Immunochemical Characterization of the Mannan Component of the External Invertase (β-Fructofuranosidase) of *Saccharomyces cerevisiae*†

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ABSTRACT: The external mannan protein invertase (β-fructofuranosidase, EC 3.2.1.26) activities were purified from three strains of *Saccharomyces cerevisiae* whose cell wall mannans have related but different carbohydrate structures. The immunochemical reactivities of the external invertase and of the cell wall mannan from each strain were compared using several anti-mannan sera that were specific for distinctive carbohydrate structural determinants. The invertase activities from each of the three strains were precipitated only by those antisera which would also precipitate the cell wall mannan of the corresponding strain. Furthermore, precipitation of each enzyme with anti-mannan serum was inhibited only by those mannan oligosaccharide haptens which would also inhibit homologous immunoprecipitation of the corresponding cell wall mannan. The mannans attached to the external β-fructofuranosidases from the *S. cerevisiae* X2180 diploid and 4484-24D-1 strains each were digested partially with a bacterial exo-α-mannanase which is effective in removing the short $\alpha(1\rightarrow2)$- and $\alpha(1\rightarrow3)$-linked side chains from the $\alpha(1\rightarrow6)$-linked mannan backbone. The modified glycoenzyme activities from both strains became precipitable by antiserum directed against a linear, unbranched $\alpha(1\rightarrow6)$-linked mannan. These studies indicate that the carbohydrate structure of *S. cerevisiae* β-fructofuranosidase mannans is similar to that of the cell wall mannan proteins, which are known to contain an $\alpha(1\rightarrow6)$-linked backbone to which are attached side chains by $\alpha(1\rightarrow2)$ and $\alpha(1\rightarrow3)$ linkages. In addition, mutations which affect the carbohydrate structure of the cell wall mannans caused similar changes in the mannan of the external yeast invertase. The external β-fructofuranosidase from *S. cerevisiae* 4484-24D-1, a strain whose cell wall mannan lacks phosphate owing to a defective mannosylphosphate transferase, was found to be completely digestible by the exo-α-mannanase. This yielded a preparation which had no detectable hexose and which was unreactive toward any anti-mannan serum. Both the specific activity of the "mannan-less" enzyme and its stability toward heat were the same as those of the untreated enzyme. Thus, the mannan that is attached covalently to the external β-fructofuranosidase does not enhance the stability of the enzyme. We also find that external invertase activity is formed by all mannan mutants selected to date, including *S. cerevisiae* X2180-1A-5 which makes a mannan that contains only traces of side chains attached to the $\alpha(1\rightarrow6)$-linked backbone. Thus, the fine structure of the polysaccharide component of this mannan enzyme that is altered in these mutants cannot be important for the translocation of the enzyme through the plasma membrane or its incorporation into the cell wall.

Using a mutant strain of yeast having a single genetic locus for β-fructofuranosidase (invertase) activity, Lampen and coworkers showed several years ago that on derepression this enzyme appears in two forms, an intracellular protein and an extracellular glycoprotein (Neumann and Lampen, 1967; Gascón and Lampen, 1968; Gascón *et al.*, 1968; Lampen, 1968). Since that time, other reports have appeared indicating that the dual nature of invertase activity is a common phenomenon in yeast (Ottolenghi, 1971a,b). External invertases contain from 50 to 70% mannose, by weight (Neumann and Lampen, 1967; Greiling *et al.*, 1969), that, depending on the yeast strain used as the source of the enzyme, is linked glycosidically to serine in the form of short oligosaccharides (Greiling *et al.*, 1969) and to asparagine through *N*-acetylglucosamine as polysaccharide chains containing up to 70 sugar units (Neumann and Lampen, 1969). However, the detailed chemical structure of the covalently linked mannan has not been investigated previously.

External β-fructofuranosidase provides a convenient marker for the study of yeast cell wall mannan biosynthesis (Lampen, 1968; Liras and Gascón, 1971). Our purpose was to determine if the mannan of the extracellular enzyme was structurally related to the total cell wall mannan; and, further, if mutations which altered the structure of the cell wall mannan (Raschke *et al.*, 1973; Ballou *et al.*, 1973; Antalis *et al.*, 1973) caused similar alterations in the structure of the mannan attached to the external enzyme. Both of these questions were answered affirmatively. The mannan components of the invertase and of the cell wall were immunochemically indistinguishable, and the mannan of both the enzyme and the wall were altered in a coordinate fashion in the mannan mutants. The invertase, from one of the mutants used in our studies, was found to be susceptible to complete enzymatic removal of the attached mannan, allowing for the first time an evaluation of the importance of attached carbohydrate in maintaining the enzymic properties of this glycoenzyme. The activity and stability of the deglycosylated enzyme were essentially identical with those of the intact glycoprotein.

Materials and Methods

Materials. DEAE-Sephadex A-50 and SP-Sephadex C-50 were obtained from Pharmacia. Ampholine carrier electrolytes, for isoelectric focusing, were from LKB. The $\alpha(1\rightarrow6)$-linked mannohexaose was prepared by Mr. Louis Rosenfeld by partial acetolysis (Jones and Ballou, 1969a) of the "back-

† From the Department of Biochemistry, University of California, Berkeley, California 94720. *Received August 23, 1973.* This work was supported by National Science Foundation Grants GB-19199 and GB-35229X, by U. S. Public Health Service Grant AM884, and by U. S. Public Health Service Postdoctoral Fellowship No. 5 F02 GM52085.

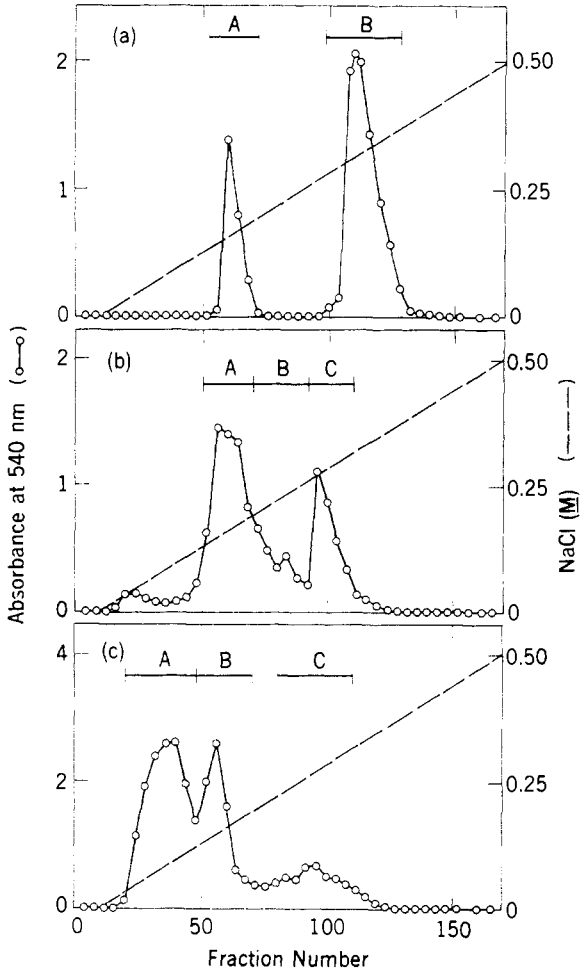

FIGURE 1: Fractionation of invertases from the ammonium sulfate step was on 4.8 × 80 cm columns of SP-Sephadex C-50. The columns were eluted with a linear gradient of 0–0.5 M NaCl in sodium citrate (pH 3.65, 0.01 M in Na^+), and 12.5-ml fractions were collected. Fractions under the bars were pooled. The invertases were from *S. cerevisiae* strains (a) X2180 diploid, (b) X2180-1A-4, and (c) 4484-24D-1.

bone" mannan obtained by enzymatic digestion (Jones and Ballou, 1968) of Red Star bakers' yeast mannan. The exo-α-mannanase from *Arthrobacter* GJM-1 cultural filtrates (Jones and Ballou, 1969b) was kindly supplied by Drs. Pauline Hsaio and W. C. Raschke. All other materials were reagent grade purchased from common sources.

Analytical Procedures. Total carbohydrate was determined by the phenol–sulfuric acid method with D-glucose as the standard (Dubois *et al.*, 1966). Protein was assayed by the method of Lowry *et al.* (1951), using bovine serum albumin as the standard, or by using a nomograph based on the extinction coefficients for enolase and nucleic acid given by Warburg and Christian (1942). The β-fructofuranosidase activity was measured by a modification of the procedure of Bernfeld (1951). The invertase preparation (10–100 μl) was added to 1.0 ml of 0.05 M sodium acetate (pH 5.0) which contained 7% sucrose at 37°; 1 ml of basic 3,5-dinitrosalicylate reagent was added at various times, the quenched reaction mixtures were heated 5 min in a boiling water bath, then diluted with 2–10 ml of distilled water, and the absorbance was read at 540 nm. In this assay, the molar extinctions of glucose and fructose were identical. One unit of β-fructofuranosidase activity was defined as 1 μmol of sucrose hydrolyzed per min at 37°.

Yeast Strains. S. cerevisiae X2180 diploid strain was obtained from a cross of the isogenic haploid strains X2180-1A and X2180-1B, which were obtained from Dr. R. K. Mortimer, Department of Medical Physics, University of California, Berkeley. *S. cerevisiae* X2180-1A-4 was selected by Raschke *et al.* (1973) and *S. cerevisiae* 4484-24D-1 by Ballou *et al.* (1973).

TABLE I: Purification of X2180 Dipold Invertase.

Fraction	Volume (ml)	Protein[a] (mg)	Activity (units)	Specific Activity (units/mg)
Streptomycin supernatant	3200	460,000	71,000	0.15
Supernatant from 50° heat treatment	2900	93,000	49,000	0.53
$(NH_4)_2SO_4$ dialysate	1100	440	21,600	49
SP-Sephadex (peak B, Figure 1a)	700	120	11,000	92
DEAE-Sephadex	230	21.2	7,680	362

[a] Protein was determined using a nomograph based on the extinction coefficients for enolase and nucleic acid (Warburg and Christian, 1942).

Immunological Methods. Antisera against *S. cerevisiae* S288C, *S. cerevisiae* X2180-1A-4, *S. cerevisiae* 4484-24D, *S. cerevisiae* 4484-24D-1, and *Kloeckera brevis* were obtained as described previously (Raschke *et al.*, 1973). Antisera specific for the α(1→6)mannan chain was prepared by adsorption of anti-*S. cerevisiae*, X2180-1A-5 serum (Raschke *et al.*, 1973) with *S. cerevisiae* X2180-1A cells (Ballou, 1970). Procedures for the preparation of yeast cell wall mannans have been described (Raschke *et al.*, 1973; Kocourek and Ballou, 1969) and methods for immunoprecipitation and inhibition reactions with cell wall mannans are detailed elsewhere (Ballou, 1970; Raschke and Ballou, 1971). Immunoprecipitation of β-fructofuranosidase activity was performed by procedures identical with those used for precipitation of cell wall mannan (Raschke and Ballou, 1971). After centrifuging the precipitin mixtures containing β-fructofuranosidase, aliquots of the supernatants were removed for enzyme assay. To obtain a value for the per cent of activity that was precipitated, the activity remaining in the supernatant was divided by the activity in the supernatant of a sample without added antiserum. No loss in β-fructofuranosidase activity was observed during the 48-hr incubation period with antiserum, and nonspecific inhibition of enzyme activity was not observed with any of the antisera used in this study.

Purification of β-Fructofuranosidase Activity from S. cerevisiae Strains X2180-1AxlB, X2180-1A-4, and 4484-24D-1. Yeast cells were grown to early stationary phase in media containing 0.3% Casamino acids, 0.5% yeast extract, and 2% sucrose at 30° and harvested to give about 1500 g of cells. The cells were suspended in 1500 ml of 0.01 M EDTA (pH 7.0) and broken by passage five times through a Gaulin paint homogenizer. Subsequent purification was carried out by procedures similar to those used by Neumann and Lampen (1967) for purification of the external β-fructofuranosidase of the FH4C mutant of *Saccharomyces* strain 303-67. A flow chart for the purification of the external β-fructofuranosidase from *S. cerevisiae* X2180 diploid is shown in Table I. Each strain tested had multiple forms of β-fructofuranosidase which

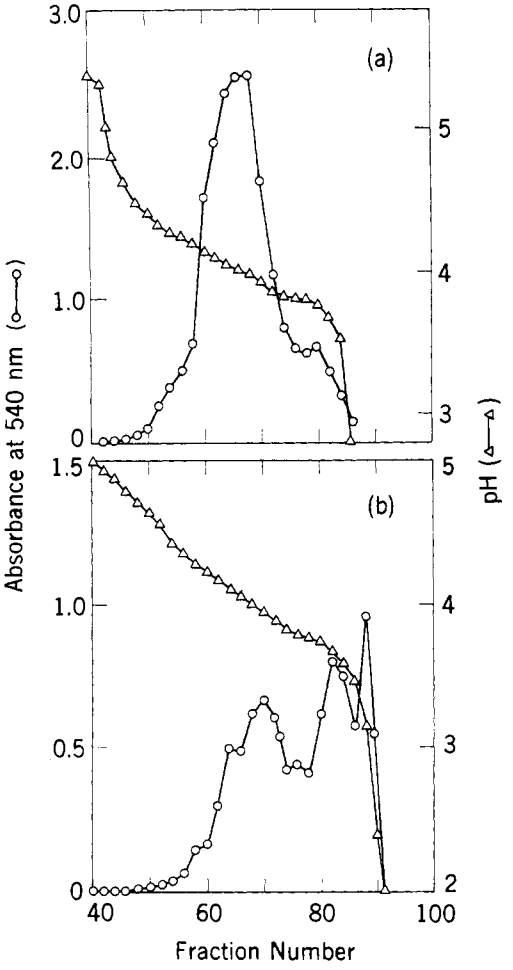

FIGURE 2: Isoelectric focusing of invertases from (a) peak B, Figure 1a, and (b) peak A, Figure 1b. Focusing was performed using a stepwise gradient from 0–100% glycerol (4% per step) containing ampholine carrier electrolytes for focusing between pH 3.0 and 6.0 in a 40-cm high jacketed column maintained at 4°. Following application of 800 V for 36–48 hr, 1-ml fractions were collected and invertase activity and pH measured. No activity was observed before fraction 40.

were soluble in 80% $(NH_4)_2SO_4$ and could be separated by chromatography on SP-Sephadex (Figure 1). The more acidic invertase activity from the *S. cerevisiae* X2180 diploid strain (peak A, Figure 1a) was unreactive with any anti-mannan serum. Therefore, subsequent immunological studies were carried out using only the activity from peak B. The multiple peaks of activity from *S. cerevisiae* X2180-A1-4 were immunochemically indistinguishable, as were those obtained from *S. cerevisiae* 4484-24D-1. Further purification of peak B from Figure 1a and peak A from Figure 1b was attempted using isoelectric focusing between pH 3.0 and 6.0 (Figure 2). Each of these preparations of β-fructofuranosidase was heterogeneous with respect to isoelectric point and no further purification was attempted. The β-fructofuranosidase activities from a given yeast strain, having different isoelectric points, were immunochemically indistinguishable with the anti-mannan sera. Each of the activities from Figure 1a–c hydrolyzed sucrose to yield glucose and fructose, raffinose to give fructose and melibiose, and failed to hydrolyze melezitose, as anticipated for β-fructofuranosidase preparations free of melibiase and β-glucosidase activities (Myrbäck, 1960). Invertase preparations used for all immunochemical work had specific activities no less than 350 units per mg of protein.

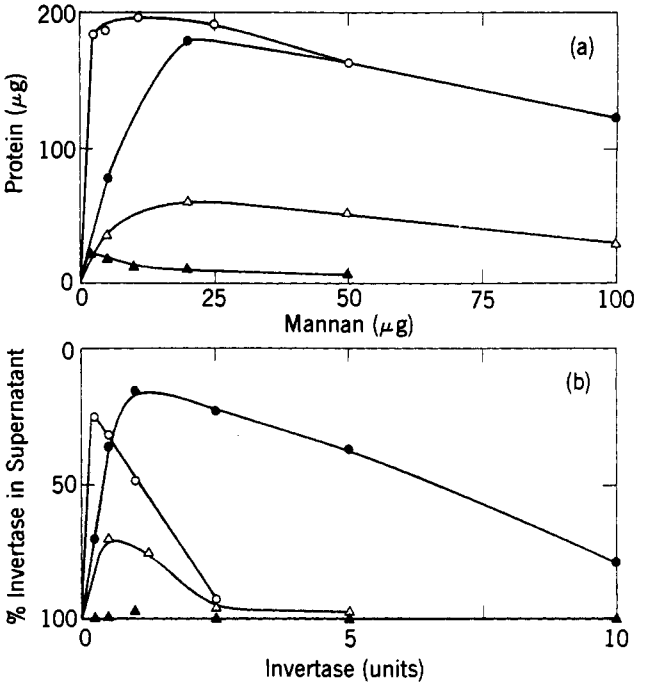

FIGURE 3: Precipitation of *S. cerevisiae* X2180 diploid (a) mannan and (b) invertase activity by anti-X2180 serum (●), anti-X2180-1A-4 serum (△), anti-X2180-1A-5 serum (▲), and anti-4484-24D-1 serum (○). Techniques used for precipitin reactions are indicated in the text.

Results

Immunochemical Properties of β-Fructofuranosidase from Saccharomyces cerevisiae X2180 Diploid Strain. Figure 3 shows the capacity of three anti-mannan sera of different specificities to precipitate the cell wall mannan and the external β-fructofuranosidase activity from *S. cerevisiae* X2180 diploid strain. Antisera having immunochemical reactivity toward α(1→3)-linked nonreducing mannose units (anti-X2180 serum) (Ballou, 1970) and toward α(1→2)-linked nonreducing mannose units (anti-4484-24D-1 serum) (Ballou *et al.*, 1973) strongly precipitated both the cell wall mannan and the invertase activity of the X2180 diploid strain. However, antiserum with specificity toward α(1→6)-linked mannose chains (anti-X2180-1A-5 serum) (Raschke *et al.*, 1973), or antiserum prepared against strain X2180-1A-4 (Raschke *et al.*, 1973), failed to react strongly with either X2180 cell wall mannan or β-fructofuranosidase.

Precipitation of X2180 cell wall mannan (Ballou, 1970) or invertase by anti-X2180 serum was completely inhibited by the tetrasaccharide αMan(1→3)αMan(1→2)αMan(1→2)-Man, as shown in Figure 4. In addition, a mixture of mannotrioses αMan(1→3)αMan(1→2)Man and αMan(1→2)-αMan(1→2)Man, and an α(1→2)-linked mannobiose, inhibited precipitation of both X2180 cell wall mannan and invertase to similar extents. A difference in solubility of the invertase–antibody complex and the cell wall mannan–antibody complex may explain the small difference in inhibition observed with low concentrations of the mannotriose inhibitor. Thus, the observed precipitation of X2180 invertase by anti-X2180 serum was due solely to the anti-mannan activity of the serum.

Treatment of X2180 invertase with exo-α-mannanase (Jones and Ballou, 1969a) resulted in a decreased capacity of the anti-X2180 serum to precipitate the enzyme and an increased reactivity of the invertase toward anti-X2180-1A-5

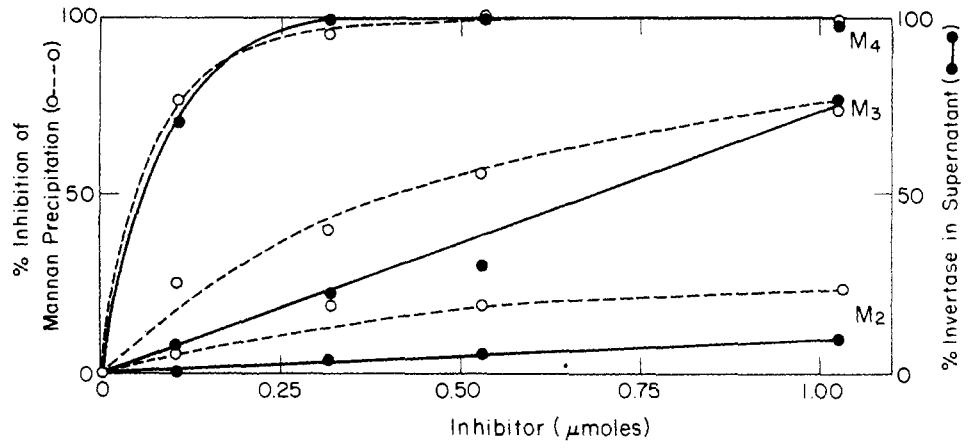

FIGURE 4: Inhibition of the precipitation by anti-X2180 antiserum of *S. cerevisiae* X2180 dipolid mannan (○) and invertase (●) with mannotetraose αMan(1→3)αMan(1→2)αMan(1→2)Man (M_4); mannotriose, αMan(1→3)αMan(1→2)Man plus αMan(1→2)αMan(1→2)Man (M_3); and mannobiose αMan(1→2)Man (M_2). Inhibition reactions were performed as indicated in the text.

serum (Figure 5). The newly acquired response of exo-α-mannanase-treated X2180 invertase was apparently brought about by partial removal of the α(1→3)- and α(1→2)-linked mannan side chains from an α(1→6)-linked mannan backbone structure. In support of this conclusion was the finding that an α(1→6)-linked mannohexaose completely inhibited the precipitation of exo-α-mannanase-digested β-fructofuranosidase by anti-X2180-1A-5 serum (Figure 6). Interestingly, no loss in sucrose-hydrolyzing activity was observed following partial digestion of the X2180 enzyme.

Immunochemical Properties of β-Fructofuranosidase from Saccharomyces cerevisiae X2180-1A-4. As with the β-fructofuranosidase activity from the diploid *S. cerevisiae* X2180, the invertases from the X2180-1A-4 strain which has the *mnn1* mutation (Raschke *et al.*, 1973) exhibited immunochemical properties similar to those of the cell wall mannan of the mutant (Figure 7). The cell wall mannan protein of this mutant strain is differentiated from X2180 mannan by the absence of α(1→3) linkages in the side chains that are attached to the α(1→6)-linked mannan backbone (Raschke *et al.*, 1973). Concomitant with the loss of α(1→3) linkages is a decrease in the mannose to phosphate ratio, and the acquisition of reactivity toward anti-mannan serum having specificity toward mannosyl phosphate determinants (C. E. Ballou, unpublished results) (*e.g.*, anti-*K. brevis*, and anti-4484-24D). The cell wall mannan and the external β-fructofuranosidase from the *mnn1* mutant were strongly precipitated by anti-X2180-1A-4 serum and were unreactive toward anti-X2180 serum. Precipitation by anti-X2180-1A-4 and anti-*K. brevis* sera of both wall mannan and invertase was inhibited approximately 50% by α-D-mannose 1-phosphate. This compound causes complete inhibition of the precipitation of the mannosyl phosphate containing *K. brevis* and *S. cerevisiae* 4484-24D cell wall mannans with anti-*K. brevis* serum (Raschke and Ballou, 1971; C. E. Ballou, unpublished results). Thus, the exact nature of the immunodominant grouping of the X2180-1A-4 mannan remains to be established. However, the similarity in immunochemical reactivities of the cell wall mannan and external β-fructofuranosidase from this *mnn1* mutant suggest that these two groups of molecules contain similar carbohydrate structures.

Immunochemical Properties of β-Fructofuranosidase from S. cerevisiae 4484-24D-1. No phosphorus is present in the cell wall mannan of this yeast strain, and genetic, chemical, and immunochemical analyses indicate that the mannan of this mutant lacks the mannosyl phosphate determinant that is characteristic of *S. cerevisiae* 4484-24D (Ballou *et al.*, 1973). The mannan consists of mannose and α(1→2)-linked mannobiose units attached α(1→2) to the α(1→6)-linked mannan backbone.

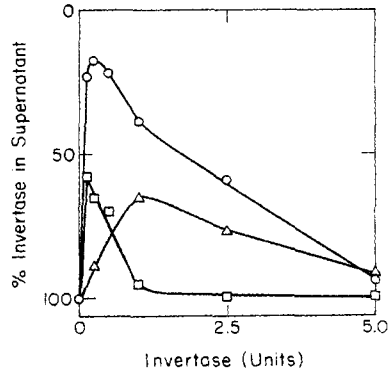

FIGURE 5: Precipitation of *S. cerevisiae* X2180 diploid invertase which had been treated with exo-α-mannanase by anti-X2180 serum (△), anti-X2180-1A-5 serum (□), and anti-4484-24D-1 serum (○). Invertase (100 units) was treated for 4 hr at 30° with *Arthrobacter GJM-1* exo-α-mannanase (5 units) in 1.0 ml of 0.05 M potassium phosphate (pH 6.8) containing 0.1 mM $CaCl_2$. Precipitin reactions on the enzymatically digested invertase were performed as indicated in the text.

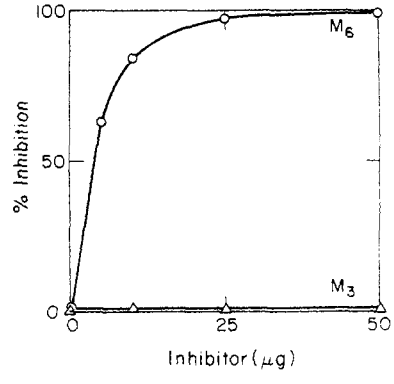

FIGURE 6: Inhibition of the precipitation by anti-X2180-1A-5 serum of *S. cerevisiae* X2180 diploid invertase, which had been treated as described in Figure 5, by α(1→6)-linked mannohexaose (○), and mannotriose, αMan(1→2)αMan(1→2)Man (△). Inhibition reactions were performed as indicated in the text.

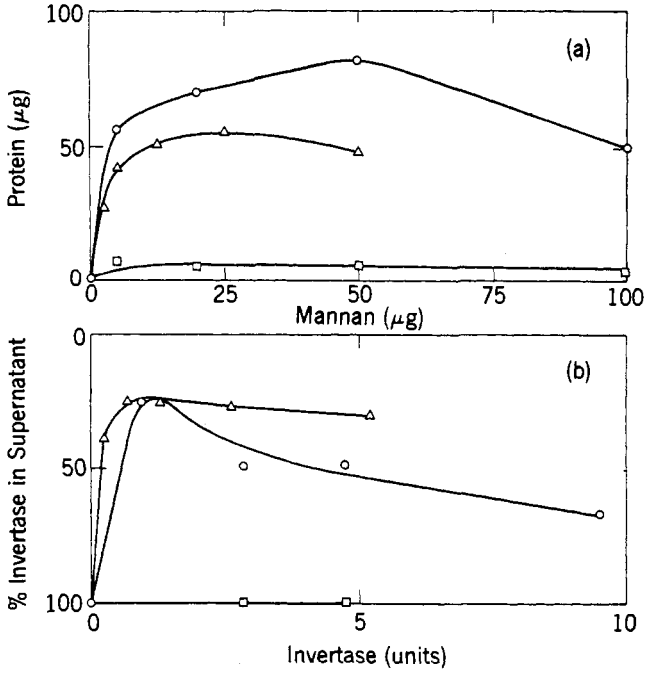

FIGURE 7: Precipitation of *S. cerevisiae* X2180-1A-4 (a) mannan and (b) invertase activity, by anti-X2180 serum (□), anti-X2180-1A-4 serum (○), and anti-*K. brevis* serum (△). Techniques used for precipitin reactions are indicated in the text.

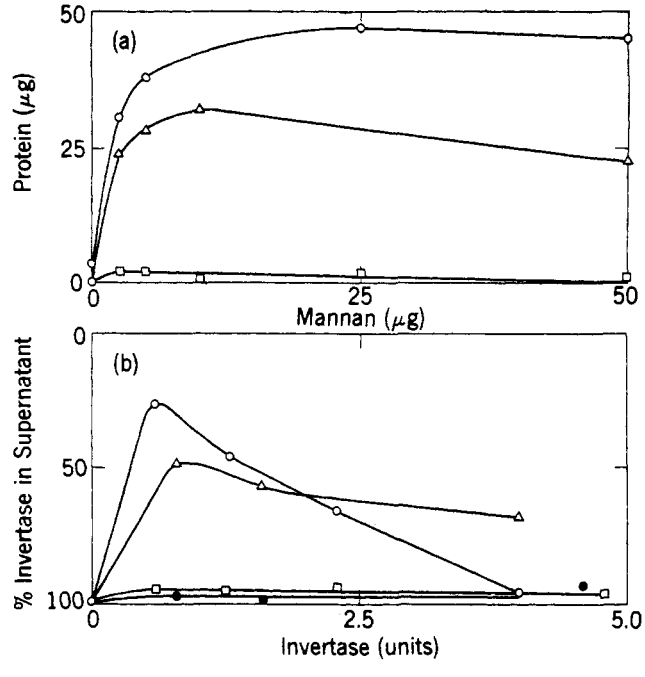

FIGURE 8: Precipitation of *S. cerevisiae* 4484-24D-1 (a) mannan and (b) invertase activity by anti-X2180 serum (●), anti-X2180-1A-5 serum (□), anti-4484-24D serum (△), and anti-4484-24D-1 serum (○). Techniques used for precipitin reactions are indicated in the text.

As with the other two strains discussed above, only those antisera which precipitated the cell wall mannan of *S. cerevisiae* 4484-24D-1 would precipitate the external invertase activity purified from this strain (Figure 8), again indicating a structural similarity between cell wall mannan and invertase mannan.

Enzymatic Digestion of the Mannan of S. cerevisiae 4484-24D-1 External β-Fructofuranosidase. Because the mannan component of the β-fructofuranosidase from this strain lacks phosphate, which normally prevents complete digestion by the exo-α-mannanase, we were able to remove all of the carbohydrate by enzymic treatment. Figure 9 shows the reactivity of 4484-24D-1 β-fructofuranosidase with anti-X2180-1A-5 serum following digestion with the exo-α-mannanase for various times up to 120 hr. The untreated enzyme was unreactive toward this antiserum which is directed against the α(1→6)-linked mannan backbone. During the first 12–48 hr, the exo-α-mannanase digested the α(1→2)-linked side chains from the backbone, exposing the α(1→6)-linked chain which has reactivity with anti-X2180-1A-5 serum. This latter reactivity was gradually lost as the exo-α-mannanase slowly hydrolyzed the α(1→6) linkages, until no significant precipitation of the exhaustively digested invertase by anti-X2180-1A-5 serum occurred after 120 hr. Before exo-α-mannanase digestion, the invertase preparation contained 2.4 μg of hexose per unit of activity. Following digestion no hexose could be detected.

To evaluate the effect that removal of mannan had on the enzymic activity, we subjected the 4484-24D-1 β-fructofuranosidase, which had been treated for various times with exo-α-mannanase, to heat treatment at 37° at pH 1.9, 3.6, 4.7, and 8.2 in 0.05 M citrate phosphate buffers as described in the legend to Table II. No inactivation of enzyme was observed after 100 min at either pH 3.6 or 4.7. However, the untreated enzyme was rapidly inactivated at pH 1.9, undergoing a first-order loss in activity with a $t_{1/2}$ of approximately 8 min. The exo-α-mannanase-digested enzyme was as stable under these acidic conditions as was the untreated β-fructofuranosidase (Table II). At pH 8.2, enzyme inactivation was again first order with a $t_{1/2}$ of approximately 320 min, and again the exo-α-mannanase-treated enzyme was as stable as the untreated enzyme (Table II). Furthermore, we found only a 5% loss in invertase activity following digestion with exo-α-mannanase for 120 hr at 22° in 0.05 M potassium phosphate (pH 6.8).

β-Fructofuranosidase Activity in Intact Cells of S. cerevisiae X2180 Diploid, X2180-1A-4, X2180-1A-5, and 4484-24D-1. External invertase activity is fully assayable in intact cells

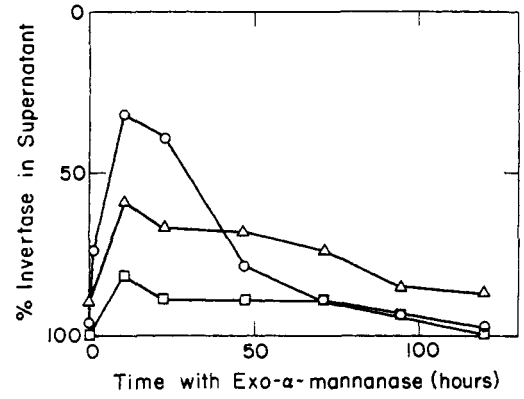

FIGURE 9: Precipitation by anti-X2180-1A-5 serum of *S. cerevisiae* 4484-24D-1 invertase which was treated for various times with exo-α-mannanase. Invertase (114 units from peaks A and B of Figure 1c) was incubated with exo-α-mannanase (16 units) in 8.0 ml of 0.05 M potassium phosphate (pH 6.8) containing 0.1 mM $CaCl_2$ and 0.5 mg per ml of bovine serum albumin. At the indicated times, an aliquot (0.9 ml) of the sample was removed and a portion was used for the precipitin reaction. At the same time exo-α-mannanase (4 units) plus 1.0 mg of bovine serum albumin, in a total volume of 0.11 ml, was added to the remaining reaction mixure. Precipitin reactions were performed as indicated in the text using 0.24 unit (□), 0.95 unit (△), and 2.4 units (○) of invertase.

TABLE II: Heat Inactivation of *S. cerevisiae* 4484-24D-1 Invertase.

Time with Exo-α-mannanase (hr)	Rate Constants for Inactivation[a] At pH 1.9 ($10^2 \times min^{-1}$)	At pH 8.2 ($10^3 \times min^{-1}$)
0	9.2	2.3
1	8.7	1.7
11	8.2	1.8
23	7.7	2.2
47	8.7	2.2
71	9.8	2.3
95	8.7	2.1
120	9.8	2.3

[a] *S. cerevisiae* 4484-24D-1 invertase was treated with exo-α-mannanase as described in the legend to Figure 9. Invertase samples (0.7 unit), which had been treated for the indicated times with exo-α-mannanase, were incubated at 37° in 1.0-ml samples of 0.05 M citrate phosphate at pH 1.9, 3.6, 4.7, and 8.2 containing 0.1 M NaCl and 1 mg per ml of bovine serum albumin. The logarithm of the enzyme activity at different times was plotted against incubation time to obtain the rate constants for inactivation. No detectable inactivation of invertase was observed at either pH 3.6 or 4.7.

(Arnold, 1972). Each of the mutant yeast strains showed invertase activity when whole cells were tested in the normal enzyme assay (Table III). In addition, none of the mutants released a significantly higher level of activity into the growth medium than did the X2180 diploid.

Discussion

Yeasts contain several extracellular hydrolytic enzymes (Lampen, 1968). Two of these, acid phosphatase and β-fructofuranosidase, have been shown to be mannan proteins (Neumann and Lampen, 1967; Boer and Steyn-Parvé, 1966); and of these, β-fructofuranosidase has been the most extensively investigated. The mannose in these invertases can be attached glycosidically as short oligosaccharides to hydroxyamino acids (Greiling *et al.*, 1969) or as polysaccharide chains to asparagine through *N*-acetylglucosamine (Neumann and Lampen, 1969). It is possible, although not established, that both types of linkages occur in a particular glycoenzyme, and that differences in the ratios of the two types of linkage may exist depending on the yeast strain used as the enzyme source.

TABLE III: Invertase Activity in Whole Cells and Growth Medium from Several *Saccharomyces cerevisiae* Strains.

S. cerevisiae Strain	Activity[a] Cells (units/g wet weight)	Growth Medium (units/ml)
X2180 diploid	90	2
X2180-1A-4	275	4
X2180-1A-5	145	2
4484-24D-1	335	0

[a] Cells were grown to late stationary phase and harvested. The cells were resuspended in an equal weight of 0.1 M sodium acetate (pH 5.0), and 50 μl of that suspension was used in the invertase assay systems. Prior to reading the absorbance at 540 nm, the diluted assay mixture was centrifuged to sediment the cells.

Our work has focused on determining the nature of the mannan of the extracellular yeast invertase, using immunochemical procedures developed in studies of yeast cell wall mannans (Ballou, 1970). Antisera with specificity toward terminal α(1→3)-linked mannose, terminal α(1→2)-linked mannose, mannosyl phosphate groups, and unsubstituted α(1→6)-linked mannan chains have been well characterized (Raschke *et al.*, 1973; Ballou, 1970; Raschke and Ballou, 1971). Our studies have shown that the yeast invertase mannan and the total cell wall mannan protein fraction are immunochemically equivalent in three *S. cerevisiae* strains which have different cell wall mannan structures. The absence of α(1→3) linkages in *S. cerevisiae* X2180-1A-4 cell wall mannan, brought about by mutagenesis of strain X2180-1A (Raschke *et al.*, 1973), caused an immunochemically equivalent change in the extracellular invertase of the mutant. Thus, the affected α(1→3)-mannosyl transferase must be involved in the synthesis both of cell wall mannan and invertase mannan. Similar conclusions were reached for the mannosyl phosphate immunochemical determinant which is lacking in the *S. cerevisiae* 4484-24D-1 strain. These results suggest that biosynthesis of all extracellular mannan utilizes the same set of enzymes, and that agents which affect synthesis of the carbohydrate moiety of yeast cell wall mannan would cause similar effects on external invertase synthesis. Therefore, selective repression of the synthesis of specific extracellular mannan proteins, such as acid phosphatase by phosphate (Rautanen and Kaerkkaeinen, 1951; Schmidt *et al.*, 1956) and β-fructofuranosidase by hexose (Liras and Gascón, 1971), must involve control over protein synthesis and not glycosylation of preformed enzyme molecules.

As has been suggested (Eylar, 1965), mannan may function in some way as a signal for enzyme secretion. However, it is difficult to understand why mannan proteins contain such a range of mannan compositions (30–90% by weight) if the only function of carbohydrate is to label glycoproteins for secretion. In addition, external invertase activity is present in whole cells of all cell wall mannan mutants studied to date, indicating that the structural features of the mannan that are altered in the mutants do not serve as a marker for the secretory process.

The specific activities of bovine pancreatic ribonucleases A and B are identical (Plummer and Hirs, 1963), although ribonuclease B contains 11% carbohydrate attached to a single asparagine residue (Plummer and Hirs, 1964) whereas ribonuclease A is not a glycoprotein. However, it was not predictable that the enzymic activity of external invertases which have been reported to contain up to 70% carbohydrate (Greiling *et al.*, 1969) attached at multiple sites, would be unaffected by complete removal of mannan. That there was no effect seems to contradict the supposition that the carbohydrate of extracellular enzymes may help maintain the stability of these glycoproteins in the extracellular environment (Berry *et al.*, 1970). Thus, the reportedly greater stability of external over internal invertase (Gascón *et al.*, 1968) is probably a consequence of differences in the amino acid composition between the two enzymes, rather than carbohydrate content. A different conclusion was reached by Arnold

(1969) who demonstrated a correlation between the carbohydrate content and stability of the polydisperse invertase that had been fractionated by DEAE-cellulose chromatography into forms that varied from 39 to 54% carbohydrate. He found that at pH 5 and 65° a fivefold range of stability paralleled this relatively small variation in carbohydrate content of bakers' yeast invertase. Clearly, factors other than carbohydrate content may have been involved in the fractionation of the invertase activity, and presumably these factors would parallel the change in stability. In our own study, we have shown that all of the carbohydrate can be removed enzymically from invertase without altering its stability at 37° and at four different pH values in the presence of bovine serum albumin. Any attempt to rationalize these two apparently conflicting sets of results must make allowances for the differences in the enzyme sources and in the design of the experiments. As an alternative function, it may be that the invertase mannan acts to anchor the enzyme in the cell wall in a form that cannot readily diffuse away. Yeasts appear to lack an outer membrane, analogous to that of Gram-negative bacteria, in which proteins could be held by hydrophobic interactions with a lipid bilayer. On the other hand, a thick glucan wall with the interspersed structural mannan protein could provide a suitable matrix in which glycoenzymes of the mannan invertase type could be entrapped and immobilized.

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