comparison of the CMCase catalytic activity and the CMCase antibody-binding capacity of the light- and heavy-vesicle fractions

Fraction[a]	Protein (mg/ml)	CMCase catalytic activity[b] (U/mg of protein)	CMCase antibody binding (^{125}I cpm/mg of protein)	^{125}I cpm/U of CMCase activity
Light vesicle (microsome rich)	4.1	16×10^{-2}	0.42×10^{6}	2.6×10^{2}
Heavy vesicle (rich in vesicles with crystalline material)	3.1	3.6×10^{-2}	0.32×10^{6}	8.9×10^{2}

[a] Fractionation was done as described in the legend to Fig. 1; direct radioimmunoassay was done with the light- and heavy-vesicle fractions.
[b] CMCase units were calculated from the catalytic activity as micromoles of glucose produced from carboxymethyl cellulose per minute.

only marginally higher, i.e., only 23% higher than that of the heavy-vesicle fraction (Table 1). The antibody-binding capacity per unit of the CMCase activity of the light vesicle fraction is much lower, i.e., only 30% of that of the heavy-vesicle fraction (Table 1). This high antibody-binding activity and low catalytic activity of the heavy-vesicle fraction indicate that the heavy vesicles contained a substantial amount of CMCase, but that the catalytic activity of this enzyme on carboxymethyl cellulose is poor.

DISCUSSION

A classical model of the secretory pathway in animal cells suggests that rER, smooth ER, Golgi bodies, condensing vacuoles, and a variety of shuttle vesicles constitute essential organelles involved in the secretion of protein molecules. Secretion may, however, proceed in many eucaryotic cells possessing only some of these organelles. Considerable differences in the organelle composition of secretory cells have been observed (8, 21; B. Hanson and S. Marple, J. Cell Biol. **97**:79a, 1983).

The data on the characteristics of the organelles derived from subcellular fractions presented in this paper corroborated the observations made by the study of intact hyphae (8, 9). Light vesicles with attached ribosomes and vesicles of differing sizes having no attached ribosomes were recovered from the subcellular fractions. Ribosome attachment to the outside membrane surface of the light vesicles suggest that these are derived from the rER, and this fraction can be considered as microsomes. However, the classical marker enzyme for rER, cytochrome *c* reductase (26), did not comigrate with this fraction; perhaps the biochemical characteristics of rER in this fungus are different from those in other systems. A significant amount of the cell-bound CMCase activity was concentrated in this light-vesicle fraction. In addition, a large amount of the CMCase activity was present in the organelle-free cytosol collected at the top of the gradient. Accumulation of the secretory product in the cytosol is unexpected. It is possible that CMCase accumulated in large vacuoles which were not preserved in this fractionation procedure; hence, the CMCase which leaked from the vacuoles appeared in the cytosol. Finally, a small amount of the CMCase activty was also found in the heavy-vesicle fraction. The proliferation of rER in a mutant which produces and secretes large amounts of CMCase and the recovery of a significant amount of the cell-bound CMCase from the rER-derived vesicles suggest that the biosynthesis of this secretory protein initiates in rER. The presence of Golgi bodies having a stacked appearance could not be demonstrated in this organism; however, a few vesiculated Golgi bodies are present (Ghosh et al., Abstr. Am. Soc. Cell Biol. **97**:305a, 1983). Golgi bodies play an important role in protein secretion; after synthesis the secretory proteins are processed and packaged inside Golgi bodies before these are secreted or stored in an intracellular storage organelle. The question remains open whether the large amount of CMCase rapidly synthesized and secreted in Rut-C30 can be handled through the rare vesiculated Golgi bodies present in this organism. It may be possible that this mutant has deregulated a bypass mechanism for rapid secretion of the CMCase.

Previous studies of protein secretion in mycelial fungi and yeasts have focused on cell wall-synthesizing enzymes (1, 2, 7, 14, 22; Hanson and Marple, J. Cell Biol. **97**:79a, 1983). In a series of yeast mutants blocked at various steps of the secretory process, secretion appears to follow a pathway typical of mammalian secretory cells, except that Golgi bodies in these cells consist of individual ER-associated saccules rather than a well-developed stack of dictyosomes (22). Cellulases destined for the cell wall are present in vesicles derived from the well-developed Golgi bodies of the oomycetes *Saprolegnia monoica* (7) and *Achlya* sp. (14). This fraction also contains elevated levels of inosine diphosphatase, ATPase, UDPglucose transferase, and carbohydrate material. Inosine diphosphatase has been localized in dictyosomes in many plants and appears to be associated with polysaccharide synthesis. However, *Achlya* sp. dictyosomes show no inosine diphosphatase, which is found instead in 150-nm vesicles derived from the Golgi bodies (14). In Rut-C30, inosine diphosphatase has been localized cytochemically in the rER (Ghosh et al., Abstr. Am. Soc. Cell Biol. **97**:305a, 1983). This, together with the morphological evidence of ER proliferation, suggests that in *T. reesei* Rut-C30 this membrane system may exhibit a broad range of biochemical activity associated with secretion. It also suggests that IDPase may be a useful marker for ER- and cellulase-associated vesicles in Rut-C30. Particle-bound ATPase comigrated with vesicles resembling plasma membrane fragments; however, the largest proportion of this activity remained with the soluble proteins, suggesting that its presence is not limited to the plasma membrane, or that it is released from the membrane during fractionation. In yeast, chitin synthetase zymogen and other secretory proteins have been localized in a membrane fraction lighter than the microsome. This light-membrane fraction, which contains vanadate-sensitive ATPase, may be associated with externalization of membrane-bound proteins and glycoprotein (Hanson and Marple, J. Cell Biol. **97**:79a, 1983). The lightest fraction of *T. reesei* vesicles may be similar to the very light membrane fraction described in yeasts.

An intriguing aspect of the fractionation of this mutant is the appearance of heavy vesicles containing crystalloid material. In thin sections, however, the presence of these vesicles can be suggested because the material inside condensing vacuoles had some order indicating the presence of

crystalline material. The presence of vesicles containing crystalloid material is often associated with some abnormality of metabolism or secretion (15, 20, 23, 28). However, crystalloid granules are also present in normal a,b cells of pancreatic islets, where they function in secretory processes (28). The CMCase antibody binding of the light- and heavy-vesicle fraction did not differ significantly. The light-vesicle fraction, however, contained much higher activity to hydrolyze carboxymethyl cellulose. This low catalytic activity of the CMCase and the crystallinity of the material present in the heavy vesicles suggest that this potently hydrolytic enzyme is stored in the heavy vesicles in a poorly active form to protect the cells from its deleterious action. In animal cells, lysosomes or zymogen granules contain a variety of inactivated hydrolytic enzymes. It is possible that hydrolytic enzymes are also stored in the vesicles of *T. reesei*. In the mutant Rut-C30, this physiological function might be exaggerated due to the overproduction of CMCase.

The hypersecretory character of Rut-C30 appears to provide a useful model for the study of cellulase secretion. Cells appear to achieve hyperproduction of the potent hydrolytic enzyme CMCase in at least two ways: sequestration as crystalloids within secretion granules and rapid externalization via the vacuole. Such a suggestion implies that mechanisms for sequestration or hypersecretion may be obligately coupled with hyperproductoin. A detailed study of the organelles involved in the externalization of CMCase will generate data on the latent capacity of microbial cells which are not specialized for secretion to develop an enhanced secretory process.

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