RELATIONSHIP BETWEEN EXTRACELLULAR ENZYMES AND CELL GROWTH DURING THE CELL CYCLE OF THE FISSION YEAST *SCHIZOSACCHAROMYCES* *POMBE* II. SUCRASE.

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By using intact cells of the fission yeast *Schizosaccharomyces pombe*, the derepressed activity of sucrase (EC 3. 2. 1. 26), as extracellular enzyme located in the periphery of the yeast, was measured during the cell cycle, with cell length serving as the measure of cell growth. Both specific activity of sucrase and average cell length were constant through asynchronous cultures. Under the same experimental conditions as for asynchronous cultures, the specific activity of sucrase showed a pattern of periodic synthesis after either selection synchronization by the gradient sedimentation method or induction synchronization by pulse treatment with hydroxyurea (HU), an inhibitor of DNA synthesis. On the other hand, the specific activity of sucrase continued to increase exponentially when HU was added to the asynchronous culture or to the selection-synchronous culture, but decreased stepwise in induction-synchronous cultures in a poor medium after selection, in which the cells divided synchronously without cell elongation. The patterns of sucrase activity in all systems were parallel to those for average cell length.

These results suggest that the synthesis of sucrase is dependent on the growth in cell length as well as acid phosphatase as we reported in a previous paper (1).

Schizosaccharomyces pombe, a fission yeast, is a rod-shaped cell which grows mainly in length during its cell cycle. This growth in length does not occur at a constant rate; cell length increases exponentially for the first three-quarters of the cell cycle and remains constant for the last quarter (2–4). Thus growth in length

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is periodic throughout the cell cycle and is proportional to increase in cell volume, which is one of the possible measurements of cell growth. Therefore, by using growth in length as a measure of total cell growth of the fission yeast, we have examined whether the synthesis of the extracellular enzyme located on the periphery (*i.e.*, outside the cell membrane, but within the cell wall) is coordinated with cell growth, or with cell division. In this experiment, in addition to selection- and induction-synchronous cultures we prepared synchronous cultures in which hydroxyurea (HU) was used in order to distinguish cell growth from cell division. In such cultures, the specific activity of acid phosphatase changed in parallel with the change in average cell length as has been previously reported (1).

In this experiment, a second extracellular enzyme, sucrase, was investigated by the same experimental systems. MITCHISON and CREANOR (5) and SISSONS *et al.* (6) have demonstrated that the potential for sucrase synthesis is associated with cell division and DNA synthesis, but not with overall cell growth. Our research, however, has resulted in somewhat different findings from the above, and has given results similar to those for acid phosphatase (1).

MATERIALS AND METHODS

Yeast strain and cultivation. Fission yeast, *Schizosaccharomyces pombe* IFO 0365, was grown in the same culture medium as previously described (1) on a reciprocating shaker (140 rpm). The incubation temperature was 32° for precultures and 28° for experimental cultures except where noted. Precultures were subsequently grown twice. A starter culture from the stock culture was inoculated into 5 ml of the culture medium and incubated for 24 hr; the culture was then diluted with fresh medium to a cell concentration of 0.4% and incubated as the second preculture. The changes in sucrase activity during the second preculturing were tested in order to determine the time when cells should be transferred to experimental media. The specific activity of sucrase increased during the lag phase, then decreased until the mid-log phase, reached a minimum between mid-log and late-log phases, and increased sharply during the stationary phase at which time reducing sugars in the medium had been reduced to less than 0.4% from the maximum concentration of 2.0%. The late-log phase cells cultured for 15 hr which showed a low level of sucrase activity were used as inoculum for the experimental cultures except where noted.

In the second preculture, we observed that sucrase activity is dependent on the sugar concentration in the medium. Accordingly, we assume that the changes in sugar concentration in the medium are caused by the inoculated cells and affect sucrase activity. Thus, the condition in which sucrase activity is unaffected by sugar concentration, that is, total sucrase activity increasing continuously and exponentially through asynchronous control cultures, had to be used. Then, the connection between initial cell concentration and total sucrase activity was tested.

As a result, at initial cell concentrations less than 2×10^6 cells/ml the patterns of total sucrase activity showed a continuous and exponential increase during the 6-hr cultivation (see Fig. 2); reducing sugar concentrations in the medium varied slightly (from 1.4% to 1.2%). The size of the inoculum into the experimental culture was therefore controlled to obtain the initial cell concentration as follows: Asynchronous control cultures were obtained by adding 10 ml of the second preculture to 150 ml of fresh medium at time zero. Synchronous cultures were produced as has been previously described (1) with the exception of the modification described below. At the point of selection synchrony, the second preculture of 120 ml was harvested, selected by the sucrose gradient sedimentation method, and suspended in 160 ml of fresh medium. In induction synchrony, the cells harvested from the second preculture of 5 ml were inoculated into 10 ml of fresh medium containing 8 mM HU, and incubated for 3 hr at 32°. The resulting oversized cells were collected and added to 160 ml of fresh medium.

The degree of synchrony was monitored by estimating the cell plate index, that is, the percentage of cells with cell plates, which is similar to the mitotic index for higher cells (7). The number of total cells and of cells with cell plates were counted on a Bürker-Türk hemocytometer slide (1).

Determination of derepressed sucrase activity. Sucrase activity was found to depend on the sugar concentration in the medium as above mentioned. Also MITCHISON and CREANOR (5) have reported that sucrase activity in *Schizosaccharomyces pombe* NCYC 132 is dependent on the glucose concentration in the medium, and that the enzyme can be rapidly derepressed by reducing the glucose concentration from 1% to 0.05%. They used the derepression procedure to measure the sucrase "potential" or capacity for synthesizing sucrase during derepression (5, 6).

In this experiment, procedures similar to those of MITCHISON and CREANOR (5) were used to measure the derepressed sucrase activity, except for Fig. 6. The rise in sucrase activity during derepression is shown in Fig. 1. The activity started to rise after a short time, followed a linear course and reached a steady-state after 3 hr. The presence of HU (8 mM) showed a slight inhibitory effect on the sucrase activity. During the depression there were small increases in cell number and in cell length. Accordingly, we followed the changes in sucrase activity during the cell cycle by measuring the activity derepressed for 3 hr in samples taken from experimental cultures. The procedure was as follows: 2-ml samples that were taken from the experimental cultures at intervals of 15 min were diluted with 40 ml of 0.85% NaCl solution (thus reducing the reducing sugars from 1.4–1.2% to 0.067–0.057%) and incubated at 28°. After a 3-hr derepression, cells were collected on a membrane filter, washed with saline and suspended in 1 ml of saline as described previously (1). The cell suspensions were used immediately thereafter for the enzyme assay.

In the culture in the poor medium (0.5% malt extract; 0.28% as to reducing

Fig. 1.

Fig. 1. Derepressed patterns of sucrose activity.

The growing culture was diluted $\times 21$ with 0.85% NaCl solution at time zero. Symbols: average cell length in the presence (▼) or the absence (▽) of 8 mM HU; cell number in the presence (▲) or the absence (△) of HU; sucrose activity in the presence (●) or the absence (○) of HU; sucrose activity of growing cells transferred to the saline solution at time zero after washing (×).

Fig. 2.

Fig. 2. Sucrase activity and cell length during asynchronous control culture.

The standard deviation in cell length was 0.09, plotted against a log scale.

sugar concentration) (Fig. 6), sucrose activity was measured without the "derepression treatment" described above, because sucrose activity could be derepressed by transferring the cells to the poor medium. Instead of the "derepression treatment," the oversized cells were incubated for 2 hr at 32° in the poor medium containing 16 mM HU before starting the experimental culture. During this incubation, the cells neither elongated nor divided. The cells in 2-ml samples taken from the experimental culture every 15 min were immediately collected, washed and suspended in 1 ml of saline to measure the enzyme activity.

Sucrase assay was as follows: The substrate mixture (0.3 ml of 0.048 M sucrose;

0.2 ml of 0.2 M acetate buffer, pH 5.0; 0.3 ml of water) was preincubated for 5 min at 37°. The reaction was started by adding the cell suspension (0.2 ml), and stopped after 10 min by raising the pH to 11.0 by the addition of 1 ml of 0.02 N NaOH. The cells were removed by centrifugation. The concentration of reducing sugars in the supernatant was determined by the SOMOGYI-NELSON procedure (8). Absorption at 500 nm was read against a blank (where the cell suspension was added after the alkali). Under this condition, the rate of product formation was linear over the 10-min period. Total enzyme activity is expressed in μg of glucose per minute per milliliter of culture; specific activity is expressed in μg of glucose per minute per cell.

RESULTS

Properties of sucrase

The hydrolytic activities of intact cells were tested for various disaccharides—sucrose, raffinose, maltose, trehalose, gentibiose, cellobiose, and melibiose. This yeast cell displayed a high specificity for sucrose and a low specificity for raffinose; the activity ratio of the former was approximately four times that of the latter. The other disaccharides examined, however, showed only slight or no cleavage. Thus, we designated the disaccharide-hydrolyzing enzyme as sucrase.

The optimum pH was found to range between 4.5 and 5.0. This value is similar to that for those saccharases in several species of *Schizosaccharomyces* determined with dried cells (9). This result is different from that for sucrase in *Schizosaccharomyces pombe* NCYC 132 with freeze-dried cells (5), where the optimum pH was 6.6. These values, however, cannot be compared directly because the activities of dried and freeze-dried cells are equal to the sum of the activities of intracellular and extracellular enzymes.

The localization of sucrase was tested by comparing its activity in intact cells with that in an equal number of cells whose permeability barriers were broken by different physical and chemical treatments—frozen-thawed 8 times in 2 min, sonicated for 2 min at 9 kHz, or incubated for 20 min in 0.65 M butanol at 32°. The activity of living cells was at a level 80% of that of the average of treated cells. These findings indicate that sucrase in this yeast is largely external; about 20% of the total sucrase may be intracellular and not accessible to the external substrate. Under our cultural conditions, sucrase was not secreted into the medium by yeast cells.

Relation between the increase in sucrase and the growth in cell length

A growing asynchronous culture was first examined as a control culture system, shown in Fig. 2. The cell number and total sucrase activity increased exponentially; both generation time and doubling time for total activity were about 145 min. Accordingly, specific activity was constant through the culture. The average cell length also remained constant.

Next, synchronous cultures were prepared by selection or by induction. In both systems, the degree of synchrony at the time of first division was very high—40–50% as shown in Figs. 3 and 4. In selection synchrony (Fig. 3), the cycle duration which was indicated by the time interval between the first and second peaks of the cell plate index was 120 min. In the induction synchrony (Fig. 4), the first two shorter cycles (90 and 105 min) were followed by normal cycle duration, as reported previously (1). The oversized cells created by induction returned to their normal size after three divisions. In both synchronous cultures, in spite of these different cycle durations the total activity of sucrase varied periodically with the same cycle as cell division, although the activity was not doubled during one cell cycle. Such specific activities varied periodically with the average cell length and gave similar patterns. The average cell length during growth in synchronous cultures approached that of a single cell (2, 3). Broad peaks for average cell length appeared just before peaks for the cell plate index, in accordance with the timing for the constant length phase in a single cell.

Fig. 3. Sucrase activity and cell length during synchronous culture produced by selection.

Vertical lines show the position of the cell plate index peaks.

Fig. 4. Sucrase activity and cell length during induction-synchronous culture. Vertical lines show the position of the cell plate index peaks.

The third experimental system in which cells continue to increase in length without division was produced by adding HU to a selection-synchronous culture (Fig. 5A) or to an asynchronous culture (Fig. 5B). In both systems (i) the first cell division occurred normally; (ii) the subsequent cell cycle was considerably delayed; and (iii) cells increased continuously and exponentially in length after cell proliferation terminated and they became oversized (1, 10). Total and specific activities of sucrase continued to increase proportionally with average cell length after cell proliferation terminated.

The last experimental situation was that in which the cells were unable to elongate but divided synchronously when the oversized cells were transferred to a poor medium as shown in Fig. 6. The average cell length decreased stepwise in relation to the two divisions.

The average cell length at division (10 μm after the first cell division) does not equal twice the average length of the daughter cells at birth (6.5 μm after the second cell division). When the correction factor (1.3 μm according to FANTES (11), 1.4 μm according to MIYATA *et al.* (12)) for the rounding off of daughter cells is subtracted from the average daughter cell length measured, the average cell length is almost half that of the mother cells. This indicates that cells show minimal longitudinal growth. In this system, the total activity of sucrase remained almost

Fig. 5. Effect of continuous treatment with HU on sucrose and cell length during growth.

(A) a selection-synchronous culture prepared by sucrose gradient sedimentation, to which HU (final concentration 8 mM) was added when cultivation was started. The vertical line shows the position of the cell plate index peak with a value of 40%. (B) an asynchronous culture in the presence of 8 mM HU.

Fig. 6. Sucrase activity and cell length in oversized cells cultured in a poor medium.

The oversized cells were produced by a 4-hr treatment at 32° with HU (16 mM) added to 13-hr-grown cells previously selected by gradient sedimentation. Then, these cells were harvested, suspended in the poor medium containing 16 mM HU and incubated at 32° for 2 hr. Finally, the cells harvested were transferred to the fresh poor medium to start the experimental culture at 32°. The vertical lines show the position of the peaks of the cell plate index.

constant throughout two cycles examined. Specific activity decreased stepwise in parallel with decrease in average cell length.

DISCUSSION

The results of the tests for localization of sucrase suggest that this fission yeast has an external and an internal enzyme, as in budding yeast (13); the greater part of the sucrase is external to the cell membrane but within the cell wall, as in *Schizosaccharomyces pombe* NCYC 132 (5). In this study, sucrase activity measured by using intact cells is that of the extracellular enzyme which is located on the periphery.

Hexose repression of sucrase formation has been described in budding yeasts (14, 15). Similar behavior was also found in the fission yeast (5). MITCHISON and CREANOR (5) and SISSONS *et al.* (6) used the rate of enzyme synthesis during derepression of samples taken from cultures as a measure of the potential for sucrase synthesis. They reported that this potential stops when DNA synthesis is stopped by continuous treatment with deoxyadenosine, although cell growth continues. Moreover, they showed that in the induction-synchronous culture prepared by pulse treatment with deoxyadenosine the potential for sucrase was synchronized to that of the step pattern during the cell cycle as in selection synchrony. These results have been interpreted to show that a potential enzyme like sucrase is associated with the DNA-division cycle in a model where a cell cycle is composed of two loosely coupled cycles, the growth cycle and the DNA-division cycle (16).

We also observed hexose repression of sucrase formation in our yeast, thus used the derepressed-sucrase activity as a measure of enzyme synthesis. The results in which sucrase synthesis in the induction synchrony is synchronized with a step pattern as in selection synchrony are similar to those for the sucrase potential in strain NCYC 132. If the synthesis of this sucrase, therefore, is associated with the DNA-division cycle, the following is predicted: (i) In an experimental system in which DNA synthesis is blocked by HU after selection synchronization, sucrase synthesis will stop although cell growth continues synchronously; whereas, (ii) in the reverse experimental system in which DNA synthesis and cell division continue synchronously although cell growth is repressed, sucrase synthesis will follow a step increase. In our results, however, the behavior of sucrase was not consistent with the above prediction. In the first system, the total activity of sucrase increased exponentially, and in the second system it remained constant through two generations. On the other hand, growth in length also continued exponentially when DNA synthesis was blocked by HU, but stopped when cell growth was repressed by nutritional limitations although the DNA-division cycle continued. Consequently, the specific activity of sucrase was not associated with the DNA-division cycle but was always proportional to the growth in length.

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