Use of the exo-β- (1→3) Glucanase from Basidiomycete QM 806 in Studies on Yeast

The standard method used for analysing yeast cell carbohydrates[1] depends on the differential solubility of glucan, mannan, and glycogen in alkali and acid. It has been found, however, that when the isolated cell wall is treated with alkali, even as dilute as 3%, most of the glucan, as well as the mannan dissolves, and a true fractionation of the carbohydrates is not obtained[2]. In addition to this, studies on yeast protoplasts frequently use the digestive juice of *Helix pomatia* to degrade the cell wall[3–6]. Apart from consisting of a large mixture of enzymes[3], this preparation has the disadvantage that in most cases it gives rise only to yeast spheroplasts and not true protoplasts.

An exo-β-(1→3) glucanase, isolated and purified from *Basidiomycete* QM 806[7] has been found to cause almost complete solubilization of the yeast cell wall. In view of this, it has been successfully used both in the analysis of the cell wall carbohydrates of *Saccharomyces cerevisiae* and in the study of protoplast formation from *S. cerevisiae* and *Wickerhamia fluorescens*.

Experimental. Cell wall analysis. Cell walls of *S. cerevisiae* were prepared free from cytoplasmic contamination by the method of MILL[8]. They were found to consist of 88–93% carbohydrate by hydrolysis and measurement of the total sugar[9,10].

Cell walls (20 mg) in 0.05 *M* acetate buffer pH 5.0 (20 ml) were incubated with 200 units of purified glucanase[11] overnight at 25°C. The mixture was centrifuged and the residue was washed and suspended in water (5 ml). The supernatant and washings were made up to 25 ml (S I). Mannan was precipitated from S I by Fehling's solution and after washing in 0.1 *N* NaOH, was dissolved in 10 *N* H_2SO_4 (0.1 ml) and made up to 10 ml with water. S I (5 ml) was also treated with ethanol (15 ml) and the precipitate was removed by centrigufation, washed in 75% ethanol and dissolved in water to 10 ml. The supernatant and washings after removal of ethanol under reduced pressure were made up to 20 ml (S II). Total

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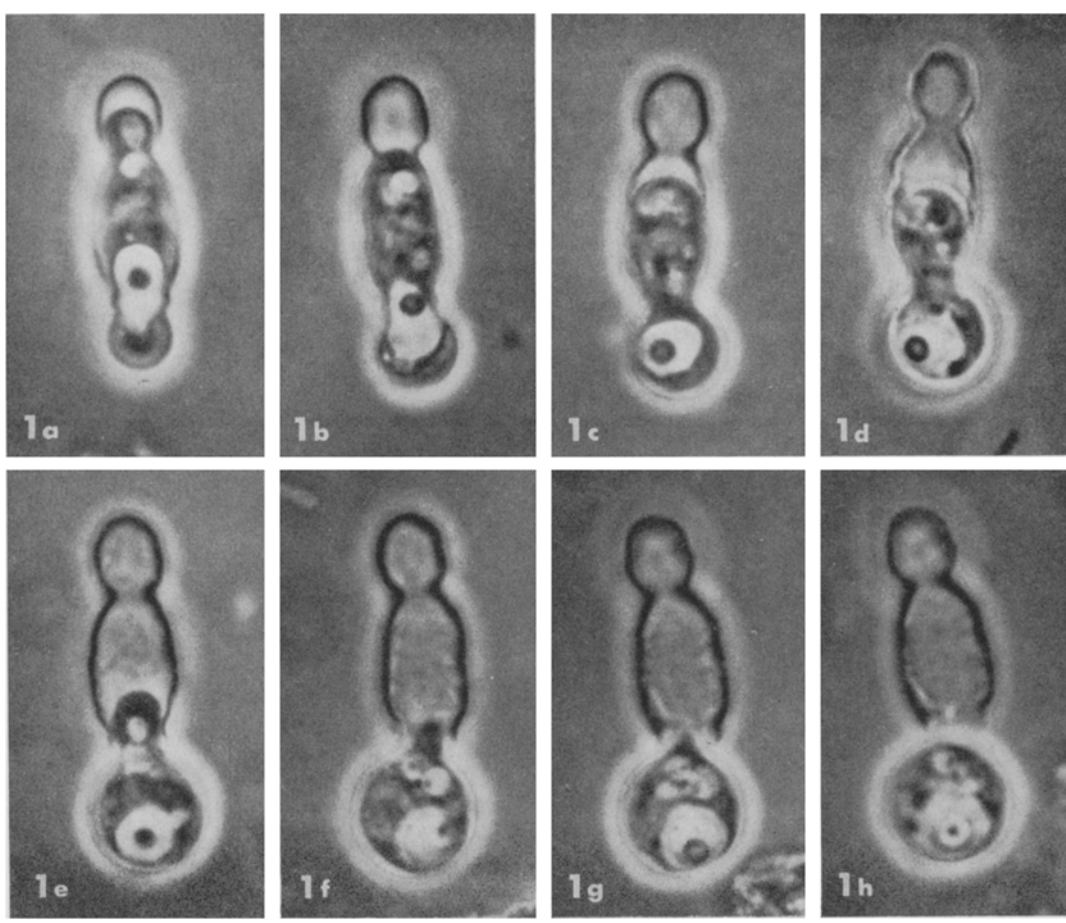

Fig. 1, a–h. Protoplast formation in *Wickerhamia fluorescens* (for explanation see text). ×1950.

Carbohydrate composition of cell wall of *Saccharomyces cerevisiae*

Fraction	Total carbohydrate (% of wall dry wt.)	Reducing groups (% of wall dry wt.)	Sugars identified by chromatography
Supernatant 1	89	40	Glucose, gentiobiose, non migrating material
Supernatant 2	49	39	Glucose, gentiobiose
Fehlings ppt.	37	Negligible	Mannose [a]
Alcohol ppt.	40	Negligible	Mannose [a]
Residue	0.5	Negligible	– [a]

[a] Fraction hydrolyzed before chromatography.

sugar was measured in all fractions by the phenol sulphuric acid method[10] and reducing groups were measured in S I and S II by the NELSON SOMOGYI method[12]. The sugars present in these fractions were identified by paper chromatography in pyridine: ethyl acetate: water (2:5:7 top layer), and were revealed by alkaline silver nitrate. The results are shown in the Table.

The results indicate glucan and mannan to be the only carbohydrates in the cell wall of *S. cerevisiae*. As only mannose was found in the hydrolysate of the alcohol ppt, a small amount of mannan must have been lost from the Fehling's ppt. The total mannan was therefore 40%. The total sugar in S II (49%) represents the total glucan of which 82% (40% of wall) was hydrolyzed by the β-(1→3) glucanase. The presence of only glucose and gentiobiose in S II indicated that the remainer of the glucan was β-(1→6) linked. Such a finding supports previous work with isolated glucan[13,14].

Protoplast Formation. S. cerevisiae and *W. fluorescens* cells from the logarithmic growth phase were harvested by centrifugation and washed in 0.1 *M* mercapto-ethanol

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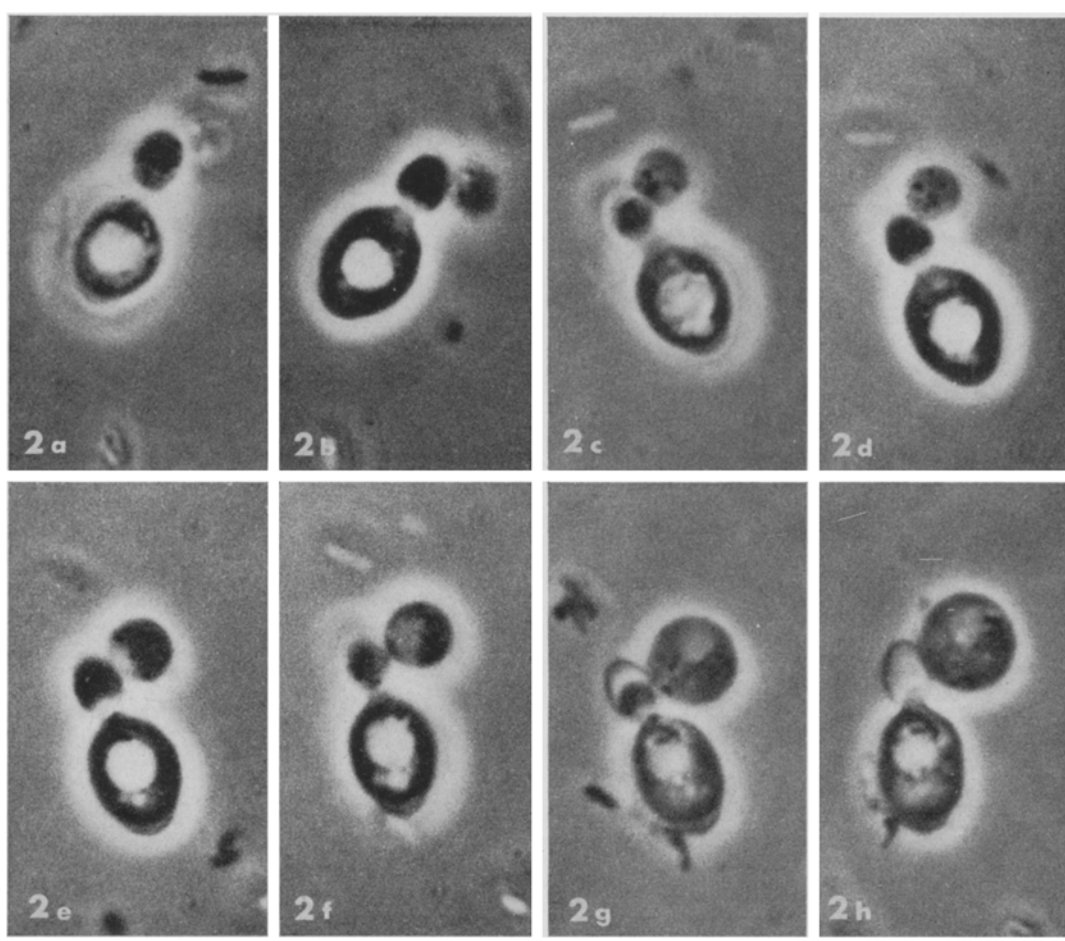

Fig. 2, a–h. Protoplast formation in *Saccharomyces cerevisiae* (for explanation see text). ×2540.

(5–10 min)[15]. After washing in 0.1 *M* cacodylate buffer, the cells were resuspended in a stabilizing protoplast medium (18 ml 0.55 *M* *D*-mannitol; 1.5 ml McIlvain's citrate phosphate buffer pH 5.6; 0.75 ml $MgSO_4$ $7H_2O$)[3]. Purified β-(1→3) glucanase (80–100 units) was added to 1 ml of cells suspended in the protoplast medium and protoplast formation was followed by phase contrast microscopy.

A polar mode of protoplast formation, suggesting a possible concentration of the β-(1→3) glucan can be observed in the first sequence of a budding cell of *W. fluorescens* (Figure 1). The cell wall has been broken open at the opposite pole to that at which bud formation takes place. As no septum has yet been formed between mother cell and bud, the cytoplasm of both mother cell and bud are incorporated into the forming protoplast. The different stages of protoplast formation can be clearly seen (Figure 1, a–h). It is interesting to note that the cell wall is almost intact at the end of protoplast formation, suggesting that degradation proceeds slowly in the protoplast medium. The fully released spherical protoplasts were obtained after 5–10 min (Figure 1, h).

In the second sequence (Figure 2), which shows a budding cell of *S. cerevisiae*, the protoplast is formed from the cytoplasm of the bud, as septum formation between bud and mother cell is already complete. In this case protoplast formation is nonpolar, which might indicate a random distribution of β-(1→3) glucan in the cell wall. The different stages of protoplast formation are shown in Figure 2, a–h. The fully released protoplast (h) is again spherical and the bud retained its original shape throughout the sequence of protoplast formation, which lasted for three minutes.

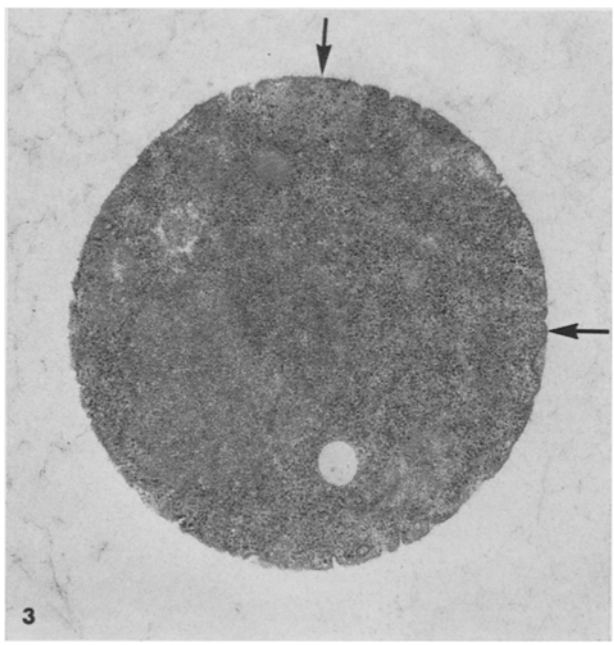

Fig. 3. Electron micrograph of a true protoplast of *Saccharomyces cerevisiae*. No adhering cell wall material is present. The arrows indicate regions where the unit membrane structure of the plasmalemma is clearly revealed. ×24,500.

The protoplasts released using β-(1→3) glucanase were examined by electron microscopy to check whether any wall material still adhered to them. Figure 3 shows a true *S. cerevisiae* protoplast obtained as previously described. The arrows indicate particularly well revealed sections of the cytoplasmic membrane, the whole of which is shown to be entirely free of adhering cell wall fragments. The β-(1→3) glucanase is thus capable of producing true protoplasts from yeast cells, even after they have been prefixed in glutaraldehyde.

Discussion. The application of the extracellular exo-β-(1→3) glucanase from *Basidiomycete* QM 806, to the analysis of yeast cell walls and the formation of yeast protoplasts has been demonstrated. The fact that this enzyme, in a purified state, entirely dissolved yeast cell walls made it an ideal tool for analysis of the carbohydrates, as it caused changes only in its specific substrate (β-1→3 glucan). In addition it gave information on the amount of this substrate present in the walls. The endo-β-(1→3) glucanases of *Bacillus circulans*[16] and *Cytophaga johnsonii*[17] were not able to cause complete solubilization of the cell walls of *S. cerevisiae* as did the *Basidiomycete* enzyme. Nor were these endoglucanases so effective in producing protoplasts from *S. cerevisiae*.

The methods described using *Basidiomycete QM 806* β-(1→3) glucanase would be useful in a comparative study of the cell wall composition and protoplast formation in different yeasts, or in a study of the effect of growth conditions and age on cell wall composition and protoplast formation. In view of the purity of the protoplasts obtained with this enzyme, it should also prove useful for studies on the protoplasts themselves.

Résumé. L'exo-β-(1→3) glucanase, isolée du *Basidiomycete* QM 806 a été utilisée avec succès pour l'analyse des polysaccharides de la paroi cellulaire de la levure *Saccharomyces cerevisiae* et pour la formation de protoplastes à partir de *Saccharomyces cerevisiae* et *Wickerhamia fluorescens.*

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Inhibition of Mammalian Acetylcholinesterase by Phenylmethanesulfonyl Fluoride[1]

The sulfonyl fluorides are powerful irreversible inactivators of many esterases[2–6]. The mechanism of their action has been well established in the case of acetylcholinesterase (acetylcholine acetyl-hydrolase, E.C. 3.1.1.7), using methanesulfonyl fluoride as inhibitor[4]. The first step is the formation of an enzyme-inhibitor complex, followed by a nucleophilic attack on the sulfur atom by the basic group of the esteratic site. The final acceptor of