SECRETION OF β-GLUCANASE BY *SACCHAROMYCES CEREVISIAE* PROTOPLASTS

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1. Introduction

Several lines of evidence indicate that β-glucanases may play an important role in budding, conjugation and cell-wall extention in yeast [1–5]. In some cases, β-glucanases were found to be associated with yeast-cell wall [4, 5] or released into the growth medium by intact yeast cells [6]. It is also known that yeast protoplasts, under appropriate conditions, synthesize and secrete most, if not all, of the cell-wall components [7, 8]. Therefore, it was of interest to examine the release of β-glucanase by growing yeast protoplasts.

2. Materials and methods

Saccharomyces cerevisiae, strain CCY 21-4-13 was grown in 2% glucose medium [9]. Washed exponential phase cells (3 g, wet wt) were suspended in 90 ml of 0.1 M phosphate buffer (pH 6) containing 0.8 M mannitol (buffer 1). Lyophilized crude snail digestive juice (1.8 g) was added and the cells were incubated at 30° for 2 hr. This treatment resulted in an almost complete conversion of the cells into protoplasts. The protoplasts were thoroughly washed with five 50 ml portions of buffer 1, always using a clean centrifuge tube, and transferred to 100 ml of a synthetic growth medium (modified medium of Johnson [10]) consisting of: yeast nitrogen base, 6.7 g; glucose, 20 g; asparagine, 2 g; KH_2PO_4, 5 g per l and supplied with 0.8 M mannitol. Concentration of protoplasts was 3.4×10^7 per ml. To an aliquot of this suspension, cycloheximide was added to 10 μg/ml.

A part of the original cells was incubated in buffer 1 for 2 hr at 30°, then washed once with the same solution and finally suspended in the synthetic growth medium supplied with 0.8 M mannitol. The inoculum was 1.2×10^8 cells per ml.

The protoplasts and the cells were cultivated with occasional stirring at 25°. At intervals, aliquots were taken and centrifuged. The supernatants were dialyzed for 4 days against four changes of 5 mM phosphate buffer (pH 5.5) containing 1 mM mercaptoethanol (buffer 2). Sedimented protoplasts were lysed with buffer 2, the lysate was sonicated at 9 kc/sec for 20 sec, centrifuged at 100,000 *g* for 30 min and the supernatant was dialyzed for 3 days as above. The contents of dialysis bags were adjusted to the same volume with buffer 2 and assayed for activities of α-glucosidase and β-glucanase.

Laminarin (Pierce Chemical Company), *p*-nitrophenyl α-D-glucoside and *p*-nitrophenyl β-D-glucoside (both from Koch-Light) at a concentration 1 mg/ml were incubated at 30° with dialyzed growth media and protoplast lysates. Digestion of laminarin was followed by increase in reducing power, measured with the Somogyi-Nelson reagent [11] using glucose as a standard. Zero-time incubation mixtures were used as reference solutions. The hydrolysis of glucosides was determined by measuring the liberated *p*-nitrophenol [12].

3. Results and discussion

A study of the secretion of autolytic cell-wall degrading enzymes by yeast protoplasts, prepared with snail digestive juice, is associated with the danger of introducing the snail enzymes into the experimental system. A careful washing of the protoplasts might not be sufficient to avoid this danger, since they are always contaminated by cell-wall remnants which may serve

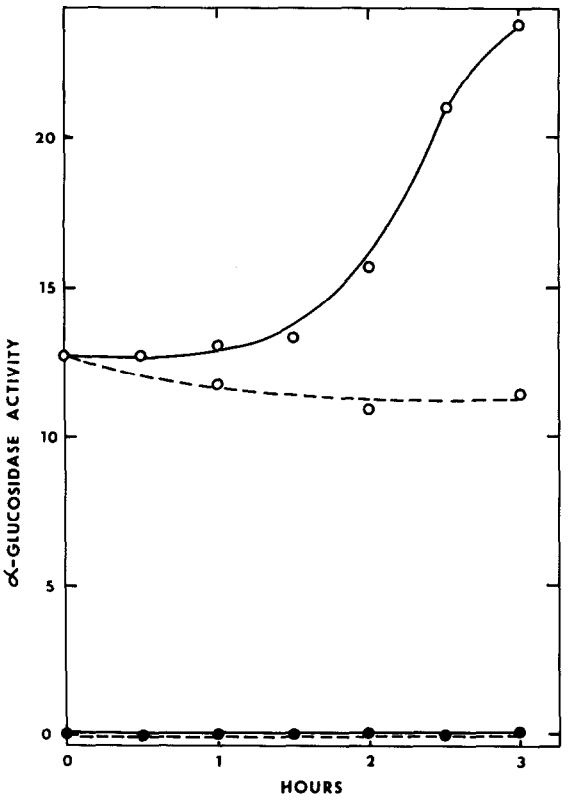

Fig. 1. α-Glucosidase activity in yeast protoplasts (○) and medium (●) during growth in the absence (—) and in the presence (---) of cycloheximide (10 μg/ml). The activity is given in μg of glucose liberated from *p*-nitrophenyl α-D-glucoside in 10 min at 30° by soluble proteins from 3.4 $\times 10^7$ protoplasts. In the case of the growth medium, no hydrolysis of the substrate occurred during 4 hr incubation.

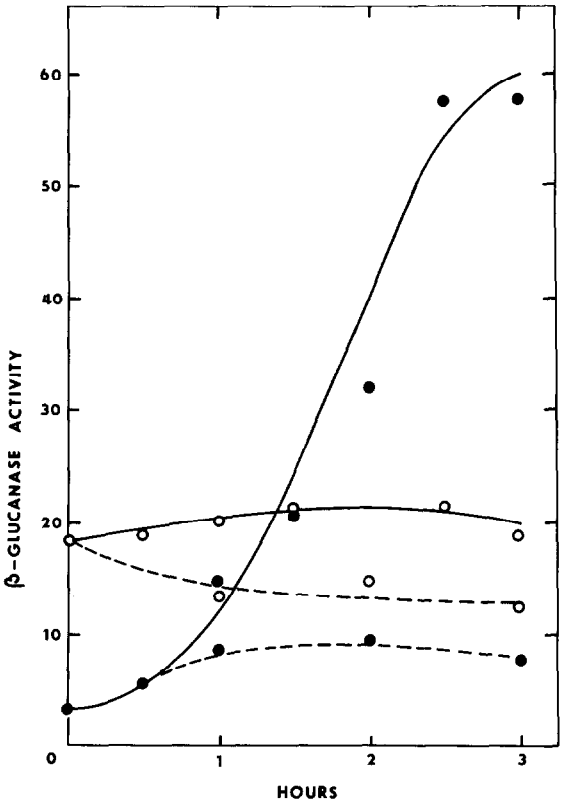

Fig. 2. β-Glucanase (laminarinase) activity in protoplasts (○) and medium (●) during growth in the absence (—) and in the presence (---) of cycloheximide (10 μg/ml). The activity is given in μg of glucose equivalents liberated from laminarin in 2.5 hr at 30° by proteins from 3.4 $\times$ 10^7 protoplasts.

as adsorbents for the snail lytic enzymes. During growth experiments a further progress in the digestion of the cell-wall material may lead to the continuous release of adsorbed enzymes into the medium. A study of the secretion of β-glucanases also requires a good stability of protoplasts since β-glucanase was reported to occur intracellularly in yeast [3, 4, 6, 12, 13]. Being aware of these facts, control experiments were carried out in order to prove that β-glucanase detected in the growth medium of protoplasts was really secreted.

An aliquot of protoplast suspension in the growth medium at zero time was lysed with buffer 2 and the lysate was centrifuged at 3000 *g*. The washed pellet was suspended in the original volume of the growth medium and incubated in parallel with protoplasts for 4 hr. The activity of β-glucanase in the pellet medium was found to be negligible in comparison with that found in the medium of intact protoplasts.

The stability of protoplasts during cultivation was checked by determination of the activity of α-glucosidase, a typical intracellular enzyme, in the growth medium [14] (fig. 1). No release of α-glucosidase occurred from protoplasts grown in the absence or in the presence of cycloheximide. The activity of intracellular α-glucosidase approximately doubled during a 3-hr growth of protoplasts. A slight decrease in the intracellular activity was recorded in the presence of cycloheximide. These results proved the good stability of protoplasts under the selected growth conditions.

Fig. 2 shows that the protoplasts of the strain of *S. cerevisiae* used secrete β-glucanase (laminarinase)

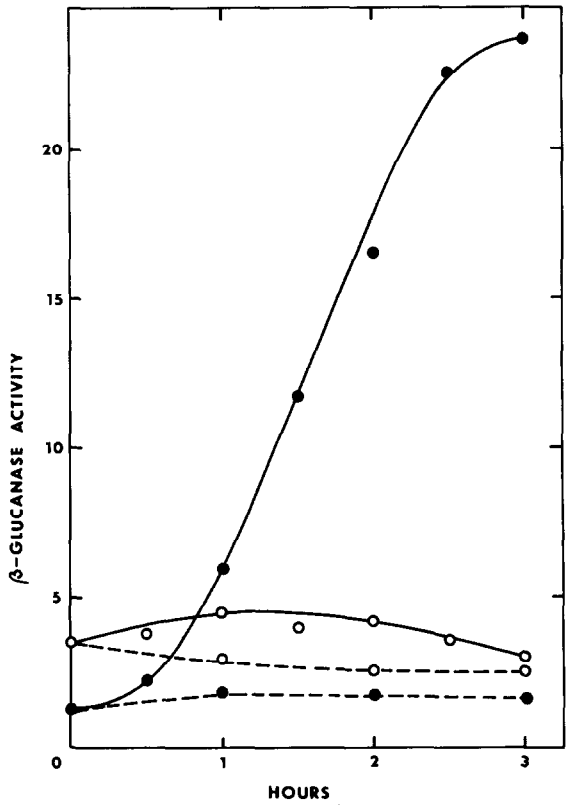

Fig. 3. β-Glucanase activity in growing protoplasts and medium assayed with *p*-nitrophenyl β-D-glucoside (symbols as in fig. 2). The activity is given in μg of glucose liberated in 2.5 hr at 30° by proteins from 3.4×10^7 protoplasts.

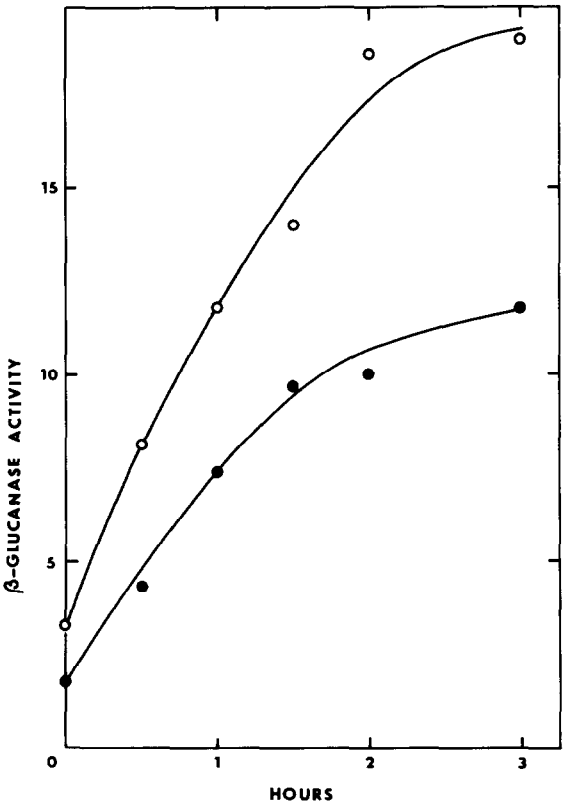

Fig. 4. Release of β-glucanase into medium by intact yeast cells during growth. The activity was assayed both on laminarin (○—○—○) and *p*-nitrophenyl β-D-glucoside (●—●—●) and is expressed in μg of glucose equivalents liberated from the substrates in 2.5 hr at 30° by proteins released by growing cells. 1.2×10^8 cells/ml was the inoculum.

into the medium. The activity of the enzyme in the medium increased with time of cultivation. De novo synthesis of the enzyme is undoubted since its extracellular appearance was strongly inhibited by cycloheximide. The level of the soluble intracellular β-glucanase did not change substantially during 3 hr. Cycloheximide brought about a slight decrease in the level of intracellular β-glucanase (fig. 2). Some β-glucanase activity was also detected in the washed particulate fraction obtained after 100,000 *g* centrifugation of lysed protoplasts. The β-glucanase secreted by the protoplasts into the medium possessed an exoglucanase activity since glucose was identified as the product of its action on laminarin. Attempts to estimate endoglucanase activity on laminarin so far gave inconclusive results.

Several examples [6, 12, 13] have demonstrated that *p*-nitrophenyl β-D-glucoside (PNPG) is the only β-glucoside serving as a substrate for yeast β-glucanases. We found that the protoplasts secrete an activity hydrolyzing PNPG but not an activity hydrolysing *o*-nitrophenyl β-D-glucoside (a chromogenic substrate for β-glucosidase), phenyl, benzyl or methyl β-D-glucosides. These results showed that the secretion of β-glucosidase did not occur and that all activity against PNPG in the growth medium was due to β-glucanase. Similar data on β-glucanase to those achieved using laminarin were obtained with PNPG as substrate (fig. 3). However, the ratio of activity on laminarin to that on PNPG was about 2–2.5:1 with secreted enzyme(s) and about 4–5:1 with the intracellular activity. This ratio was reported to

be 15:1 and 22:1 for the purified intracellular β-glucanase from *S. cerevisiae* [6] and *S. lactis* [12], respectively. A value close to unity was found with another *S. cerevisiae* strain [13]. Our ratios are in the range of values reported by Matile et al. [4]. The different ratios of activities on laminarin and PNPG found outside and inside the protoplasts indicate the existence of more than one enzyme or of different properties of the intracellular and extracellular enzymes. In analogy with other enzymes secreted by protoplasts [7], one may assume that also β-glucanase occurs in yeast in both light and heavy glycoprotein forms, the latter being transported through the plasmalemma. This assumption is, however, in contrast to the findings of Abd-El-Al and Phaff [6] that intracellular and extracellular (secreted by intact cells) β-glucanases of *Fabospora fragilis* had the same elution volume on a Sephadex column. Our preliminary results using gel filtration also did not reveal essential differences in molecular weight between the external and internal β-glucanases of the protoplasts.

We also found that β-glucanase was also released to a considerable extent by intact cells grown under the same conditions as the protoplasts (fig. 4). 1.2×10^8 cells (inoculum) secreted about half of the activity secreted by 3.4×10^7 protoplasts. This result, indicating that a portion of the β-glucanase is retained by the cell wall, should be treated with caution since the secretion of extracellular enzymes may be controlled differently in the presence and in the absence of the cell wall.

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References

[1] T.D. Brock, J. Bacteriol. 90 (1965) 1019.
[2] B.F. Johnson, J. Bacteriol. 95 (1968) 1169.
[3] C. Shimoda and N. Yanagishima, Physiol. Plant. 24 (1971) 46.
[4] P. Matile, M. Cortat, A. Wiemken and A. Frey-Wyssling, Proc. Natl. Acad. Sci. U.S. 68 (1971) 636.
[5] D.R. Barras, Antonie van Leeuwenhoek, J. Microbiol. Serol. 38 (1972) 65.
[6] A. Abd-El-Al and H.J. Phaff, Biochem. J. 109 (1968) 347.
[7] J.O. Lampen, Antonie van Leeuwenhoek, J. Microbiol. Serol. 34 (1968) 1.
[8] O. Nečas, Bacteriol. Rev. 35 (1971) 149.
[9] P. Biely, Z. Krátký, J. Kovařík and Š. Bauer, J. Bacteriol. 107 (1971) 121.
[10] B.F. Johnson, J. Bacteriol. 94 (1967) 192.
[11] L.G. Paleg, Anal. Chem. 31 (1959) 1902.
[12] M.A. Tingle and H.O. Halvorson, Biochim. Biophys. Acta 250 (1971) 165.
[13] T.D. Brock, Biochem. Biophys. Res. Commun. 19 (1965) 623.
[14] S.-C. Kuo and J.O. Lampen, J. Bacteriol. 106 (1971) 183.