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Relationship of Large and Small Invertases in *Saccharomyces*: Mutant Selectively Deficient in Small Invertase

BEATRICE B. ABRAMS, RICHARD HACKEL,† TAKEMITSU MIZUNAGA, AND J. OLIVER LAMPEN*

Waksman Institute of Microbiology, Rutgers—The State University, Piscataway, New Jersey 09954

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A mutant strain of *Saccharomyces cerevisiae* (D10-ER1) has been isolated after a two-step mutagenesis of strain 4059-358D (*SUC 1*) using ethyl methane sulfonate. Cells of this new strain produced a level of total invertase equaling that of 4059 but contained only trace amounts of the small, internal, aglycan form of the enzyme (<0.1% of total in D10-ER1 compared with 6% in 4059). When D10.ER1 was crossed with an invertase-hyperproducing strain dgr3 (*SUC3*), progeny were isolated (HZ400-5A and HZ400-2C) in which levels of total invertase had at least quadrupled. The percentage of small invertase, however, remained insignificant. Levels of small invertase in strain HZ400-5A were determined by affinity chromatography on conconavalin A-Sepharose, gel permeation chromatography, and isopycnic centrifugation in CsCl. The large invertase of the *SUC1* yeasts described here was found to contain a form apparently greater in size than the large invertase of the *SUC2* strain FH4C; this probably reflects a higher content of carbohydrate. The overall results of this study do not support a direct structural relationship between large and small invertases. The implications on invertase biosynthesis and structure are discussed.

Two forms of invertase (β-D-fructofuranosidase, EC 3.2.1.26) exist in *Saccharomyces*: a large, secreted mannoprotein of 270,000 molecular weight, 50% carbohydrate by weight (22), and a smaller, intracellular aglycan enzyme with a molecular weight of 135,000 (9). Although the function of the large enzyme is obvious, that of the smaller is not because sucrose, its logical substrate, is not available within the cell (6).

Invertase activity is dependent on one of six nonallelic sucrose genes denoted *SUC1-6* (21), and no mutants containing only one form of the enzyme have been described (9, 14). This fact, coupled with the uncertain function of the small enzyme, its structural and immunological similarities with the large enzyme (10), and the similar kinetic behavior of the two enzymes (2, 10, 12, 23, 28), has led to suggestions that the small enzyme may be a precursor or degradative product of the large enzyme (3, 16, 20) or that the two forms may share a common catalytic subunit (10). Evidence establishing these hypotheses has been difficult to obtain. Data on the temporal sequence of synthesis of the two forms indicate that they may be unrelated (8), and studies using pulse-chase labeling or inhibitors of glycosylation have failed to demonstrate a definite precursor-product relationship (8, 16, 20). Because of these difficulties, the recent isolation of mutant strains possibly lacking small invertase (R. Hackel, Ph.D. thesis, City University of New York, New York, 1977) prompted a more careful biochemical investigation. This paper presents evidence for the independent nature of the two forms of invertase by describing mutant strains of yeast deficient in small invertase, yet producing or hyperproducing the large enzyme. The mutants lacking small invertase were compared with the parent strain (*SUC1*) from which they were derived and with the well-studied strain FH4C (*SUC2*) from which both large and small invertases have been purified and characterized (9, 22).

† Present address: Graduate Department of Biochemistry, Brandeis University, Waltham, MA 02154.

MATERIALS AND METHODS

Yeast strains, growth conditions, and harvest. Descriptions of the strains used are presented in Table 1. Stock cultures were maintained on slants of Difco yeast extract (0.3%), peptone (0.5%; Bacto, Difco); glucose (2%) and agar (1.5%; Bacto, Difco). Large and small invertases have different sensitivities to catabolite repression (9, 11), and yeasts were grown under conditions designed to maximize total invertase production. FH4C cells were grown overnight at 28°C in 600 liters of 1% yeast extract–0.2% ammonium sulfate–4% glucose medium. D10-ER1 cells were grown in 30 liters of yeast extract (1%), peptone (3%; Bacto, Difco), and fructose (0.5%) at 28°C overnight. Both strains were harvested in a Sharples model 16 centrifuge (13,200 × *g*), and the yeast pastes were stored at

TABLE 1. *Characteristics of yeast strains studied*

Strain	Gene specifying invertase activity	Invertase activity in crude extracts (U/mg of protein)	General description and lineage	Source of strain or reference
FH4C	*SUC2*	45	Hyperproducer of small and large invertase; resistant to catabolite repression	19
4059-358D (4059)	*SUC1*	3	Ultimate parent of strain D10-ER1; large and small invertases present	Hackel, Ph.D. thesis
D10-ER1	*SUC1*	4	Mutant obtained from an invertase-negative mutant strain (D10) of 4059 by treatment with ethyl methane sulfonate	This paper
dgr3	*SUC3*	31	2-Deoxy-D-glucose-resistant mutant; hyperproducer of large and small invertases	This paper
HZ400-5A	*SUC1*	16	Clone from tetrad 5[a] *SUC1 MAL1 dgr*	This paper
HZ400-2A	*SUC1*	4	Clone from tetrad 2[a] *SUC1 MAL1 DGR*	This paper
HZ400-2B	*SUC3*	2	Clone from tetrad 2[a] *SUC3 mal0 DGR*	This paper
HZ400-2C	*SUC1*	25	Clone from tetrad 2[a] *SUC1 MAL1 dgr*	This paper
HZ400-2D	*SUC3*	8	Clone from tetrad 2[a] *SUC3 mal0 dgr*	This paper

[a] Tetrads derived from mass mating of derivatives of dgr3 × D10-ER1. See text for details.

−80°C. Other strains were grown in 1.5 liters of medium containing yeast extract (0.3%), peptone (0.5%; Bacto, Difco), and fructose (0.5%) at 28°C on a rotary shaker at 180 rpm. The usual inoculum was 0.5% (vol/vol) of a stationary phase culture. The organisms were harvested by centrifugation at 8,300 × *g* for 20 min and processed immediately.

Mutagenesis and mating. The procedure for ethyl methane sulfonate mutagenesis was a modification of the method of Fink (7). Ethyl methane sulfonate was given in a dose sufficient to kill about 50% of the cells treated (29). D10-ER1 (a revertant that ferments sucrose and raffinose) was obtained by plating 1.2×10^7 ethyl methane sulfonate-treated cells of strain D10 (an invertase-negative mutant of strain 4059; Hackel, Ph.D. thesis) on raffinose agar plates (yeast extract, 0.5%; peptone, 1%; raffinose, 2%; and agar, 1.5%). These plates were incubated for 1 week at 25°C. Revertant colonies were tested for sucrose fermentation, and positive isolates were purified by restreaking on raffinose agar plates. Crosses to genetically characterized nonfermenting or sucrose-fermenting yeast strains indicated that revertant D10-ER1 probably contains a *SUC1* allele tightly linked to the *MAL1* locus (Hackel, Ph.D. thesis).

The invertase-hyperproducing mutant dgr3 was derived from strain EK-6B (*SUC3 MAL3*) by selection on a medium containing 2-deoxy-D-glucose (30; Hackel, Ph.D. thesis). Cells grown on a high-glucose medium repressive for invertase synthesis were spread on raffinose agar plates. After the plates were dried, a central well was cut to which was added 5 mg of deoxyglucose in 0.2 ml of water, and a few crystals of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine were placed nearby. The plates were incubated at 30°C for several days, and deoxyglucose-resistant colonies were isolated and purified.

Examination of the invertase patterns in mutant D10-ER1 was facilitated by crossing this strain to the hyperproducing strain dgr3. Before this cross, strain D10-ER1 was crossed to a segregational sucrose nonfermenter (EZ-5B; *suc0 mal0* [Hackel, Ph.D. thesis]) to introduce the **a** mating type. Strain dgr3 was mated to the *SUC3 mal0 DGR* strain R.S. 5-1 to obtain a *dgr* yeast unable to ferment maltose. Because the *SUC3* and *MAL3* loci in these strains are tightly linked (as are *SUC1* and *MAL1* [21]), the absence of maltose fermentation would distinguish *SUC3* from *SUC1* strains.

Crosses between haploid strains of opposite mating type (D10-ER1-3A, **a** *SUC1 MAL1 DGR*; and dgr3-3B, *α SUC3 mal0 dgr*) were initiated by mixing the cells in a tube of 1% yeast extract–2% peptone–2% glucose broth. Diploids were selected on an appropriate selection medium (7). Sporulation of diploids was induced on potassium acetate (17). Asci were dissected by the method of Johnston and Mortimer (15). Because the gene conferring invertase hyperproduction (catabolite repression resistance) is not linked to the *SUC3 MAL3* loci present in strain dgr3 (Hackel, Ph.D. thesis), it was possible to identify and isolate segregants bearing the revertant *SUC1* gene (linked to *MAL1*) and containing the *dgr* genotype. Strains HZ400-2C and HZ400-5A are such segregants.

Preparation of crude extracts. Yeast were homogenized in 0.2 M sodium acetate buffer (pH 4.9) containing 3 mM phenylmethanesulfonyl fluoride (added immediately before homogenization) in a vol-

ume of buffer equal to that of the cell paste, but at least enough to yield a final volume of 20 ml. The suspension was shaken in a Braun homogenizer with 0.45-mm glass beads for 3 min while being cooled with liquid CO_2 and then centrifuged at 4°C for 45 min at 12,000 × *g*. The supernatants were collected and used immediately.

Invertase assays. Invertase was assayed at 30°C in the presence of 5 mg of bovine serum albumin per ml as described by Goldstein and Lampen (12).

Protein. Protein was assayed by the turbidity method of Bucher (4).

Column chromatography. Two columns of Bio-Gel A-0.5m (200 to 400 mesh) were used for gel permeation chromatography (see figure legends). One column (2 by 90 cm) was equilibrated and developed with 50 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, pH 7.3; the other (2 by 95 cm) was equilibrated and developed with the same buffer containing 0.5 M NaCl. Samples were loaded in 1 to 3 ml, and the columns was developed at 4°C at 6 to 12 ml/h as indicated in the figure legends. Fractions of approximately 2 ml were collected. Because there are no appropriate molecular weight standards for large glycoproteins, the results are expressed as partition coefficients [$K_{av} = (V_e - V_o)/(V_t - V_o)$, where V_o = void volume of the column, V_t = total volume, and V_e = elution volume].

For the separation of large and small invertases, diethylaminoethyl (DEAE)-Sephadex A-50 columns (approximately 1.2 by 14 cm) were equilibrated with 50 mM Tris-hydrochloride buffer (pH 7.3), and crude extracts containing approximately 11 mg of protein were applied. Invertases were eluted with a 60-ml linear gradient from 0 to 0.4 M NaCl in Tris-hydrochloride buffer (pH 7.3), and 1-ml fractions were collected. Large invertase elutes at 0.13 M NaCl, and small invertase elutes at >0.3 M.

Concanavalin A (ConA)-Sepharose was used to selectively bind large invertase. The ConA was purified from jack bean meal by the method of Agrawal and Goldstein (1) and was coupled to Sepharose 6B by the method of March et al. (18). ConA-Sepharose columns (0.5 by 8 cm) in Pasteur pipettes were washed with 5 ml of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 0.1 M NaCl, 10 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.5% α-methyl-D-mannoside to remove uncoupled ConA, and then with 5 ml of the same buffer without α-methylmannoside. Columns were operated at room temperature to minimize hydrophobic bonding, and the samples were collected in ice. Experiments with successive additions of crude extracts showed that no breakthrough of large invertase occurred when 100 U of invertase or 10 mg of protein was applied.

Cesium chloride gradient centrifugation. Yeast crude extracts containing approximately 1 U of invertase in 25 mM sodium phosphate buffer (pH 7.4) were adjusted to densities of 1.36 g/cm^3 by the addition of solid CsCl and centrifuged at 4°C for 45 h at 35,000 rpm in a Beckman SW 50.1 rotor. Fractions of 12 drops were collected from the bottom of the tube. Densities were calculated from refractive indices (27), and samples were assayed for invertase activity.

Endo-β-*N*-acetylglucosaminidase H treatment. Endo-β-*N*-acetylglucosaminidase H was purified from *Streptomyces plicatus* by the procedures of Tarentino and Maley (25) and was a gift of Jan Tkacz of this institute. Samples of FH4C or HZ400-5A large invertase from the peak of invertase activity which eluted from DEAE-Sephadex with 0.13 M NaCl (15 to 25 U in 1 to 3 ml) were dialyzed overnight against 1 liter of 0.1 M sodium citrate buffer (pH 5.5) at 4°C. The enzyme solutions were incubated at 37°C for 24 h with endoglucosaminidase H (0.76 μg in 0.02 ml per 15 U of invertase) and 50 μg/ml each of chloramphenicol and cycloheximide.

Materials. Bio-Gel A-0.5 m was purchased from Bio-Rad Laboratories, Richmond, Calif.; DEAE-Sephadex A-50 and Sepharose 6B were from Pharmacia Fine Chemicals, Inc., Piscataway, N.J.; jack bean meal, bovine serum albumin, and phenylmethanesulfonylfluoride were from Sigma Chemical Co., St. Louis, Mo.; and CsCl was from Gallard-Schlesinger, Carle Place, N.Y. Large invertase was purified from *Saccharomyces* strain FH4C by the method of Goldstein and Lampen (12).

RESULTS

Characterization of invertases in yeast mutants. (i) Behavior on ConA-Sepharose. ConA-Sepharose specifically binds molecules containing terminal α-mannosyl (or sterically related) residues (13). Material not binding to the column presumably lacks such carbohydrates, and Gallili has shown by the criteria of gel permeation and ion-exchange chromatography that the portion of the invertase from *Saccharomyces* strain 1710 not bound by ConA-Sepharose columns is small invertase (G. Gallili, Ph.D. thesis, Rutgers University, New Brunswick, N.J., 1975). Large invertase is bound irreversibly. Table 2 illustrates the interactions of various invertase preparations (from late-exponential-phase cells) and ConA-Sepharose. When crude extracts of strain 4059 were passed through a ConA/Sepharose column in less-than-saturating quantities, 6% of the total invertase activity did not bind. Under similar conditions, 3% of the invertase activity of a crude extract from FH4C and 4% of that from dgr3 did not bind. In contrast, only 0.1% of the total activity of a crude extract of D10-ER1 and less than 0.2% of that from HZ400-5A passed through the column. Virtually no small invertase was detected even in early to mid-exponential-phase cultures of strain HZ400-5A by chromatography of extracts on ConA-Sepharose (Table 3). (The cells in this experiment were grown in 2% glucose instead of 0.5% to partially repress large invertase synthesis, and the low specific activities reflect this repression.) The ConA-Sepharose columns were run at room temperature to lessen hydrophobic interactions between invertase and ConA; when 5 mg of bovine serum albumin per ml was added to column buffers to further re-

TABLE 2. *Levels of total and small invertase in crude extracts of yeast as determined by chromatography on ConA-Sepharose*[a]

Strain/tetrad	Amt applied to column		Small invertase recovered		Invertase (U/mg of protein) in crude extract	
	Protein (mg)	Invertase (U)	U	% of total invertase	Total invertase	Small invertase
FH4C	2.0	97	3.0	3	48	1.5
4059	0.41	1.6	0.09	6	3.9	0.22
dgr3	0.14	4.4	0.16	4	31	1.1
D10-ER1	10	72	0.10	0.1	7.2	0.01
HZ400-5A	0.35	10	<0.02	<0.2	33	0.06
HZ400-2A[b]	0.54	2.0	<0.004	<0.2	3.7	<0.01
HZ400-2B[b]	0.45	1.1	0.07	6	2.4	0.16
HZ400-2C[b]	0.22	5.6	<0.01	<0.2	25	<0.05
HZ400-2D[b]	0.54	4.2	0.13	3	7.8	0.24

[a] Columns (0.5 by 8 cm, Pasteur pipettes) were washed at room temperature with 5 ml of 0.02 M α-methyl-D-mannoside in 50 mM Tris-hydrochloride buffer (pH 7.5) containing 1 mM $MgCl_2$, 10 mM $CaCl_2$, and 0.1 M NaCl followed by 5 ml of the buffer without α-methylmannoside. Samples were applied and eluted at room temperature in the second buffer and collected in ice.

[b] See footnote *a*, Table 1.

TABLE 3. *Effect of culture age on small invertase production in HZ400-5A as determined by chromatography on ConA-Sepharose*[a]

Turbidity (Klett units)[b]	Total invertase (U/ml)	Total protein (mg/ml)	% Small invertase[c]
74	27	5.1	0
119	59	14.1	0.3
144	70	20.3	0.3

[a] Twenty liters of yeast extract (0.3%), peptone (0.5%; Bacto, Difco), and glucose (2%) medium in a 30-liter fermentor were inoculated with 5% (vol/vol) of a stationary phase culture of HZ400-5A and incubated at 30°C. Samples (1.5 liters) were withdrawn at various times, the cells were harvested and broken, and the crude extract was assayed as described in the text.

[b] Measured with a Klett filter (red no. 66) with water as a blank.

[c] Invertase not bound to ConA-Sepharose.

duce hydrophobic binding, there was no significant change in the results (data not shown).

The binding of invertase to ConA-Sepharose was also used to investigate the relationship among the *SUC1* gene, small invertase deficiency, and invertase hyperproduction. The introduction of the *dgr* locus into either *SUC1* or *SUC3* yeasts increased total invertase activity four- to sixfold (Table 1). Furthermore, in four sister ascospores (HZ400-2A, B, C, D) obtained from mating mutant dgr3 (*SUC3*) × D10-ER1 (*SUC1*), the deficiency of small invertase segregated with the *SUC1* locus and independently of the *dgr* locus (Table 2). It is also of interest that the specific activity of small invertase in the new mutant strains is significantly less than normal regardless of the state of the *DGR* gene.

(ii) Behavior during gel permeation chromatography. Crude extracts of FH4C chromatographed on Bio-Gel A-0.5 m yielded a single, broad peak of large invertase (peak L-F, Fig. 1A) and a peak of small invertase (peak S-F, Fig. 1A) equaling 3% of the total activity. When a crude extract of strain 4059 was chromatographed, 10% of the activity recovered eluted as small invertase (peak S-4, Fig. 1C). The large invertase eluted as a complex peak, part of the activity (peak VL-4, Fig. 1C) eluting ahead of FH4C invertase and thus probably a molecule of greater size (Table 4). Bio-Gel chromatography of crude extracts from the new mutant strains revealed that both D10-ER1 and HZ400-5A contain at least two size classes of large invertase (VL and L, Fig. 1B and D) similar to those in the parent 4059, but contain only trace amounts of small invertase (S-D and S-H, Fig. 1B and D), specifically 0.02 to 0.05% of the total activity in the case of D10-ER1 and 0.4% for HZ400-5A. In the initial studies, Bio-Gel columns were developed in 50 mM Tris-hydrochloride buffer, pH 7.3 (Fig. 1A and B). Later columns were run in Tris buffer containing 0.5 M NaCl to counteract nonspecific ionic interactions because this increased the separation between large and small invertases (Fig. 1C and D).

(iii) CsCl equilibrium centrifugation. Because proteins and carbohydrates vary widely in buoyant density (values around 1.3 g/cm^3 are characteristic for proteins, whereas those around 1.6 g/cm^3 are associated with carbohydrates [5]), isopycnic centrifugation in CsCl was used to confirm the virtual absence of small invertase in strain HZ400-5A. Large invertase secreted by

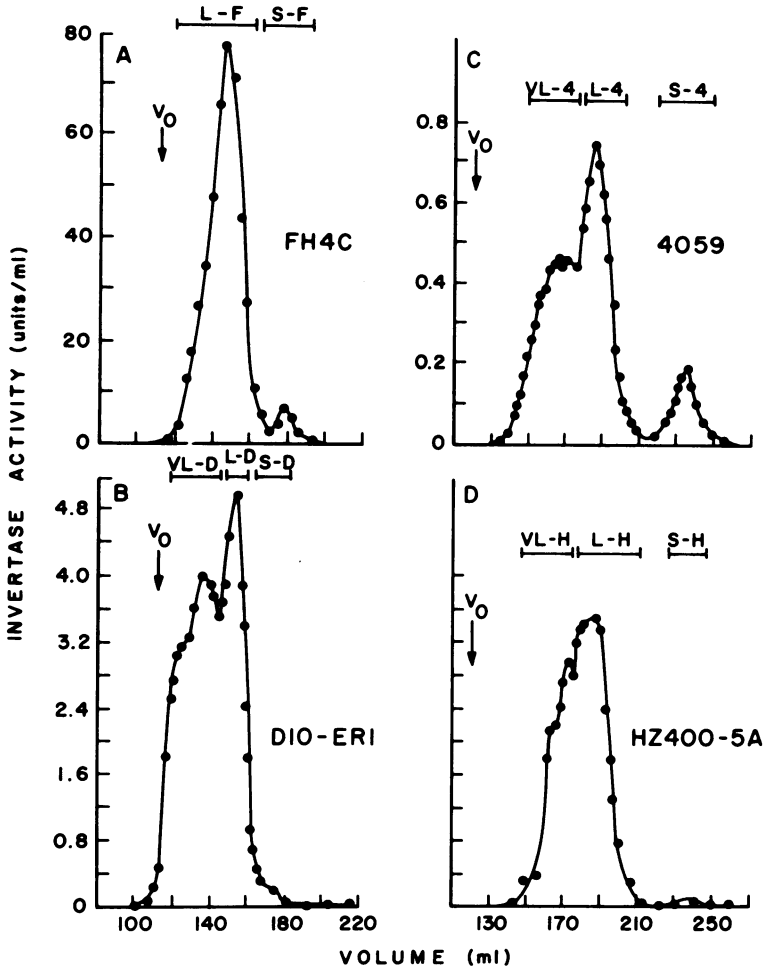

FIG. 1. *Comparison by gel permeation chromatography of invertases in crude extracts of various yeast strains. (A) A crude extract of strain FH4C (1,900 U of invertase, 40 mg of protein) was loaded on a column (2 by 90 cm) of Bio-Gel A-0.5 m which was developed in 50 mM Tris-hydrochloride buffer (pH 7.3) at 12 ml/h (see text). (B) A crude extract of strain D10-ER1 (200 U of invertase, 55 mg of protein) was chromatographed as in (A). (C) A crude extract of strain 4059 (45 U of invertase, 26 mg of protein) was loaded on a column (2 by 95 cm) of Bio-Gel A-0.5 m which was developed in 50 mM Tris-hydrochloride buffer (pH 7.3) containing 0.5 M NaCl at 10 ml/h. (D) A crude extract of strain HZ400-5A (174 U of invertase; 11 mg of protein) was chromatographed as in (C).*

the *SUC1* parent strain 4059 and by HZ400-5A band as broad peaks with peak densities of 1.43 and 1.45, respectively (Fig. 2). Purified FH4C large invertase yields a more compact peak with a peak density of 1.45 (data not shown). Strain 4059 also has an invertase with a peak density of 1.31. The denser invertase would be the glycosylated form, whereas the lighter would be the aglycan small invertase. In HZ400-5A, the denser form is present, but the lighter invertase species is undetectable. By centrifugation in CsCl, then, no small invertase is apparent in cell extracts of HZ400-5A.

Further characterization of the large invertase from strain HZ400-5A. When crude extracts of FH4C are chromatographed on Bio-Gel A-0.5 m in the presence of 0.5 M NaCl (plot not shown), a single peak of large invertase emerges similar to peak L-F shown in Fig. 1A but with a K_{av} of 0.36 (Table 4). Large invertase from strain HZ400-5A (or any other of the *SUC1* strains studied), however, elutes in 0.5 M NaCl as a complex peak primarily composed of two components with K_{av} values of 0.30 and 0.37 (Fig. 1C and Table 4). The enzyme in the first peak of the complex (VL) should be greater in

TABLE 4. *Comparison of K_{av} values[a] for large and small invertases*

Strain	K_{av}		
	VL[b]	L[b]	S[b]
Crude extracts			
FH4C		0.36 (97)[c]	0.70 (3)
4059	0.28 (41)	0.36 (48)	0.64 (10)
HZ400-5A	0.30 (50)	0.37 (50)	0.65 (0.4)
Partially purified large invertases[d]			
FH4C		0.35[e]	
HZ400-5A	0.31[f]		

[a] K_{av} values (partition coefficients) were obtained after chromatography on a Bio-Gel A-0.5 m column (2 by 95 cm) in 50 mM Tris-hydrochloride buffer (pH 7.3) containing 0.5 M NaCl and at a flow rate of 7 to 10 ml/h. See also legends to Fig. 1 and 2.

[b] See Fig. 1.

[c] Numbers in parentheses indicate percentage of invertase activity recovered in the peak.

[d] Invertases were partially purified over DEAE-Sephadex (see text). Large invertases were treated with endo-β-*N*-acetylglucosaminidase H as described in text.

[e] Before treatment with endoglucosaminidase; after treatment, K_{av} = 0.54.

[f] Before treatment, with endoglucosaminidase; after treatment, K_{av} = 0.50.

size than the large invertase of FH4C (peak L). To see if this difference in K_{av} is still apparent after removal of most of the covalently linked carbohydrate, large invertases from the two yeasts were treated with endo-β-*N*-acetylglucosaminidase H (an enzyme which cleaves the di-*N*-acetylchitobiosyl residue in the core region of the polysaccharide [25]) and were then rechromatographed on Bio-Gel.

Crude extracts from the two yeast strains were initially passed over DEAE-Sephadex columns to remove the bulk of the noncovalently bound polysaccharides and to partially purify the enzymes. Samples from the first peak of invertase activity (eluting from DEAE-Sephadex at 0.13 M NaCl) were then chromatographed on Bio-Gel A-0.5 m columns. The invertase from strain HZ400-5A eluted as a single peak with a K_{av} of 0.31 (Table 4; Fig. 3A) corresponding to peak VL-H found after Bio-Gel chromatography of the crude extracts (Table 4; Fig. 1D). In the case of FH4C invertase, a single peak eluted from Bio-Gel with a K_{av} of 0.35 (Table 4; Fig. 3B). The K_{av} values of invertases eluting from DEAE-Sephadex at higher salt concentrations were not determined because 0.13 M NaCl had eluted the forms of special interest.

After treatment with endoglucosaminidase H, the enzymes from the two strains chromatographed on Bio-Gel as much sharper peaks. Their K_{av} values, however, still differed; 0.54 for FH4C invertase and 0.50 for HZ400-5A invertase (Table 4; Fig. 3A and B).

DISCUSSION

The formation of large invertase in yeast has been a useful model for the study of glycoprotein biosynthesis in eucaryotes. The occurrence of an internal, nonglycosylated form of invertase, however, has created a problem. From the evidence cited above, it has been difficult to ascertain whether the large and small forms of invertase (i) share a precursor-product relationship; (ii) share a common subunit; or (iii) are structurally unrelated. We have now shown that cells of the new mutant strain D10-ER1 and of the derived invertase-hyperproducing strain HZ400-5A which probably bear a revertant form of the *SUC1* gene (Hackel, Ph.D. thesis) contain large invertase but only barely detectable amounts of small invertase. Our conclusion is based on examination of the invertases from HZ400-5A after

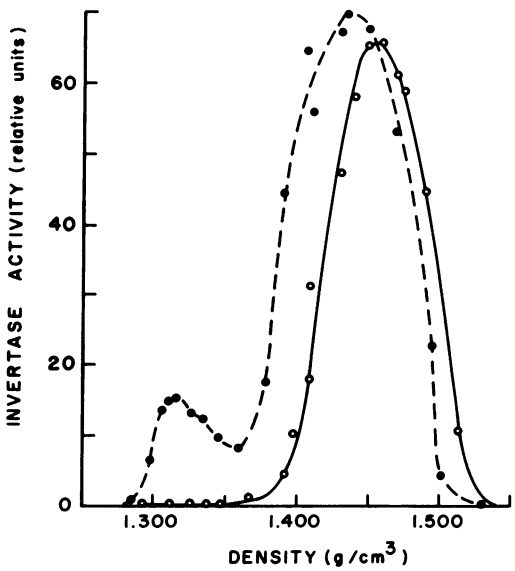

FIG. 2. *Buoyant densities of the invertases in strain 4059 and in the derived mutant HZ400-5A. Crude extracts of strain 4059 (●- - -●) or HZ400-5A (○——○) containing approximately 1 U of invertase were adjusted with solid CsCl to an initial density of 1.36 g/cm³, centrifuged to equilibrium, and fractionated as described in the text. Specific gravity of the CsCl fractions was calculated from refractive index, and samples were assayed for invertase. Invertase activity was inhibited about 20 to 30% by the amounts of CsCl encountered in these experiments, and data are therefore expressed as relative units.*

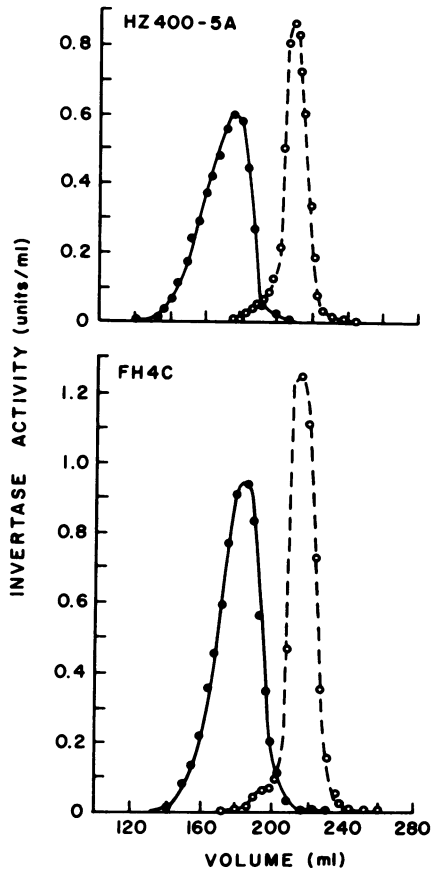

FIG. 3. *Characterization of partially purified large invertases from strains HZ400-5A and FH4C by chromatography on Bio-Gel A (A-0.5m) before and after treatment with endo-β-N-acetylglucosaminidase H. Samples of the large invertase peaks which eluted from DEAE-Sephadex in 0.13 M NaCl were either treated with endoglucosaminidase or left untreated and then chromatographed on a Bio-Gel A-0.5 m column (2 by 95 cm) equilibrated with 50 mM Tris-hydrochloride buffer (pH 7.3) containing 0.5 M NaCl and developed at a flow rate of 7 ml/h. Strain HZ400-5A (see text for digestion conditions): Samples (15 U of invertase in 3 ml) before* (●——●) *and after treatment* (○- - -○). *Strain FH4C: Samples (25 U in 1.5 ml) before* (○- - -○) *and after treatment* (●——●).

isopycnic centrifugation, ConA binding, and gel permeation chromatography. These methods depend on three different molecular properties (buoyant density, specific affinity, and molecular size). Strain HZ400-2C is also deficient in the small enzyme. Furthermore, HZ400-5A cells from either early or late-exponential-phase cultures contain only trace amounts of small invertase (Table 3). When these results are examined in light of other available information, the idea that the small and large enzymes are structurally distinct seems the most tenable.

Large and small invertases appear to be kinetically identical and antigenically related (2, 10, 23, 24, 28), although their amino acid compositions differ (10). Gascon et al. (10) suggested that small invertase might be composed of a subunit identical with one in the large enzyme (presumably containing an active site) and a second subunit unique to the small form; this structure might account for the differences in amino acid content between the two invertases. The amount of active small enzyme might then be limited selectively if the dissimilar subunit is absent or defective.

The idea of shared subunits, however, has become less tenable in light of recent discoveries. Large invertase appears, by a variety of criteria, to be a dimer composed of two identical subunits (26) and to contain approximately six cysteine residues per dimer (22; B. B. Abrams, unpublished data). Because small invertase lacks cysteine (2, 9), it seems highly improbable that one of its subunits is identical with those in the large form. Limited proteolysis of the subunit of large invertase to remove cysteine residues also seems improbable, because the proteolytic cleavage would have to remove three cysteine residues per subunit. The reactivities of the cysteine residues and their distribution in the cyanogen bromide cleavage products from large invertase suggest that the cysteine residues do not lie close together (22; Abrams, unpublished data).

Another possibility is that the trace amounts of small invertase activity present in strains D10-ER1 and HZ400-5A are the result of a mutation in the structural gene for small invertase, so that the enzyme being synthesized is only marginally active, although the actual protein may be present in substantial quantities. The mutation, in this case, would probably involve the enzyme's active center. This explanation would also mean that large and small invertases are not products of the same structural gene because the activity of the large enzyme appears unimpaired. An immunoassay specific for determinants outside the active center of small invertase might resolve this issue.

Our results, then, lend support to the idea that the two forms of invertase are distinct and make it unlikely that they share a common structural gene even though antigenically cross-reactive. Large and small invertases do appear to share regulatory genes, however, although they are not equally sensitive to catabolite repression. For example, strains which are unable to ferment sucrose lack both large and small invertases (10, 14) and revertants usually regain both forms of the enzyme (14). Mutant D10-ER1 is a

revertant from an invertase-negative yeast (D10) and is unique in that it has regained the ability to form large invertase in an amount equaling that of the original parent, while producing no more than trace amounts of a small enzyme. When D10-ER1 was crossed with the hyperproducing strain dgr3, both total invertase and small invertase activity rose, thus indicating that the *dgr* mutation, at a locus distinct from *SUC1*, had affected a common regulatory function. Specifically, when two *SUC1 dgr* progeny from tetrad dissections (HZ400-5A or -2C) were tested, the clones produced four to six times more total invertase than D10-ER1, although much less than the *SUC3* parent strain (dgr3). Similarly, the level of small invertase in HZ400-5A was at least 10-fold that in D10-ER1, although it remained significantly below that of either dgr3 or 4059 (0.2 to 0.4% compared with 4 to 10% in dgr3 and 4059).

The large invertase from the *SUC1* strains HZ400-5A and 4059 includes active material that elutes on gel permeation chromatography before the large invertase from FH4C. This observation was of interest because differences in size among large invertases from strains with the various *SUC* genes have not been documented previously. (The gel filtration columns used in earlier studies excluded FH4C large invertase and would not have resolved molecules of even greater size.) When the very large invertase from HZ400-5A was treated with endo-β-*N*-acetylglucosaminidase H to remove most of the carbohydrate, the product eluted ahead of the treated FH4C enzyme. Thus, the HZ400-5A enzyme might have a larger protein core, or the larger size may simply reflect a less complete removal of the carbohydrate. It has already been shown that complete removal of carbohydrate from the undenatured purified FH4C invertase by endoglucosaminidase H is not possible (26). The bulk of the remaining carbohydrate can, however, be removed by treatment in the presence of sodium dodecyl sulfate (26), and it has recently been found that the core produced under these conditions from the purified large invertase of strain 4059 is the same size as that from FH4C large invertase (T. Mizunaga, unpublished data). We infer from these findings that the very large components in the *SUC1* glycoprotein invertases contain protein cores of the same size as in the main large fractions, but have a higher content of carbohydrate.

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