Isolation of Glucanase-Containing Particles from Budding Saccharomyces cerevisiae

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ABSTRACT In an investigation of the role of glucanases in modifying yeast cell walls at the location of new buds, vesicles derived from the endoplasmic reticulum, which are secreted locally into the cell wall of growing buds and may be involved in the secretion of glucanases, have been isolated.

In yeast, exo-β,1,3-glucanase (EC 3.2.1.6) is present both extra- and intracellularly. Exponentially growing cells contain about 16% of the enzyme activity intracellularly (within the plasmalemma). Most, if not all, of this intracellular glucanase is sedimentable. Of the three classes of subcellular particles that contain glucanase, one is almost completely absent from stationary phase cells and almost absent from cells of the late budding phase of the budding cycle. These particles were isolated from budding cells by combined differential and density gradient centrifugation. They contain exo- and endo-β,1,3-glucanases, mannan, and protein. The isolate consists mainly of membrane-bounded particles with diameters corresponding to those of the secretory vesicles observed in situ. It is concluded that these particles are identical with the vesicles derived from the endoplasmic reticulum.

In budding yeasts like Saccharomyces cerevisiae, cell division involves the modification of cell wall properties at the location of new buds. Whether this modification consists of local dissolution or merely softening of the existing wall is not clear, but in any case it is thought to be caused by enzymes acting on cell wall constituents. According to Nickerson (1), protein disulfide reductase (EC 1.6.4.4) is responsible for cleaving disulfide bridges that link together glucosaminoglycan complexes of the yeast cell wall. On the other hand, Johnson has proposed (2) that synthesis of glucan in yeast cell walls occurs by insertion of glucose into glucan chains at cuts made by extracellular glucanases. That β-glucanase softens the cell wall has been demonstrated indirectly by blocking the synthesis of glucan (3) and directly by adding glucanase to the medium (4).

From the cytological viewpoint, the problem of what enzyme system is involved in the budding process is less important than the question of how the mother cell is able to concentrate the enzyme action at the site of the bud. Moor (5) has observed striking proliferations of the endoplasmic reticulum (ER) that result in the formation of numerous small vesicles, which are secreted into the wall at the location of the new buds. The secretion of ER-derived vesicles continues until the growth of the daughter cell is completed. According to Moor (5), these vesicles may be involved in the secretion of disulfide reductase, whereas Sentandre and Northeote have concluded (6) that they carry cell wall material into the wall of the growing bud.

The present report deals with an attempt to isolate the ER-derived vesicles in order to solve the problem by direct biochemical analysis.

METHODS

Baker's yeast (Saccharomyces cerevisiae) strain 1022, Inst. Microbiol. Swiss Fed. Inst. Technol.) was cultured, and protoplasts were prepared, as described previously (7).

Cell fractionation

Cells from the first exponential or stationary-growth phase were ruptured by vigorous shaking in the presence of Ballotini beads and sorbitol medium (0.5 M sorbitol, 0.05 M Tris·HCl buffer pH 7.2, 1 mM EDTA) for 7 sec. After low-speed centrifugation (10 min at 3,000 × g) the extracts were fractionated by differential centrifugation into sediment 1 (20 min, 20,000 × g), sediment 2 (20 min, 55,000 × g), sediment 3 (60 min, 150,000 × g), and soluble fraction. For density equilibrium centrifugation in isopycnic gradients of Urografin (8), 4.5-ml gradients loaded with 1.0 ml of suspended particles were centrifuged for 2.5 hr at 39,000 rpm in a Spinco SW-39 rotor.

Enzyme assays

Exoglucanase (laminarinase) activity (EC 3.2.1.6) was assayed by measurement of glucose liberated from laminarin (9) in a hexokinase plus glucose-6-phosphate dehydrogenase system, or by the method of Brock (10) with p-nitrophenyl-β-glucopyranoside as substrate. Endoglucanase activity was assayed by recording viscosity changes in the presence of carboxymethylpachyman (11). Standard methods were employed for the assay of other enzyme activities.

Determinations

Protein was determined by the method of Lowry et al. (12). Polysaccharide was estimated with the anthrone reagent after heat-denatured samples, previously pipetted into glass filter discs, had been washed with ethanol–water 4:1.

Electron microscopy

Cells and isolated particles were fixed with glutaraldehyde (13) and the sections were contrasted with uranyl acetate and lead citrate.
**RESULTS**

**Correlation between budding and glucanase activity**

Any successful cell-fractionation work must be based on a meaningful biochemical or morphological marker. Of the two prospective marker enzymes, disulfide reductase and glucanase, the first has proved to be absent from the strain of yeast used. Preliminary experiments with exoglucanase, however, showed a correlation between its activity and the condition of the cell population. In contrast to the report of Abd-El-Al and Phaff (9), we found that in stationary-phase cells with practically no budding cells present the specific β-glucanase activity is much lower than in exponentially growing cells. Moreover, analysis of Wiemken's (14) synchronously budding cultures showed a sudden increase of glucanase activity just before the budding phase.

Exoglucanase activity can be estimated (by means of the substrate p-nitrophenyl-β-glucoside) in intact *Saccharomyces* cells. Therefore at least some of it is outside the plasmalemma.

Since it is not secreted into the culture medium the enzyme must be confined to the cell wall. Extracellular localization is further indicated by the ability of yeast cells to grow on laminarin as the sole carbon source.

**Intracellular β-glucanase**

The presence of intracellular β-glucanase is shown by the difference of activities measured in intact and ruptured cells. These differences depend to some extent on the method of rupturing employed. The highest values, about 16%, could be obtained after repeated freezing and thawing of the cells. This value corresponds roughly to the loss of glucanase activity upon converting cells into naked protoplasts.

In extracts from exponentially growing cells, 14–15% of the total activity is sedimentable (80 min at 150,000 × g). This value is highly reproducible; since less than 0.5% of the total exoglucanase activity is associated with purified cell walls, it indicates that most if not all of the intracellular enzyme is
FIG. 2. Electron micrographs of Saccharomyces cells and isolated glucanase particles. A, young bud containing numerous secretory vesicles. ×40,000. B, in the mature daughter cell (double-cell phase) secretory vesicles are almost absent. ×19,600. C, glucanase particles present in the fractions 4–6 of density gradients as illustrated in Fig. 1D. Note the absence of mitochondria and the presence of large contaminating membrane fragments, probably plasmalemma. ×32,000. D, isolated glutaraldehyde-fixed glucanase particles contrasted negatively with phosphotungstic acid. ×32,000.

sedimentable. In extracts from stationary-phase cells, only about 7% of exogluccanase activity is sedimentable.

**Glucanase particles**

If the whole sedimentable material present in an extract from exponentially growing cells is subjected to density gradient centrifugation, the distribution of exogluccanase activity suggests the association of this enzyme with three distinct classes of particles (Fig. 1A). In order to find out the possible relation of one of these classes with the ER-derived vesicles, we performed identical fractionations with exponentially growing and stationary-phase cells. The result (Fig. 1B) shows clearly that the exogluccanase peak at about 1.14 g/cm³ (fractions 8–10) is almost completely absent from stationary-phase cells having almost no budding cells. The same peak is much lower for late budding cells than for initial budding cells from a synchronous culture (14) (Fig. 1C). It seems, therefore, that this peak marks particles, which we will hereafter call glucanase particles, that are connected with the budding process. [The heaviest class of particles containing glucanase has been identified earlier with fragments of the plasmalemma (15) and the fraction with the lowest density probably represents fragments of the ER since it also contains NADH–dichlorophenol–indophenol oxidoreductase activity (Fig. 1A).]

The position in the Urografin gradients of glucanase particles corresponds to that of the mitochondrial enzyme cytochrome oxidase, EC 1.9.3.1 (Fig. 1A). Therefore, a differential centrifugation to eliminate the mitochondria had to pre-
cede the gradient centrifugation. Most mitochondria, as well as some plasmalemma fragments, are contained in the 20,000 × g sediment, whereas most of the glucanase particles are present in sediment 2 (see Methods). Fig. 1D shows the distribution of sediment, 2 particles in the type of Urograin gradient used for the purification of glucanase particles. It appears that these particles contain polysaccharide and a little protein in addition to the exo-β-glucanase. Acid hydrolysis of the polysaccharide yields mannose exclusively, which indicates that it consists of mannan. The presence of polysaccharide in the glucanase particles is further indicated by the absence of the 1.14 g/cm² peak if stationary-phase cells are fractionated.

The availability of a limited amount of carboxymethyl-

pectin allowed the estimation of endo-β-1,3-glucanase in various preparations; Table 1 shows that glucanase particles not only contain exo- but also some endoglucanase activity. Attempts to determine endoglucanase activity by calculating the difference between total reducing groups and glucose formed upon incubation in the presence of laminarin have yielded positive but only qualitatively reliable results. For isolated cell walls, it seems to be the only way of estimating endoglucanase activity; in contrast to the exoglucanase, which is released upon repeated washings, a considerable amount of endoglucanase remains firmly associated with the cell walls.

**Morphology of glucanase particles**

In thin sections of budding Saccharomyces cells, the ER-derived vesicles (5) become visible as membrane-bounded circles with a diameter of about 0.1 μm and an electron-transparent content (Fig. 2A). Abundant during the initial budding phase, these vesicles are absent in the late budding phase just before the septum is formed (Fig. 2B). Figure 2C shows the center of a pellet of glucanase particles collected from several density gradients. This fraction consists largely of small vesicles. Mitochondria are completely absent but large membrane fragments, possibly from the plasmalemma, represent a conspicuous impurity. If sectioned equatorially (distinct membrane contours) the small vesicles appear to have the same diameter (0.1 μm) as the ER-derived vesicles *in situ*. A better estimate of vesicle diameters can be derived from electron micrographs of the flattened negatively-stained particles (Fig. 2D); the calculated diameter of the corresponding sphere is 0.06–0.15 μm.

**DISCUSSION**

The data presented strongly suggest that local modifications of *Saccharomyces* cell walls are caused by the secretion of vesicles that contain β-glucanase into the wall at the site of new buds.

Marchant and Smith (16) have described bud formation in *S. cerevisiae* to occur by an extension of the entire parent cell wall. On the other hand, labeling of the parent wall with fluorescent antibodies has shown that upon budding the cell wall of the daughter cell is largely unlabeled (17). It thus seems that the production of cuts in glucan chains by the action of secreted glucanase (2) results in an extensible cell wall which is, however, continuously supplied with new glucose residues for the insertion into existing glucan chains and with mannan secreted together with glucanases. Morphological observations (5) suggest that this process continues until the growth of the daughter cell is completed. Although secretion of glucanases into the wall is probably limited to budding cells, stationary cells do contain high amounts of extracellular glucanase. If in resting cells these enzymes have access to the glucan molecules, the cell wall would appear as the result of a continuous process of destruction and repair of one of its principal constituents, and budding would represent merely a local intensification of this process.

A fraction of membrane-associated glucanase is recovered in fragments of the plasmalemma. This unexpected finding could be explained by the fusion of glucanase vesicles with the plasmalemma, a process which contributes to the growth of this membrane. The presence of glucanases in the plasmalemma fraction would then suggest the initial association of these enzymes with the membranes of the glucanase particles. There are probably two or more exoglucanases, which differ in their substrate affinity: the ratio of activity on laminarin to

**Table 1. Purification of glucanase particles by combined differential and isopycnic density gradient centrifugation**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Exo-β-1,3 glucanase</th>
<th>Endo-β-1,3-glucanase</th>
<th>Polysaccharide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laminarin</td>
<td>Substrate</td>
<td>PNPG</td>
<td>Substrate</td>
</tr>
<tr>
<td>% of</td>
<td>units/mg</td>
<td>% of</td>
<td>units/mg</td>
</tr>
<tr>
<td>total</td>
<td>protein</td>
<td>total</td>
<td>protein</td>
</tr>
<tr>
<td>activity</td>
<td>activity</td>
<td>activity</td>
<td>activity</td>
</tr>
<tr>
<td>Cell-free extract</td>
<td>100</td>
<td>108</td>
<td>100</td>
</tr>
<tr>
<td>Sediment 1 (20,000 × g)</td>
<td>3.0</td>
<td>94</td>
<td>2.4</td>
</tr>
<tr>
<td>Sediment 2 (55,000 × g)</td>
<td>9.0</td>
<td>69</td>
<td>9.2</td>
</tr>
<tr>
<td>Sediment 3 (150,000 × g)</td>
<td>2.4</td>
<td>13</td>
<td>4.0</td>
</tr>
<tr>
<td>Soluble fraction</td>
<td>85.6</td>
<td>181</td>
<td>86.0</td>
</tr>
</tbody>
</table>

Exoglucanase activities are given in micrograms of glucose produced per hour at 37°C. Endoglucanase activities are given in arbitrary units.

*P-Nitrophenyl-β-glucopyranoside.

† These fractions contain only part of the material loaded on the density gradients. They correspond to fractions 1 and 2 (plasmalemma), 4–6 (glucanase particles), and 9–11 (light membranes) of the gradient depicted in Fig. 1D.
that on p-nitrophenyl-β-glucoside is markedly lower in the glucanase particles than in the plasmalemma (Fig. 1C), which suggests a preferential binding of the former activity to the membrane. It may further be hypothesized that the third class of glucanase-containing particles (light membranes) consists of endoplasmic reticulum and represents the precursor structure of glucanase particles.

Morphologically, the budding process could be conceived as a special case of tip growth in hyphal fungi. Recent reports (18–21) have shown that the so called “Spitzenkörper” of many filamentous fungi represents an accumulation of numerous vesicles that are secreted into the wall at the hyphal tip. It may be speculated that these vesicles correspond functionally to the glucanase particles of yeast.

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