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METABOLISM OF 2-DEOXY-D-GLUCOSE BY BAKER'S YEAST

IV. INCORPORATION OF 2-DEOXY-D-GLUCOSE INTO CELL WALL MANNAN*

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SUMMARY

1. *Saccharomyces cerevisiae* grown in the presence of 2-deoxy-D-glucose incorporate this glucose and mannose analogue into cell wall polysaccharides. Fractionation of cell walls to mannan- and glucan-containing fractions followed by analysis for glucose, mannose and deoxyglucose showed that deoxyglucose was incorporated mainly, if not exclusively, into cell wall mannan.

2. Mild acid hydrolysis of the mannan-containing fraction isolated from deoxyglucose-grown cells afforded besides free deoxyglucose two oligosaccharides containing both mannose and deoxyglucose. They were tentatively identified as 3-*O*-α,D-mannopyranosyl-2-deoxy-D-glucose and a trisaccharide containing the former disaccharide and an additional mannose residue at non reducing terminal.

3. The results demonstrate direct interaction of deoxyglucose metabolites with the enzyme system of *Saccharomyces cerevisiae* responsible for the cell-wall mannan biosynthesis.

INTRODUCTION

In our previous paper[1] it was reported that *Saccharomyces cerevisiae* grown in the presence of 2-deoxy-D-glucose incorporate this analogue of glucose and mannose into cell wall material. In the present paper the identification of the cell wall polysaccharide containing deoxyglucose is described. The finding is discussed in view of the inhibitory effect of deoxyglucose on yeast growth.

MATERIALS AND METHODS

Yeast and cultivation

S. cerevisiae, strain CCY 21-4-13 was grown in a medium containing 2 % glucose, 0.2 % $(NH_4)_2SO_4$ and 0.75 % brewer's yeast extract on a shaker at 28°. Yeast was grown also in the same medium containing 0.05 or 0.2 % deoxyglucose.

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Preparation and fractionation of cells walls

Cell walls of control yeast and yeast grown with deoxyglucose were prepared as described in our previous paper[1]. Lyophilized cell walls were fractionated using ethylenediamine extraction into mannan–protein (Fraction A), glucan–mannan–protein (Fraction B) and glucan–protein (Fraction C) complexes according to KORN AND NORTHCOTTE[2].

Gel filtration of Fraction A

Fraction A, the mannan–protein complex, isolated from the cell walls of deoxyglucose-grown cells was chromatographed on a Sephadex G-200 column (68 cm × 2 cm). Elution was carried out with 0.1 M NaCl. Fractions of 5 ml were collected and analyzed for protein ($A_{280\ nm}$), mannose (anthrone) and deoxyglucose (see below). Fractions containing the high molecular mannan–protein complex were pooled, desalted by dialysis (3 days, 4°) and freeze-dried. This material, further referred to as fraction A', was found to be homogeneous in free boundary electrophoresis (1 % solution in 0.05 M borate buffer, pH 9.29). Nitrogen content of Fraction A' was 3.3 % (Dumas).

Preparation of protein-free mannan

500 mg of Fraction A isolated from deoxyglucose-grown yeast cell walls was dissolved in 10 ml of 5 M NaOH and heated at 100° for 15 min. After cooling the solution was diluted with 40 ml of water and poored through a column of cation and anion exchange resins (Bio-Deminrolit and Dowex 50 (H^+)). Neutral eluate was dialyzed against water, concentrated in a vacuum and lyophilized to give 135 mg of mannan-containing deoxyglucose. This sample gave a single peak in free boundary electrophoresis (1 % solution in 0.05 M borate buffer, pH 9.29). Analysis showed that the mannan prepared in this way contained 0.26 % of nitrogen (Dumas).

Isolation of mannose and deoxyglucose-containing oligosaccharides

Both Fraction A' and mannan from cell walls of yeast grown in the presence of 0.05 and 0.2 % deoxyglucose was hydrolyzed in 0.02 M HCl at 100° (1 h, 10 mg/ml) to liberate free deoxyglucose and deoxyglucose and mannose-containing oligosaccharides. The hydrolysate was chromatographed on Schleicher and Schüll 2043 B chromatographic paper in ethyl acetate–pyridine–water (8:2:1, by vol., Solvent A). 2–3 succesive developments, each for 24 h, were used. The oligosaccharides were located by diphenylamine–aniline reagent [3] and by $HClO_4$ in acetone[4]. They were eluted and chromatographed a second time on the same paper in ethyl acetate–pyridine–water (5:3:2,by vol. Solvent B) and for a third time in Solvent A (two developments for 24 h). The oligosaccharides were obtained also from extracellular polysaccharides isolated from the cultivation medium. The medium neutralized with KOH was evaporated *in vacuo* to a small volume and dialyzed against running tap water for 4 days. To the dialyzed solution NaOH was added to give 1 M concentration and the solution was heated at 80° for 2 h. After cooling the extracellular polysaccharides were precipitated with 2 vol. of ethanol at 4° for 2 h. After reprecipitation in the same way they were dissolved in water and dialyzed against running tap water for 4 days and finally freeze-dried. Hydrolysis of this material with 0.02 M HCl (100°, 1 h) yielded besides free two deoxyglucose-containing oligosaccharides with identical chromato-

graphic mobility as those liberated upon the same treatment from Fraction A' and protein-free mannan.

Methods used for identification of deoxyglucose-containing oligosaccharides

Acid hydrolysis. Sugar components of the two oligosaccharides were identified after hydrolysis with 0.25 M H_2SO_4 (100°, 1 h). The hydrolysates were neutralized with Amberlite IRA-402 (OH^-) prior to chromatographic resolution.

Reduction with $NaBH_4$. Oligosaccharides were treated with an excess of $NaBH_4$ in water. After 16 h the solutions were passed through a Dowex 50 (H^+) column and the eluates were evaporated to dryness in a vacuum. Boric acid was removed by three successive evaporations with methanol. Resulting residue was dissolved in water, chromatographed with Solvent B and the products detected[3,4].

A similar treatment of oligosaccharides with $Na_4B_2O_7$ instead of $NaBH_4$ led to recovery of the original substances.

Enzymic hydrolysis. Both oligosaccharides were hydrolyzed with α-mannosidase isolated from mannan-containing cultural medium of Arthrobacter GJM-1 (ref. 5). The 60 % $(NH_4)_2SO_4$ precipitate prepared as described by JONES AND BALLOU[5] was dialyzed against 0.01 M phosphate buffer (pH 6.8) containing 10^{-4} M $CaCl_2$ (24 h, 4°) and used as the enzyme preparation. The same buffer was used in the incubation mixtures of the enzyme with deoxyglucose-containing oligosaccharides (37°). Aliquots of the incubation mixtures were examined for liberation of free sugars by paper chromatography in Solvent A and Solvent B.

Molar ratio of sugars. Molar ratio of mannose to deoxyglucose was estimated on the basis of different color products of mannose and deoxyglucose with anthrone reagent. Color product of mannose shows a maximum absorption at 620 nm while that of deoxyglucose at 520 nm. Mannose, deoxyglucose, their mixtures in the ratio 1:1 and 2:1 and the deoxyglucose and mannose containing oligosaccharides (in 1 ml volume) were mixed with 2 ml of 0.2 % anthrone in concentrated H_2SO_4 and heated at 100° for 16 min. The ratio of absorbances found at 520 nm and 620 nm was calculated for each sample. Comparison of the values for oligosaccharides with those of standard samples gave the molar ratio of mannose to deoxyglucose.

Analytical procedures. Glucose and mannose were determined by the procedure used previously[1]. Since the walls and the wall fractions contain large amounts of carbohydrates which consume periodate, the original method of WARAVDEKAR AND SASLAW[6] for determination of 2-deoxy sugars employing low concentration of HIO_4 had to be modified. These samples were analyzed for deoxyglucose as follows:

Samples (5 mg) were suspended (cell walls and Fractions B and C) or dissolved (Fraction A) in 5 ml of 62.5 mM H_2SO_4 and heated at 100° for 10 min. This time of hydrolysis was found to be sufficient to liberate deoxyglucose into solution quantitatively. After this treatment 0.1 ml aliquots were diluted to 0.7 ml with distilled water, 0.1 ml of 0.1 M $NaIO_4$ was added and the sample was kept at room temperature for 30 min. Then 0.2 ml of 10 % Na_3AsO_3 in 0.05 M H_2SO_4 was added followed by addition of 50 μl of 2.5 M H_2SO_4. After the disappearance of a transitory brown color due to iodine in solution, 2 ml of 0.6 % thiobarbituric acid were added. The mixture was then heated at 100° for 20 min, and after cooling the absorbance was measured at 532 nm. A standard curve for deoxyglucose was prepared in the same manner.

Glucose and mannose in the above described procedure produced a yellow

colored product ($A_{max,\ 450\ nm}$) having a small absorbance at 532 nm, which interfered with determination of deoxyglucose. The cell walls and wall fractions from control yeast were therefore run simultaneously with the samples from yeast grown in the presence of deoxyglucose. The absorbance of the samples from control yeast at 532 nm was subtracted from the absorbance of corresponding samples containing deoxyglucose, in order to obtain an exact value for the deoxyglucose content.

The above procedure is based on the formation of malonaldehyde by periodate oxidation of deoxyglucose. This means, that the method estimates only deoxyglucose which is not substituted in polysaccharides at C-3 and C-4 with sugar other than deoxyglucose. It should be noted, therefore, that the real content of deoxyglucose in analyzed samples may be higher than the values obtained. Mild acid hydrolysis used prior to periodate oxidation splits only the acid labile glycosidic linkage of deoxyglucose. Stronger acids to achieve total hydrolysis of polysaccharides could not be used since deoxyglucose is rapidly decomposed during heating in more acidic solutions (Fig. 1).

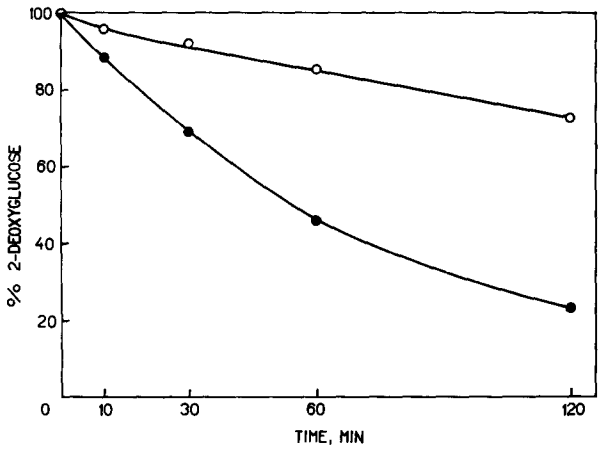

Fig. 1. Decomposition of deoxyglucose during heating in diluted H_2SO_4 at 100°. ○—○, 0.0625 M H_2SO_4; ●—●, 0.25 M H_2SO_4. Deoxyglucose was determined according to WARAVDEKAR AND SASLAW[6].

RESULTS AND DISCUSSION

Localization of deoxyglucose in yeast cell wall

The evidence that *S. cerevisiae* grown in the presence of deoxyglucose incorporate this glucose analogue into cell wall material[1] led us to establish which cell wall component deoxyglucose entered. Isolated cell walls of control yeast and inhibited yeast grown with deoxyglucose were separated into three fractions according to KORN AND NORTHCOTTE[2] (Table I). The low yield of fraction A representing mannan–protein complex obtained in the case of the cell walls from yeast grown in the presence of deoxyglucose is in agreement with the results of chemical analysis of cell walls[1] which showed reduced content of mannose in the cell walls from yeast inhibited with deoxyglucose.

The cell walls and wall Fractions A, B, C from yeast grown in the presence of

0.2 % deoxyglucose were analyzed for glucose, mannose and deoxyglucose (Table II). The highest amount of deoxyglucose was present in fraction A which represents the mannan-protein complex from the cell walls. Approximately the same ratio of mannose to deoxyglucose in the cell walls as well as in the wall fractions demonstrated that deoxyglucose was incorporated mainly into cell wall mannan.

Similar results were obtained with samples isolated from yeast grown in the presence of 0.05 deoxyglucose. The ratio of mannose to dGlc was determined to be

TABLE I

FRACTIONATION OF CELL WALLS WITH ETHYLENEDIAMINE

Yields are given in percent from lyophilized material.

Cell walls	*Fraction A*	*Fraction B*	*Fraction C*
From control yeast	25.5	1.1	70
From yeast grown with 0.05% deoxyglucose	20.5	4.9	63.4
From yeast grown with 0.2% deoxyglucose	16.2	9.1	62.5

TABLE II

HEXOSE CONTENT OF CELL WALLS AND CELL WALL FRACTIONS FROM YEAST GROWN WITH 0.2% 2-DEOXYGLUCOSE

Values are given in percent from lyophilized material.

Sample	*Glucose*	*Mannose*	*Deoxyglucose*	*Molar ratio mannose/deoxyglucose*
Cell walls	50	17	0.9	19
Fraction A	0	60	2.6	23
Fraction B	5	3	0.18	17
Fraction C	76	6.5	0.27	24

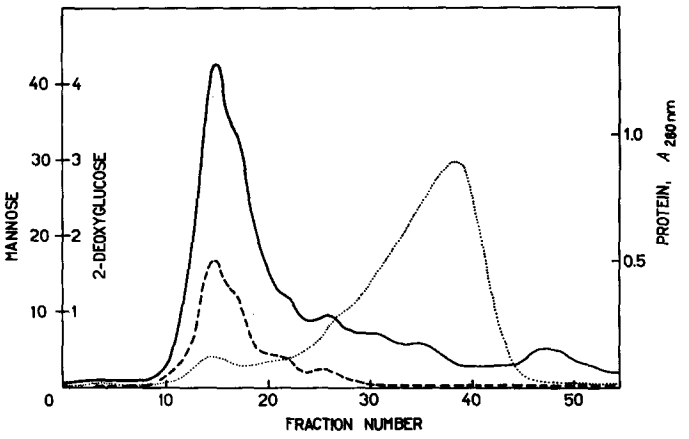

Fig. 2. Gel filtration of Fraction A from cell walls of yeast grown with 0.05% deoxyglucose on a Sephadex G-200 colomn. Fractions were analyzed for protein (———), mannose (— — —) and deoxyglucose (---------). Values of mannose and deoxyglucose are given in μg/50 μl of the effluent.

also about 20:1 in the cell walls as well as in Fraction A, however, the value 13:1 was found in the case of Fraction C.

Thus, the presence of deoxyglucose in cell wall glucan can not be excluded on the basis of the present results. Furthermore, the analytical procedure used for determination of deoxyglucose does not estimate such molecules of deoxyglucose which a resubstituted with natural hexoses at C-3 and C-4. $\beta(1\rightarrow3)$ linkages apart from $\beta(1\rightarrow6)$ linkages are characteristic for yeast glucan structure.

A proof that deoxyglucose is present in fraction A as a part of a heteropolysaccharide with mannose was attained by gel filtration of fraction A on a Sephadex G-200 column (Fig. 2). Analysis of the chromatographic effluent for mannose and deoxyglucose showed that deoxyglucose together with mannose were present in a high molecular weight polysaccharide–protein complex which emerged in the void volume of the column. Fractions 10–20 were pooled, desalted, freeze–dried (Fraction A') and subjected to hydrolysis with 0.02 M HCl at 100°. Paper chromatographic examination of the hydrolysate showed that during hydrolysis of Fraction A' deoxyglucose and two deoxyglucose-containing oligosaccharides were formed besided small amounts of mannose (Fig. 3). The two oligosaccharides (I and II) were not observed in the hydrolysates of Fraction A' from control cell walls mixed with appropriate amount of free deoxyglucose.

The same compounds were liberated from protein-free mannan treated in the same way. Hydrolysates of extracellular polysaccharides isolated from the growth medium containing deoxyglucose gave the same chromatographic pattern. This result supports the view that the extracellular polysaccharides of yeast occurring in growth media possess the same structure and are synthesized by the same enzymic

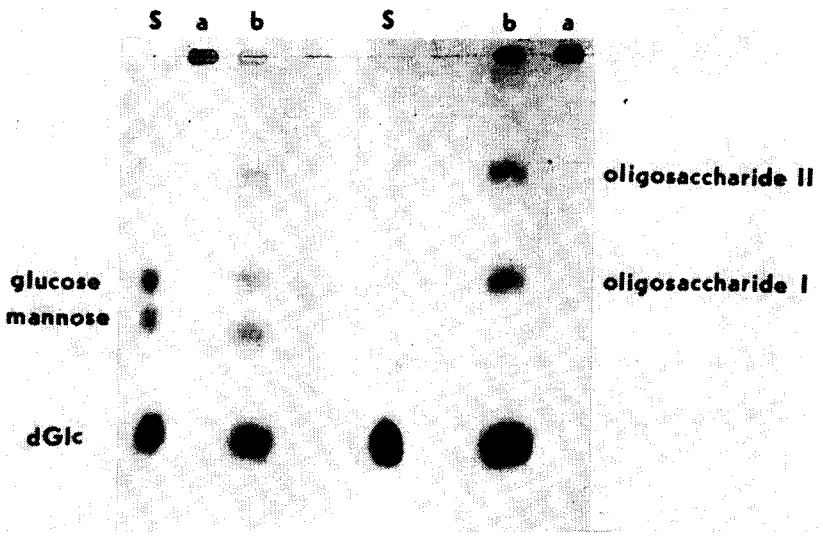

Fig. 3. Hydrolysis of Fraction A' isolated from cell walls of yeast grown with 0.05% deoxyglucose (0.02 M HCl, 100°, 1 h) followed by paper chromatography on Schleicher and Schüll 2043 B paper in ethyl acetate–pyridine–water (5:3:2, by vol.). Left side of the chromatogram is detected with diphenylamine–aniline reagent[3], right side with $HClO_4$ in acetone[4]. S, standards; a, Fraction A' before hydrolysis; b, Fraction A' after hydrolysis.

systems as the cell wall polysaccharides. The extracellular polysaccharides apparently originate in the cell wall.

Identification of oligosaccharides containing deoxyglucose

The oligosaccharides I and II were liberated from yeast mannan under very mild acid hydrolysis. Glycosidic bonds of 2-deoxy sugars are known to be very acid labile in comparison with those of neutral sugars[7,8]. This fact in itself is a proof that each oligosaccharide contains only one deoxyglucose residue which is present on reducing end of the molecule. The oligosaccharides were identified based upon these criteria:

(1) Both oligosaccharides gave positive test for 2-deoxy sugars with $HClO_4$ in acetone[4] and a characteristic red color of 2-deoxy sugars with diphenylamine–aniline reagent[3].

(2) Treatment of both oligosaccharides with $NaBH_4$ led to complete loss of their specific reactions characteristic for 2-deoxy sugars as a consequence of the reduction of deoxyglucose on reducing end of the oligosaccharides.

(3) Oligosaccharide I was partially split with 0.25 M H_2SO_4 (100°, 1 h) to give mannose and deoxyglucose (deoxyglucose was partially decomposed during this treatment). Under the same conditions oligosaccharide II partially gave oligosaccharide I, mannose and deoxyglucose. Oligosaccharide II seems to contain oligosaccharide I as a part of its structure.

(4) Hydrolysis of oligosaccharide I with α-manosidase produced by Arthrobacter GJM-1 (ref. 5) yielded mannose and deoxyglucose. A short time treatment of oligosaccharide II led to the formation of oligosaccharide I, mannose and deoxyglucose. A long term enzymic hydrolysis of oligosaccharide II gave mannose and deoxyglucose. This showed that the sugar residues in deoxyglucose-containing oligosaccharides are connected with α-linkages.

(5) Periodate oxidation of both oligosaccharides followed with thiobarbituric acid test[6] showed almost no formation of malonaldehyde. The formation of malonaldehyde during periodate oxidation was observed only after previous hydrolysis of oligosaccharides in 0.25 M H_2SO_4 (100°, 30 min). This result demonstrated that deoxyglucose is in both oligosaccharides substituted with mannose at C-3 or C-4. Since no (1→4) linkages have been found in *S. cerevisiae* mannan, it may be concluded that mannose is linked to deoxyglucose with (1→3)linkage.

(6) Chromatographic mobility of oligosaccharide I correspondend to that of a disaccharide. Chromatographic mobility of oligosaccharide II corresponded to that of a trisaccharide. This presumption was supported by estimation of the mannose to deoxyglucose ratio (Table III). Oligosaccharide I contains one residue of mannose per one residue of deoxyglucose. Oligosaccharide II most likely contains two residues of mannose per one residue of deoxyglucose.

Based on the above criteria and regarding the present information of *S. cerevisiae* mannan structure[9], the structure of oligosaccharide I may be suggested as 3-O-α,D-mannopyranosyl-2-deoxy-D-glucose. The oligosaccharide II contains the former disaccharide and an additional mannose residue probably at non-reducing terminal linked with α(1→2) or α(1→3) glycosidic bond. The possibility, that oligosaccharide II is a mixture of two compounds with different linkages of terminal mannose residue, can not be excluded.

TABLE III

ESTIMATION OF THE MOLAR RATIO MANNOSE/2-DEOXYGLUCOSE IN OLIGOSACCHARIDES I AND II

Estimation was based on difference between color products of mannose and deoxyglucose with anthrone reagent. For details see MATERIALS AND METHODS.

Sample	$A_{520\ nm}/A_{620\ nm}$ *of the color product with anthrone reagent*	*Estimated ratio mannose/deoxyglucose*
Deoxyglucose	3.85	
Mannose	0.73	
Mannose/deoxyglucose 1:1	1.78	
Mannose/deoxyglucose 2:1	1.37	
Oligosaccharide I	1.62	1:1
Oligosaccharide II	1.17	2:1

Although the low yields of the oligosaccharides, in comparison with free deoxyglucose, liberated on mild acid hydrolysis of yeast mannan, did not permit the establishment of their definite structure, these results clearly show that deoxyglucose was incorporated into yeast cell wall mannan. The incorporation proceeds apparently *via* GDP–deoxyglucose[10,11].

In terms of the accepted structure of *S. cerevisiae* mannan it is obvious that free deoxyglucose and the two oligosaccharides could be liberated by cleavage of deoxyglucosyl bonds in the side chains of the mannan molecule. The length to the released fragments corresponds to the length of the side chains in natural polysaccharide.

Under the assumption that the mechanism of the mannan biosynthesis was not altered in the presence of deoxyglucose metabolites, the liberation of the trisaccharide composed of one residue of deoxyglucose and two mannose residues under mild acid hydrolysis indicates that, in natural cell wall mannan (1→3)-linked mannose residues do not have to be located strictly on non-reducing terminals and may be further substituted with additional mannose residues. The problem of the localization of (1→3) linkages was touched in the work of KOCOUREK AND BALLOU[9] who, based on the gas–liquid chromatography data of BHATTACHARJEE AND GORIN[12], suggested that some side chains are connected to the (1→6)-linked backbone by (1→3) linkages.

Possible consequences of the deoxyglucose incorporation into mannan

It is apparent that the incorporation of deoxyglucose may influence the natural structure of the polysaccharide. Residues of deoxyglucose in mannan can not serve as acceptors for further carbohydrate units to be linked with (1→2) linkage. This may cause a reduced branching of the mannan molecule. The fact that the occurrence of oligosaccharides in comparison to free deoxyglucose in mild acid hydrolysates was very small (Fig. 3) illustrates that only small portion of deoxyglucose attached to mannan was further substitued with mannose.

Though the incorporation of deoxyglucose into *S. cerevisiae* mannan demonstrates a direct interaction of deoxyglucose metabolites with the mannan synthesizing system of the above yeast, the changes in the mannan structure, due to the deoxyglucose incorporation, do not appear to be primarily responsible for the effect of deoxyglucose on cell wall formation in *S. cerevisiae*. This effect of deoxyglucose was

shown to be dependent upon the concentration of the inhibitor in the growth medium[1]. In the cell wall mannan–protein complex (Fraction A) isolated from yeast grown in the presence of 0.2 and 0.05 % deoxyglucose, however, almost the same ratio of mannose to deoxyglucose was estimated. In this connection it should be noted that no evidence has so far been obtained for the entrance of deoxyglucose into cell wall material of *Schizosaccharomyces pombe*[11], a yeast more deoxyglucose-sensitive than *S. cerevisiae*[13,11].

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