BIOSYNTHESIS OF β-GLUCANS CATALYZED BY A PARTICULATE ENZYME PREPARATION FROM YEAST

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1. Introduction

The β-glucan represents one of the major components of the yeast cell walls. In *Saccharomyces cerevisiae* cell walls the glucan has been found to be composed of two fractions a high mol. wt. β(1→3) linked glucan containing small proportion of β(1→6) interchain linkages [1]. The minor fraction is a polymer consisting of predominantly β(1→6) linked glucosyl units containing about 20% of β(1→3) interchain and inter-residue linkages [2].

The biosynthesis of fungal cell wall glucans in a cell free system has been studied to some extent in *Phytophtora cinnamomi* [3] and *Cochliobolus miyabeanus* [4,5]. In yeast, where the glucan represents some 30–50% of dry weight of the cell walls the molecular mechanism of its biosynthesis is so far unknown, while the biosynthesis of other yeast cell wall polysaccharides mannan and chitin has been subject of numerous studies [6–9].

This paper describes isolation of an enzyme system from yeast, which catalyzes the transfer of glucosyl units from GDP-[U-^{14}C]glucose and UDP-[U-^{14}C] glucose into polymers tentatively characterized as β-glucans containing both β(1→3) and β(1→6) glycosidic linkages.

Abbreviations: α-amylase, 1,4-α-D-Glucan glucanohydrolase (EC 3.2.1.1); β-amylase, 1,4-α-D-Glucan maltohydrolase (EC 3.2.1.2); β-glucosidase, β-D-Glucoside glucohydrolase (EC 3.2.1.20); Endo-1,3-β-glucanase, 1,3-β-D-Glucan glucanohydrolase (EC 3.2.1.39); Exo-1,3-β-glucosidase, 1,3-β-D-Glucan glucohydrolase (EC 3.2.1.58); Endo-1,6-β-glucanase, 1,6-β-D-Glucan glucanohydrolase (EC 3.2.1.75)

2. Materials and methods

GDP-D-[U-^{14}C]glucose (spec. act. 100 mCi/mmole) was purchased from New England Nuclear. UDP-D-[U-^{14}C]glucose (spec. act. 310 mCi/mmole) and ADP-D-[U-^{14}C]glucose (spec. act. 276 mCi/mmole) were from the Radiochemical Centre, Amersham. GDP-D-glucose, UDP-D-glucose, ADP-D-glucose and TDP-D-glucose were from Sigma Chemical Co. Oligosaccharides of β(1→3) and β(1→6) series were prepared from laminarin and/or pustulan by their partial hydrolysis with fuming HCl according to Feingold et al. [10].

Endo-1,3-β-glucanase was prepared from the culture fluid of yeast [11]. Salivary α-amylase was prepared according to Algranati and Cabib [12], β-amylase from barley was purchased from Koch-Light Ltd. Partially purified exo-1,3-β-glucosidase and endo-1,6-β-glucanase were prepared from the culture fluid of *Penicillium brefeldianum* (Š. Bálint, V. Farkaš, unpublished results).

The particulate enzyme preparation was obtained as follows: *Saccharomyces cerevisiae,* strain CCY 21-4-13 was grown at 25°C on a shaker in a semi-synthetic medium containing 2% glucose, 0.7% yeast autolysate and 0.2% ammonium sulfate (in w/v) [13]. The cells were harvested in mid logarithmic phase and washed twice with ice-cold water. All subsequent operations were carried out at 0–4°C. The thick suspension of cells with ballotini beads (size 0.5 mm) in 0.05 M Tris-HCl buffer (pH 7.2) containing 0.5 M mannitol and 1 mM EDTA was disintegrated for 2 min at 3500 rev/min in a rotatory disintegrator cooled from the outside by a mixture of dry-ice and

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ethanol. The degree of cell disintegration was checked microscopically. When at least 95% cell rupture was achieved, usually after 1.5–2 min disintegration the thick slurry was decanted several times with the above buffered solution. The membrane fraction sedimenting between 10 000 and 100 000 *g* during 30 min centrifugation was used as the enzyme source. The washed enzyme was resuspended in redistilled water. The protein content in the individual enzyme preparations was 20–40 mg/ml.

The standard incubation mixture with GDP[U-^{14}C] glucose contained 2 μmol of Tris-HCl buffer (pH 7.2), 1 μmol $MnCl_2$, 1 nmol of GDP-[U-^{14}C]glucose (97 000 cpm) and 0.2 mg of enzyme protein in a total volume of 50 μl.

The standard incubation mixture with UDP-[U-^{14}C] glucose contained 2 μmol of imidazole-HCl buffer (pH 6.5), 1 μmole $MgCl_2$, 1 nmol of UDP-[U-^{14}C] glucose (91 000 cpm) and 0.2 mg of enzyme protein in total volume of 50 μl.

The incubation was carried out at 30°C for 30 min. The reaction was terminated by addition of 1 ml of cold ethanol–1 M ammonium acetate solution (2:1, by vol.) and after washing the precipitate three times with the same solution its radioactivity was determined in the liquid scintillation spectrometer by suspending the sample in 2 ml of water and gelling it by addition of 5 ml of Instagel emulsifier (Packard Instrument Co.). The counting was performed at 8–10°C.

The scaled up incubations for batch preparations of radioactive polysaccharides were carried our for 2.5 h at 30°C and the products were washed as described above. The total incorporation of radioactive glucose into the particulate fractions in these incubations represented about 10% of the radioactivity initially present in the incubation mixture as GDP-[U-^{14}C] glucose or UDP-[U-^{14}C]glucose.

3. Results and discussion

Particulate enzyme preparation from the yeast *Saccharomyces cerevisiae* catalysis the transfer of [^{14}C]glucose from GDP-[U-^{14}C]glucose and UDP-[U-^{14}C]glucose into the fraction insoluble in 66% ethanol.

As can be seen in table 1 of the sugar nucleotides tested the best glucosyl donor under the given conditions was GDP-[U-^{14}C]glucose. The UDP-[U-^{14}C] glucose was by about 50% less effective. The reaction with GDP-[U-^{14}C]glucose required the presence of Mn^{2+} ions while the reaction with UDP-[U-^{14}C] glucose was stimulated equally well both by Mn^{2+} and Mg^{2+} ions. The addition of an equimolar concentration of UDP-[U-^{14}C]glucose to the incubation mixture with GDP-[U-^{14}C]glucose had additive effect on the amount of glucose incorporated into the polymer while the addition of unlabelled UDP-glucose was without effect on the amount of radioactivity incorporated from GDP-[U-^{14}C]glucose. The same result was obtained when unlabelled ADP-glucose and/or TDP-glucose were added to the standard incubation mixture. These observations indicate that in case of mixed-substrate incubations two simultaneous

Table 1
Specifity of glucosyl transfer

Substrate	pmoles of [^{14}C]glucose incorporated	Percentage of incorporation with GDP-glucose
GDP-[U-^{14}C]glucose	4.88	100
UDP-[U-^{14}C]glucose	2.38	49
ADP-[U-^{14}C]glucose	1.07	22
GDP-[U-^{14}C]glucose + UDP-[U-^{14}C]gluocse	7.92	162

The incubation mixture contained 2 μmol of imidazole-HCl buffer (pH 6.5), 0.5 μmol of $MnCl_2$, 0.5 μmol of $MgCl_2$ 0.1 nmole of the respective nucleoside-diphosphate-[U-^{14}C]glucose (45 000–51 000 cpm), and 0.3 mg (as protein) of the particulate enzyme preparation in 50 μl volume. The incubation was carried out at 30°C for 15 min.

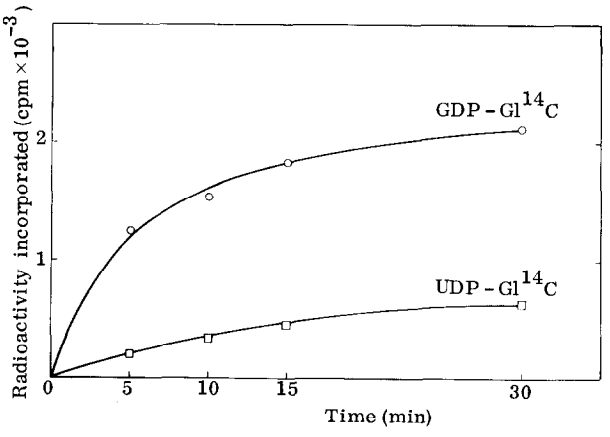

Fig.1. Time course of [^{14}C]glucose incorporation from GDP-[U-^{14}C]glucose and UDP-[U-^{14}C]glucose into fraction insoluble in 66% ethanol.

reactions proceeded, one with UDP-[U-^{14}C]glucose and the other one with GDP-[U-^{14}C]glucose as respective substrates.

Fig.1 depicts the time course of the glucosyl transfer from GDP-[U-^{14}C]glucose and UDP-[U-^{14}C] glucose into the products insoluble in 66% ethanol. The extent of incorporation was directly proportional to the amount of enzyme present in the incubation mixture.

Total acid hydrolysis of the products carried out according to Peat et al. [14] released free radioactive glucose. β-Elimination [15] of the radioactive products of transglycosylic reactions with GDP-[U-^{14}C]glucose and/or UDP-[U-^{14}C]glucose liberated 40–50% and 10% respectively of the incorporated radioactivity. Paper chromatography revealed the presence of glucose and oligosaccharides of β(1→3) and β(1→6) series in the β-eliminable portions.

It could be therefore anticipated that a large proportion of incorporated glucosyl units especially in the product from GDP-[U-^{14}C]glucose is linked directly to protein, most probably to serine and/or threonine, similarly as in yeast mannan [15].

Partial acid hydrolysis [1] of the macromolecular residues after β-elimination afforded a mixture of radioactive glucose and oligosaccharides of β(1→3) and β(1→6) series. Acetolysis of the nondiffusible residue after β-elimination of the product from GDP-[U-^{14}C] glucose carried out according to Wolfrom and Thompson [16] afforded in 60% yield a mixture of oligosaccharides and glucose that cochromatographed with β(1→3) and β(1→6) oligosaccharide markers. On the other hand the product from UDP-[U-^{14}C]glucose released by acetolysis only 30% of radioactivity mainly as glucose. The radioactive spot migrating together with authentic laminaribiose was eluted from the chromatogram of the acetolysate of the GDP-[U-^{14}C]glucose product and its structure was confirmed by $NaBH_4$ reduction and by periodate oxidation [17]. Periodate oxidation, sodium borohydride reduction and acid hydrolysis of the disaccharide showed the presence of radioactive ribitol and glycerol indicating the presence of a (1→3) glucosidic bond. The radioactive disaccharide when incubated with β-glucosidase was completely hydrolysed to glucose.

On the basis of above evidence the radioactive disaccharide which represents a fragment from the radioactive polymer synthesized in vitro from GDP-[U-^{14}C] glucose has been identified as laminaribiose, i.e. 3-*O*-β-D-glucosyl-D-glucose.

As an important point in the characterization of the synthesized polymers was the determination of their susceptibility to specific glucanohydrolases. A 24 h treatment of the radioactive polymers with the mixture of α- and β-amylase released about 10–15%

Table 2
Percentage of radioactivity released from [^{14}C]polysaccharides under the action of various β-glucan hydrolases

Polysaccharide synthesized in vitro from	β-Glucan hydrolase		
	exo-1,3-	endo-1,3-	endo-1,6-
GDP-[U-^{14}C]glucose	25	4	26
UDP-[U-^{14}C]glucose	70	25	12

of radioactivity from both polymers. After subsequent dialysis the radioactive polymers were incubated with different β-glucanases. The degree of dissolution of each polymer was determined after removal of low mol. wt. fragments by dialysis (see table 2). The nature of the liberated oligosaccharides was determined by paper chromatography. Partially purified yeast endo-1,3-β-glucanase liberated from the UDP-[U-^{14}C] glucose polymer glucose and a mixture of radioactive oligosaccharides while the product of glucosyl transfer from GDP-[U-^{14}C]glucose was not susceptible to this enzyme. A partially purified exo-1,3-β-glucosidase liberated from the UDP-[U-^{14}C]glucose product free glucose while the polymer prepared from GDP-[U-^{14}C] glucose released a mixture of higher oligosaccharides (DP>6) that could not be resolved by paper chromatography.

Although the last result is difficult to interpret, the preliminary evidence shows, that the product synthesized from UDP-[U-^{14}C]glucose is most probably a β(1→3) glucan containing some (1→6) linkages at branching and interchain points [1]. The product synthesized from GDP-[U-^{14}C]glucose appears to be a β(1→6) glucan containing some β(1→3) linkages in the side chains [2]. The presence of (1→3) bonds is confirmed by the finding of laminaribiose in the acetolysis and partial acid hydrolysis products. The presence of β(1→6) bonds is judged from the relatively high susceptibility of the polymer to acetolysis which preferentially cleaves (1→6) glycosidic bonds [18].

In addition, the results obtained by β-elimination of the products enable us to assume that they are heterogenous in nature containing different proportions of glucosyl units covalently linked to a peptide. The presence of a glucan-protein fraction in the yeast cell walls has been described by Korn and Northcote [19] in baker's yeast and by Kolarová et al. [20] in *Candida albicans*, however, the nature of the linkage between protein and polysaccharide moieties has not been clearly established.

Although there remains some uncertainty about the precise structure of the glucose polymers synthesized in vitro the experiments presented here demonstrate the possibility to study the biosynthesis of yeast cell wall glucans also at the molecular level. The significance of such experiments for understanding the molecular basis of yeast morphogenesis is undoubted.

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