Biochemical Studies on Walls Synthesized by *Candida utilis* Protoplasts

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SUMMARY

Protoplasts of *Candida utilis* obtained by treatment with helicase built a new wall in a liquid medium. The spherical protoplast was converted to a tubular form which later changed to an ellipsoidal yeast. Walls isolated at different stages of regeneration were analysed. Glucan (42 to 48 %), mannan (25 to 31 %) and protein (8 to 9 %) were the main constituents of walls of normal and completely regenerated ellipsoidal forms. Walls of the tubular forms differed markedly by having a high content of chitin (12 to 18 %) and of protein (20 %); mannose was present only as traces. The glucan content was similar in normal, tubular and ellipsoidal regenerating yeasts. These results demonstrate that some modifications occur in the structural polysaccharides of the yeast wall which can be correlated with morphological changes in the reversion process. The significance of glucose, mannose and acetylglucosamine polymers is discussed in relation to the biosynthesis of regenerated walls.

INTRODUCTION

Necas (1956) and Eddy & Williamson (1959) found that yeast protoplasts were capable, under well defined conditions, of forming new wall structures and of regeneration. However, relatively few chemical studies on this phenomenon have been reported. Recently, Garcia-Mendoza & Novaes-Ledieu (1968) described the *de novo* synthesis of wall structures by *Candida utilis* protoplasts incubated in a liquid medium and noted a high content of chitin in walls of the first regenerating tubular forms. The aim of the present study was to investigate the composition of the wall synthesized newly by protoplasts throughout the regeneration process and to discuss the chemical differences in relation to the morphology.

METHODS

Growth conditions and preparation of protoplasts

As in our previous experiments (Garcia-Mendoza & Novaes-Ledieu, 1968), *Candida utilis* CECT 1061 was grown in Winge medium in a reciprocal shaker at 28°. Yeast was harvested by centrifugation at 10,000g for 10 min., when an optical density of O.D._{600 mμ} of 0.6 was reached, and washed with distilled water. Then the yeast was washed once with 0.01M-2-mercaptoethanol in 0.01M-citrate buffer (pH 5.8). Treated yeast was washed twice with this buffer, resuspended in the digestion medium containing 0.8M-mannitol in citrate buffer (pH 5.8), containing 1 to 2 mg./ml. dried helicase

(Industrie Biologique Française, Gennevilliers, France) and shaken at 28°. After 3 hr all the yeast was converted into protoplasts as seen by examination under a phase microscope.

Cultivation of protoplasts in nutrient medium

Protoplasts prepared from different quantities (5 to 100 mg. dry wt) of normal yeast, prepared as described above, were washed twice with 0.8M-mannitol, suspended in 500 ml. of Winge medium containing 0.55M-MgSO₄·7H₂O (iso-osmotic for this strain) and incubated at 28° with gentle shaking. In a series of experiments the initial protoplast inoculum used was minimal (about 5 mg. dry wt) and regenerating yeast was collected after 18, 30, and 64 hr of incubation. In other series larger amounts of initial protoplast inoculum (about 100 mg. dry wt) were used and samples were collected after 4, 18, and 36 hr; moreover, regenerating yeasts in their final logarithmic phase (after about 20 hr incubation) were centrifuged and a portion of the deposit was incubated again for 6 or 16 hr in a fresh Winge medium to obtain sufficient yeast at different stages of logarithmic reproduction. During incubation, wall regeneration and cell budding was followed under a phase microscope.

Preparation of walls

Yeast at different stages of regeneration was collected by centrifugation and treated in a Braun cell homogenizer using no. 12 ballotini beads. To purify the material, the crude wall preparations were suspended several times in 60 and 10% sucrose in water and centrifuged at 10,000 g for 20 min. Subsequent treatments with 10 and 1% NaCl were performed. Finally, the walls were washed with distilled water and dried in a vacuum.

Analytical methods

Nucleic acids were estimated from the ultraviolet spectra of extracts obtained by treating samples with 0.5 N-perchloric acid at 75 to 80° for 30 min. (Gale & Folkes, 1953). All preparations showing more than 0.5% nucleic acids were eliminated because they could have been contaminated with cytoplasmic materials.

Protein was determined by the procedure of Lowry, Rosebrough, Farr & Randall (1951) performed on cold N-KOH extracts of walls: casein was the reference standard.

Amino acids were characterized by chromatography of hydrolysates (6 N-HCl at 105° for 24 hr) on Whatman paper no. 1; excess HCl was removed in vacuum over NaOH. Chromatograms were run for 18 hr with *n*-butanol:formic acid:water (75:15:10, v/v); amino acids were detected with ninhydrin (0.2%, w/v) in acetone. Three groups of amino acids: arginine, lysine and histidine; aspartic acid, serine and glycine; glutamic acid and threonine were eluted separately with water from chromatograms and characterized by electrophoresis on Whatman no. 1 paper at pH₆ 11.5, 2.25 and 6.5 respectively.

Neutral carbohydrates were determined using an anthrone reaction (Chung & Nickerson, 1954). Solutions containing known quantities of glucose and mannose were estimated simultaneously. Identification of monosaccharides was by paper chromatography of hydrolysates prepared in sealed tubes at 105° with N-HCl for 4 and 6 hr. Excess HCl was removed in a desiccator over NaOH. The paper chromatograms (Whatman no. 1) were run for 24 hr with *n*-butanol:acetone:water (4:5:1, v/v). Sugars were detected with aniline hydrogen phthalate in *n*-butanol. Semiquantitative deter-

mination of monosaccharides was made on chromatograms as described by Gottschalk & Ada (1965).

For total amino sugars, samples were hydrolysed in sealed tubes with 6 N-HCl at 105° for 6 hr. The insoluble materials were separated by filtration and HCl was removed in a vacuum over NaOH pellets. Neutral sugars, neutral amino acids and condensation products of amino acids were removed by passage of the hydrolysate through a column of Dowex 50-X8 (H⁺) resin prepared by the method of Boas (1953), then total hexosamine was determined by the method of Randle & Morgan (1955) using glucosamine HCl as a standard. Amino sugars were resolved by chromatography on Whatman no. 1 paper with *n*-butanol:pyridine:0.1 N-HCl (5:3:2, v/v) as solvent. Hexosamine was located by using a modification of the Elson-Morgan reagent (Partridge, 1948).

For estimation of chitin, yeast walls (5 to 10 mg.) were shaken for 6 hr at 37° with 2 mg. fungal chitinase (Koch Light) in ammonium acetate buffer 0.03 M (pH 5.8). Chromatograms on Whatman no. 1 paper were run with *n*-butanol:acetic acid:water (3:1:1, v/v) for 24 hr. N-Acetylglucosamine, chitobiose and chitotriose were detected with diphenylamine trichloroacetate reagent (Hough, Jones & Wadman, 1950). The intensity of colour and the size of N-acetylglucosamine (NAGA) spot were compared with spots produced by known amounts of standard NAGA. The spots corresponding to chitobiose and chitotriose were eluted from chromatogram with water and hydrolysed with 2 N-HCl for 3 hr at 105°. Glucosamine-HCl, liberated by acid hydrolysis, was estimated semiquantitatively on chromatograms (for conditions, see above). Part of the enzymic hydrolysate was dried in a vacuum and then hydrolysed with 2 N-HCl for 4 hr at 105°. Excess HCl was removed by repeated evaporations over NaOH and the residue was passed through cationic resin (Dowex 50). Glucosamine-HCl was estimated by means of a modification of the method of Randle & Morgan as described by Ghuysen, Tipper & Strominger (1966).

Lipids were isolated by ether extraction after a preliminary treatment with methanol to disrupt any lipoprotein complexes. The extracts were centrifuged to remove traces of suspended material, evaporated and dried in a vacuum over P₂O₅.

Solubility of chitin in acids and alkalis

Samples of yeast walls regenerated for 18 hr were treated for 30 min. with cold N-HCl, then treated 30 min. at 100°. The residual walls were incubated with N-HCl at 100° for 30 min. Residues and supernatants were dried in a vacuum.

Enzymic treatments of walls of regenerating cells

Parallel to the chemical studies, regenerating yeasts were treated with helicase or with chitinase and the attack followed under a microscope. Conditions of enzymic digestion were the same as those described above for the treatment of normal yeast and walls.

Enzymic pretreatments of yeast protoplasts

To eliminate possible contaminants attached to the surface of protoplasts and to study the consequences of this cleaning on successive regenerating phases, the following two procedures were used.

Chitinase treatment: Large amounts of protoplasts prepared with helicase were

incubated for 3 hr in 0.8 M-MgSO₄·7H₂O in 0.03 M-acetate buffer (pH 5.8), and 1 mg. chitinase/ml. The reaction mixture was centrifuged and the pellet treated twice more in the same way. The three supernatants were collected and kept to analyse.

Lysozyme treatment. Large amounts of protoplasts prepared with helicase were incubated once for 30 min. in 0.8 M-sucrose and 0.05 M-MgCl₂ in 0.03 M-tris-HCl (pH 7.5) and 200 µg. lysozyme/ml.

Controls without enzyme were included. Both preparations of protoplasts were washed to eliminate the residual enzymes and inoculated in reversion medium as described above.

RESULTS

Chemical analyses

Examination under a light microscope of different stages of protoplast regeneration showed that all the initially spherical protoplasts showed signs of growth, seeming to bud after 2 to 4 hr; afterwards they changed to tubular forms from which new normal

Table 1. *Chemical composition of Candida utilis walls*

About 5 mg. protoplasts were inoculated in 500 ml. Winge medium.

	Normal cells 24 hr growth	18 hr re- generating cells* (tubular form)	30 hr re- generating cells† (yeast form)	64 hr re- generating cells‡ (yeast form)
Total carbohydrate (anthrone)	78-80	50-51	75-78	78-79
Glucose	48	48	46	47
Mannose	30	1-2	29	30
Total hexosamine (Rondle & Morgan)	0.2-0.5	16	2-3	2-3
Protein (Lowry)	8-9	20	9	9
Chitin (chitinase)	0.1-0.2	18	1-3	1-2
Free and bound lipids (gravimetrically)	1	0.5-1	1	1

* Logarithmic phase yeast.

† Final logarithmic phase yeast.

‡ Stationary phase yeast.

shaped yeasts later originated. A homogeneous population of ellipsoidal-shaped yeast was not complete until after 30 to 36 hr of incubation although the first ellipsoidal forms appeared after 20 to 24 hr.

Garcia-Mendoza & Novaes-Ledieu (1968) showed the composition of walls of tubular forms (18 hr incubation) to be very different from that of the parent yeast. These results have been confirmed (see Table 1): a high content of chitin (18 %) and a small amount of mannose were found in the tubular form. Normal yeast walls contained large amounts of mannose and small amounts of chitin. The samples between 20 and 30 hr incubation were discarded because the cultures were showing a heterogeneous appearance.

In contrast with the above finding, the wall material from minimal inocula, prepared from final logarithmic phase cells (after 30 hr incubation) and stationary phase cells (64 hr incubation) had a composition grossly similar to that of normal yeast (Table 1).

Walls of 4 hr regenerating yeast were therefore analysed. Preliminary experiments

performed with these structures showed three principal components: glucan, protein and chitin (this last over 12 %). This suggested that a complex of glucan plus chitin could be formed during the early stage of wall biosynthesis.

Wall material, from large inocula, prepared from stationary-phase yeast from the 36 hr culture differed largely from material obtained above with 30 hr walls (see Tables 1, 2). In the former there was as high a proportion of mannose as in normal walls, but the chitin content remained approximately the same as that in tubular forms, possibly as a consequence of the exhausted medium, so there were few opportunities for the regenerating yeast to bud. In these preparations the protein content was reduced to the value of normal yeast walls. Chemical analyses of logarithmic phase yeast walls (20 hr plus 6 hr, and 20 hr plus 16 hr), both from ellipsoidal cells, revealed compositions similar to that of normal yeast walls, suggesting that the newly formed generation contained only traces of chitin, as in normal walls.

Table 2. *Chemical composition of Candida utilis* walls

About 100 mg. protoplasts were inoculated in 500 ml. of Winge medium.

	Normal cells 24 hr growth	18 hr re- generating cells* (tubular form)	36 hr re- generating cells† (yeast form)	20+6 hr regenerat- ing cells‡ (yeast form)	20+16 hr regenerat- ing cells‡ (yeast form)
Total carbohydrate (anthrone)	78-80	50-51	65-68	78-80	78-80
Glucose	48	48	42	48	48
Mannose	31	1-2	25	30	30
Total hexosamine (Rondle & Morgan)	0.2-0.5	16	16	1-1.2	0.4-0.8
Protein (Lowry)	8-9	20	9	9	9
Chitin (chitinase)	0.1-0.2	18	18	1.5-1.8	0.3-0.5
Free and bound lipids (gravimetrically)	1	0.5-1	0.5-1	0.6-1	0.5-1

* Logarithmic phase yeast.

† Stationary phase yeast.

‡ Logarithmic phase yeast from two-stage culture.

Analyses of the various stages of regenerated yeast walls and normal walls have shown that all the usual amino acids were present and in similar proportions. The $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in the regeneration medium did not have an influence on the growth of yeast and on the composition of walls because analyses of normal cell walls incubated in Winge medium and in the same medium but containing MgSO_4 were similar.

Solubility of regenerated cell walls in acids and alkalis

Treatment with N-HCl, in cold and at 100° solubilized only a small portion of tubular walls. The soluble material corresponded to 15 to 20 % of total wall and was glucan and protein. In the insoluble part an acid-insoluble glucan and the chitin were found. Treatment of the residue with N-KOH at 100° dissolved the glucan alone, the glucosamine polymer being extraordinarily resistant toward acid and alkali. In normal walls, 70 % of anthrone-positive products were soluble in hot HCl.

Effect of selected enzymes on regenerated walls

Phase-microscope examination showed that the walls of 18 hr tubular forms treated with helicase were damaged to some degree and that a small proportion of these

tubes were transformed into protoplasts. Treatment with chitinase damaged the wall structures to a lesser degree. Regenerating ellipsoidal cells harvested during the logarithmic phase of growth (30 hr incubation, 20 hr plus 6 hr incubation, or 20 hr plus 16 hr incubation) and treated with helicase were markedly attacked, giving a high percentage of protoplasts, suggesting that the carbohydrate chains present in their walls are similar to those in normal cells. As was expected, treatment with chitinase did not appear to affect the wall structure.

Regeneration of enzymic pretreated protoplasts

After pretreatment with chitinase, examination under a phase microscope showed that protoplasts had difficulties in building a new wall. Growth was slower and after 18 hr incubation in the usual conditions, numerous protoplasts were observed; these were often deformed but without clear appearance of reversion. Some tubular forms with thick and less compact walls were also encountered. After 24 hr incubation the preparations appeared more normal, showing a high percentage of tubular forms. The first yeast forms were observed after 40 to 44 hr incubation. Cultures which had regenerated for 18 hr and 24 hr presented generally a heterogeneous picture, thus making it difficult to obtain a pure preparation of tubular form walls. However, preliminary chemical analyses were carried out on samples of walls obtained from tubular yeasts and from 36 hr regenerating yeasts (stationary phase forms described in Table 2). We expected that both walls would be rich in chitin; however, the chitin content was lower than that expected.

Microscopic examination showed that pretreatment with lysozyme also interfered with reversion of protoplasts. Estimation of chitin in the walls of 18 hr tubular forms and 36 hr regenerating ellipsoidal yeasts (stationary phase forms described in Table 2) gave percentages lower than those found when protoplasts were incubated without pretreatment with lysozyme.

DISCUSSION

Protoplasts of our strain of *Candida utilis* synthesized walls *de novo* despite earlier reports that a solid medium was necessary for regeneration of protoplasts of yeasts whose walls contain mannan. Protoplasts of mannan-free yeasts regenerate either in liquid or on solid media (Svoboda, 1965; Necas & Svoboda, 1965). The composition of the regeneration medium is probably important; presumably the osmotic stabilizer plays a role preventing leakage of intermediates of mannan synthesized extracellularly (Kozak & Bretthauer, 1968). This may be one reason why Eddy & Williamson (1959) did not obtain reversion of protoplasts of *Saccharomyces carlsbergensis*, though they observed tubular forms like those we saw. Alternatively, their regeneration medium may have been nutritionally inadequate.

The walls of *Candida utilis*, when fully regenerated, had the chemical constitution of normal walls, but the intermediate tubular forms were rich in glucan and chitin but deficient in mannan. Mannan formation was associated with restoration of the normal ellipsoid form. We suggest, therefore, that primary formation of a glucan-chitin matrix gives the organism rigidity and provides a basis for mannan deposition, which confers the ellipsoid form. This view is consistent with Bartnicki-Garcia & Nickerson's (1962) report of extra mannan in walls of yeast-like forms of *Mucor rouxii* and with Chattaway, Holmes & Barlow's (1968) report that the mycelial form

of *C. albicans* is relatively rich in chitin. Chitin formation is associated with budding in yeasts (Bacon, Davidson, Jones & Taylor, 1966; Bacon, Jones & Ottolenghi, 1969).

The glucan-chitin matrix is probably identical with the fibrillar network revealed by electron microscopy of early regenerating protoplasts (Kopecka, Ctrvrtnicek & Necas, 1965; Necas, Svoboda & Havelkova, 1968; Uruburu, Elorza & Villanueva, 1968) and previously believed to be composed of glucan; it is presumably closest to the protoplast membrane in the normal yeast.

The regenerating protoplasts in our work and in other reports probably possessed residual wall components which acted as primers for synthesis of new polymer (Glaser & Brown, 1957; Garcia-Mendoza, Garcia Lopex, Uruburu & Villanueva, 1968; Streiblova, 1968; Bacon *et al.* 1969; Darling, Theilade & Birch-Andersen, 1969). The delaying effect on regeneration of pretreatment with lysozyme or chitinase is probably due to removal of primer by these enzymes (Cherkazov & Kravchenko, 1968).

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