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Preliminary evidence for a glucan acceptor in the yeast *Candida albicans*

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Two membrane preparations containing glucan synthase activity were obtained by lysis of regenerating sphaeroplasts (enzyme A) or mechanical breakage (enzyme B) of yeast (*Candida albicans*) cells. The reaction products of both enzymes (glucans A and B respectively) were characterized as linear β-1,3-linked glucans on the basis of chemical and enzymic analysis. In addition, two pools of glucan could be distinguished in glucan A preparations on the basis of their susceptibility to an exoglucanase. In no case were the reaction products synthesized *de novo*; rather the radioactive chains were added to the non-reducing end of non-radioactive preformed glucan chains or to an acceptor of a different nature. At least some of the preformed chains of glucan A, but not those of glucan B, showed a free reducing terminal. Glucan A preparations were endowed with endoglucanase activity, which, under appropriate conditions, released glucose, laminaribiose and laminaritriose. These sugars were also found in cell-wall autolysates. On the basis of the origin of both enzyme preparations it is suggested that glucan molecules are synthesized while they are bound to a non-glucan acceptor that is subsequently excised, presumably by cell-wall-associated glucanases.

INTRODUCTION

The cell walls of fungi are primarily composed of polysaccharides. Of these glucans, mannans and chitin are by far the most widespread among the different fungal species. Most of our knowledge about the structure and biosynthesis of fungal polysaccharides relies on studies carried out in baker's yeast (*Saccharomyces cerevisiae*). However, in the last few years a great deal of attention has been paid to the study of the cell wall of *Candida albicans*, a dimorphic human pathogenic fungus.

As in *S. cerevisiae*, some form of glucan appears to be the structural component responsible for the integrity of *C. albicans* cell wall. Early reports by Bishop *et al.* (1960) and Yu *et al.* (1967) identified an alkali-soluble glucan containing predominantly β-1,6 linkages. More recently, by using the fractionation procedure described by Manners *et al.* (1973) for *S. cerevisiae*, Gopal *et al.* (1984*b*) have separated the alkali-insoluble glucan into acid-soluble and acid-insoluble fractions. The former consisted of a highly branched polymer with a high ratio of β-1,6 to β-1,3 linkages (8.4). This ratio was much lower (1.7) in the insoluble glucan.

Methods for the preparation of enzyme fractions which catalyse an efficient transfer of glucose from UDP-glucose to a linear β-1,3-glucan have been described in *S. cerevisiae*. Shematek *et al.* (1980) lysed protoplasts in the presence of EDTA. The subsequent particulate fraction was very active when supplemented with exogenous factors (glycerol, bovine serum albumin, and ATP or GTP). On the other hand, Larriba *et al.* (1981) broke cells in the presence of 1 M-sucrose and obtained a membrane preparation of similar efficiency. By using the second method, Orlean (1983) succeeded in synthesizing a β-1,3-glucan from *C. albicans* and extended some of the observations reported in *S. cerevisiae* to this enzyme.

An important difference between the *Saccharomyces* glucans synthesized by both kinds of preparations concerns the accessibility of the reducing end of the molecule. Thus, whereas Shematek *et al.* (1980) found that the sugar at the reducing end came from the exogenous substrate and concluded that the polymer was synthesized *de novo*, Larriba *et al.* (1981) failed to detect a free reducing end in the molecule and suggested that the glucan synthesized by their preparations was bound to an acceptor of a different nature.

In the present work we have analysed and compared the nature of the glucans synthesized by similar enzyme preparations derived from yeast cells of *C. albicans*. Our results suggest that the molecule is synthesized while bound to an acceptor, other than glucan, which is subsequently excised, presumably during its transfer to the cell-wall glucan. We have also found that glucanases are associated with glucan preparations synthesized by the method of Shematek *et al.* (1980).

MATERIALS AND METHODS

Materials

UDP-[U-^{14}C]glucose (268 mCi/mmol) and NaB^3H_4 (14 Ci/mmol) were purchased from The Radiochemical Centre (Amersham, Bucks., U.K.). UDP-glucose, GTP, EDTA, $NaBH_4$ and α-amylase were from Sigma. Zymolyase 5000 was from Seikagaku Kogyo. Bio-Gel P-2 was from Bio-Rad and Sephacryl S-200 from Pharmacia. Toluene (scintillation grade) was from Merck. Omnifluor was from New England Nuclear.

Organisms and culture conditions

Candida albicans strain 3153, serotype A, was obtained from the Pasteur Institute (Paris, France) and used throughout this work. Yeast cells were prepared from a liquid culture, inoculated at a density of 25000 cells/ml

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in 500 ml flasks containing 150 ml of YED (2% glucose, 1% yeast extract) and grown at 28 °C in a rotatory shaker. Mid-exponential-phase cells ($A \simeq 2.0$) were harvested and washed by centrifugation.

Cladosporium resinae was grown as described by Walker & Hare (1977) to provide optimal yields of 1,3-α-D-glucanase. *Sporotrichum dimorphosporum* (Basidiomycete QM 806) was grown as described by Reese & Mandels (1959) for 12 days. At that time the culture medium contained high levels of exo-1,3-β-D-glucanase.

Purification of enzymes

Purification of α-glucanase was carried out essentially as described by Walker & Hare (1977), except that the electrofocusing step was replaced by two cycles of gel filtration through a column (90 cm × 2 cm) of Sephacryl S-200 equilibrated with 50 mM-phosphate buffer, pH 6. The purified enzyme did not show appreciable activity towards laminarian or starch. Culture fluids from Basidiomycete QM 806 were dialysed against 50 mM-acetate buffer, pH 5.5, and, in some cases, the exoglucanase was partially purified by gel filtration on the same Sephacryl column equilibrated with acetate buffer. This step completely removed a contaminant β-glucosidase from the exoglucanase. During purification, hydrolases were assayed with α-1,3-glucan [obtained from *Schizosaccharomyces pombe* as described by Manners & Meyer (1977)] or laminarin as substrates by measuring the liberation of glucose and/or reducing power. A unit of glucosidase liberates 1 μmol of glucose or reducing power (expressed as glucose equivalent)/min at 30 °C.

β-1,3-Glucan synthase preparations

Two methods for the preparation of 1,3-β-D-glucan synthase (EC 2.4.1.34) were used. Enzyme A was obtained by a modification of the method described by Shematek *et al.* (1980) for *S. cerevisiae*. Protoplasts were prepared from yeast cells by using zymolyase 5000 (Hernández *et al.*, 1986). They were regenerated in osmotically stabilized (1 M-sorbitol) YED medium for 30 min and then lysed at 4 °C in 4 mM-EDTA in 50 mM-Tris/HCl buffer, pH 7.8, and homogenized at the same temperature in a glass homogenizer. The whole homogenate as centrifuged at 4 °C for 1 h at 50000 *g* and the pellet resuspended in cold Tris/HCl buffer, homogenized, and referred to as 'enzyme A'.

Enzyme B was prepared by the method of Larriba *et al.* (1981). Cells were resuspended in Tris/HCl buffer containing 1 M-sucrose, mixed with glass beads (0.45–0.5 mm diameter) and disrupted in a Braun homogenizer (Braun, Melsungen, Germany). Residual whole cells and cell walls were sedimented by centrifugation at 3000 *g* for 5 min, and this supernatant was centrifuged at 50000 *g* for 1 h. The final pellet was resuspended in the disrupting buffer, homogenized, and used as a source of enzyme B.

Polymers synthesized by enzymes A and B will be referred to as 'glucan A' and 'glucan B' respectively.

Glucan synthase assay and large-scale preparation of glucan *in vitro*

For glucan A the standard reaction mixture contained (final volume 125 μl): 0.4 mM-UDP-[U-^{14}C]glucose (0.22 Ci/mol), α-amylase (20 μg/ml), 44 mM-EDTA, 100 μM-GTP, 8% glycerol, 50 mM-Tris/HCl, pH 7.8, and enzyme fraction (100–500 μg of protein). For glucan B the reaction mixture was the same, except that 0.8 M-sucrose replaced the glycerol. Reaction mixtures were incubated at 24 °C for 30 min. Then 2 vol. of 10% (w/v) trichloroacetic acid were added and the whole mixture was filtered through glass-fibre filters (Whatmann GF/C). The filters and retained material were washed with 10 ml each of 5% trichloroacetic acid and 66% (v/v) ethanol, dried, and the radioactivity was determined.

For large-scale preparation of radioactive polymers the standard reaction mixtures were scaled up 50-fold. After 1 h of incubation 2 vol. of 100% ethanol were added and the suspension centrifuged at 12000 *g* for 20 min at 4 °C. The pellet was washed several times with distilled water (until supernatants were devoid of radioactivity) and then treated with 1% SDS for 5 min at 50 °C. This treatment solubilized additional label. After a further centrifugation followed by two washes with water the pellet was resuspended in distilled water and kept at −20 °C.

Modified Smith degradation of the polymers synthesized *in vitro*

Samples of glucan containing up to 3000 μg of carbohydrate were incubated with 100 μmol of sodium metaperiodate in the dark for 15 h at room temperature. Subsequent reduction and hydrolysis were carried out as previously described (Guillén *et al.* 1985), except that 4 M-H_2SO_4 instead 4 M-HCl was used. There was a 75% recovery of radioactivity before the paper-chromatography step.

Labelling of the reducing terminus

Samples of glucans A and B of known specific radioactivity were reduced in 0.1 M-$NaBH_4$ in either water (adjusted to pH 9 with a drop of NH_3) or 0.1 M-NaOH in glass-stoppered test tubes (final volume 200 μl). When indicated, 0.1 M-NaB^3H_4 (5 mCi, sp. radioactivity 466 mCi/mmol) was used as a reducing agent. After 18 h at room temperature the reaction was stopped by the addition of 200 μl of acetic acid and the glucan pelleted by centrifugation, washed three times with distilled water and hydrolysed in 2 M-HCl at 100 °C for 2 h. The cooled hydrolysate was applied to a column of Amberlite MB-3 resin and the column washed with 4 vol. of distilled water. The eluate was evaporated to dryness, resuspended in water and analysed by paper chromatography in solvent B. Authentic [^{14}C]sorbitol and [^{14}C]mannitol were prepared by reduction of the corresponding [^{14}C]aldoses with $NaBH_4$.

Cell-wall purification

About 3.5×10^{10} cells were broken in a Braun homogenizer in two steps of 45 s each. The extent of breakage was greater than 98%. The homogenate was centrifuged at 3000 *g* and the pellet washed four times with acetate buffer (pH 5.2). Then it was resuspended in 50% ethanol and left for 2 h in the cold. The 3000 *g*-sedimentable material was washed with the same buffer and then resuspended in 50 ml of 1% SDS and maintained at 50 °C for 5 min. After a further centrifugation at 3000 *g* the pellet was washed twice with acetate buffer, pH 5.2. Cell-wall purity was checked through the process by examination under the phase-contrast microscope for absence of unbroken cells.

Table 1. Chemical analysis of different lots of glucans A and B

For details, see the text.

Sample	Specific radioactivity (c.p.m./μg of glucose)	Percentage of labelled sugars after the Smith degradation			Length of the radioactive chains*
		Glucose	Erythritol	Glycerol	
A_1	2.9	89	2	1.0	45
A_2	1.6	88	0	1.5	30
A_3	2.8	94	2	1.5	32
B_1	11.4	92	2	6.0	9
B_2	45.0	94	1	5.0	10
B_3	29.0	86	3	2.5	23

* This parameter was tentatively calculated as c.p.m. in glucose plus 2 × c.p.m. in glycerol divided by 2 × c.p.m. in glycerol.

Table 2. Effect of specific glucosidases in the reaction products of enzymes A and B

Samples of glucans A_1 and B_1 were precipitated with trichloroacetic acid, washed twice with water and incubated for 24 h with the indicated enzymes in a final volume of 100 μl. Reaction mixtures were then centrifuged and radioactivity in supernatants and pellets determined. Notes: [a]130 m-units; [b]4 units; [c]50 units; [d]10 m-units; [e]acetate buffer, 50 mM, pH 5.5; [f]citrate buffer, 50 mM, pH 5.2.

Treatment	Radioactivity (c.p.m.)			
	Glucan A		Glucan B	
	Supernatant	Pellet	Supernatant	Pellet
Trichloroacetic acid	146	8935	185	7844
Water	187	7304	199	7059
Exo-β-glucanase[a,e]	5208	3637	7708	106
Zymolyase[b,e]	4803	2609	6316	1851
α-Amylase[c,e]	258	7375	230	7181
α-Glucanase[d,f]	314	7846	—	—

General

Paper chromatography was performed on Whatman no. 1 paper in solvents A (butan-1-ol/pyridine/water, 6:4:3, by vol.), B (ethyl acetate/pyridine/water, 10:4:3, by vol.), C (butanol/acetone/water, 4:5:1, by vol.) or D (butan-2-one/H_3BO_3-saturated water/acetic acid, 10:1:1, by vol.). Unlabelled sugars and polyalcohols were detected with $AgNO_3$ reagent (Trevelyan *et al.*, 1950). Radioactive spots in chromatograms were located by cutting strips of paper into 1 cm portions and determining the radioactivity associated with each one.

Sugars in solution were determined by the phenol/H_2SO_4 method (Dubois *et al.*, 1956) and protein by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Reducing sugars were measured by the method of Somogyi (1952) as modified by Nelson (1975). Glucose was determined with glucose oxidase coupled to peroxidase.

Gel filtration for analysis of sugars was performed in a Bio-Gel P-2 column (196 cm × 1 cm), with 50 mM-ammonium acetate as eluent at a flow rate of 0.2 ml/min. Fractions (1.5 ml) were collected.

Radioactivity was determined in a Beckman LS 100C liquid-scintillation spectrometer. Samples containing both ^{14}C and ^{3}H were counted in the appropriate channels and values corrected by use of a standard quench calibration curve.

RESULTS

Chemical characterization of the reaction products

Enzymes A and B incorporated, under standard conditions, up to 40 and 20% of the substrate respectively into a material insoluble in trichloroacetic acid.

Three different samples of glucan A (A_1, A_2 and A_3) and three of glucan B (B_1, B_2 and B_3) were synthesized and subjected to chemical and enzymic analysis. The products synthesized by enzymes A and B were insoluble in water, ethanol and acid (5% trichloroacetic acid or 0.5 M-acetic acid). About 70% of them were solubilized in 0.3 M-NaOH. In this case, acidification of the medium with acetic acid reprecipitated the bulk of the radioactivity.

Neither native nor trichloroacetic acid-precipitated glucans released soluble radioactivity when incubated with sodium metaperiodate. After periodate treatment, reduction with $NaBH_4$, acid hydrolysis and paper chromatography (solvent A), almost all the radioactivity co-chromatographed with glucose, but a small amount of

label was detected in the glycerol and, in some cases, erythritol areas. On the basis of the glycerol/glucose ratio and assuming only one non-reducing end per molecule (see below), average chain lengths of 30–45 and 9–23 radioactive glucose units were calculated for the different lots of glucans A and B respectively (Table 1). These values are minimal estimations, since part of the glycerol could arise during the acid hydrolysis. However, in the case of glucan A_2, it was possible to assume that all the glycerol arose from the non-reducing terminal, since hydrolysis of the native polysaccharide only yielded glucose.

The specific radioactivities of glucans A and B (c.p.m./μg of glucose) were calculated after Smith degradation in order to exclude the possible contribution of some contaminating mannan to carbohydrate determinations by the phenol/H_2SO_4 method. They ranged from 1.6 to 2.9 for glucan A and from 11.4 to 45 for glucan B (Table 1). Since the specific radioactivity of the substrate was about 2333, it follows that both enzyme preparations, but specially enzyme A, contained high levels of unlabelled glucan synthesized before the addition of exogenous substrate.

Enzymic analysis

Treatment of the reaction products with exogenous glucanases indicated that, under standard conditions of enzyme concentration and with incubation periods shown to be effective in degrading a glucan synthesized by membrane fractions of *S. cerevisiae* (Larriba *et al.*, 1981), only glucan B was degraded to the extent expected from the above results. No more than 50–60% of the radioactivity associated with glucan A was solubilized by partially purified exoglucanase, zymolyase or a combination of these together or in sequence (Table 2). Addition of chitinase or proteinase to exoglucanase-treated glucan, or vice versa, did not significantly improve the amount of label solubilized (results not shown). On the other hand, both polymers were completely resistant to α-amylase and to α-1,3-glucanase.

In order to analyse further the apparent inability of the exoglucanase to hydrolyse glucan A, a sample of this polymer (A_1), containing 9000 c.p.m. and 3340 μg of carbohydrate (as glucan), was subjected to repeated additions of enzyme. Each addition was followed by incubation and separation of the soluble products by centrifugation. As shown in Fig. 1, the rate of release of soluble radioactivity was fairly constant during the initial steps, but then slowed down steadily until the end of the experiment. The solubilization rate of glucose or total carbohydrate did not match very well that of the radioactivity, its decline being much slower. As a consequence the specific radioactivity of the glucose released decreased during the course of the experiment, indicating that the glucanase preferentially hydrolysed the glucan synthesized *in vitro*. In agreement with this result the specific radioactivity of the insoluble residue (2300 c.p.m. and 1660 μg of glucose) decreased to 1.39. The same behaviour was exhibited by other glucans after appropriate exoglucanase treatments. Thus the specific radioactivity of glucan A_3 decreased from 2.8 to 0.7 and that of glucan B_3 from 29 to 1.3. Fig. 1 also shows that the exoglucanase solubilized larger amounts of total carbohydrate than of reducing equivalents, the latter exceeding that of free glucose. Since all the radioactive soluble material was identified as glucose (see below), this result suggests that short chains of unlabelled glucan also came into solution.

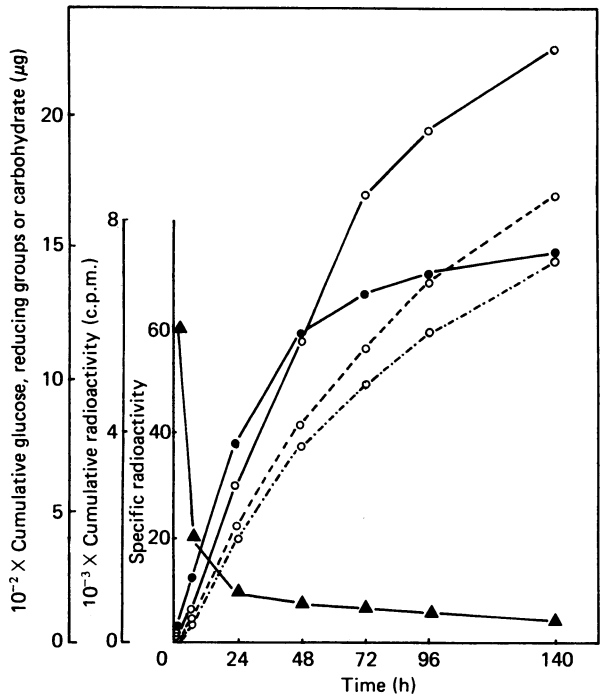

Fig. 1. Time-course of hydrolysis of glucan A by the action of the exoglucanase

A sample of trichloroacetic acid-precipitated glucan A_1 (9000 c.p.m. and 3340 μg of glucose as glucan) was supplemented with 0.5 ml of exoglucanase (130 m-units) in acetate buffer and incubated at 30 °C. At the indicated times, the suspension was centrifuged at 12000 *g* and the pellet was supplemented with an equivalent amount of exoglucanase. A portion of each supernatant was transferred to scintillation vials to determine radioactivity (●) and the rest was used to measure total carbohydrate (○——○), reducing power (○--○) and glucose (○-·-○). The specific radioactivity of the glucose released is also represented (▲).

In a parallel experiment different samples of glucan were supplemented with exoglucanase. At different times the samples were centrifuged and soluble products analysed as described above. Although the insolubility of glucan precluded an accurate sampling, the results, when expressed as a percentage of solubilized label (glucose or carbohydrate), were similar to those shown in Fig. 1.

All these results are indicative of the presence of two pools of glucans in glucan A preparations on the basis of their susceptibility to the exoglucanase. The most resistant pool could be, however, completely hydrolysed by increasing the amount of exoglucanase (× 10) during four incubation periods of 24 h, thus indicating that most, if not all, of the linkages were of the β-1,3 type (not shown).

Since the exoglucanase used breaks all β-1,3 bonds with affecting β-1,6 linkages (Nelson *et al.*, 1969), it was used to investigate the presence of β-1,6 ramifications (Lopez-Romero & Ruiz-Herrera, 1977). Samples of glucans A and B containing 40000 c.p.m. each were exhaustively treated with exoglucanase until no more radioactivity was solubilized. The soluble products were concentrated, desalted in a MB-3 resin column, and

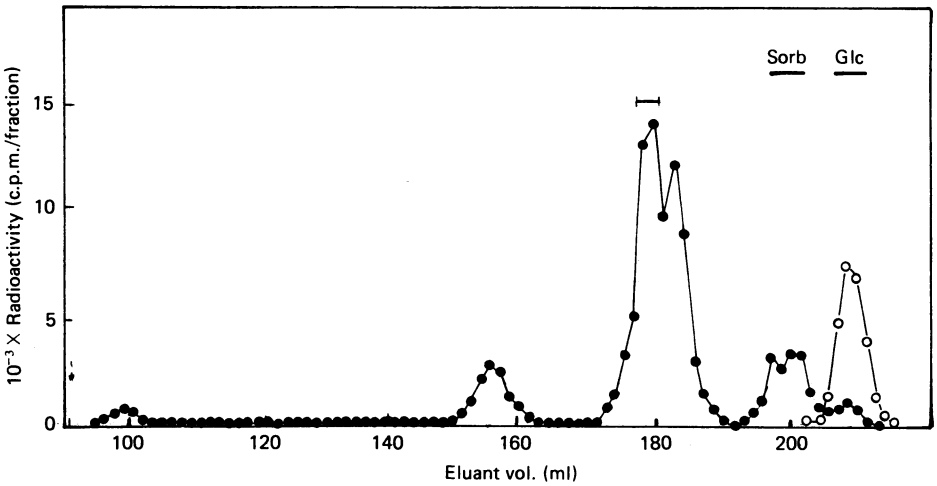

Fig. 2. Bio-Gel P-2 column of the products released by the exoglucanase from NaB^3H_4-reduced glucan A molecule

A sample of trichloroacetic acid-precipitated glucan A (30000 c.p.m.) was labelled with NaB^3H_4 and then subjected to four successive treatments of 24 h with exoglucanase (500 m-units each) as indicated in the legend of Fig. 1. The combined supernatants were pooled and passed through the column. The void volume is indicated with an arrow. The position of authentic standards [glucose (Glc) and sorbitol (Sorb)] are indicated with bars. ●, ^3H; ○, ^{14}C.

filtered through a Bio-Gel P-2 column. In both cases only one radioactive peak, further identified as glucose by paper chromatography, was eluted from the column.

Analysis of the reducing end

Treatment of glucan A_3 with $NaBH_4$ in water (pH 9) or alkali (0.1 M-NaOH) followed by total acid hydrolysis only yielded [^{14}C]glucose. The absence of [^{14}C]sorbitol argues against the synthesis *de novo* of glucan A unless the reducing terminal was bound to an acceptor of a different nature through an alkali-resistant bond. However, when the reduction step was carried out with NaB^3H_4 in addition to [^{14}C]glucose, [^3H]sorbitol was produced in the subsequent hydrolysis, indicating that at least some molecules of unlabelled glucan A have free reducing ends. Some [^3H]glucose was also produced under our experimental conditions. It may be ascribed to the exchange of protons between the reducing mixture and free hydroxy groups (C-2, C-4 and C-6) of glucose units in the native glucan.

Since glucan A samples had not been purified, it appeared possible that we were labelling the reducing end of some contaminant glycogen. Thus, samples of NaB^3H_4-reduced glucan were incubated with α-amylase and exoglucanase. Only the second enzyme solubilized, on addition of [^{14}C]glucose, tritiated low-M_r compounds. One of them, representing about 5% of the total ^3H, was identified as sorbitol by paper chromatography (solvent D). The others appeared to correspond to reduced higher oligosaccharides on the basis of their elution profile in the Bio-Gel P-2 column (Fig. 2). Interestingly, neither sorbitol nor the putative reduced oligosaccharides carried the ^{14}C label. The radioactive material included in the major peak (fractions 179–180 of Fig. 2) was transformed into [^3H]sorbitol by the action of the exoglucanase, as deduced by the change in its elution pattern in the Bio-Gel P-2 column and mobility in paper chromatography (solvent D) (result not shown). By contrast, it remained unaltered after treatment with α-amylase.

The sorbitol/glucose ratio increased 2–3-fold when the reduction step was carried out in 0.1 M-NaOH (results not shown). This result may point to the existence of an alkali-labile bond between the reducing terminals of some glucan chains and another component. However, it is more likely to be a reflection of either the solubility of glucan or the stability of $NaBH_4$ in alkali.

When glucan B was reduced and then hydrolysed, no traces of [^{14}C]- or [^3H]-sorbitol were detected under any of the conditions described above. We conclude that glucan B was not synthesized *de novo* either (unless it was bound to an acceptor other than glucan) and that the unlabelled glucan present in enzyme B was bound to an acceptor through an alkali-stable bond. Addition of a small portion of the cell-wall fraction to enzyme B followed by incubation with UDP-[^{14}C]glucose resulted in a polymer whose reducing end could be now labelled with NaB^3H_4.

In order to check whether glucan B and/or part of glucan A were bound to chitin, glucans A_3 and B_3 were subjected to the same treatments described above but the hydrolysis steps were carried out in 4 M- instead of 2 M-HCl. Under these conditions an authentic-sorbitol control was degraded to the extent of nearly 40%. Accordingly the amount of [^3H]sorbitol yielded by glucan A_3 was much lower. However, in no case was [^3H]glucosaminitol detected among the hydrolysis products of any of the glucans. Glucan B_3 did not release [^{14}C]- or [^3H]-sorbitol either.

Presence of glucanase activity in enzyme A preparations

Time-course-incorporation kinetics of enzymes A and B are shown in Fig. 3. After the onset of the incorporation, a gentle, but reproducible, decrease in the reaction product of enzyme A was observed, whereas that of enzyme B remained unchanged during the whole period tested. This result suggested that glucanase activity bound to glucan A during, or immediately after, its synthesis. In order to check such a possibility, samples of glucan A_1 were incubated in acetate buffer. Controls of the same material previously treated with trichloroacetic acid were supplemented with acetate buffer or

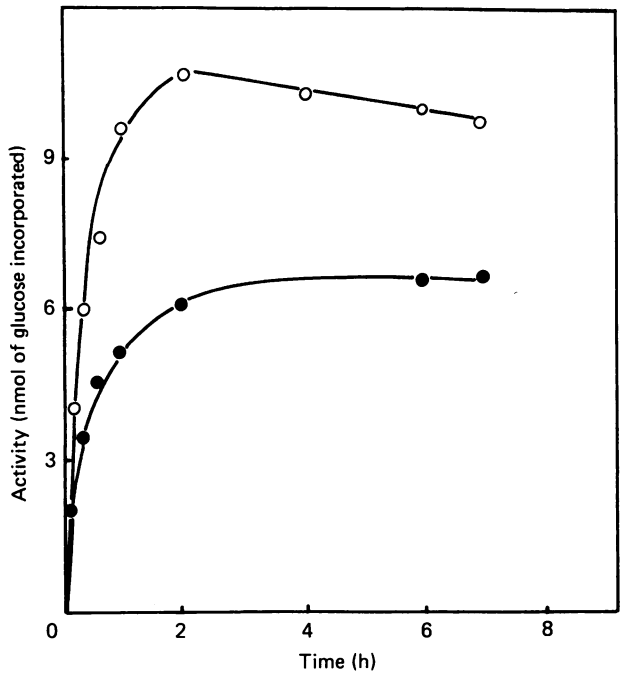

Fig. 3. Time course of glucan synthetase A (○) and B (●) preparations in standard reaction mixtures

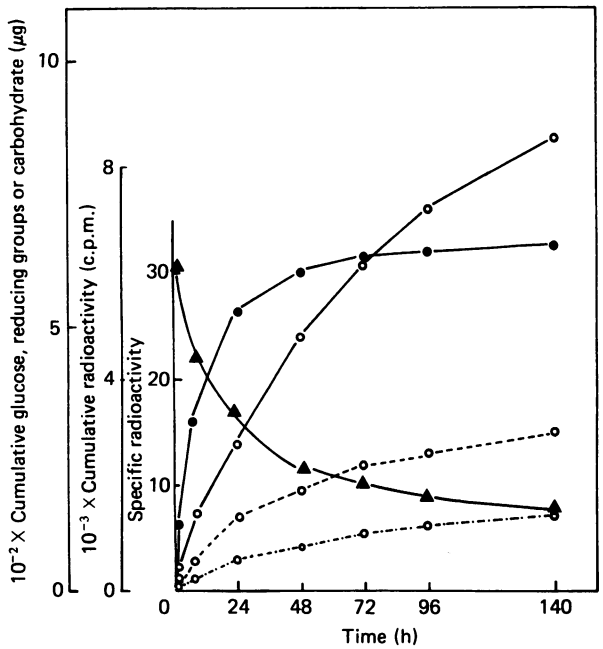

Fig. 4. Time-course of hydrolysis of glucan A by endogenous glucanase

The experiment was performed as indicated in Fig. 1, except that glucan had not been precipitated with trichloroacetic acid, and acetate buffer, instead of exoglucanase, was added at the indicated times. Symbols are as in Fig. 1.

exoglucanase from Basidiomycete QM. Native glucan, when resuspended in buffer, released about 65% of the radioactivity in 24 h. By contrast, no soluble labelled material was released when glucan A had been treated with trichloroacetic acid or boiled before the incubation. As expected, the radioactivity of this glucan could however, be solubilized by exoglucanase.

The fact that the 'endogenous' glucanase remained associated with the reaction product during the preparation of the latter (washings plus SDS treatment) suggested that it was firmly attached to it. The tightness of the binding was confirmed by experiments in which repeated additions of buffer to a sample of glucan were followed, each time, by incubation and separation of soluble products by centrifugation. As shown in Fig. 4, the enzyme was not detached from the substrate (even after four incubation cycles), as deduced from the release of soluble radioactivity during the next incubation. In a parallel experiment, exoglucanase was repeatedly added to a trichloroacetic acid-treated sample of the same material. An example of the pattern of release has been shown in Fig. 1.

Solubilization of radioactivity during time-course hydrolysis or repeated incubations was accompanied by the release of carbohydrate (Fig. 4). Since the specific radioactivity of the products solubilized by the 'endogenous' glucanase decreased as incubations were prolonged, it may be concluded that, as shown for the exoglucanase, this enzyme also preferentially attacks the glucan synthesized *de novo*.

The material solubilized after 2, 7 and 24 h of incubation was analysed by paper chromatography (solvents B and C). In all the cases glucose, laminaribiose and laminaritriose were the only radioactive products detected. The relative proportions of the three sugars did not vary significantly during the period studied; nevertheless, a small decrease in the amount of laminaritriose and an increase in the level of glucose was observed in the 24 h sample.

Glucan B, when allowed to autohydrolyse under the conditions described for glucan A, neither released soluble radioactivity nor carbohydrate.

On the nature of the 'endogenous' glucanase

Since the sphaeroplasts used for the preparation of enzyme A had been obtained by the action of zymolyase, it appeared possible that the glucanase component of the complex (Z-glucanase) became attached to the residual glucan on the sphaeroplast surface and ended up in enzyme A preparations. If so, this enzyme would be responsible for the autolysis observed in the above experiments. This possibility was reinforced by the fact that, when assayed with trichloroacetic acid-treated glucan A, the zymolyase complex released laminaribiose and glucose (24 h incubations), shorter incubation times (2 h) also releasing traces of laminaritriose. In other experiments, samples of trichloroacetic acid-treated glucan A from different lots were incubated for 10 min in the cold with zymolyase and then pelleted by centrifugation. Upon resuspension in acetate buffer and incubation at 30 °C, all of them released soluble radioactivity.

However, other evidence suggests that an endogenous glucanase could be responsible for the hydrolysis of glucan A. During the preparation of a mixed membrane fraction, a portion of the cell-wall fraction was added to its own supernatant and then the membrane fraction was obtained in the usual way. When the glucan synthesized by this preparation was subjected to autohydrolysis, it released soluble radioactivity, whereas a glucan B control obtained under standard conditions

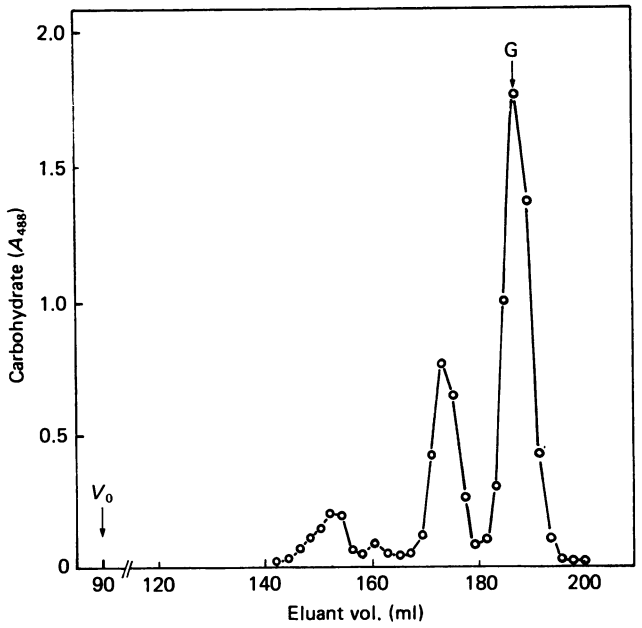

Fig. 5. Bio-Gel P-2 column chromatography of diffusible products arising from cell-wall autolysates

The void volume (V_0) and elution position of glucose (G) are indicated with arrows. For details, see the text.

did not. Glucose and laminaribiose accounted for 50 and 30% of the label released respectively. Thus cell walls contain an endogenous glucanase similar to that responsible for the degradation of glucan A. The absence of laminaritriose and the high ratio glucose/laminaribiose as compared with that yielded by autolysis of glucan A may be ascribed to the inclusion of an additional glucanase in the cell wall. In another experiment cell walls were obtained, subjected to autohydrolysis in a dialysis bag for 3 days, and diffusible material concentrated and passed through the Bio-Gel P-2 column. The elution profile of phenol/H_2SO_4-positive material is shown in Fig. 5. Material included in peaks I, II and III was further analysed by paper chromatography in solvents B and C. Peak I contained glucose and mannose, whereas peak II yielded primarily laminaribiose and *N*-acetylglucosamine together with traces of glucose, mannose and laminaritriose. The low amount of material associated with peak III prevented its unambiguous identification. On the basis of the R_F of oligosaccharides of the laminarin series, it corresponded to the hexasaccharide. Thus, at least two, and possibly the three, sugars released by autolysis of glucan A were also released by autolysis of the cell wall.

DISCUSSION

Two enzyme preparations from *Candida albicans* similar to those currently used for synthesizing β-1,3-glucan from *Saccharomyces* also catalysed an efficient transfer of glucose from UDP-glucose into trichloroacetic acid-insoluble material. Both reaction products were identified as linear β-1,3-glucans by chemical and enzymic analysis. The resistance of the products to α-amylase was indicative that no glycogen was synthesized by any of the enzymes. On the other hand, the β-1,3 linkage was deduced from their resistance to periodate and susceptibility to β-glucanases. Nevertheless, some radioactive material travelling as erythritol in paper chromatography was yielded by some glucan preparations after Smith degradation. The source of that material does not appear to be radioactive glycogen for several reasons: (*a*) the reaction mixtures contained α-amylase; (*b*) no label was solubilized by treatment of the products with α-amylase; and (*c*) similar proportions of erythritol were yielded by the residue left by the previous treatment after Smith degradation. As mentioned above, it could arise from breakage of glucose units during the hydrolysis step.

The resistance of glucan A to hydrolysis by the exoglucanase was not due to the presence of α-1,3 linkages. It could be explained, instead, by the peculiar kinetics of release of radioactivity and carbohydrate caused by the exoglucanase. The presence of glucanase-resistant glucan cores in enzyme A, but not enzyme B, preparations is not surprising, since the former originate from sphaeroplasts obtained by the action of zymolyase. Some of the radioactive units are probably added to those zymolyase-resistant cores, giving rise to the radioactive resistant pool which, in all the cases studied, amounted to a percentage smaller than its carbohydrate counterpart. The demonstration that higher levels of exoglucanase, when acting in successive periods, could accomplish total hydrolysis of the radioactive polysaccharide, yielding glucose as the only labelled end product, led us to the conclusion that we had synthesized a linear β-1,3-glucan.

The absence of [^{14}C]sorbitol after reduction and hydrolysis of glucans A and B indicates that the newly formed chains were bound either to unlabelled glucan or to an acceptor of a different nature. In the latter case they must be bound through an alkali-resistant linkage, since reduction in the presence of 0.1 M-NaOH also failed to yield [^{14}C]sorbitol. Some, at least, of the glucan chains associated with enzyme A have free reducing ends, since they could be labelled with NaB^3H_4. By contrast, we were unable to detect free reducing ends in glucan B, in spite of the fact that enzyme B also contained (although to a lesser extent of glucan A) appreciable levels of unlabelled glucan. Accordingly, enzyme B preparations appear to contain exclusively, or at least be enriched in, glucan molecules bound to an acceptor of a different nature.

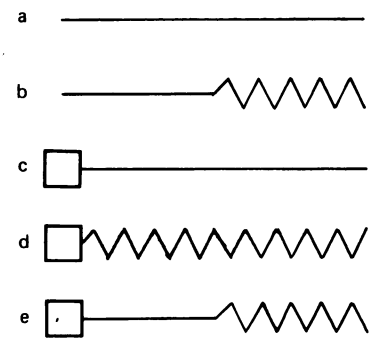

Fig. 6. Models for glucan molecules of glucan A and B preparations

a and/or b models are always present in glucan A; c, d and e may co-exist with them. c, d and e (but not a or b) may co-exist in glucan B. Symbols: □, acceptor; ——, non-radioactive glucan; ⩙ radioactive glucan.

This acceptor is not a polysaccharide with mannose or *N*-acetylglucosamine at its reducing terminal. The possibilities envisaged by us are shown in Fig. 6.

With those models in mind, the decrease in specific radioactivity of the glucose released during enzymic hydrolysis may be explained by the mode of action of the exoglucanase used. As shown by Nelson (1975), this enzyme proceeds from the non-reducing terminal by a multiple-attack mechanism removing an average of four glycosyl residues per encounter. Thus the release of glucose of maximal specific radioactivity during the initial phases of hydrolysis indicates that the polymer grew towards the non-reducing end. In subsequent steps such a rate would decrease as the radioactive 'tips' (models b and e of Fig. 6) or chains (model d) were being hydrolysed.

Our glucan A models also explain the solubilization of ^{3}H-labelled oligosaccharides lacking the ^{14}C label after the action of the exoglucanase on samples of that glucan previously reduced with $NaB^{3}H_4$. This observation is, in turn, in agreement with the results shown in Fig. 1, in which the values of total carbohydrate released by the exoglucanase exceeded those of free glucose, in spite of the fact that [^{14}C]glucose was the only radioactive product solubilized by the exoglucanase.

Regardless of its origin, the presence of glucanase activity associated with the enzyme A (but not enzyme B) preparations might explain the discrepancies found in *Saccharomyces* concerning the state of the reducing end of the glucan molecules synthesized *in vitro*. It is possible that glucanases from the enzymic complex used by Shematek *et al.* (1980) for the preparation of sphaeroplasts (cytohelicase) or another endogenous glucanase secreted by *Saccharomyces*, ended up in their glucan synthase preparations. If so, they could be responsible for the presence of [^{14}C]sorbitol after reduction and hydrolysis of the polysaccharide, which, in turn, would lead to the erroneous conclusion that the polysaccharide was synthesized *de novo*.

The nature of the enzyme preparations used may help us to rationalize the results presented here. As described for *S. cerevisiae* (Shematek *et al.*, 1980), the bulk of active glucan synthase of *C. albicans* regenerating protoplasts may be attached to the plasma membrane, giving rise to a glucan net (Gopal *et al.*, 1984*a*), which would become incorporated into enzyme A preparations. Some of the chains could remain associated with the synthase after lysis of protoplasts and continue their elongation upon the addition of exogenous substrate, whereas others probably represent finished products. By contrast, during the preparations of enzyme B, mature glucan molecules with free reducing ends are eliminated with the cell-wall fraction, and the membrane-associated glucan probably represents molecules that are being synthesized while bound to an acceptor. During normal cell growth or protoplast regeneration the acceptor could serve as a signal for the transfer (*en bloc*?) of the newly synthesized chains to the glucan net present in the cell wall or protoplast surface respectively. Glucanases associated with that structure (Notario, 1982; the present paper) could play a role in such a transfer and/or in the processing of the final glucan molecules.

There is growing evidence that glycogen, previously thought to be an homopolysaccharide, belongs to the family of proteoglycans, its biosynthetic origin being a protein on to which the polymer is built (Aon & Curtino, 1984, 1985; Blemenfeld & Krisman, 1985; Rodriguez & Whelan, 1985). Thus it would not be surprising if, by analogy, the synthesis of yeast glucan involved an intermediate of similar nature. Some direct evidence has been presented here.

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