Subcellular Localization of Glucanase and Cellulase in *Saprolegnia monoica* Pringsheim

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(1→3)-β-Glucanase and cellulase, thought to be involved in the growth and branching processes of *Saprolegnia monoica* hyphae, were mainly localized at the edge of the colony. Autolysis of purified cell walls occurred preferentially in newly-formed walls. This process was also more important in walls of branched hyphae than in those of unbranched mycelium.

Intracellular fractions were separated and characterized by density gradient ultracentrifugation, enzymic tests and electron microscopy. The fractions rich in β-glucanase and cellulase contained dictyosomes and apical vesicles. In agreement with cytological descriptions of the apex, the present results confirm the role of these 'morphogen' enzymes in hyphal morphogenesis.

INTRODUCTION

Wall lytic enzymes may be involved in the process of hyphal morphogenesis. Recent results suggest or support their participation in wall growth. There is a positive correlation between the level of (1→3)-β-glucanase, cellulase and/or proteases and apical growth and branching of the mycelia of *Neurospora crassa* (Mahadevan & Mahadkar, 1970), the germination of conidia of *Microsporum gypseum* (Page & Stock, 1974), the hormone-induced branching of Achlya (Thomas & Mullins, 1967, 1969; Mullins, 1973) and the budding phase of yeast (Maddox & Hough, 1971; Cortat, 1971). Enzymes capable of degrading wall polymers [proteases and/or (1→3)-β-glucanase] have been isolated from the hyphal walls of *Neurospora crassa* (Mahadevan & Mahadkar, 1970; Mehta & Mahadevan, 1975) and from the yeast walls of Schizosaccharomyces (Barras, 1972; Fleet & Phaff, 1974).

The presence of inhibitors of protein synthesis in the growth medium of *Saprolegnia monoica* leads to the complete loss of branching capacity of the mycelium and a corresponding decrease in cellulase and (1→3)-β-glucanase activities (Fèvre, 1972, 1976). In this paper, I describe an attempt to elucidate the location of these two 'morphogen' enzymes in the mycelium of *Saprolegnia monoica.*

METHODS

Culture methods. The strain, *Saprolegnia monoica* Pringsheim no. 539 67 Dick, obtained from CBS, Baarn, The Netherlands, was maintained on a wheat flour medium. Liquid cultures were grown in 250 ml Erlenmeyer flasks by inoculating 100 ml of the liquid medium of Machlis (1953) with mycelial discs (4 mm diam.) from plate cultures, and incubating at 23 °C for 4 days. The mycelium was harvested by filtration through paper in a Buchner funnel and washed with cold distilled water. Plate cultures were grown by inoculating mycelial discs into the centre of Petri dishes which contained Machlis (1953) medium supple-

mented with 12 g agar l^{-1} and covered by a Cellophane film. After 8 days, the colonies were cut in concentric zones with sterile microscalpels, following guidelines drawn under the dishes.

Preparation of extracts. Mycelia from each concentric zone were freeze-dried then ground in a mortar with purified sand (3 parts by weight) and 0·01 M-Tris/HCl buffer pH 7·2 (4 parts by volume) to make a thick slurry. After centrifugation (20000 *g*, 20 min, 4 °C) the pellet was discarded and the supernatant was kept in ice until used.

For experiments requiring isolated wall fractions, the lyophilized mycelium was ground with sand and buffer as above, and then centrifuged at 1000 *g* for 10 min; the pellet was washed eight times with 0·4 M-sucrose and distilled water.

Cytoplasmic particles were obtained by mixing and grinding the fresh mycelia in buffer (0·01 M-Tris/HCl, 0·5 M-sorbitol, 0·001 M-EDTA, pH 7·2) in a Virtis homogenizer for three periods of 30 s at 4 °C. The wall fraction was discarded after centrifugation at 1000 *g* for 10 min and the particulate fraction was obtained by centrifuging the supernatant at 48000 *g* for 30 min. For sucrose density gradient analysis, the particulate fraction, suspended in 2 ml buffer, was layered on to a 30 ml sucrose gradient (20 to 50 %, w/v) or Urografin (Schering) gradient (containing 0·01 M-Tris/HCl pH 7·2, 0·001 M-EDTA) and centrifuged at 4 °C in a Beckman L5.50 or MSE centrifuge (SW27 rotor) at 25000 rev. min^{-1}. Tests indicated that isopycnic sedimentation of particles was obtained after 4 h centrifugation. Fractions of 25 drops were collected and analysed for enzyme activities.

Enzyme assays. β-Glucanase activity [EC 3.2.1.6; 1,3-(1,3;1,4)-β-D-glucan 3(4)-glucanohydrolase] was assayed by incubating 100 to 300 μl of enzyme preparation with 1 ml of glucan substrate (3 mg laminarin ml^{-1} in McIlvaine buffer pH 5) at 37 °C and determining the amounts of reducing sugars or glucose in the mixture after incubation. For each reaction, corresponding controls (lacking enzyme or substrate) were prepared and their activity was subtracted from the activity of the enzyme sample. Cellulase activity [EC 3.2.1.4; 1,4-(1,3;1,4)-β-D-glucan 4-glucanohydrolase] was assayed by measuring the loss of viscosity caused by 0·25 ml of enzyme preparation in a 2 % (w/v) solution of hydroxyethylcellulose in McIlvaine buffer pH 5 at 40 °C (Courtois & Bui, 1967). One unit was taken as the amount of enzyme that caused a 1 % decrease in viscosity relative to that of a blank. Acid phosphatase [EC 3.1.3.2; orthophosphoric-monoester phosphohydrolase (acid optimum)] and alkaline phosphatase [EC 3.1.3.2; orthophosphoric-monoester phosphohydrolase (alkaline optimum)] were assayed as described by Schurr & Yagil (1971). ATPase [EC 3.6.1.3; ATP phosphohydrolase] was assayed as described by Nombela, Uruburu & Villanueva (1974) in the presence of oligomycin (Kulaev, 1973). Succinic INT reductase was determined according to the method of Morré (1971). NADH cytochrome *c* reductase [EC 1.6.99.3; NADH:cytochrome *c* oxidoreductase] was determined using the methods described by Tolbert (1974) and Shore & MacLachlan (1975). β-Glucosidase [EC 3.2.1.21; β-D-glucoside glucohydrolase] was assayed as described by Cortat (1971).

Protein was determined by the method of Lowry *et al.* (1951). Reducing sugars were estimated with the dinitrosalicylic acid reagent (Miller, 1959), and glucose with the Gluco-kit (DB Merieux) and Glucostat (Worthington) according to the manufacturers' instructions. Total sugars were determined by the anthrone method (Fairbairn, 1953).

Electron microscopy. Gradient fractions were fixed in 2 % (v/v) glutaraldehyde buffered with 0·1 M-potassium phosphate pH 7·2, containing 0·5 M-sorbitol, for 2 h at 4 °C, then postfixed for 45 min in buffered 1 % (w/v) OsO_4. After dehydration in ethanol, pellets were embedded in Epon. Cytoplasmic membranes in thin sections were stained using alkaline lead citrate.

RESULTS

Localization of glucanase and cellulase in the fungal colony

Mycelia grown on Cellophane in Petri dishes were cut in concentric zones and analysed for enzyme activities. (1→3)-β-Glucanase and cellulase were mainly localized at the edge of the colony (the extension and branching zone) and their activities decreased with increasing age of the zone. The activities (per mg freeze-dried mycelium) in the oldest mycelial zone were only 12 % (cellulase) and 40 % (glucanase) of those in the hyphal tip area (Fig. 1*a*). The specific activities (activity per mg protein) showed the same trend (Fig. 1*b*): activities in the oldest zone were 50 % (glucanase) and 20 % (cellulase) of those in the youngest zone (Fig. 2). The distribution of other enzymes was different. Acid phosphatase, in particular, and, to a lesser extent, β-glucosidase were localized in the older parts of the mycelium.

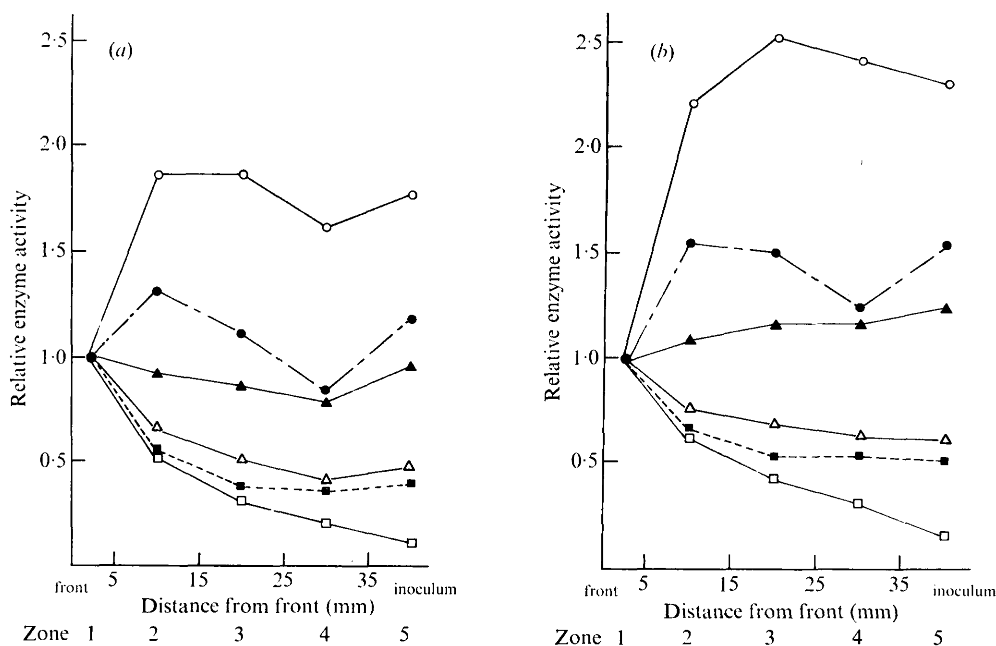

Fig. 1. Enzyme activities in different parts of the colony of *Saprolegnia monoica*. Mycelia grown on Cellophane in Petri dishes were cut in concentric zones, freeze-dried and assayed for enzyme activities, as described in Methods. The activity found in each zone is expressed relative to that found in the apical zone: these activities were expressed (*a*) per mg freeze-dried mycelium, or (*b*) per mg protein. ○, Acid phosphatase; ●, succinic INT reductase; ▲, β-glucosidase; □, cellulase; ■, (1→3)-β-glucanase (dinitrosalicylic reagent); △, (1→3)-β-glucanase (DB Merieux kit).

Glucanases of cell walls – autolysis

Differential centrifugation has shown that glucanase and cellulase are bound to the cell walls and cytoplasmic particles (Fèvre, Turian & Larpent, 1974). Wall enzymes can be liberated by the mechanical action of freezing and thawing. The amount of released glucanase is related to the morphology of the mycelium, branched hyphae containing more β-glucanase than unbranched mycelium (Fèvre, 1976).

Purified walls incubated in McIlvaine buffer pH 5 at 37 °C released soluble sugars into the supernatant. Analysis of the products (Fig. 2*b*) showed that glucose (tested by Glucostat) represented only a small part of the total sugar released (tested by the anthrone method), suggesting that the major products of autolysis are long-chain soluble glucans, produced by the action of an endo-glucanase in the cell wall. At the same time (1→3)-β-glucanase was released (Fig. 2*b*). Comparison with results for mycelia grown in the presence of DL-*p*-fluorophenylalanine (Fig. 2*a*) showed that branched mycelia have a higher autolytic capacity and release more glucanase than unbranched hyphae.

Localization of the sites of autolysis

Purified walls obtained from different zones of the fungal colony were tested for the release of sugars by autolysis (Table 1). The total release of sugar from the youngest zone was nine times greater than that from the oldest zone. In each zone, glucose was always released in small quantities, confirming the endo-mechanism of the autohydrolytic process. Wall preparations also hydrolysed laminarin; the ratio of (1→3)-β-glucanase activity in the youngest part of the colony to that in the oldest part was about 2:1. Thus, laminarin was more readily degraded than the macromolecules of the wall. It seems that walls in

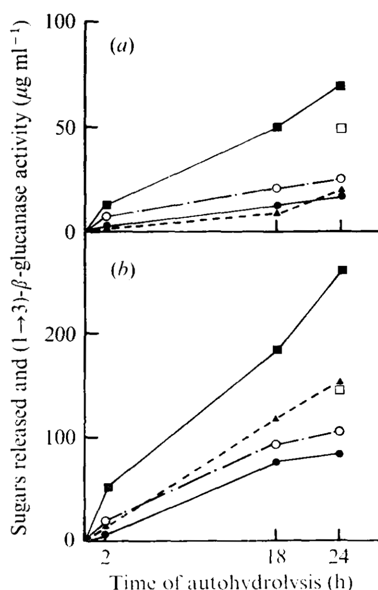

Fig. 2. Autolysis of purified walls of *Saprolegnia monoica*: (*a*) walls of unbranched mycelia, grown with DL-*p*-fluorophenylalanine; (*b*) walls of branched mycelia. Sugars released by autolysis were tested with: anthrone (■) for total sugar; dinitrosalicylic acid reagent (▲) for reducing sugar; Gluco-kit (○) or Glucostat (●) for glucose. The activity of (1→3)-β-glucanase liberated after 24 h autohydrolysis was tested against laminarin using dinitrosalicylic reagent (□).

Table 1. *Total sugars and glucose released by autolysis and* (1→3)-*β-glucanases of purified cell walls of different parts of the colony of Saprolegnia monoica*

Colonies grown on Cellophane film in Petri dishes were cut in concentric zones. The walls of the mycelium of each zone were purified and tested for their autolytic (200 mg walls, 4 ml, 18 h) and glucanase (100 mg walls, 1 ml, 1 h) activities. Results for the different analysis methods are all expressed as glucose (μg) equivalents.

Zone	Distance from colony edge (cm)	Autolysis			(1→3)-β-Glucanase
		Anthrone	Gluco-kit	Glucostat	Gluco-kit
1	0–1	712	372	24	350
2	1–2	456	292	33	300
3	2–3	424	260	28	286
4	3–inoculum	84	96	10	202

the younger part of the mycelium could be attacked more easily than older walls, because the difference in sugar released by autolysis between zones 1 and 4 was greater than the difference in glucanase activity.

Subcellular location of glucanase and cellulase

Cytoplasmic particles sedimented by centrifugation at 48000 *g* were layered on a continuous gradient of sucrose or Urografin. Glucanase was distributed in three different density bands, corresponding to those of the plasma membrane (1·19 g cm^{-3}), the Golgi apparatus (1·14 g cm^{-3}) and the endoplasmic reticulum (1·10 g cm^{-3}) (Cortat, 1971) (Fig. 3). Glucanase-rich particles from unbranched mycelium were less abundant and the difference was obvious for the particles of density 1·14 to 1·11 g cm^{-3} (Fig. 4). These densities correspond to those of the cytoplasmic vesicles isolated from yeast buds (Cortat, 1971). Cellulase activity was also distributed in three different density zones. The presence of

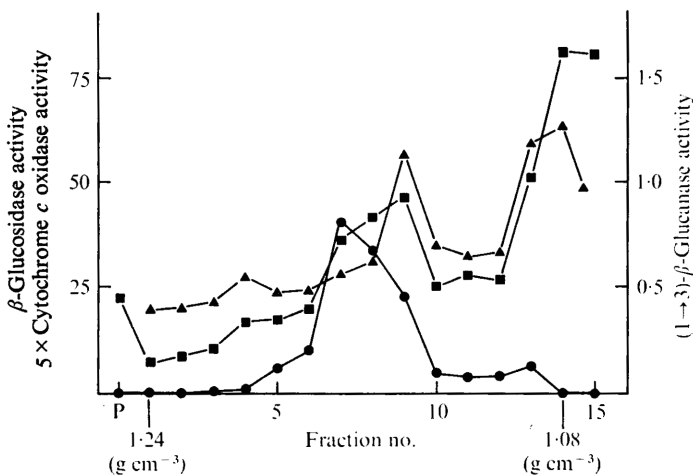

Fig. 3. Distribution of enzyme activities in a continuous density sucrose gradient: ●, cytochrome *c* oxidase; ■, β-glucosidase; ▲, (1→3)-β-glucanase. Cytoplasmic particles, prepared as described in Methods, were layered on the sucrose gradient and centrifuged (4 h, 26000 rev. min^{-1}, SW27 MSE rotor). Activities of cytochrome *c* oxidase and β-glucosidase are expressed as change in extinction units per fraction and glucanase activity is expressed as mg glucose released per fraction.

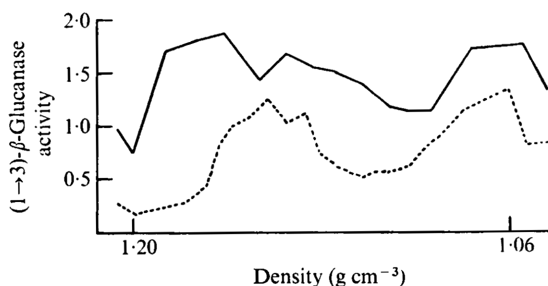

Fig. 4. Distribution of glucanase activity in a continuous density Urografin gradient. Cytoplasmic particles, prepared from branched mycelium (—) or unbranched mycelium, grown with DL-*p*-fluorophenylalanine (---), were layered on the Urografin gradient and centrifuged (4 h, 26000 rev. min^{-1}, SW27 MSE rotor). (1→3)-β-glucanase activities, expressed as mg glucose released in 6 h per fraction, were corrected to the same amount of protein layered on the gradients.

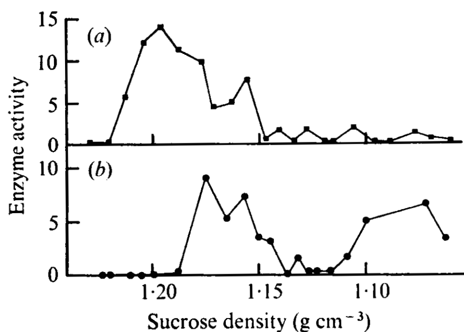

Fig. 5. Distribution of cellulase activity in a continuous density sucrose gradient: (*a*) $MgCl_2$ present in the extraction buffer and in the gradient; (*b*) $MgCl_2$ absent. Cytoplasmic particles were layered on the sucrose gradient and centrifuged (4 h, 26000 rev. min^{-1}, SW27 Beckman rotor). Cellulase activities are expressed in arbitrary units.

Table 2. *Distribution of glucanase and cellulase activities among membrane fractions*

Cytoplasmic particles were prepared as described in Methods and centrifuged on a discontinuous density sucrose gradient. Enzymes were assayed against laminarin (glucanase) and hydroxyethylcellulose (cellulase). For each enzyme, activities are expressed as a percentage of the total activity of that enzyme found in the gradient.

Interface (g cm^{-3})	(1→3)-β-Glucanase	Cellulase
Soluble/1·08	1·3	10
1·08/1·10	11·8	12
1·10/1·13	30·7	22
1·13/1·16	34·6	31
1·16/1·17	16·3	15
1·17/1·20	5·3	10

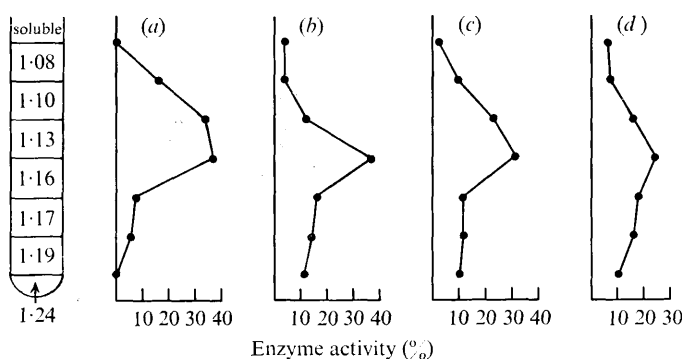

Fig. 6. Distribution of marker enzyme and cellulase activities among subcellular fractions of *Saprolegnia monoica*: (*a*) NADH cytochrome *c* reductase; (*b*) alkaline phosphatase; (*c*) cellulase; (*d*) Mg^{2+}-dependent ATPase (plus oligomycin). Cytoplasmic particles were prepared as described in Methods and centrifuged on a discontinuous density sucrose gradient. For each enzyme, activities are expressed as a percentage of the total activity of that enzyme in the gradient.

$MgCl_2$ in the buffer resulted in a shift of cellulase from density 1·10 to 1·20 g cm^{-3} (Fig. 5), which indicates that the enzyme is present in the rough endoplasmic reticulum; according to Shore & MacLachlan (1975) a high Mg^{2+} concentration conserves the integrity of rough endoplasmic reticulum and leads to its sedimentation at high density.

The distribution of cellulase and glucanase activities in the discontinuous sucrose gradient is shown in Table 2; 34 % of the glucanase activity and 31 % of the cellulase activity were in the layer of density 1·16 g cm^{-3}.

Mg^{2+}-stimulated ATPase (a plasma membrane marker) showed a higher concentration at the bottom than at the top of the gradient; NADH cytochrome *c* reductase (an endoplasmic reticulum marker) was mainly located near the top of the gradient (Fig. 6). Alkaline phosphatase, demonstrated *in situ* in Achlya dictyosomes (Dargent, 1975), sedimented at medium density (1·16 g cm^{-3}), which corresponds to the maximum of cellulase and glucanase activities found in the gradient. Electron microscopy of the 1·16 g cm^{-3} fraction, the richest in 'morphogen' hydrolases, showed dictyosomes that had probably been dissociated during homogenization. Cytoplasmic vesicles were also present and these were of a similar size to those found in the growing centres (apex and branch sites) (Fig. 7). The 1·13 g cm^{-3} fraction showed larger vesicles which could be fragments of endoplasmic reticulum or tonoplast. The 1·20 g cm^{-3} fraction showed double membrane vesicles which may have been associated with plasma membrane. A few dictyosomes were also present.

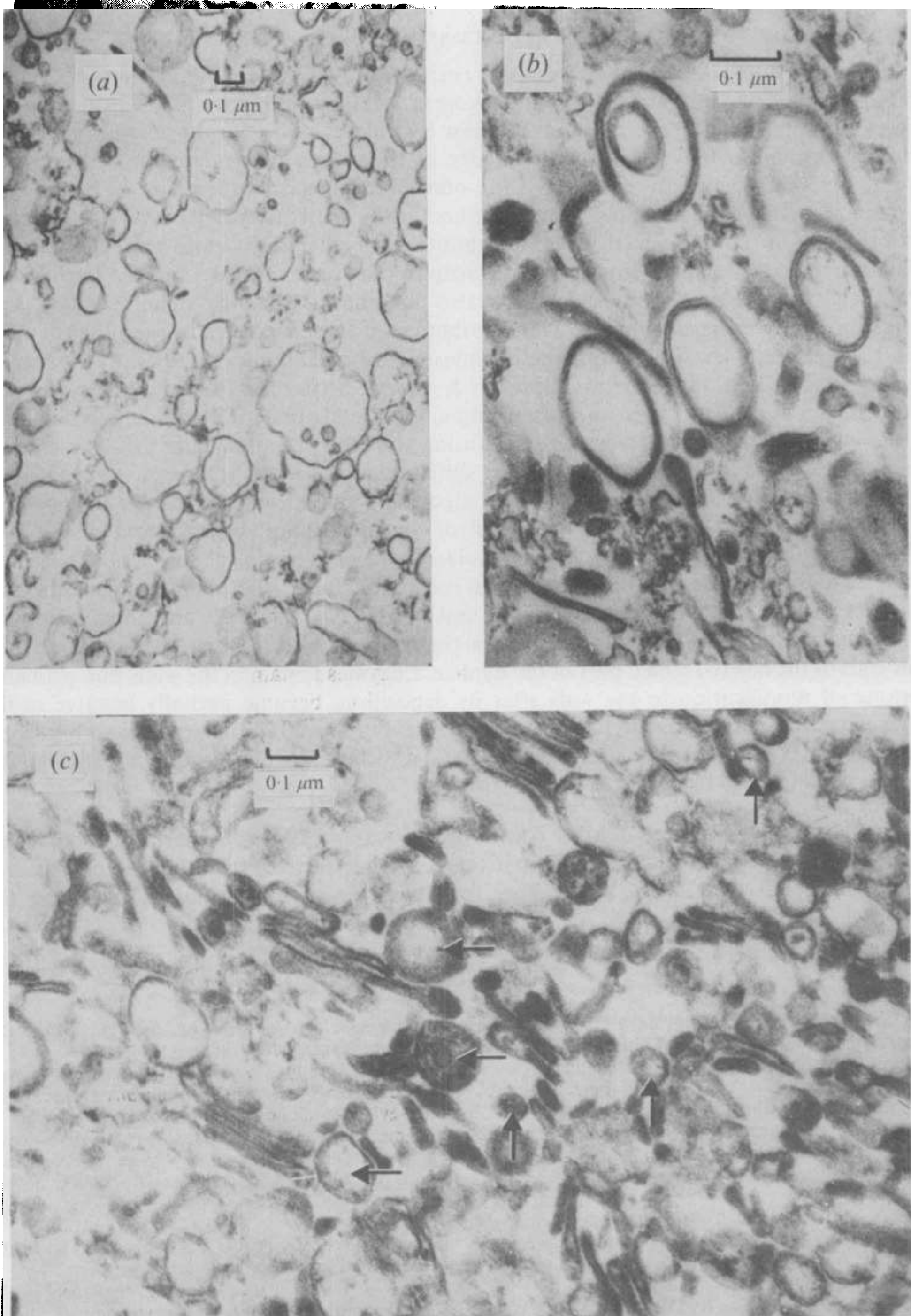

Fig. 7. Electron micrographs of cytoplasmic membranes obtained by centrifugation in a discontinuous density sucrose gradient. (*a*) Light membranes, endoplasmic reticulum and tonoplast (interface 1·08/1·13 g cm^{-3}). (*b*) Plasma membrane contaminated by isolated Golgi saccules (interface 1·16/1·20 g cm^{-3}). (*c*) Dictyosomes and Golgi-derived cytoplasmic vesicles (interface 1·13/1·16 g cm^{-3}). Horizontal arrows indicate large vesicles and vertical arrows indicate small ones.

DISCUSSION

Previous results (Fèvre, 1972) showed a correlation between the hyphal morphogenesis of Saprolegnia and the activity of 'morphogen' enzymes (cellulase and glucanase). The present results confirm the relation between hydrolases and morphogenesis. Glucanase and cellulase are mainly localized at the edge of the colony where growth and branching of the hyphae occur. In common with other fungal species, hyphae of Saprolegnia contain enzymes which can hydrolyse their own wall polymers. Autolysis occurs preferentially in newly-formed walls; and branched hyphae have more autolytic enzymes than unbranched hyphae. Intracellular glucanase and cellulase are mainly localized in the Golgi apparatus although they are also present in fractions rich in endoplasmic reticulum and plasma membrane. The distribution of these enzymes in the fungus colony and the hyphae is consistent with the cytoplasmic differentiation described by cytologists (Grove & Bracker, 1970; Grove, Bracker & Morré, 1970; Heath, Gay & Greenwood, 1971). Growth centres (apex and branching sites) are characterized by an accumulation of vesicles derived from dictyosomes and fusing with the plasma membrane. These apical vesicles are involved in wall growth of the differentiation centres (Dargent, 1972; Mullins & Ellis, 1974) where they probably secrete precursors and enzymes necessary for growth.

The present results give some indication of the biochemical organization of the apex of Saprolegnia. Dictyosomes, rich in hydrolases and derived apical vesicles, may secrete cellulase and glucanase into the walls. By an endo-mechanism, these 'morphogen' enzymes may act on the wall polymers, softening and plasticizing the wall, and thus permitting extension and growth. Enzymes trapped in the wall network may be responsible for the autolysis of the newly-formed part of the hyphae. Enzymes remain in the walls but, probably because of modification to the wall after its deposition, become partially inactive in the older part of the mycelium. Lytic enzymes are essential to wall growth (apex and branches) but wall synthesis must occur simultaneously and a 'delicate balance' must exist (Bartinicki-Garcia & Lippman, 1972).

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