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

VI. A STUDY ON CELL WALL MANNAN

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SUMMARY

The incorporation of 2-deoxy-D-glucose into cell wall mannan of growing *Saccharomyces cerevisiae* proceeded continuously during culture growth and followed the cell multiplication. About 10% of mannan labelled with deoxyglucose was concurrently released into the medium. The distribution of deoxyglucose between the side-chains and the main chain of mannan has been established. Approximately 90% of deoxyglucose present in the polysaccharide was bound in the side-chains and only 10% was located in the (1 → 6)-linked main chain. This result suggested that deoxyglucose metabolites serving as glycosyl donors in mannan biosynthesis were much worse substrates for the enzyme(s) responsible for the formation of the main chain of the polysaccharide than for the mannosyl transferases involved in the formation of the mannan side-chains. Degradation of deoxyglucose-containing mannan by α-mannosidase of Arthrobacter GJM-1 stopped at the deoxyglucosyl residues.

INTRODUCTION

The analogue of glucose and mannose, 2-deoxy-D-glucose, was found to be incorporated into cell wall mannan of *Saccharomyces cerevisiae* grown in 2% glucose medium [1]. During mild acid hydrolysis of mannan isolated from such cells, free deoxyglucose, 3-*O*-α,D-mannopyranosyl-2-deoxy-D-glucose and a 3-*O*-mannobiosyl-2-deoxy-D-glucose were liberated [1]. In view of the accepted structure of *S. cerevisiae* mannan [2] these substances could originate only in the side chains of the mannan molecule by the splitting of the acid-labile glycosidic bond of deoxyglucose. The previous study [1] has not provided data on the presence of deoxyglucose in the (1 → 6)-linked backbone and, in which phase of the growth cycle deoxyglucose incorporation took place since only stationary phase cells were used for the isolation of the deoxyglucose-containing polysaccharide. The present study was undertaken to look at these points, particularly in order to get some information about the biosynthetic system of *S. cerevisiae* mannan based on its behaviour towards the mannose analogue.

MATERIALS AND METHODS

Chemicals

Deoxy-[U-^{14}C]glucose (0.14 Ci/mole) was synthesized in our laboratory from [U-^{14}C]glucose (Research Institute for Development and Production of Radioisotopes, Praha) by the glycal method. Deoxy-[1-^{3}H]glucose (24.4 Ci/mmole) was purchased from the Radiochemical Centre, Amersham.

Yeast, cell walls and deoxyglucose entrance into wall material

S. cerevisiae, strain CCY 21-4-13 was grown in 2% glucose medium [3] supplied with 0.05% deoxyglucose unless otherwise stated. Growth was followed by measuring the absorbance at 420 nm of the cell suspensions. The large-scale preparation of cell walls, isolation and purification of the mannan–protein complex on Sephadex G-200 and preparation of protein-free mannan were described previously [1]. To follow the entrance of radioactive deoxyglucose into cell walls during the growth cycle, the cells were disintegrated on microscale in the following manner: 2-ml aliquots of the cultures were taken at intervals, the cells were centrifuged (supernatants were saved), washed twice with ice-cold water, mixed with glass beads (diameter around 0.5 mm) in test-tubes and shaken three times for 1 min using a vibratory shaker, type VT, Mikrotechna, Praha. The first two shakings were followed by centrifugation of the mixture to sediment the portion of unbroken cells present on the tube walls. Microscopic observation showed that the above procedure yielded practically complete disintegration (more than 99.9%). After separation of the glass beads the cell walls were centrifuged, washed 5 times with a large excess of ice-cold water and transferred to Whatman No. 1 paper strips for measurement of the radioactivity.

The supernatants obtained after the centrifugation of cells were used for the determination of mannan released into the growth medium during the growth experiment. They were dialyzed against running tap water for 8 days, the content of dialysis bags was concentrated in vacuo over P_2O_5 and transferred to paper strips for the determination of radioactivity.

Alkali treatment of mannan–protein containing deoxy-[^{14}C]glucose

Fraction A purified on a Sephadex G-200 column [1] was subjected to β-elimination under conditions used by Sentandreu and Northcote [4]. At intervals aliquots were neutralized with diluted acetic acid and dialyzed twice against 20 vol. of water (24 h, 4 °C). The dialyzates, after being concentrated, were used for the determination of the portion of deoxyglucose bound alone or as a part of the mannose oligosaccharides directly to the peptide through the O-glycosidic linkages [4].

Determination of the distribution of deoxyglucose within mannan

Mannan or mannan–protein isolated from the cell walls of *S. cerevisiae* grown with deoxy-[^{14}C]glucose were hydrolyzed with 0.02 M HCl at 100 °C at a concentration of 10 mg/ml. At intervals 50-μl samples were taken, neutralized with diluted ammonia, applied to Whatman No. 1 paper and chromatographed in ethyl acetate–pyridine–water (5 : 3 : 2, v/v/v) for 20 h. After detection of the guiding strips with $HClO_4$ in acetone [5], the areas at the origin and the areas corresponding to deoxy-

glucose, 3-*O*-mannosyl-deoxyglucose and 3-*O*-mannobiosyl-deoxyglucose were cut out for the determination of radioactivity (see below). The portion of radioactivity which could not be liberated from mannan in the form of the above three fragments and which stayed at the origin of the chromatogram was considered to correspond to the deoxyglucose present in the (1 → 6)-linked main chain. The liberation of deoxyglucose and deoxyglucose-containing oligosaccharides during mild acid hydrolysis may be apparent from Fig. 1. It should be emphasized that the liberation of deoxyglucose and lower oligosaccharides from the main chain is not considered since the samples of polysaccharides used never contained more than one deoxyglucose residue per 15–18 residues of mannose. The macromolecular residue of deoxyglucose-containing mannan obtained after mild acid hydrolysis showed in fact the same elution profile on a Sephadex G-50 column as the original sample.

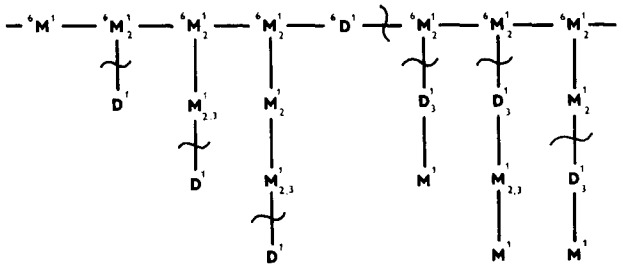

Fig. 1. Schematic representation of deoxyglucose-containing mannan of *S. cerevisiae*: M, mannosyl residues; D, deoxyglucosyl residues; swung dashes indicate the bonds which are split during mild acid hydrolysis. The actual ratio of mannose to deoxyglucose in the polysaccharide is not respected.

Determination of radioactivity

Radioactive samples present on Whatman No. 1 paper were subjected to oxygen flask combustion [6]. ^{3}H-labelled water was absorbed in dry methanol and $^{14}CO_2$ in 12% ethanolamine in methanol [6]. An aliquot of these solutions was mixed with a toluene scintillation liquid (5 g PBP and 100 mg POPOP per 1 of toluene) and the radioactivities were determined with a Nuclear Chicago counter, type Mark I, combined with a table computer Olivetti, type Programma 101.

Hydrolysis with α-mannosidase

A sample of cell wall mannan prepared from control yeast or from deoxyglucose-grown yeast was dissolved in 0.05 M phosphate buffer (pH 6.8) containing 10^{-4} M $CaCl_2$ to give a concentration of 1 mg/ml. An appropriate amount of α-mannosidase of Arthrobacter GJM-1 [7] prepared previously [1] was added and the mixture was incubated at 38 °C. Aliquots (100 μl) were taken for the determination of reducing sugars [8] and for chromatographic examination of the liberated hexoses on Whatman No. 1 paper in ethyl acetate–pyridine–water (5 : 3 : 2, v/v/v).

RESULTS AND DISCUSSION

Incorporation of deoxyglucose into mannan during the growth cycle

To establish the distribution of deoxyglucose within the mannan molecule we considered it important to find out whether the incorporation of the analogue into

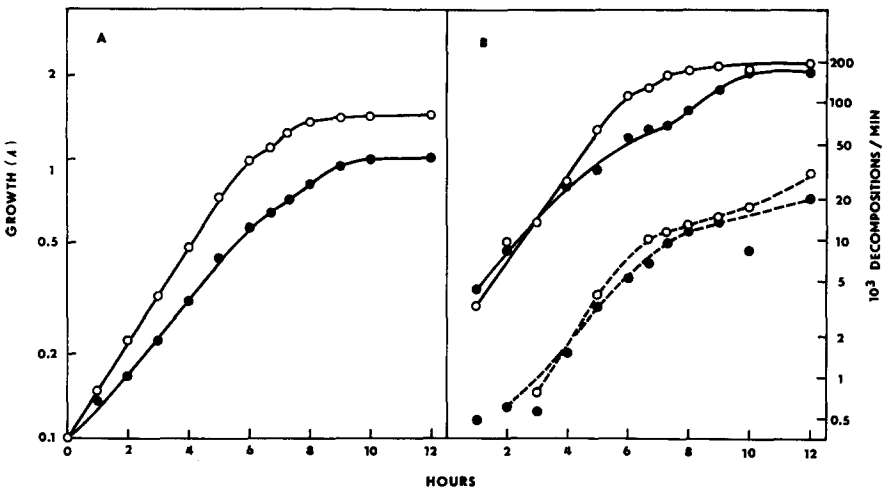

Fig. 2. Part A. Growth of *S. cerevisiae* in 2 % glucose medium supplied with deoxy-[^{3}H]glucose: 0.01 % and 0.6 μCi/ml (○); 0.05 % and 2.7 μCi/ml (●). Part B. Concurrent incorporation of deoxy-[^{3}H]glucose into isolated cell walls (full line) and extracellular polysaccharides (dashed line). Other symbols as above. The radioactivities correspond to the amount of material isolated from 0.4 ml of the growth medium.

mannan occurs in a particular growth phase of yeast, e.g. in the late exponential phase when glucose is becoming exhausted in the medium. The results in Fig. 2 show that deoxyglucose incorporation into the wall and extracellular polysaccharides (from 90% into mannan) proceeded continuously during culture growth and followed the pattern of cell multiplication well. It seems, therefore, that the position of incorporated deoxyglucose in mannan will not depend on the growth phase of yeast. About 10% of mannan synthetized during the experiment was concurrently released into the growth medium. The slope of the curves indicates that this release did not cease completely in the stationary phase of growth.

Distribution of deoxyglucose within mannan and mannan–protein

Only 1% of radioactivity in the form of dialyzable material was liberated from the mannan–protein complex labelled by deoxy-[^{14}C]glucose during 48-h treatment with 0.1 M NaOH at 22 °C. This finding pointed out that the only negligible portion of deoxyglucose was linked directly or as a part of mannose oligosaccharides to the peptide of the mannan–protein complex and that deoxyglucose was incorporated almost exclusively into the high molecular polysaccharide.

On mild acid hydrolysis of the mannan–protein complex (Fig. 3) 76% of deoxyglucose was liberated as free sugar, 8.7% as disaccharide and 6.6% as trisaccharide. Thus approximately 90% of the analogue was present in the side chains of the mannan molecule. Only 15% of total deoxyglucose was further substituted with additional mannose residues at C-3. About 10% of the radioactivity could not be released from the mannan–protein by prolonged hydrolysis. It is concluded, therefore, that this portion of deoxyglucose was incorporated into the main chain of the polymer.

A similar distribution of deoxyglucose between the main chain and the side

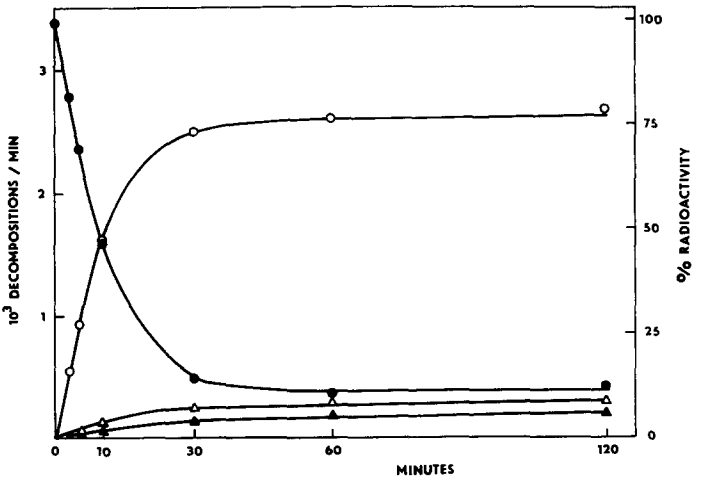

Fig. 3. Time course of liberation of deoxyglucose (○), mannosyldeoxyglucose (△) and mannobiosyldeoxyglucose (▲) from the purified mannan–protein complex extracted from the cell walls of *S. cerevisiae* grown with deoxy-[^{14}C]glucose during hydrolysis with 0.02 M HCl at 100 °C. The radioactivity remaining in the macromolecular residue is also plotted (●). Radioactivities correspond to 10 μg of mannan–protein complex.

chains and an almost identical portion of deoxyglucose substituted with mannose residues were obtained with protein-free mannan prepared from the above used sample of mannan–protein as well as with mannan isolated from yeast grown with 0.009% deoxyglucose (the concentration which inhibits the growth rate only slightly and does not induce lysis of the cells in the exponential phase of growth) (Table I). These results showed that the distribution of deoxyglucose in mannan is apparently only the function of the mannan synthesizing system and its response to the modification in mannose at C-2.

The distribution of deoxyglucose in mannan is in contrast with that of mannose

TABLE I

RADIOACTIVITY IN FRAGMENTS AND HIGH MOLECULAR RESIDUE OBTAINED AFTER MILD ACID HYDROLYSIS OF DEOXY[^{14}C]GLUCOSE-CONTAINING MANNAN

Samples were hydrolyzed for 2 h and processed as described in Materials and Methods. Radioactivities of starting samples are taken for 100 %.

Sample	Concentration of deoxyglucose in medium (%)	% deoxyglucose liberated			% deoxyglucose in macromolecular residue
		as free sugar	as biose	as triose	
Mannan-protein	0.05	76	8.7	6.6	11
Mannan*	0.05	80	8.3	5.6	10.2
Mannan**	0.009	76	7.6	4.8	10.1

* Prepared from the quoted mannan–protein with the mannose: deoxyglucose ratio 18.
** Mannose: deoxyglucose ratio about 40.

in natural polymer. According to several reports [9–13] approximately two thirds of mannose residues in *S. cerevisiae* mannan are bound in the side chains, the rest form the (1 → 6)-linked backbone. Since there is no doubt about the existence of the (1 → 6)-linked main chain [12, 14] the reason for the above discrepancy should be a different affinity of individual mannan-synthesizing enzymes [15] to mannosyl and deoxyglucosyl donors in the polysaccharide biosynthesis. A comparison of the ratios of mannose and deoxyglucose units in the side chains and in the main chain indicates that deoxyglucose (intermediates) is at least a three times worse substrate for the system responsible for the formation of (1 → 6) linkages than for the system constructing the side chains with (1 → 2) and (1 → 3) linkages.

Results in Fig. 3 and Table I also show that deoxyglucose after being incorporated into mannan remains for the major part unsubstituted. This is most probably the consequence of the inability of deoxyglucose residues to serve as acceptors for further carbohydrate units to be linked with the (1 → 2) linkage. Preferential incorporation of deoxyglucose to the ends of the mannan side chains cannot be excluded However, due to the extreme acid lability of the deoxyglucosyl bond and the acid lability of deoxyglucose itself, classical methods for the structural analysis of deoxyglucose-containing mannan could not be applied, so that the actual reason of the low degree of deoxyglucose substitution remains unknown.

One point which we would like to stress again is that the liberation of the trisaccharide with deoxyglucose at the reducing end on mild acid hydrolysis (Fig. 1) represents evidence that (1 → 3) linkages do not only connect the terminal mannosyl residues in the side chains of mannan of our strain of *S. cerevisiae.*

Action of α-mannosidase on deoxyglucose-containing mannan

The enzyme was able to split off 80% of mannose from the mannan isolated from control cells. Deoxyglucose-containing polysaccharide was less accessible to the digestion; only 62% of the mannose was liberated. The ratio of mannose: deoxyglucose in the sample decreased from 18 to 9 after enzymic treatment. Examination of the released sugars by paper chromatography showed the presence of mannose only. Deoxyglucose residues were not attacked by the enzyme. Mild acid hydrolysis of the polysaccharide treated once with α-mannosidase released only free deoxyglucose but no deoxyglucose-containing oligosaccharides. Further application of α-mannosidase to the sample with removed terminal deoxyglucosyl units led to the liberation of an additional 15% of mannose originally masked by deoxyglucose. This observation suggested that a part of deoxyglucose was located at the terminal position of longer side chains. The proportion of (1 → 2) and (1 → 3) linkages connecting such deoxyglucose residues remains still unknown, however.

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REFERENCES

1 Biely, P., Krátký, Z. and Bauer, Š. (1972) Biochim. Biophys. Acta 255, 631–639
2 Ballou, C. E. (1970) J. Biol. Chem. 245, 1197–1203
3 Biely, P., Krátký, Z., Kovařík, J. and Bauer, Š. (1971) J. Bacteriol. 107, 121–129
4 Sentandreu, R. and Northcote, D. H. (1968) Biochem. J. 109, 419–432
5 Weidemann, G. and Fischer, W. (1964) Z. Physiol. Chem. 336, 192–194
6 Kalberer, F. and Rutschmann, J. (1961) Helv. Chim. Acta 44, 1956–1966
7 Jones, G. H. and Ballou, C. E. (1969) J. Biol. Chem. 244, 1043–1051
8 Paleg, L. G. (1959) Anal. Chem. 31, 1902–1904
9 Haworth, W. N., Heath, R. L. and Peat, S. J. (1941) J. Chem. Soc. 833–842
10 Cifonelli, J. A. and Smith, J. (1955) J. Am. Chem. Soc. 77, 5682–5684
11 Peat, S., Turvey, J. R. and Doyle, D. (1961) J. Chem. Soc. 3918–3923
12 Stewart, T. S., Mendershausen, P. B. and Ballou, C. E. (1968) Biochemistry 7, 1843–1854
13 Bush, D. A. and Horisberger, M. (1973) J. Biol. Chem. 248, 1318–1320
14 Jones, G. H. and Ballou, C. E. (1969) J. Biol. Chem. 244, 1052–1059
15 Ballou, C. E., Kern, K. A. and Raschke, W. C. (1973) J. Biol. Chem. 248, 4667–4671