THE EFFECT OF DITHIOTHREITOL ON EXTERNAL YEAST INVERTASE

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SUMMARY: *Saccharomyces cerevisiae* external β-D-fructofuranosidases (EC 3.2.1.26) when treated with dithiothreitol are included to a greater extent during gel filtration on Sephadex G-200 than the untreated enzymes. Reduced invertase retains its reactivity toward antiserum directed against the attached mannan polymer. Dithiothreitol treatment has little effect on the heat stability of the enzyme. Yeast cells treated with dithiothreitol release varying amounts of invertase at rates dependent on the structure of the cell wall mannan. Our results suggest that a disulfide bond in yeast invertases may function, in concert with the carbohydrate attached to the proteins, in anchoring these glycoenzymes in the yeast cell wall.

Yeast external invertases are glycoproteins containing 50-70% by weight of D-mannose (1,2). The attached mannan polymer is similar to that of the cell wall structural mannan (3). However, the function of the enzyme-bound carbohydrate is unclear because removal of the mannan affects neither the activity nor stability of invertase (3,4). Neumann and Lampen (1) showed that the purified enzyme from the *Saccharomyces* strain FH4C (5) contains 5 half-cystine residues but that only 3 of these are reactive with sulfhydryl reagents such as iodoacetate or 5,5'-dithiobis(2-nitrobenzoic acid). These data, coupled with the observations that invertase can be released from whole yeast cells following treatment with mercaptans (6-8), suggested that the enzyme might contain disulfide bonds. Our results confirm this conclusion and suggest a function for both the disulfide bonds and the attached carbohydrate in helping to retain the invertase in the cell wall.

EXPERIMENTAL

Sephadex G-200 (fine) was from Pharmacia and Bio-Gel A5m from Bio-Rad Laboratories. [^{3}H]-Iodoacetic acid (357 Ci/mole) and [2-^{3}H-ethyl]-N-ethyl maleimide (75 Ci/mole) were purchased from New England Nuclear. Dithiothreitol was obtained from Sigma Chemical Company. All other chemicals were reagent grade obtained from common commercial sources.

Cell wall mannan mutants of *S. cerevisiae* X2180-1A were those isolated previously (9,10).

Exo-α-mannanase was purified from cultural filtrates of *Arthrobacter* GJM-1 (11), whereas external β-D-fructofuranosidases were prepared from *S. cerevisiae* strains X2180-1Ax1B and 4484-24D-1 (3). Digestion of *S. cerevisiae* 4484-24D-1 invertase with exo-α-mannanase was performed by procedures detailed elsewhere (3). Invertase activity was measured by the modification (3) of the procedure of Bernfeld (12).

The preparation of antisera against *S. cerevisiae* X2180-1A-5 and *S. cerevisiae* 4484-24D-1 (9,10) and procedures for immunoprecipitation of external β-D-fructofuranosidase (3) followed published methods.

The partially purified invertase (500-1500 units), from which the carbohydrate had been removed by exo-α-mannanase treatment, was incubated for 1 hr at 23° either with 10 μmoles of iodoacetic acid in 10 ml of 0.1 M sodium phosphate, pH 8.0, or with 10 μmoles of N-ethyl maleimide in 1.0 ml of 0.05 M sodium phosphate, pH 6.8. The reactions were stopped by addition of 100 μmoles of DTT and the solutions adjusted to pH 5 with 1 M acetic acid. The products were dialyzed at 4° against 3 changes of 10 l of 0.01 M sodium acetate, pH 5.0, and the nondialyzable material was lyophilized. No loss of activity was observed and the invertase chromatographed with the reduced enzyme on Sephadex G-200. Incubation of the iodoacetate-treated invertase with [^{3}H]-iodoacetate (1 μmole) was carried out for 3 hr at 23° in 1.0 ml of sodium phosphate, pH 8.0. N-Ethyl maleimide-treated invertase was incubated for 1.2 hr at 23° with [2-^{3}H-ethyl]-N-ethyl maleimide (0.3 μmoles) in 1.0 ml of 0.05 M sodium phosphate, pH 6.8.

RESULTS AND DISCUSSION

Prolonged treatment of *S. cerevisiae* 4484-24D-1 external invertase with exo-α-mannanase (120 hr) removed all of the mannose from the enzyme (3). The size of the enzyme, judged by chromatography on Bio-Gel A5m, was decreased by this treatment (Fig. 1). Tarentino *et al.* (4) removed the carbohydrate from

ABBREVIATIONS. DTT is for dithiothreitol.

yeast invertase using an endo-β-N-acetylglucosaminidase and also observed that the "mannanless" enzyme appeared smaller than the native enzyme on a column of Sepharose 6B. In contrast to its properties on agarose, *S. cerevisiae* 4484-24D-1 invertase from which the carbohydrate had been removed appeared slightly larger than the untreated enzyme following gel filtration on Sephadex G-200 (Fig. 2). After limited exo-α-mannanase digestion (4 hr), this effect was

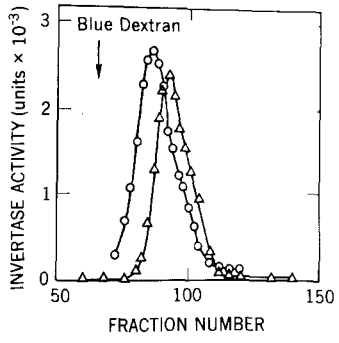

Fig. 1. Chromatography of invertases on Bio-Gel A5m (2 x 90 cm). Elution was with 0.1 M sodium acetate, pH 5.0, and 2 ml fractions were collected and assayed for invertase activity. Approximately 10,000 units of 4484-24D-1 invertase were applied to the column in 2 ml; untreated invertase (0-0), and invertase treated for 120 hr (8) with exo-α-mannanase (Δ-Δ). Blue dextran was included as an internal standard.

enhanced. The mannan attached to the native enzyme apparently interacted with the dextran polymer thereby retarding elution. Partial removal of the carbohydrate decreased this interaction; and, on complete removal of carbohydrate, the gel filtration property of invertase closely approached that of a globular protein of equivalent molecular weight.

Interestingly, this anomalous Sephadex filtration behavior was not observed with invertases which had been preincubated in the presence of DTT. The 4484-24D-1 invertase treated with DTT showed an expected decrease in size on Sephadex G-200 chromatography following exo-α-mannanase digestion (Table I). This decrease in size parallels the degree of digestion (3). Similar results were obtained with the external invertase isolated from *S. cerevisiae* X2180-

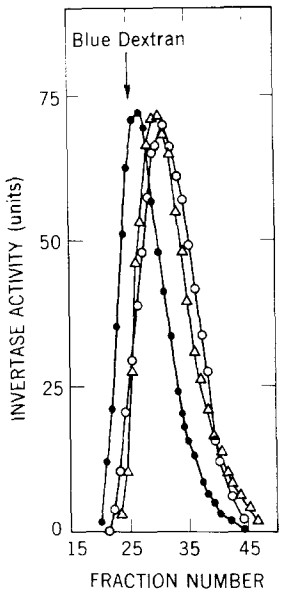

Fig. 2. Chromatography of invertases on Sephadex G-200 (2 x 46 cm). Elution was with 0.1 M sodium acetate, pH 5.0, and 2 ml fractions were collected and assayed for invertase activity. Approximately 500 units of 4484-24D-1 invertase were applied to the column in 2.0 ml; untreated invertase (0-0); invertase after 4 hr with exo-α-mannanase (●-●); invertase after 120 hr with exo-α-mannanase (Δ-Δ). Blue dextran was included in each run as an internal standard.

1Ax1B (Table II). Repeated attempts to label the DTT-treated mannanless *S. cerevisiae* 4484-24D-1 invertase either with [2-^{3}H-ethyl]-N-ethyl maleimide or [^{3}H]-iodoacetic acid were unsuccessful. Numerous examples exist of the unreactivity of protein sulfhydryl groups toward alkylating reagents (13) and N-ethyl maleimide (14), and the reduced disulfide bonds of the core protein of *Hansenula wingei* 5-factor are similarly unreactive (15). We conclude that DTT does reduce interchain disulfide bonds in the invertase but that the resulting SH groups are not reactive under the mild alkylation conditions employed.

The DTT-treated invertase from *S. cerevisiae* 4484-24D-1 was precipitated by antiserum directed against the mannan portion of the molecule (Fig. 3). Thus, the catalytic subunit, like the native enzyme, must be a mannan protein. Furthermore, the stability at 37° at pH 2.0, 3.5 or 7.8 of the enzyme was unchanged following DTT treatment (Table II). The heat stability of invertase from

TABLE I

Effect of DTT on elution of invertase following exo-α-mannanase digestion.

Time with exo-α-mannanase (hr)	Ve/Vo	
	With DTT	Without DTT
S. cerevisiae 4484-24D-1		
0	1.34	1.21
1	1.41	1.06 (4 hr)
11	1.56	-
23	1.59	-
47	1.61	-
71	1.63	-
95	1.66	-
120	1.75	1.17
S. cerevisiae X2180-1Ax1B		
0	1.26	1.23
4	1.60	1.14

Invertase from *S. cerevisiae* 4484-24D-1 was incubated with exo-α-mannanase for the indicated times. Samples (1 ml) were removed and treated with or without 10 mM DTT for 1 hr at 30° and applied to a 2 x 46 cm column of Sephadex G-200 in 0.1 M sodium acetate, pH 5.0, at 4°. Blue dextran was added to each sample as an internal standard. Fractions of 2 ml were collected and assayed for invertase activity and absorbance at 660 nm. Ve/Vo is the ratio of the elution volume of invertase activity to the elution volume of blue dextran.

which the carbohydrate had been removed by exo-α-mannanase digestion was also unaffected by DTT treatment and was similar to the native enzyme.

That dissociation of invertase by DTT treatment did not affect its catalytic activity or stability suggested that disulfide bonds might function in some capacity other than catalysis. In confirmation of the results of others (6-8), we

TABLE II

Effect of DTT on heat stability of S. cerevisiae 4484-24D-1 invertases.

Invertase	$t_{1/2}$ (min) pH 2.0	pH 7.8
Untreated	22	620
Untreated + DTT	22	680
Exo-α-mannanase-treated (120 hr)	25	620
Exo-α-mannanase-treated + DTT (120 hr)	19	660

Invertases (0.1 units) were incubated at 37° in 1.0 ml of 0.05 M citrate phosphate containing 1 mmole of NaCl and 1 mg of bovine serum albumin, either with or without 10 μmoles of DTT, at pH 2.0, 3.5 and 7.8. The logarithm of the enzyme activity at various times was plotted against incubation time to obtain the $t_{1/2}$ for inactivation. No detectable inactivation was observed at pH 3.5.

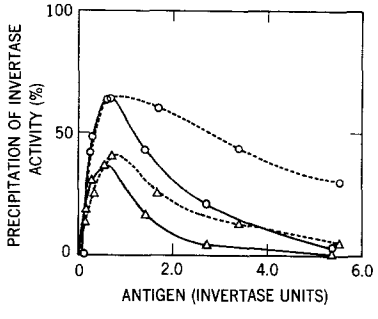

Fig. 3. Effect of DTT treatment on the precipitation of *S. cerevisiae* 4484-24D-1 invertase by mannan antisera. Invertase which had been treated 4 hr with exo-α-mannanase was incubated with (dashed lines) and without (solid lines) 10 mM DTT for 1 hr at 30°. Precipitation of the invertase by 4484-24D-1 antiserum (0-0) and X2180-1A-5 antiserum (Δ-Δ) was performed as indicated in the experimental section. Dialysis of the DTT-treated enzyme (1 ml) versus 3 changes of 1 l of 0.1 M sodium acetate, pH 5.0, over a 3 day period did not lead to reassociation of the enzyme as judged by chromatography on Sephadex G-200.

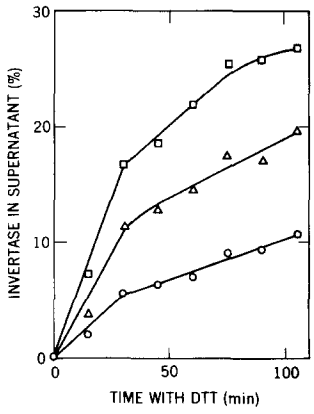

Fig. 4. Release of invertase from cell wall mannan mutants by DTT treatment. Yeast cells, grown to log phase in 5% D-glucose, 0.5% yeast extract and 0.3% Casamino acids, were harvested and washed with 1% KCl. The cells were suspended in an equal weight of 0.05 M sodium phosphate, pH 7.0, and incubated at 37° for the indicated times. Aliquots (100 µl) were removed and added to 100 µl of 50 mM N-ethyl malemide in 0.05 M sodium phosphate, pH 7.0. Activity was determined using this cell suspension and the supernatant obtained after centrifuging the cells, and the percent of invertase released was determined by comparing these values. Yeast strains: *S. cerevisiae* X2180-1A (0-0), *S. cerevisiae* X2180-1A-5 (Δ-Δ) and *S. cerevisiae* X2180-1A-4 (□-□).

found that a portion of the external invertase was released from whole yeast cells by DTT treatment (Fig. 4). Both the rate and extent of invertase release under these conditions were dependent on the mannan structure of the yeast strain. Cell wall mannan of the wild type *S. cerevisiae* X2180-1A has a backbone of α1→6-linked mannose units to which are attached, through α1→2 linkages, mannose, mannobiose and mannotriose sidechains. Some of the latter are substituted by mannobiosylphosphate groups. *S. cerevisiae* X2180-1A-5, which lacks these oligosaccharide side chains in its mannan, released more invertase than the wild type cells. *S. cerevisiae* X2180-1A-4 mannan lacks mannotriose sidechains and has a large proportion of mannosylphosphate groups linked to mannobiose sidechains. This latter strain released invertase most readily of those tested. The results on invertase release from whole cells indicate that the polysaccharide side chains of the cell wall structural mannan proteins and the yeast mannan invertase function to retain the extracellular glycoenzyme in the cell wall matrix. That purified invertase can be dissociated by DTT treatment suggests that the disulfide bonds in this enzyme may

play a role in anchoring it in the cell wall. It will be of interest to determine whether the external invertase secreted by yeast protoplasts (16) contains an intact disulfide bond or whether the enzyme is secreted in the reduced form and linked to the cell wall by the action of a sulfhydryl-disulfide interchange enzyme (17).

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