JOURNAL OF BACTERIOLOGY, Sept. 1979, p. 924-931
0021-9193/79/09-0924/08$02.00/0

Vol. 139, No. 3

Synthesis of 1,3-β-Glucanases in *Saccharomyces cerevisiae* During the Mitotic Cycle, Mating, and Sporulation

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Received for publication 13 February 1979

Upon fractionating *Saccharomyces cerevisiae* asynchronous cultures by sucrose density gradient centrifugation in a zonal rotor and examining the exo-1,3-β-glucanase and deoxyribonucleic acid content of the cells, a periodic step increase in the activity of this enzyme was observed, indicating a discontinuous pattern of synthesis or activation of exo-1,3-β-glucanase during the mitotic cycle at the transition from the S to the G_2 phase. Similar results were obtained for endo-1,3-β-glucanase by assaying activity against oxidized laminarin in permeabilized cells, suggesting that the synthesis of endo-1,3-β-glucanase is controlled in the same way. When **a** and α strains were mated, the specific activity of cell extracts against laminarin, oxidized laminarin, and pustulan remained constant while zygote formation was taking place. However, when growth resumed, active synthesis of 1,3-β-glucanases took place as shown by the occurrence of a significant increase in the specific activity against the three substrates. Specific changes in the level of glucan degradative enzymes, not observed in a haploid parental strain, occurred when the diploid *S. cerevisiae* AP-1 was induced to sporulate. The sporulation process triggered the activation of first the pustulan degradative capacity and then the capacity to hydrolyze oxidized laminarin. The specific activity against this substrate was 10 times higher than that against pustulan.

It has been reported that the synthesis of yeast 1,3-β-glucanases is coupled to the morphogenetic events in which these enzymes are presumably involved (12). Cortat et al. (10) claimed that, during the mitotic cycle of *Saccharomyces cerevisiae*, the exo-1,3-β-glucanase content reaches a maximum in the cell at the time of bud emergence and suggested that the synthesis of this enzyme probably took place as a preliminary step for budding. On the other hand, studies carried out by Brock (6) showed that exo-1,3-β-glucanase synthesis in the yeast *Hansenula wingei* is activated after the mating process, indicating that the enzyme is needed for the concomitant cell fusion.

However, these data concern only exo-1,3-β-glucanase, and in *S. cerevisiae* at least another 1,3-β-glucanase (with an endohydrolytic mode of action) is produced during growth (4, 12, 33). Moreover, we have shown that exo-1,3-β-glucanase does not play a critical role in growth or mating of *S. cerevisiae* and postulated that this critical function is probably reserved for the endo-1,3-β-glucanase (33; T. Santos, F. del Rey, J. R. Villanueva, and C. Nombela, submitted for publication). As another approach towards the clarification of the functionality of glucan degradative enzymes, we have undertaken a systematic study to follow the evolution of the glucan degradative capacity during the life cycle, which includes the mitotic cycle and the mating and sporulation processes, in *S. cerevisiae*.

MATERIALS AND METHODS

Chemicals. Laminarin was purchased from Koch-Light Laboratories, Colnbrook, Buks, England. *p*-Nitrophenyl-β-D-glucoside (*p*-NPG), calf thymus DNA, and bovine serum albumin were from Sigma Chemical Co. Periodate-oxidized laminarin was prepared by the method of Hay et al. (19), and pustulan was prepared as described by Reese and Mandels (30). All other reagents were of analytical grade from commercial sources.

Organisms, media, and culture conditions. Three haploid strains and a diploid were used. *S. cerevisiae* S288C (α mating type) and *S. cerevisiae* C32C (**a** mating type) (33) were from our collection. *S. cerevisiae* A364 A (haploid) and *S. cerevisiae* AP-1, a diploid obtained in a cross of *S. cerevisiae* α, 131-20, and A364A (9), were kindly supplied by Dominguez and Haber, respectively.

Stock cultures were maintained on slants of YEDP agar (4% glucose, 4% peptone, 2% yeast extract, and 2% agar) (13), and liquid cultures were grown in flasks containing one fourth of their capacity of YED × 2 medium (4% glucose and 2% yeast extract) (13). The

flasks were incubated at 28°C with shaking in an orbital incubator (Gallenkamp). The composition of the presporulation medium was 1% potassium acetate, 0.6% yeast nitrogen base (Difco), 0.5% yeast extract, 0.5% peptone, 1.02% potassium biphthalate, 40 μg of uracil per ml, and 40 μg of adenine per ml, the pH being adjusted to 5.5 by the addition of dilute NaOH. The sporulation medium contained only 1% potassium acetate. When desired, it was buffered at pH 6.5 with 0.2 M morpholinepropanol sulfonic acid. Sporulation was induced by growing the diploid strain *S. cerevisiae* AP-1 in the presporulation medium (up to a density of 5×10^7 cells per ml) and then centrifuging and suspending the cells (3.5×10^7 cells per ml) in the sporulation medium, after washing them with portions of the same medium. Only 1/10th of the capacity of the flask was filled with medium. Presporulating and sporulating cultures were shaken at 28°C in an orbital incubator (Gallenkamp). Cells, zygotes, and asci were counted under a phase-contrast microscope by visual inspection in a THOMA hemocytometer Slide (E. Harthack, Germany) after fixation in 3.7% formaldehyde and 0.1 M NaCl.

Extract preparation. For the preparation of extracts, the cells were harvested by centrifugation at 4°C, washed with 50 mM sodium acetate buffer (pH 5.5), and suspended in this buffer. Ballotini glass beads were added, and the suspension was homogeneized in a Braun homogenizer for 75 to 90 s under dry ice refrigeration. The extracts were dialyzed overnight against 50 mM acetate buffer (pH 5.5). If required, the cells were frozen at −20°C and stored before breakage.

Zonal centrifugation in sucrose density gradients. The fractionation of asynchronous cultures was carried out by zonal centrifugation in sucrose gradients (34). A sucrose gradient (28 to 40%), generated with the use of a gradient mixer and peristaltic pump (131900 DESAGA, Heidelberg, Germany), was prepared at 4°C in a Sorvall SZ-14 zonal rotor of 1,300 ml. The gradient was generated with the rotor running at 1,000 rpm. *S. cerevisiae* S288C was grown up to an optical density of 8 U at 600 nm, growth was stopped by the addition of three volumes of cold water plus 50 μg of cycloheximide per ml, and 300 mg (dry weight) of cells were suspended in 40 ml of 7% sucrose, applied to the zonal rotor, and finally dispersed by adding 40 ml of sterile cold water. After this, the rotor was speeded up to 2,000 rpm and after 8 min decelerated, without using the break, until it stopped. The gradient was fractionated into 25-ml fractions with the use of the peristaltic pump and an Ultrorack LKB fraction collector. The first fractions corresponded to the most dense ones (see Fig. 1)

Enzyme assays and other determinations. 1,3-β-Glucanases were assayed either in cell-free extracts or in intact or permeabilized cells when the size of the sample, as in the case of the synchronized populations, was too small to permit the preparation of extracts. When the enzymes were assayed in extracts, the substrates used were laminarin, oxidized laminarin, and pustulan. An exo- and an endo-1,3-β-glucanase are produced by vegetatively growing *S. cerevisiae*. Both of them attack laminarin; oxidized laminarin is preferentially degraded by the endo-1,3-β-glucanase, and the exo-1,3-β-glucanase is also active on pustulan (1,6-β-glucan) (12, 33). Due to the periplasmic localization of exo-1,3-β-glucanase and to the fact that it is active against the synthetic derivative *p*-NPG (12), it may be assayed in intact cells with this substrate (33). Endo-1,3-β-glucanase was assayed in cells, permeabilized with toluene and ethanol as described by Sentandreu et al. (35) with oxidized laminarin as the substrate (33). This assay accounts for essentially all the endo-1,3-β-glucanase activity which can be detected in cell extracts as indicated by the fact that the values obtained when assaying either extracts or permeabilized cells of a mutant lacking exo-1,3-β-glucanase and retaining endo-1,3-β-glucanase (33) with this substrate were very similar (data not shown).

The assays were based on the release of either reducing sugar groups from laminarin, oxidized laminarin, and pustulan or of *p*-nitrophenol from *p*-NPG. In all cases the reaction mixtures contained, in 1 ml of 50 mM acetate buffer (pH 5.5), the substrate at a concentration of 0.25% and the corresponding enzyme preparation. The incubations were carried out at 37°C for periods of time ranging from 30 to 120 min, and the reaction was stopped by heating in a boiling-water bath for 3 min. The cells or precipitated materials were removed by centrifugation, and the supernatant fluids were used to determine either reducing sugars by the method of Somogyi (36) and Nelson (28) or *p*-nitrophenol by adding 4.5 ml of 4% Na_2CO_3 to 0.5 ml of the supernatant fluid and determining the optical density at 420 nm. One unit of enzyme will liberate 1 nmol of the corresponding reaction product per min under the conditions of the reaction. Protein was determined by the method of Lowry et al. (24) with bovine serum albumin as standard, and the specific activity was expressed as units of enzyme per milligram of protein. DNA was measured by the diphenylamine method of Burton (8) modified by Herber et al. (20).

RESULTS

Synthesis of 1,3-β-glucanases during the vegetative cell cycle. Two enzymes, capable of degrading β-glucans, have been found to be produced by vegetatively growing cells of *S. cerevisiae* (4, 33). One is an exo-1,3-β-glucanase, active against laminarin and *p*-nitrophenyl-β-D-glucose, which can also hydrolyze 1,6-β-glucan (pustulan). The other is an endo-1,3-β-glucanase, which hydrolyzes laminarin and oxidized laminarin but neither *p*-NPG nor pustulan. To study the production of these enzymes during the cell cycle, we have followed, slightly modified, the procedure described by Sebastian et al. (34), which consists of fractionating asynchronous cultures of *S. cerevisiae* by sucrose gradient zonal centrifugation to obtain populations of cells corresponding to the different stages of the mitotic cycle (18) which were used to measure their content in 1,3-β-glucanases (Fig. 1 and 2). As shown in Fig. 1a, when an asynchronous culture of *S. cerevisiae* S288C was fractionated, the top fractions (fraction 40 to 36) contained

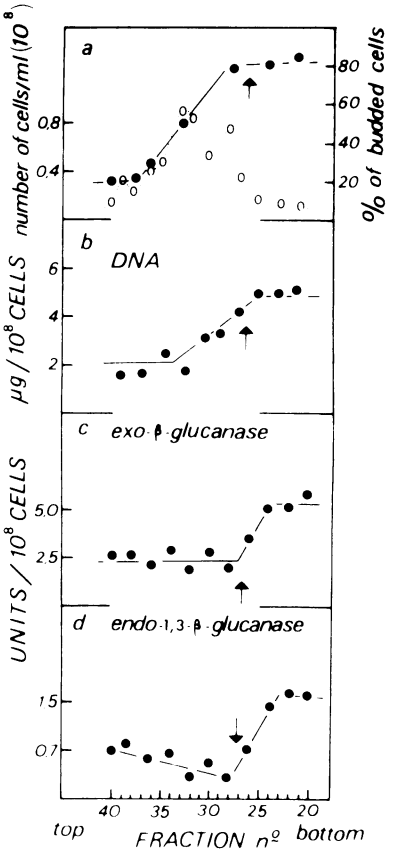

FIG. 1. *1,3-β-glucanase content of cell populations of S. cerevisiae S288C fractionated by zonal centrifugation (a) Cell concentration of the fractions (○) and percent by budded cells (●). (b) DNA content. (c) Exo-1,3-β-glucanase content (assayed against p-NPG in whole cells). (d) endo-1,3-β-glucanase content (assayed in permeabilized cells against oxidized laminarin). Arrows mark the time of induction of exo- and endo-1,3-β-glucanase.*

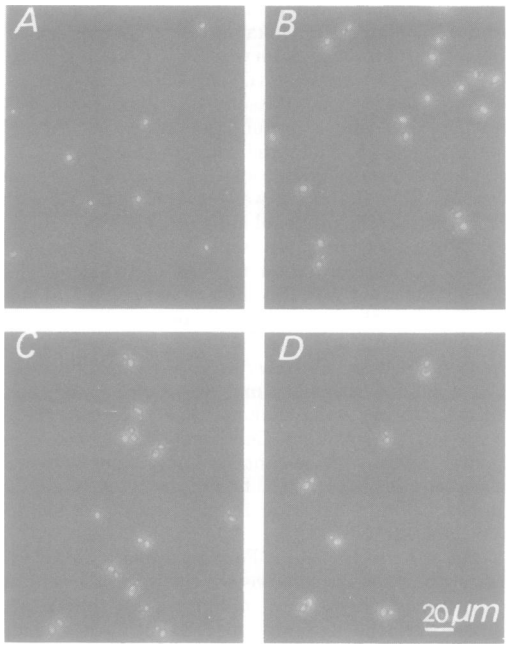

FIG. 2. *Phase-contrast micrographs of cell populations fractionated by zonal centrifugation, corresponding to the experiment of Fig. 1. (a) Fraction 39; (b) fraction 31. Note the abundance of cells with a tiny bud in (c) fraction 27 (beginning of glucanase synthesis, see Fig. 1). (d) Fraction 25.*

almost exclusively unbudded cells (Fig. 2a). The percentage of budded cells increased from fraction 36 to 28. Note the tiny buds of at least 50% of the cells of fraction 31 (Fig. 2b). Finally, the fractions at the bottom (fractions 28 to 20) contained mostly budded cells, with buds of a larger size, closer to that of a daughter cell (Fig. 2c and d).

The DNA content of this haploid strain was of about 2.5 ± 0.08 µg/10^8 cells in fractions 40 to 33 and doubled between this latter fraction and 26 (Fig. 1b). These results indicated that fractions 40 to 33 mostly contained cells at different stages of the G_1 phase; the cells synthesizing DNA (at the S phase) appeared from fraction 33 to 26, and the most dense fractions, 26 to 20, consisted of cells at the G_2 and ND (nuclear division) phases and beginning of G_1 again.

The exo-1,3-β-glucanase content, measured in whole cells against *p*-NPG, was about 2.5 ± 0.08 U/10^8 cells from fraction 40 to 27; it reached a double value from fraction 27 to 24 and maintained it in the rest of the most dense fractions (Fig. 1c). Something similar was observed when the activity against oxidized laminarin was determined in permeabilized cells of the different fractions (Fig. 1d). The activity content doubled (0.7 ± 0.02 to 1.5 U/10^8 cells) from fraction 27 to 24, although in this case the level decreased from fraction 40 to 28, suggesting a certain degree of inactivation during the periods preceding synthesis. These results are consistent with the idea that both 1,3-β-glucanases of *S. cerevisiae* are synthesized or activated once per mitotic cycle after the DNA synthesis period, at the beginning of the G_2 phase.

Synthesis of 1,3-β-glucanases during mating. The synthesis of 1,3-β-glucanases during the mating process was also examined. In *S. cerevisiae* the hormone of the opposite mating type arrests cell growth at the G_1 phase (5, 7, 17). In agreement with this, we observed that when populations of **a** and α mating types were mixed together, during the first 250 min the

optical density of the mixed cell suspension increased very slowly (Fig. 3a) while zygote formation was taking place (Fig. 3b). The level of specific activity against laminarin, oxidized laminarin, and pustulan remained constant or even decreased to a small extent (Fig. 3c). However, when zygote formation had finished and growth resumed, as shown by the significant increase in the turbidity of the cell suspensions, this was also accompanied by an increase in the levels of specific activity against the three substrates. It is therefore clear that no active synthesis of 1,3-β-glucanases took place at the moments preceding or during mating but only when the process was finished and growth resumed.

Synthesis of 1,3-β-glucanases during sporulation. The sporulation process consists of a sequence of events leading to meiosis and ascus formation (15). In view of the morphogenetic activity which it involves, we decided to examine the variations in the levels of activities responsible for glucan degradation throughout this process. As shown in Fig. 4b, under our experimental conditions almost 75% of the cells of the diploid strain *S. cerevisiae* AP-1 were induced to sporulate. This was accompanied by very significant changes in the specific activity of cell extracts against laminarin and oxidized laminarin (Fig. 4c). Initially, it was practically undetectable but, after 18 h in sporulation medium, it had reached a value of almost 16 U/mg of protein, which is higher than we have ever observed in cell extracts of *S. cerevisiae* during vegetative growth. Also significant were the

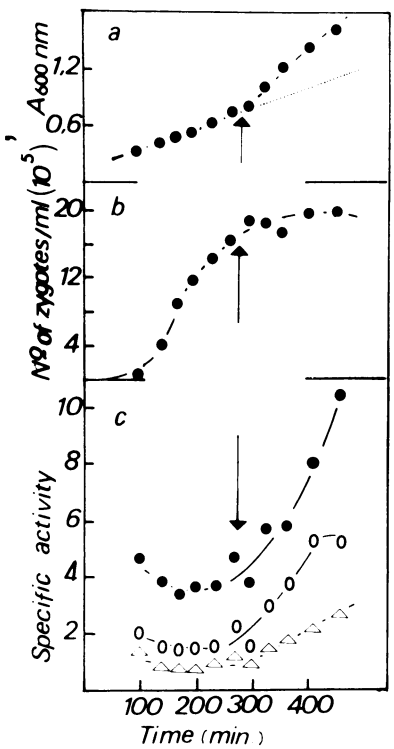

FIG. 3. *Synthesis of 1,3-β-glucanases during the mating process. Actively growing cultures of S. cerevisiae S288C and S. cerevisiae C32C were mixed together, in equal proportions to a concentration of* 5.6×10^6 *cells per ml. at zero time. (a) Optical density of cell cultures at 600 nm. (b) Zygote concentration. (c) Specific activity of cell extracts against laminarin (●), oxidized laminarin (○), and pustulan (△).*

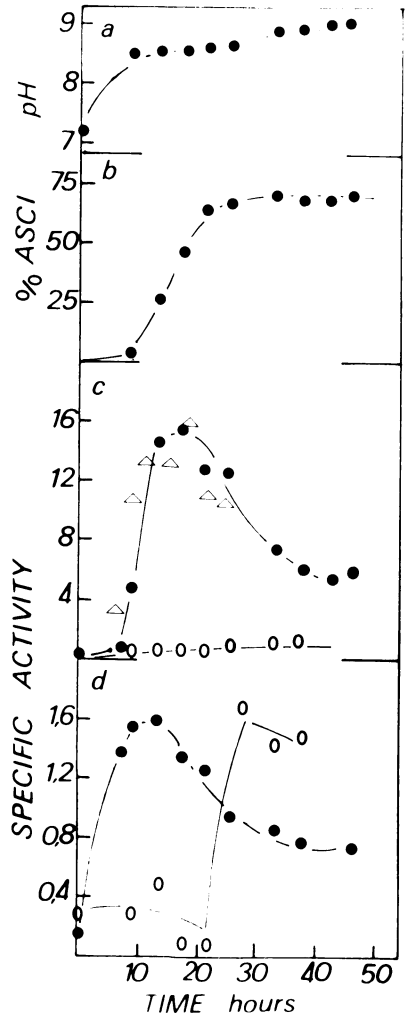

FIG. 4. *Synthesis of 1,3-β-glucanases by S. cerevisiae AP-1 during sporulation. (c) Specific activity of cell extracts against laminarin (△) and oxidized laminarin (●). Control: specific activity against oxidized laminarin (○) in cell extracts of the haploid strain S. cerevisiae A364A. (d) Specific activity against pustulan in cell extracts of S. cerevisiae AP-1 (●) and A364A (○).*

changes in specific activity against pustulan (Fig. 4d). This activity was very low at the time of transfer of the cells to the sporulation medium, reached a maximum after 10 h (when complete asci commenced to be observed), and decreased thereafter. As a control, cells of the haploid strain *S. cerevisiae* A364 A, one of the parents of diploid *S. cerevisiae* AP-1, were incubated under sporulation conditions. Almost no oxidized laminarin-hydrolyzing activity was found in its extracts after 40 h of incubation in the sporulation medium, and only activity against pustulan was observed, but much later than in the diploid (Fig. 4d).

This evidence clearly shows that the sporulation process leads to the sequential induction of glucan degradative enzymes; the activity against pustulan appeared first, followed by the activity against oxidized laminarin. The sequential induction of the two enzymes is more clearly illustrated in Fig. 5, where the rates of variation of the two specific activities were plotted against time. The maximum rate of increase occurred about 4 h later in the case of activity against oxidized laminarin than in the case of the pustulan-hydrolyzing enzyme.

Finally, Fig. 6 shows the results of an experiment, similar to that represented in Fig. 4, but carried out in a buffered medium to exclude the possibility that pH changes caused the inactivation of the enzymes and might be responsible for the variations in the levels of glucan degradative activities. Under these conditions, the percentage of sporulating cells was less than 50% and the capacity to degrade oxidized laminarin was also lower, although it showed a similar pattern of variation. The specific activity against pustulan reached, in the first 10 h, a value close to that of the previous case; then it diminished, and a new increase was observed later on.

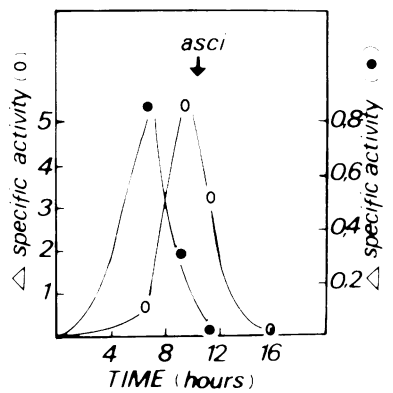

FIG. 5. *Rate of increase in specific activity against pustulan* (●) *and oxidized laminarin* (○) *during sporulation of S. cerevisiae AP-1. Data of Fig. 4 were used. Each value was calculated dividing the increments in specific activity by the time (hours).*

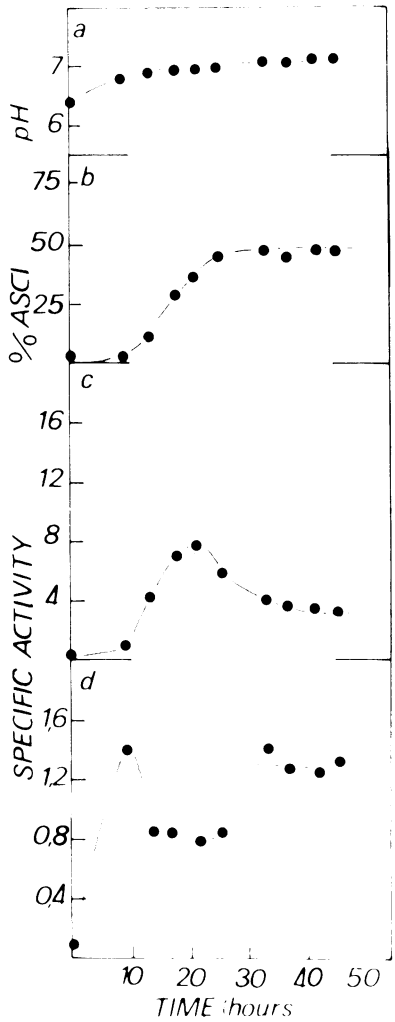

FIG. 6. *Synthesis of 1,3-β-glucanases by S. cerevisiae AP-1 during sporulation in a buffered medium. Specific activity against oxidized laminarin (c) and pustulan (d) in cell extracts of S. cerevisiae AP-1.*

DISCUSSION

S. cerevisiae produces two 1,3-β-glucanases (4, 33), both of which are glycoproteins. The most abundant is an exo-1,3-β-glucanase which attacks the polymer at the nonreducing ends and is also active against 1,6-β-glucan. The other enzyme is the endo-1,3-β-glucanase which splits internal linkages and is therefore active against periodate-oxidized laminarin (12, 33). In contrast to some other fungal species, synthesis of 1,3-β-glucanases in *S. cerevisiae* is constitutive during

vegetative growth and not catabolite repressible (31).

Data on the regulation of the production of these enzymes during the cell cycle are scarce; Cortat et al. (10) found that the exo-1,3-β-glucanase synthesis parallels emergence of the bud. It, therefore, would be predicted that the synthesis of exo-1,3-β-glucanase during the cell cycle must take place at the end of the G_1 phase or at the beginning of the DNA synthesis period (18).

To carry out a study of the synthesis of enzymes involved in glucan degradation during the yeast mitotic cycle, we have fractionated synchronous cultures by sucrose density gradient centrifugation in a zonal rotor as described by Sebastian et al. (34). This procedure has already been used with success by other workers for the analysis of different aspects of the yeast cell cycle (29, 37). Rate sedimentation in gradient zonal rotors is an accurate method to separate cells on the basis of size and, thus, age (34, 37), so that upon centrifugation of a logarithmic population of cells, the cell cycle is represented across the rotor. Analysis of cell cycle-dependent phenomena by this procedure avoids many of the difficulties associated with synchronously growing cultures (34, 37).

In contrast to the findings of Cortat et al. (10), we observed that the level of exo-1,3-β-glucanase was the same in all the fractions of cells at different stages of the G_1 and S phases; it started to increase in the last fraction of cells synthesizing DNA and reached a double value in those fractions which contained mostly cells with a big bud. A similar result was obtained when the activity against oxidized laminarin was measured in permeabilized cells. The amount of enzyme in cells at G_2 and ND phases was twice that of unbudded cells, and the step occurred in the same fractions as in the case of exo-1,3-β-glucanase. The only difference was that the level of activity decreased from the unbudded cells fractions through the ones corresponding to the next stages of G_1 and to the S phase until the corresponding step was observed.

We believe that these results are consistent with the notion that, as in the case of other enzymes, the synthesis of both 1,3-β-glucanases of *S. cerevisiae* is discontinuous during the cell cycle (16, 27), and it takes place once per cycle at the beginning of the G_2 phase. However, as we measure enzyme content in cells at different stages of the cell cycle instead of enzyme synthesis, we cannot rule out the alternative hypothesis that the step increase in enzyme activity could be due to periodic activation rather than periodic synthesis (11). The decrease in the level of endo-1,3-β-glucanase at the stages preceding its synthesis could indicate a certain degree of inactivation, probably due to the lower stability of this enzyme compared with that of exo-1,3-β-glucanase. The reason for the discrepancy between our results and those of Cortat et al. (10) is not apparent to us. Although these workers employed a different method consisting of isolating a fraction of cells, growing them in synchrony for several generations, and determining the enzyme content in samples taken during the growth cycles, it is difficult to imagine how this could affect the result. In any case, it is clear that the synthesis of 1,3-β-glucanases at G_2 or at any other phase of the cycle can provide the cell with enough of the enzymes for bud emergence or for any other function that 1,3-β-glucanases might serve, such as cell wall extension or daughter cell separation (1, 21). Moreover, we have shown that exo-1,3-β-glucanase is not critically needed for bud emergence in *S. cerevisiae* (33).

To follow the change in the levels of 1,3-β-glucanases during mating, we also measured the activity against laminarin, oxidized laminarin, and pustulan in cell extracts of mixed populations of cells of opposite mating types. No significant variation in the specific activity against each one of the three substrates was observed upon mixing the cells and during the period of zygote formation. But upon resumption of growth, active production of the enzymes took place, as shown by the significant increase in the specific activities which occurred thereafter. These results are consistent with the evidence presented above regarding the synthesis of 1,3-β-glucanases during the cell cycle. Upon mixing populations of cells of opposite mating types, growth is arrested at the G_1 phase (5, 7, 17) while mating takes place and leads to zygote formation. Under these conditions one may expect that no synthesis of exo- and endo-1,3-β-glucanase will occur until zygote and cells which have not mated resume growth. Brock (6) reported that after conjugation of two strains of *Hansenula wingei* the level of exo-1,3-β-glucanase attained a higher value, suggesting that an activation of the synthesis of the enzyme takes place some time during the mating process. These experiments were carried out in a nitrogen-free medium, which excludes the possibility of resuming growth, but where *H. wingei* (not *S. cerevisiae*) is capable of mating. Therefore, these results are difficult to compare with ours, and it is possible that exo-1,3-β-glucanases of *H. wingei* and *S. cerevisiae* are controlled in a different way.

In any case, it is clear that the conclusions

which attribute a certain function to glucanases on the basis of the time of their synthesis should be regarded with caution. Exo-1,3-β-glucanase does not seem to be a critical enzyme for mating in *S. cerevisiae*, since mutants lacking this enzyme mate normally (Santos et al., submitted for publication). Endo-1,3-β-glucanase might be necessary, but the level attained during vegetative growth may be high enough to serve any function in which it might be involved during the morphogenetic changes which accompany the mating process.

A third important part of the *S. cerevisiae* life cycle is sporulation which takes place in diploid cells. This involves a sequence of events which determine important changes from the morphological and biochemical point of view. Ascus formation is accompanied, for example, by an increase in the levels of degradative enzymes such as proteases (3, 23), RNAses (2), and glycogen degradative activities (9). It is interesting to note that during the meiotic cycle, many enzymes which do not seem to be specifically involved in sporulation are also synthesized in an ordered sequence which very closely reproduces the timing of the mitotic cycle (25, 26). Because the biosynthesis of the ascospore cell wall (14, 22, 32) might demand the participation of 1,3-β-glucanases, it was of interest to follow the evolution of glucan degradative activities.

A very different picture from the situation in the mitotic cycle emerged from these results. Significant increases in the level of specific activities against pustulan, laminarin, and oxidized laminarin took place during sporulation. However, the activation of the enzymes was not simultaneous, but rather was sequential. First, a maximum in the pustulan degradative capacity was reached, followed by the increase in the capacity to degrade oxidized laminarin. The specific activity against this latter substrate was much higher than that against pustulan. These results indicate that the enzymes involved in glucan degradation are induced during the sporulation process in a specific fashion. These enzymes synthesized during meiosis could be the same as those produced during the mitotic cycle. If this is the case, then it is interesting that their relative proportions and times of synthesis are clearly altered upon sporulation. But it is also possible that the production of a new enzyme(s) with 1,3-β-glucanase activity is induced in the process of forming of asci. This possibility is currently being tested in our laboratory.

ACKNOWLEDGMENTS

F. del Rey and T. Santos were recipients of fellowships from Ministerio de Educación y Ciencia. This investigation was aided by a grant from Comisión Asesora de Investigación Científica.

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