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Glycogenolytic Enzymes in Sporulating Yeast

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During meiosis in *Saccharomyces cerevisiae*, the polysaccharide glycogen is first synthesized and then degraded during the period of spore maturation. We have detected, in sporulating yeast strains, an enzyme activity which is responsible for the glycogen catabolism. The activity was absent in vegetative cells, appeared coincidently with the beginning of glycogenolysis and the appearance of mature ascospores, and increased progressively until sporulation was complete. The specific activity of glycogenolytic enzymes in the intact ascus was about threefold higher than in isolated spores. The glycogenolysis was not due to combinations of phosphorylase plus phosphatase or amylase plus maltase. Nonsporulating cells exhibited little or no glycogen catabolism and contained only traces of glycogenolytic enzyme, suggesting that the activity is sporulation specific. The partially purified enzyme preparation degraded amylose and glycogen, releasing glucose as the only low-molecular-weight product. Maltotriose was rapidly hydrolyzed; maltose was less susceptible. Alpha-methyl-D-glucoside, isomaltose, and linear α-1,6-linked dextran were not attacked. However, the enzyme hydrolyzed α-1,6-glucosyl-Schardinger dextrin and increased the β-amylolysis of β-amylase-limit dextrin. Thus, the preparation contains α-1,4- and α-1,6-glucosidase activities. Sephadex G-150 chromatography partially resolved the enzyme into two activities, one of which may be a glucamylase and the other a debranching enzyme.

Meiosis in *Saccharomyces cerevisiae* is initiated when diploid yeast cells heterozygous for the mating-type alleles a and α are shifted to a nitrogen-free medium containing a respirable carbon source. Mature asci containing four haploid ascospores appear in the culture 8 to 10 h after initiation in acetate medium and can comprise up to 95% of the total cell population when sporulation is complete (ca. 24 h).

Sporulation is marked by such biochemical events as increased respiration (7, 8, 11, 19), DNA synthesis (11, 19, 34), and turnover of RNA (11, 19), protein (11, 19, 29), and glycogen (6, 19, 20).

Previous workers (7, 20, 35) have shown that sporulating yeast undergo an increase in dry weight, most of which is due to synthesis of the cell wall polymers glucan and mannan and of the storage carbohydrates trehalose and glycogen. Kane and Roth (20) found that glucan, mannan, trehalose, and glycogen are synthesized to the same extent in both sporulating and nonsporulating strains. However, only in sporulating cells was glycogen synthesis followed by a period of breakdown coinciding with the appearance of mature spores.

Recently, we have studied the regulation of glycogen metabolism during meiosis in bakers' yeast, focusing in particular on the catabolism of this polysaccharide. In this communication, we report the presence and some of the properties of the glycogen-degrading enzyme activities which appear during the yeast sporulation process.

(Portions of this work were presented at the 77th Annual Meeting of the American Society for Microbiology, New Orleans, La., 8-13 May 1977, and at the Molecular Biology of Yeast Symposium at Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 18-22 August 1977.)

MATERIALS AND METHODS

Yeast strains and culture conditions. Most of this work was done with *S. cerevisiae* strain AP1 a/α, an adenine auxotroph derived from the haploids A364A (a *ade1 ade2 ura1 his7 lys2 tyr1 gal1*) and α_1 131-20 (α *ade2* [non-complementary with *ade2* from A364A] *ura3 cyh2 can1 leu1*) (19). Other strains occasionally used were X-14 a/α, Y-55 a/α, and a nonsporulating yeast AP1 α/α derived by a mitotic crossover from AP1 a/α (19). Stock cultures were maintained at 4°C on slants containing 1% yeast extract, 1% peptone, 3% glucose, and 1.7% agar.

Vegetative (presporulation) medium (PSP2) contained 6.7 g of yeast nitrogen base (with amino acids), 1.0 g of yeast extract, 10 g of potassium acetate, and 50 mg of adenine sulfate per liter of 50 mM potassium phthalate, pH 5.1. Sporulation medium (SP2) con-

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tained 3.0 g of potassium acetate and 0.2 g of raffinose per liter of distilled water. The procedures for growth, sporulation, and measurement of sporulation percentages were as described in a previous communication (6).

Preparation of extracts. Cells were harvested from sporulation medium (SP2) (150 to 250 ml) on Millipore filters (pore size, 0.22 μm), washed on the filter with 100 ml of ice-cold distilled water, and then stored at −20°C. When needed, the cells were resuspended in 5 ml of ice-cold 0.1 M citrate buffer, pH 6.2 (buffer A), and then transferred to a Braun homogenization flask containing 6 to 7 g of Glasperlen glass beads (0.45-mm diameter; B Braun Melsungen AG, Germany). The protease inhibitors phenylmethylsulfonyl fluoride (in absolute ethanol) and Trasylol were added to final concentrations of 0.3 mg/ml and 185 U/ml, respectively, and the cells were then homogenized for 2 to 4 min in a CO_2-cooled Braun homogenizer. Breakage was monitored microscopically and was always >95%. The homogenate was centrifuged first for 20 min at 12,000 × *g* on a Sorvall RC2B centrifuge and then for 90 min at 176,000 × *g* on a Beckman model L2-65B ultracentrifuge, and the clarified supernatant was stored at −20°C. Protein was measured by the procedure of Lowry et al. (27), with bovine serum albumin as a standard.

Preparation of enzyme from isolated ascospores. Y-55 cells harvested after 22.5 h at 30°C in SP2 (containing ~87% asci) were washed as above with distilled water and incubated at 30°C in 0.1 M tris(hydroxymethyl)aminomethane, pH 9.3, with 0.5 M sodium thioglycolate. After 40 min, the cells were recovered, washed with ice-cold water, and then resuspended in distilled water to a final volume of 50 ml. To half of the cells (25 ml) was added 0.5 ml of glusulase. The suspension was incubated with shaking at 30°C until lysis of vegetative cells and digestion of ascus walls were complete (~70 min), as determined by microscopy. The glusulase digest was diluted with 15 volumes of ice-cold distilled water and centrifuged to pellet the free ascospores. The diluted lysate supernatant was brought to 80% saturation with ammonium sulfate and kept at 0°C. After 18 h, precipitated protein was recovered by centrifugation, washed twice with 100% ammonium sulfate, dissolved in buffer A, and stored at −20°C. The ascospore pellet from the glusulase digest was washed six times with ice-cold distilled water (200 ml per wash) by vigorous stirring with a glass rod followed by centrifugation. The washed spores were subjected to Braun homogenization as described above, the homogenate was clarified by centrifugation, and the extract was brought to 80% saturation with ammonium sulfate. The protein pellet was recovered by centrifugation, washed twice with 100% ammonium sulfate, dissolved in buffer A, and then stored at −20°C. The remainder of the original suspension of whole asci (25 ml) was processed simultaneously and exactly as described above for the ascospore pellet except that glusulase treatment was omitted.

Partial purification of glycogen-degrading enzymes. A 20-g amount (wet weight) of AP1 a/α cells containing ~70% asci was harvested after 21 to 25 h of incubation in SP2 and resuspended in 45 ml of buffer B (0.1 M citrate buffer, pH 6.2, with 20% glycerol, 1 mM dithiothreitol, 0.9 mg of phenylmethylsulfonyl fluoride, and 5,000 U of Trasylol). All subsequent steps were carried out at 0 to 4°C. The cells were broken as described above. From the clarified extract, the activity precipitating between 33 and 66% ammonium sulfate was recovered, dissolved in buffer B, and dialyzed against 50 mM tris(hydroxymethyl)aminomethane, pH 7.7, with 5% glycerol + 0.5 mM dithiothreitol (buffer C). The preparation was applied to a column (2.4 by 19 cm) of diethylaminoethyl-cellulose (DE52) and eluted between 0.1 and 0.2 M NaCl with a 0 to 0.5 M linear NaCl gradient in buffer C. Enzyme-containing fractions were pooled and dialyzed against buffer C minus dithiothreitol. The activity was precipitated with 66% ammonium sulfate, redissolved in buffer C, and chromatographed on a column (2.5 by 50 cm) of Sephadex G-200 equilibrated with 0.1 M tris(hydroxymethyl)aminomethane, pH 7.7, with 5% glycerol. Peak fractions were pooled, dialyzed against buffer A, concentrated with Aquacide II (Calbiochem, LaJolla, Calif.), dialyzed a second time against buffer A, then made 5% with respect to glycerol, and stored at −20°C. The final material (designated Sephadex fraction B) was ~15-fold purer than the crude extract.

Glycogen determinations. The procedures for extraction, isolation, and measurement of cellular glycogen were as described in a previous communication (6).

Enzyme assays. Glycogenolytic enzyme activity was diluted with buffer A to a volume of 0.1 to 0.2 ml and then preincubated without substrate at 30°C. After 2 min, 0.4 to 0.5 ml of 0.5 to 1.0% oyster glycogen (in buffer A) was added, and all samples were incubated at 30°C. At timed intervals, reactions were stopped by boiling the tubes for 15 min. (When necessary, the samples were centrifuged to remove denatured protein.) Glucose in the samples (or sample supernatants) was measured with a coupled assay system containing glucose oxidase (EC 1.1.3.4), peroxidase (EC 1.11.1.7), and *o*-dianisidine (15). One unit of activity was defined as the amount of enzyme which released 1 μmol of glucose from glycogen per min at 30°C.

The protocol was modified for monitoring column fractions: up to 100 μl of column effluent was combined with 0.4 ml of the glucose oxidase–peroxidase–*o*-dianisidine mixture, followed by addition of 0.5 ml of 0.4% glycogen in buffer A. All samples were incubated at 30°C for 20 to 60 min, followed by addition of 1.5 ml of 6 N HCl. The optical densities of all samples were read against glucose standards at 540 nm on a Bausch & Lomb Spectronic 70.

Enzymatic hydrolysis of glycogen and other substrates. Substrates were made up to the desired concentrations in buffer A and equilibrated at 30°C. After 2 to 3 min, reactions were begun by adding an appropriate amount of enzyme; incubation was at 30°C. At timed intervals, 200-μl portions were removed, diluted 1:5 with distilled water, and boiled 15 min on a water bath. Glucose in each sample was measured as described.

Sugar analyses. Total carbohydrate was measured by the phenol-sulfuric acid method of Dubois et al. (9) or with the tryptophan-borosulfuric acid pro-

cedure of Badin et al. (1). Glucose was used as a standard.

Reducing sugars were determined by the procedure of Nelson (30) with glucose and maltose as standards.

Glucose-1-phosphate (G-1-P) was measured with a coupled enzyme system consisting of phosphoglucomutase (EC 2.7.5.1), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), and nicotinamide adenine dinucleotide phosphate. Reactions were carried out in triethanolamine buffer, pH 7.6, containing 1.6 mM $MgCl_2$, 0.9 mM ethylenediaminetetraacetic acid, 18.6 μM glucose-1,6-diphosphate, 0.4 mM nicotinamide adenine dinucleotide phosphate, and 0.15 and 0.30 U of phosphoglucomutase and glucose-6-phosphate dehydrogenase, respectively, in a final volume of 1.0 ml. After addition of samples, all assay mixtures were incubated for 10 min at 30°C and then read at 340 nm on a Gilford spectrophotometer against identically processed G-1-P standards.

Chromatography was performed in a descending mode on either Whatman no. 1 or Whatman 3MM paper (W. & R. Balston, Ltd., England) or in an ascending mode on Eastman Chromagram thin-layer sheets with a cellulose adsorbent (Eastman Kodak Co., Rochester, N.Y.). Two solvent systems were used: *n*-butanol–ethanol–water (3:1:1; solvent system 1) and *n*-butanol–pyridine–water (6:4:3; solvent system 2). Samples were desalted with ion exchangers (AG-50W-X8, H^+ form, and AG1-X8, HCO_3^- form; Bio-Rad Laboratories) before application. Papergrams were developed at room temperature for 39 to 61 h and then air dried. Thin-layer plates were subjected to double-ascent chromatography at room temperature and then air dried. Sugar spots were located with alkaline $AgNO_3$ (40); background was removed by dipping the chromatograms in 4% $Na_2S_2O_3$.

Reagents. Ingredients for yeast culture media were obtained from Difco Laboratories. Oyster glycogen, potato amylose, maltose, maltotriose, α-Schardinger dextrin, G-1-P, glucose-1,6-diphosphate, α-methyl-D-glucoside, *o*-dianisidine, phenylmethylsulfonyl fluoride, nicotinamide adenine dinucleotide phosphate, and the enzymes glucose-6-phosphate dehydrogenase, phosphoglucomutase, peroxidase, glucose oxidase, and β-amylase were obtained from Sigma Chemical Co. Trasylol and isomaltose were purchased from Calbiochem; high-molecular-weight dextrans were obtained from Pharmacia Fine Chemicals, Inc. Alpha-glucosyl-Schardinger dextrin (α-GSD) was a generous gift of E. Y. C. Lee, University of Miami Medical School. Glusulase was obtained from Endo Laboratories.

Beta-limit dextrin was prepared by exhaustive hydrolysis (42%) of oyster glycogen at 30°C with sweet potato β-amylase in 50 mM acetate buffer, pH 4.8. Fresh enzyme was added at 5, 10, and 20 h, and incubation continued until release of maltose was complete (30 h). The digest was boiled for 45 min, and the dextrin was precipitated with 2 volumes of 95% ethanol, redissolved, dialyzed against buffer A, and then stored at −20°C.

All other chemicals used were reagent grade.

RESULTS

Figure 1 compares the glycogen contents of sporulating AP1 **a**/α cells and nonsporulating

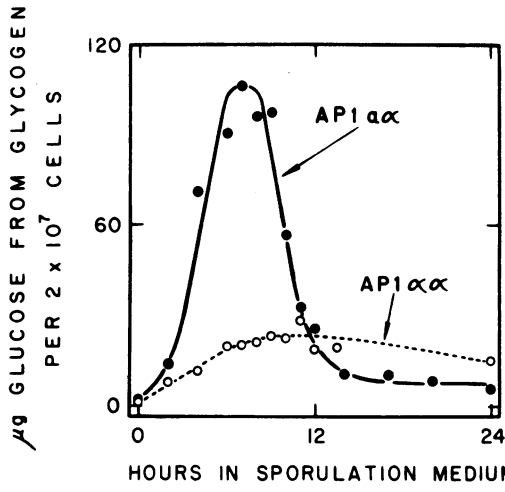

FIG. 1. *Glycogen synthesis and breakdown by AP1* **a**/α *and AP1* α/α *cells in sporulation medium. Vegetative cells of both strains were shifted to SP2 and incubated at 30°C. At designated intervals, 12- and 15-ml portions of the* **a**/α *and* α/α *cultures, respectively, were harvested and washed on Millipore filters. Glycogen was isolated and enzymatically degraded, and the resulting glucose was measured with glucose oxidase. Ascus formation was 69.6 and 0% at 24 h in the* **a**/α *and* α/α *cultures, respectively.*

AP1 α/α cells. In both strains, the quantity of glycogen is initially very low; however, synthesis of the polysaccharide begins by about 2 h. In the sporulating cells, glycogen increases sharply, reaching a maximum at 7 h, followed by a rapid breakdown which eventually reduces the glycogen content to near-presporulation levels. Ascospores begin to appear during this period of glycogen catabolism. In the nonsporulating strain, glycogen is also synthesized, but in lesser amounts. However, very little of the accumulated glycogen is degraded during the remainder of the incubation period; instead, the glycogen content remains substantial and, by 24 h, is more than 10-fold higher than the zero-time glycogen level.

It has been suggested (20) that because both sporulating and nonsporulating strains make glycogen, synthesis may be a nonspecific response to nitrogen deprivation in sporulation medium. Because extensive breakdown occurs only in sporulating cells (19, 20), glycogenolysis, by contrast, may be a sporulation-specific event.

To determine whether glycogen breakdown was regulated by one or more glycosidic enzymes produced during sporulation, cells from several yeast strains were shifted to SP2 as usual and incubated at 30°C. At various times, portions were harvested, washed, and subjected to Braun homogenization, and the extracts were tested for activity against glycogen.

Table 1 shows that 12- and 24-h extracts of AP1 a/α cells and two other sporulating strains, X-14 and Y-55, contain enzyme which is active against glycogen. However, only traces of activity were detected in 12- and 24-h cells of the asporogenous AP1 α/α strain, consistent with the observation (Fig. 1) that glycogenolysis is not extensive in this strain. The near absence of activity in the latter plus the observation that extracts of zero-time cells of all strains tested do not degrade glycogen support the view that glycogenolysis is mediated by one or more glycosidases which may be specific to the sporulation process.

To determine whether the activity is present within the yeast ascospores, extracts were made from spores isolated from whole asci of strain Y-55. Table 2 shows that glycogenolytic enzyme is in fact present in yeast ascospores; however, the specific activity of the enzyme was about threefold higher in the intact asci than in the isolated spores. Furthermore, of the total activity recovered from the sporulated culture, >71% of the enzyme was present in the spore-free epiplasm.

Timing of appearance of glycogenolytic activity. Glycogenolytic enzyme was absent in AP1 a/α cells harvested as late as 6 h after the shift to sporulation medium (Fig. 2). However, activity could be detected by 8 h, coincident with the appearance of mature ascospores and the beginning of glycogenolysis. By 18 h, the enzyme level became maximal and remained approximately constant until 24 h. The appearance of activity at 8 h agrees with the data of Magee and Hopper (29), who observed that sporulating yeast exposed to cycloheximide 6 h after initiation of meiosis fail to degrade their accumulated glycogen pools.

Mode of glycogenolysis. Because yeasts are known to produce both glycogen phosphorylase (12, 25) and phosphatases (4, 26, 28), it was

TABLE 1. *Glycogenolytic activity in representative strains of S. cerevisiae during incubation in sporulation medium*[a]

Yeast strain	h in sporulation medium	% Sporulation[b]	Sp act (mU/mg of protein)
AP1 a/α	0	0	0
	12	40.8	23.1
	24	69.6	44.0
X-14 a/α	0	0	0
	12	39.6	24.3
	24	76.9	37.9
Y-55 a/α	0	0	0
	12	47.1	67.0
	24	69.2	77.1
AP1 α/α	0	0	0
	12	0	1.0
	24	0	1.2

[a] Cells from each strain were shifted to SP2 and incubated at 30°C. At the indicated times, 150 ml of cells was removed from each culture, harvested, washed on Millipore filters, and stored at −20°C. Each cell sample was subjected to homogenization, and the clarified extracts were tested for glycogenolytic activity.

[b] Sporulation percentages were determined by counting 200 to 1,100 cells from each strain under a microscope with an oil immersion lens.

TABLE 2. *Glycogenolytic activity in ascal fractions from S. cerevisiae strain Y-55 a/α*[a]

Ascal fraction	Glycogenolytic activity (mU/mg of protein)[b]	Total activity recovered (mU)	% Total activity
Whole asci	92.1	2,661	100
Spore-free epiplasm	ND[c]	1,958[d]	71.5
Isolated ascospores	32.3	768 (703[e])	28.5

[a] Cells were incubated at 30°C for 22.5 h in SP2, then harvested, and stored at −20°C. Cell fractions were prepared as described in the text. Sporulation at 22.5 h was 87%.

[b] One unit of activity was defined as the amount of enzyme which released 1 μmol of glucose from glycogen per min at 30°C.

[c] ND, Not determined.

[d] Corrected for glycogenolytic activity contributed by glusulase.

[e] Determined by difference between whole ascal and epiplasmic enzyme.

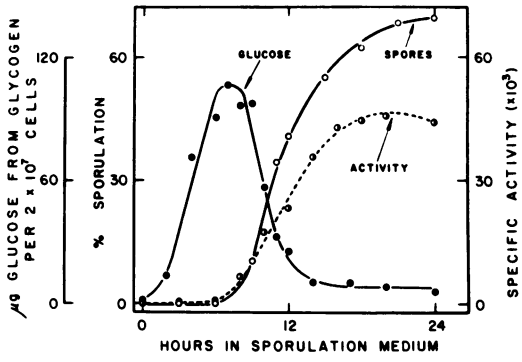

FIG. 2. *Timing of appearance of glycogenolytic activity in sporulating AP1 a/α cells. At the indicated times, 163 ml was withdrawn from the culture. One milliliter was fixed with 4% Formalin and used to determine percentage of sporulation. A 12-ml amount was harvested and washed on Millipore filters and then used to measure cellular glycogen. The remaining cells were homogenized, and the extracts were tested for enzyme activity and protein.*

possible that the disappearance of glycogen during sporulation was due to phosphatase(s) acting on G-1-P generated from the polysaccharide by glycogen phosphorylase. Such a mechanism requires P_i for glycogenolysis. We therefore measured the rate of glucose production in phosphate-free enzyme digests containing glycogen, G-1-P, or a combination of glycogen plus G-1-P (Fig. 3). Under these conditions, G-1-P cannot be formed and any phosphatase(s) present should hydrolyze it to glucose and P_i. In all digests, substrate concentrations were made equimolar with respect to anhydroglucose. After 24 h, only ~5% of the available hexose was released from G-1-P, and >95% of the original G-1-P was recovered in unaltered form. During the same period, glycogen was ~85% hydrolyzed. Glucose production from the glycogen + G-1-P mixture was higher than that from glycogen alone, the increment being approximately equal to the glucose released from G-1-P. Because the rate of glucose formation from G-1-P cannot account for the release of glucose from an equivalent amount of glycogen, glycogenolysis cannot be due to glycogen phosphorylase plus phosphatase.

Similarly, when we measured the release of glucose from enzyme digests containing equivalent concentrations of either glycogen or maltose, we found that, although the latter was hydrolyzed (Fig. 4), the reaction rate was insufficient to account for the release of glucose from an equivalent amount of glycogen. Furthermore, chromatography of an identical glycogen hydrolysate (data not shown) revealed that glucose was the only sugar enzymatically released. Therefore, an amylase + maltase combination is not responsible for glycogenolysis.

In contrast to maltose, the trisaccharide maltotriose was hydrolyzed by the enzyme preparation at nearly the same rate as glycogen during the first 2 to 4 h of incubation (data not shown). By 23 h, ~38% of the available glucose had been released. Two possibilities could account for this observation: either 38% of the maltotriose had been hydrolyzed completely to glucose, or all of the trisaccharide had been hydrolyzed to maltose and glucose. Chromatography of the hydrolysate revealed substantial amounts of the latter sugars (Fig. 5); maltotriose, which was most plentiful at 8 to 10 h, diminished progressively with incubation time and was barely detectable by 25 h, demonstrating that all of the maltotriose is hydrolyzed to glucose and maltose, the latter serving as a secondary substrate for the enzyme.

The enzyme preparation extensively hydrolyzed amylose, the linear, unbranched component of starch (data not shown), demonstrating that α-1,4 linkages can be cleaved in glucans lacking the highly branched, arboreal architecture characteristic of glycogens and amylopectins.

Demonstration of α-1,6-glucosidase ac-

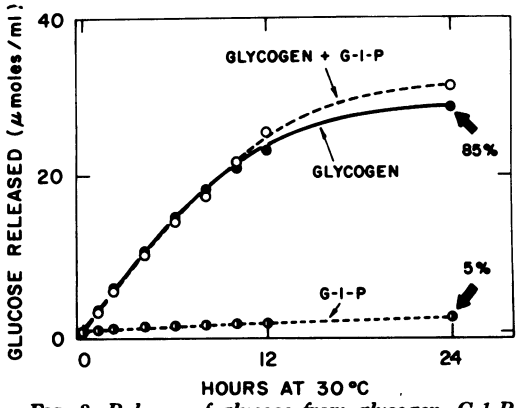

FIG. 3. *Release of glucose from glycogen, G-1-P, and a glycogen + G-1-P mixture by glycogenolytic enzyme from sporulating AP1 a/α cells. Final substrate concentrations in the glycogen and G-1-P hydrolysates were 33.7 mM with respect to anhydroglucose; the glycogen + G-1-P mixture contained 67.4 mM anhydroglucose, half in the form of glycogen, the rest as G-1-P. Enzyme used was a 66% ammonium sulfate precipitate from a crude extract of a 24-h SP2 culture of AP1 a/α containing 59% asci. A total of 120 mU of glycogenolytic activity was added per digest, each at a final volume of 2.6 ml. Glucose was measured as described. Arrows denote percent hydrolysis at 24 h.*

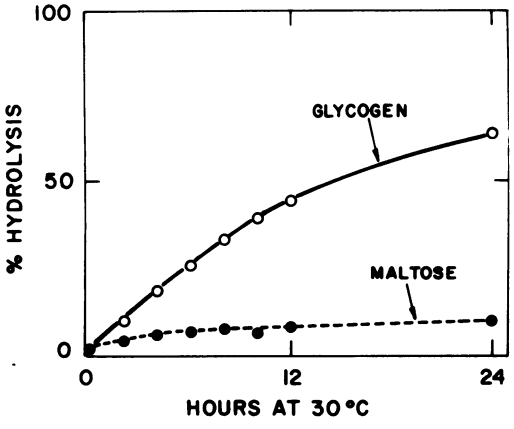

FIG. 4. *Hydrolysis of glycogen and maltose by glycogen-degrading enzyme from sporulating AP1 a/α cells. Hydrolyses were conducted in 0.1 M citrate, pH 6.2, in final volumes of 2.0 and 1.5 ml of glycogen and maltose, respectively. Final substrate concentrations in both digests were 35 mM with respect to anhydroglucose. A total of 24 mU of enzyme (Sephadex fraction B) was added per ml of final hydrolysate volume.*

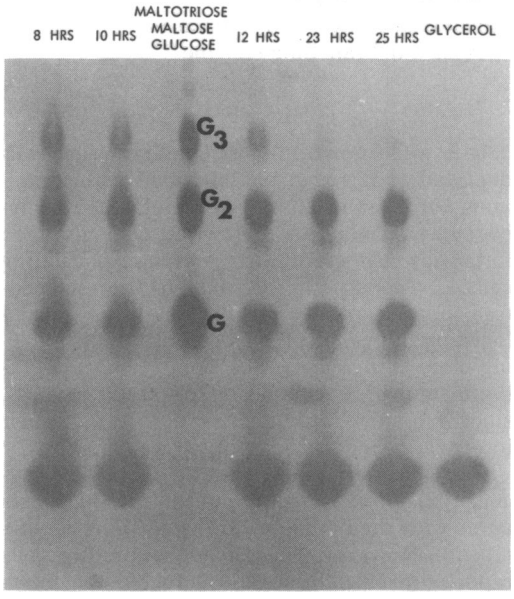

FIG. 5. *Thin-layer chromatogram of portions from enzymatic hydrolysis of maltotriose. The hydrolysate contained 8.9 mM maltotriose in a final volume of 1.5 ml plus 48 mU of enzyme (Sephadex fraction B). All samples were desalted with ion exchangers. The chromatogram was spotted with 30 μg of carbohydrate from each maltotriose sample, 35 μg each of glucose (G), maltose (G_2), and maltotriose (G_3), and 25 μg of glycerol. The latter was present in the original enzyme preparation and thus appeared in all but the standard samples. Development was with solvent system 1. Direction of migration: from top to bottom.*

tivity. The extent of hydrolysis of glycogen (Fig. 3 and 4) suggested that both an α-1,4- and an α-1,6-glucosidase activity were present. Attempts to demonstrate the latter on isomaltose and linear poly-α-1,6-linked dextran were unsuccessful (data not shown). In a further attempt, we used as a substrate α-GSD (Fig. 6), a cyclic dextrin which is formed by the action of microbial enzymes on starch (14, 38) and which consists of six glucose monomers joined by α-1,4 bonds with a glucose stub joined to the ring by an α-1,6 linkage. Because the dextrin ring is susceptible to very few enzymes, this substance is a highly specific α-1,6-glucosidase substrate (14, 24, 38). Figure 6 shows that α-GSD is slowly hydrolyzed by the enzyme. Chromatography revealed glucose as the only reducing sugar present in the hydrolysate; furthermore, because the enzyme was inactive on the unsubstituted cyclic dextrin, we conclude that glucose was released from α-GSD by specific cleavage of the α-1,6-glucosidic bond. The slow rate of hydrolysis of this substrate may be due to its unusual cyclic structure.

To determine whether the α-1,4- and α-1,6-glucosidase activities were due to one or two enzymes, Sephadex fraction B was chromatographed on Sephadex G-150, and the active material was divided into eight fractions (Fig. 7) which were tested on another α-1,6-glucosidase substrate, β-limit dextrin, prepared by treating glycogen with β-amylase. The latter releases maltose by hydrolyzing α-1,4 bonds of glycogen; however, it cannot hydrolyze α-1,6 bonds or cleave α-1,4 linkages of glucose chains beyond the α-1,6-branch points. Therefore, exhaustive

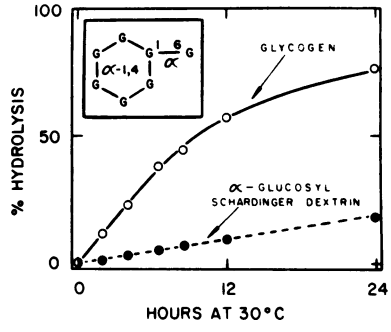

FIG. 6. *Hydrolysis of glycogen and α-1,6-GSD by glycogenolytic enzyme from sporulating AP1 a/α cells. The glycogen hydrolysate contained 33.5 mM anhydroglucose in a final volume of 1.5 ml; the α-GSD hydrolysate contained the heptasaccharide at a concentration of 7.1 mM in a final volume of 1.5 ml. Enzyme dosages (Sephadex fraction B) were 48.4 and 72.6 mU for the glycogen and α-GSD hydrolysates, respectively. Inset: Structure of α-GSD.*

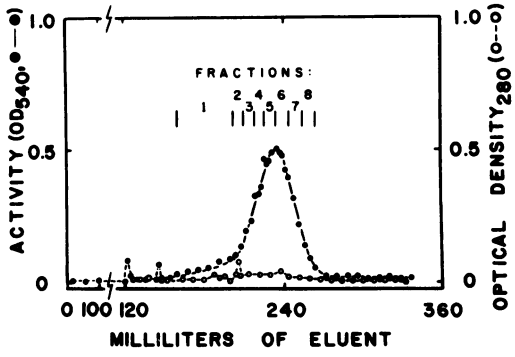

FIG. 7. *Sephadex G-150 chromatography of glycogen-degrading enzyme from sporulating AP1 a/α cells. The enzyme (Sephadex fraction B) was concentrated from 7.1 to 3.0 ml with Aquacide II and then applied to a Sephadex G-150 column (2.5 by 90 cm) and eluted with buffer A. Fractions of 2 ml were collected and monitored for glycogenolytic activity and optical density at 280 nm. Active fractions were combined as shown, forming eight major fractions which were concentrated with Aquacide II and stored at −20°C.*

β-amylolysis of glycogen yields a refractory β-limit dextrin enriched in α-1,6 bonds.

When the fractions in Fig. 7 were tested with β-limit dextrin as substrate, a diminishing activity was observed across the Sephadex peak (data not shown), suggesting that the early fractions contained α-1,6-glucosidase. This idea was tested by determining the change in β-amylolysis of the dextrin after pretreatment with either fraction 1 or fraction 8 (i.e., the most widely separated fractions) or a mixture of fractions 1 and 8. Figure 8 shows that after preincubation with fraction 1 or fractions 1 and 8, β-limit dextrin is again very susceptible to β-amylase, whereas dextrin treated with fraction 8 alone is no more susceptible than the untreated material. The data indicate that the early Sephadex fractions contain enzyme which acts on α-1,6 bonds, thus exposing new α-1,4 linkages for cleavage by β-amylase. The α-1,6-glucosidase activity is apparently lacking in the later Sephadex fractions. Our data therefore suggest that glycogenolysis in sporulating yeast is due to two enzymes. One of these acts on α-1,4 bonds and is probably a glucamylase; the other acts on α-1,6 bonds and may be a debranching enzyme.

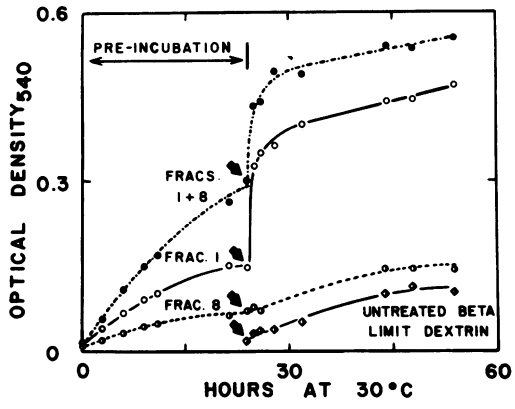

FIG. 8. *β-Amylolysis of glycogen β-limit dextrin after pretreatment with glycogenolytic enzyme fractions from Sephadex G-150. Each hydrolysate (containing 0.4% β-limit dextrin in a final volume of 2.0 ml of buffer A) was preincubated at 30°C with 3 mU of either Sephadex G-150 fraction 1 or fraction 8 or a mixture of 3 mU each of fractions 1 + 8 (see Fig. 7). After 24 h, the hydrolysates were boiled for 10 min and cooled, and to each was added 45 U of sweet potato β-amylase. The hydrolysates were returned to incubation at 30°C and monitored for release of reducing power. Untreated β-limit dextrin (0.4% in buffer A) plus 45 U of sweet potato β-amylase was used as a control. Arrows denote addition of β-amylase. Glucose release during preincubation was monitored with glucose oxidase; amylolysis was measured by release of reducing sugar (shown as optical density at 540 nm). Glucose values from preincubation were converted to reducing sugar optical density equivalents.*

DISCUSSION

It is well known (22, 36, 39) that yeast cells deprived of nitrogen but provided with exogenous carbon and energy accumulate both glycogen and trehalose, suggesting that glycogen formation by sporulating and nonsporulating cells is a nonspecific response to the nitrogen-free sporulation medium. Although the physiological significance of glycogen metabolism during meiosis in yeast is obscure, glycogen degradation appears to be specific to sporulating cells, since **a**/**a** and α/α diploids do not degrade their internal pools of this carbohydrate (19, 20).

Our observations on the metabolism of glycogen by sporulating and nonsporulating yeasts agree with the findings of previous investigators (19, 20). However, whereas nonsporulating cells reportedly (19, 20) make 60 to 80% as much glycogen as do sporulating yeast, we have found that glycogen synthesis in AP1 α/α cells is consistently and markedly less than that observed in **a**/α cells (Fig. 1). This discrepancy may result from slight differences in culture conditions or, in the case of AP1 α/α, a variation in this strain since the original observations were made (19). Moreover, because yeast store glycogen in both alkali-soluble and alkali-insoluble pools (5, 17, 39), it is possible that α/α cells produce ample amounts of the polysaccharide but store most of it in a form not readily isolable by our extraction procedure.

The enzyme preparation from our sporulating yeast cells extensively hydrolyzes glycogen, releasing glucose as the only low-molecular-weight product. The hydrolysis was shown not to result from combinations of phosphorylase and phosphatase (Fig. 3) or amylase and maltase (Fig. 4). Further evidence on the latter point is that maltose was chromatographically undetectable in glycogen digests even after brief periods of enzymolysis.

In addition to glycogen, the enzyme preparation is very active on amylose, the linear component of starch, and thus contains an α-1,4-glucosidase capable of hydrolyzing α-1,4-glucans lacking α-1,6 bonds and/or branch residues. Alpha-methyl-D-glucoside is not attacked (data not shown); however, maltotriose is quantitatively hydrolyzed to glucose plus maltose. The latter is a very poor substrate for the α-1,4-glucosidase (Fig. 4) and defines the lower limit of substrate size specificity for the enzyme.

The ability of the enzyme preparation to release glucose from α-GSD demonstrated the presence of an α-1,6-glucosidase activity. Glu-

cose production from enzymatic cleavage of the dextrin ring was unlikely, because the enzyme was inactive against unsubstituted α-Schardinger dextrin (data not shown). Furthermore, because the enzyme acts on maltotriose and because ring cleavage would yield a substituted maltosaccharide, attack on the ring would lead to more extensive hydrolysis than was observed (cf. Fig. 6) and would also generate a homologous series of oligosaccharides. Glucose, however, was the only reducing sugar detected on chromatography of the α-GSD hydrolysate.

Sephadex G-150 chromatography resolved the glycogenolytic activity into eight fractions whose ability to hydrolyze β-amylase-limit dextrin diminished inversely with elution volume (data not shown). Beta-amylolysis of the dextrin following pretreatment with early and late G-150 fractions (Fig. 7 and 8) confirmed that two enzymatic activities, an α-1,6- and an α-1,4-glucosidase, had been partially resolved. The latter may be a glucamylase, an enzyme which splits glucose from starch, dextrins, etc., starting like β-amylase at the nonreducing ends of α-1,4-polyglucose chains. Such an enzyme has been described in *Saccharomyces diastaticus* (18).

The α-1,6-glucosidase might be an amyloglucosidase with dual specificity for α-1,4- and α-1,6-glycosidic bonds. However, the inability of the enzyme to degrade glycogen completely (Fig. 3 and 4) and its slight activity on maltose (normally a good amyloglucosidase substrate [21, 33]) argue against this possibility. The inability of the enzyme to hydrolyze isomaltose and poly-α-1,6-linked dextran suggests that the enzyme cannot cleave α-1,6 linkages in small or large substrates lacking proximal α-1,4-glycosidic bonds.

Another α-1,6-glucosidase from yeast, isoamylase, increases the β-amylolysis limit of glycogen by hydrolyzing the interchain α-1,6 bonds, releasing a mixture of maltosaccharides (2, 16). These would be susceptible to hydrolysis by the α-1,4-glucosidase, resulting in an accumulation of maltose. However, because neither maltose nor higher maltosaccharides were detected on chromatograms of glycogen hydrolysates, the α-1,6-glucosidase cannot be isoamylase. Limit dextrinase, another α-1,6-glucosidase, is also present in yeast (2). However, it is specific for α-amylase-limit dextrins and is thus an unlikely candidate for the α-1,6-glucosidase demonstrated here.

A more likely possibility is a debranching enzyme (amylo-1,6-glucosidase-oligo-1,4 → 1,4-glucantransferase; EC 2.4.1.25 + 3.2.1.33) which catalyzes first the transfer of an oligosaccharide segment from a glycogen side chain to another nonreducing end of the polysaccharide and then hydrolysis of the remaining glucose stub involved in the α-1,6 linkage. The enzyme, which has been purified from vegetative yeast (23) and described in rabbit muscle (31, 32) and other sources (3, 10), releases only glucose from glycogen, increases the β-amylolysis limit of the polysaccharide, and hydrolyzes α-GSD. Another possibility is an amylo-1,6-glucosidase which, although inactive on intact glycogen, reportedly hydrolyzes terminal α-1,6-linked glucose residues of glycogen phosphorylase-limit dextrin (41), thereby increasing the β-amylolysis limit of this polymer. Like the debrancher, amylo-1,6-glucosidase also releases α-1,6-linked glucose from α-GSD (2); both enzymes are thus similar to one of the enzymes described here.

A key issue is whether the glycogenolytic enzymes in our yeast are truly sporulation-specific gene products. The glycogenolytic activity begins to appear at the time of major protein synthesis during sporulation (19) and rises coincidentally with the percentage of asci; furthermore, the activity was found in three *S. cerevisiae* strains, demonstrating its probable ubiquity in sporulating yeast. With glycogen as substrate, only traces of activity were found in α/α cells 12 and 24 h after shift to sporulation medium, and no activity was found in vegetative cells of any strain at the time of shift. Furthermore, when enzyme from active extracts was mixed with protein from each of the inactive extracts, no variation in the level of enzyme activity was observed even when inactive protein was present in as much as a 7.3-fold excess over the active material. Therefore, the absence of enzyme in the inactive preparations is not due to the presence of endogenous inhibitors or the lack of activators.

This evidence suggests that at least one of the enzymes responsible for glycogenolysis, namely, the α-1,4-glucosidase, is sporulation specific. Of the aforementioned α-1,6-glucosidases which are possible, the amylo-1,6-glucosidase-oligo-1,4 → 1,4-glucantransferase (i.e., debranching enzyme) of Lee et al. (23, 24) is known to be active on native glycogen. Hence, this enzyme, if present in vegetative cells, would have been detected even in the absence of the α-1,4-glucosidase. Therefore, the absence of activity observed with extracts of our vegetative yeast means either that the α-1,6-glucosidase is sporulation specific or that the enzyme is in fact present in vegetative cells but has a peculiar specificity which precludes its detection when glycogen is used as a substrate in the absence of the α-1,4-glucosidase. The ambiguity could be settled by testing vegetative cell extracts on more specific substrates, e.g., β-limit dextrin or α-GSD; however, we have not done these experiments. Nevertheless, the

absence of detectable α-1,4-glucosidase activity in vegetative cells means that the ability to degrade glycogen appears only during sporulation.

The sporulation-specific enzyme may exist in vegetative cells as a zymogen which is specifically activated during sporulation; however, the observation (29) that cycloheximide prevents glycogenolysis in sporulating yeast suggests that the enzyme is a product of de novo protein synthesis during meiosis. Nevertheless, an extracellular glucamylase and debrancher have been reported in vegetative cells of *S. diastaticus* (18), and an intracellular debrancher (23, 24) and amylo-1,6-glucosidase (2) have been described in vegetative cells of *S. cerevisiae*. It is therefore possible that the absence of glycogenolytic activity which we observed in vegetative cells is a function of the acetate presporulation medium employed (6), and variations of the latter and/or use of culture conditions promoting glycogen accumulation might elicit formation of one or both of the enzymes in vegetative cells.

The enzymes are present in both isolated ascospores and the spore-free epiplasm. Before breakage, the former were thoroughly washed to remove enzymatic activity contributed by both glusulase and ascus cytoplasm. Nevertheless, some of the spore-associated activity may have been due to contaminating enzyme (from glusulase and/or ascoplasm) nonspecifically adsorbed by the spores, which are "sticky" in nature (13, 37). However, because the two values for the spore-associated enzyme (determined by direct assay and by calculating the difference between total and epiplasmic enzyme; Table 2) were in good agreement, this possibility is remote.

The total activity of the enzymes in the spore-free epiplasm is ~71% of that in the intact asci. Based on measurements taken from representative light micrographs of mature asci and assuming that asci and ascospores are regular ellipsoids, we calculated that four ascospores collectively comprise on the average 35% of the total volume of the ascus. This figure agrees well with that obtained for the percentage of the activity found in the spores (~29%; cf. Table 2), suggesting that the enzymes are uniformly distributed throughout the ascus and are nonselectively packaged within the spores during spore wall formation.

The α-1,4-glucosidase activity (and possibly the α-1,6 activity) thus appear to be sporulation-specific gene products in *S. cerevisiae* cells induced to sporulate by the acetate-to-acetate regimen. Because the α-1,4-glucosidase is reasonably stable, it may be readily purifiable. As such, it may be a very useful specific gene product marker for the complex regulatory system leading from premeiotic DNA synthesis and recombination through meiosis to ascospore formation. No other molecular entities specific to this process have hitherto been described.

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