DEMONSTRATION OF A FIBRILLAR COMPONENT IN THE CELL WALL OF THE YEAST *SACCHAROMYCES CEREVISIAE* AND ITS CHEMICAL NATURE

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ABSTRACT

The ultrastructure of isolated cell walls of *Saccharomyces cerevisiae* from the log and stationary phases of growth was studied after treatment with the following enzymes: purified endo-β-(1 → 3)-glucanase and endo-β-(1 → 6)-glucanase produced by *Bacillus circulans;* purified exo-β-glucanase and endo-β-(1 → 3)-glucanase produced by *Schizosaccharomyces versatilis;* commercial Pronase. While exo-β-glucanase from *S. versatilis* had no electron microscopically detectable effect on the walls, Pronase removed part of the external amorphous wall material disclosing an amorphous wall layer in which fibrils were indistinctly visible. Amorphous wall material was completely removed by the effect of either endo-β-(1 → 3)- or endo-β-(1 → 6)-glucanase of *B. circulans* or by a mixture of the two enzymes. As a result of these treatments a continuous fibrillar component appeared, composed of densely interwoven microfibrils resisting further action by both of the *B. circulans* enzymes. The fibrillar wall component was also demonstrated in untreated cell walls by electron microscopy after negative staining. Because of the complete disappearance of the fibrils following treatment with the *S. versatilis* endo-β-(1 → 3)-glucanase it can be concluded that this fibrillar component is composed of β-(1 → 3)-linked glucan. Bud scars were the only wall structures resistant to the effect of the latter enzyme.

INTRODUCTION

It is now generally accepted that the cell wall of eucaryotic plants is built up of fibrillar and amorphous components (18). However, evidence has been reported against the presence of a fibrillar component in yeast cell walls. Bowden and Hodgson (4) failed to detect a fibrillar component in either native cell walls or in cell walls after treatment with various agents. Their findings are in accordance with the earlier work of Houwink and Kreger (9), who reported that native cell walls, in contrast to those boiled in hydrochloric acid, revealed only a diffuse X-ray diffraction diagram. However, the conspicuous fibrillar component which is synthesized in regenerating yeast proto-

plasts (19, 10) and observations of microfibrils occasionally made in yeast cell walls digested by snail enzymes (23), by phosphomannanase (16), or by Pronase (24) led us to the conclusion that yeast cell walls might well contain such a fibrillar component.

The rigidity and shape of yeast cell walls are ascribed to several glucan components containing both β-(1 → 3)- and β-(1 → 6)-linked glucose units (21). We selected four β-glucanases (see Materials and Methods), on the basis of their availability in highly purified form in the Davis laboratory, for treatment of purified cell walls of baker's yeast in the hope that selective hydrolyses would provide new insights into cell wall structure.

In the present paper the existence of a fibrillar wall component will be demonstrated in isolated cell walls of *Saccharomyces cerevisiae* (i) by treatment with endo-β-(1 → 3)- and with endo-β-(1 → 6)-glucanases from *Bacillus circulans* and (ii) by negative staining of the native cell walls. The chemical nature of the fibrillar wall component was inferred from its digestibility by an endo-β-(1 → 3)-glucanase from *Schizosaccharomyces versatilis*. The structure of the yeast cell wall is discussed on the basis of the results obtained.

MATERIALS AND METHODS

Isolation of the Cell Walls

Cell walls were prepared from commercial baker's yeast cells (Red Star Yeast Co., Oakland, Calif.) disrupted in a Bronwill cell homogenizer as described in detail by Fleet and Phaff (7).

Preparation of Bacterial Endo-Glucanases

B. circulans strain WL-12 was grown on 0.3% baker's yeast cell walls in a buffered synthetic medium at pH 6.7 (25). After 3 days of shaking on a rotary shaker (200 rpm) at room temperature the culture fluid was separated by centrifugation. After dialysis at 1°C against 5 mM sodium succinate buffer, pH 5.0, the solution was lyophilized. Purification of the endo-β-(1 → 3)- and endo-β-(1 → 6)-glucanases was carried out by a procedure different from one previously developed in our laboratory (25). Full details of the procedure will be published elsewhere (Fleet and Phaff[1]). In brief, the two endoglucanases were separated from each other and from accompanying undigested cell wall mannan by passing the enzyme solution over a column of Sephadex G-100. The endo-β-(1 → 3)-glucanase was further purified by chromatography over columns of DEAE-cellulose and hydroxyapatite. The endo-β-(1 → 6)-glucanase, after separation on Sephadex G-100, was further purified by chromatography over DEAE cellulose. The purified enzymes were specific for β-(1 → 3)-linked and β-(1 → 6)-linked glucans, respectively, and were free of amylase, mannanase, phosphomannanase, phosphatase, and protease activity.

[1] Fleet, G. H., and H. J. Phaff. 1974. *J. Bacteriol.* In press.

Preparation of Exo-β-Glucanase from Schizosaccharomyces Japonicus Var. Versatilis

This yeast (culture no. 60-255) was obtained from the yeast collection of the Department of Food Science and Technology, University of California, Davis. For convenience it will be referred to in the remainder of the text by the older name *Schizosaccharomyces versatilis*. The enzyme was prepared from the cell homogenate of this yeast according to the procedure of Fleet and Phaff (7). Its specific activity was 180 U/mg of protein. The enzyme successively removes single glucose units by hydrolysis from the nonreducing end of β-(1 → 3)-linked glucans. It also hydrolyzes β-(1 → 6)-linked glucans at one hundredth of the rate obtained with β-(1 → 3)-glucan.

Preparation of Endo-β-(1 → 3)-Glucanase from Cell Walls of S. Versatilis

This enzyme, together with exo-β-glucanase, is tightly bound to the cell wall of this yeast (7). The enzymes can be relased from the walls by autodigestion in sodium succinate buffer at pH 5.0 and we have obtained the endo-β-(1 → 3)-glucanase in an electrophoretically pure state (8). The sp act of the purified enzyme was 6.0 U/mg of protein. The enzyme is specific for β- (1 → 3)-glucans and it hydrolyzes these substrates in a random manner to laminaribiose and glucose.

ACTIVITY UNITS: All glucanase activity units reported are expressed in terms of μmoles of reducing groups (expressed as glucose) released per minute at 30°C from laminarin (for endo-β-(1 → 3)-glucanase and exo-β-glucanase) or from pustulan (for endo-β-(1 → 6)-glucanase) at their respective pH optima.

PRONASE TREATMENT: This proteolytic enzyme was a commercial preparation (Grade B, Calbiochem, San Diego, Calif.). Its concentration was 5 mg/ml of distilled water. The cell walls were suspended in the solution and incubated at 37°C for 24 hrs. The remaining wall material then was washed by centrifugation.

GLUCANASE TREATMENT: Weighed portions (5 mg/ml) of dried cell walls were suspended in distilled water by ultrasonic vibration for 1 min and then heated to 100°C for 5 min to inactivate wall-associated glucanases. The walls were recovered by centrifugation and resuspended in the desired enzyme solution (0.5 U/ml of buffer). Next, the mixtures were placed in stoppered test tubes after adding 0.01% sodium azide as an antimicrobial agent and they were incubated at 30°C on a slowly

revolving Rollordrum to keep the cell walls in suspension. Portions of the reaction mixture were removed for analysis of reducing sugars and total carbohydrate released from the walls by the particular glucanase treatment. Reducing sugars were determined after centrifugation of the residual wall material by the Nelson-Somogyi procedure (22). Glucose was used as the standard. Total carbohydrate was measured by the phenol sulfuric acid method (5).

ELECTRON MICROSCOPY: Enzyme-treated cell walls, suspended in distilled water, were placed on copper grids covered by Formvar supporting film (Belden Mfg. Co., Chicago, Ill.). After drying, the preparations were shadowed with carbon and platinum.

Negative staining of untreated cell walls was done with a 1% phosphotungstic acid solution at pH 7.1. The samples were examined under a Tesla electron microscope.

RESULTS

Cell walls of *S. cerevisiae* incubated in buffer and used as controls had a smooth external surface (Fig. 1) without microfibrils. However, the internal wall surface revealed fine, somewhat blurred microfibrils (Fig. 1). Houwink and Kreger (9) also mentioned the presence of such fibrils on the inner walls of *Candida tropicalis* and baker's yeast and they pointed out that these fibrils were all but concealed in an amorphous material. The internal wall surface of whole cells is smooth and has prominent rodlike invaginations into the cytoplasm as shown in freeze-etched preparations (17, 23). Presumably a part of this amorphous inner layer is removed during mechanical cell disruption, revealing the somewhat blurred microfibrils.

The amorphous material on the external surface of the stationary phase cell walls was progressively removed by treatment with endo-β-(1 $\rightarrow$ 3)-glucanase of *B. circulans* (Fig. 2) or by the action of endo-β-(1 $\rightarrow$ 6)-glucanase of *B. circulans* (Fig. 3) and by a mixture of both enzymes (Figs. 4, 5). Similar observations were made with cell walls from log phase cells. As Fig. 2 shows, digestion usually begins at the external margin of bud scars and proceeds in all directions; it also starts in the region of mechanical breakage of the walls. During the enzymatic reaction densely interwoven microfibrils begin to appear underneath the layer of amorphous material. Prolonged treatment of the cell walls with each enzyme alone or with their mixture ultimately resulted in almost complete removal of the amorphous surface material. The newly revealed continuous fibrillar component retained the shape of the original cell wall. Not only was external amorphous material removed by the effect of each enzyme or their mixture, but also the microfibrils on the internal wall surface became more clearly and completely disclosed (compare Fig. 1 and Fig. 4). Fig. 4 also shows that the arrangement of the microfibrils on both the external and internal surfaces of the fibrillar layer was similar.

Each enzyme alone or their mixture was able to remove completely the amorphous material from the bud scars. Underneath this amorphous material circularly oriented microfibrils were observed first (Figs. 2, 3), and later, after complete removal of all amorphous material, distinct microfibrils appeared which were similar in character to those in the rest of the cell wall (Figs. 3, 5). It is clear that bud scar rings and plugs, besides having a high chitin content (1), are also made up of microfibrils of the fibrillar wall component (Fig. 5), and that microfibrils of scar rings are not circularly oriented.

In contrast to the fibrillar component of regenerating yeast protoplasts formed in liquid media (10, 11), microfibrils of the fibrillar cell wall component are not arranged in bundles but as separate densely interwoven microfibrils without forming interfibrillar spaces. This arrangement is similar to that in yeast protoplasts regenerating their walls inside a gelatine medium (20).

The question might be raised whether the fibrillar component which becomes visible after treatment of the cell walls with endo-β-glucanases from *B. circulans* is actually present in the untreated cell walls or whether the fibrils originate in vitro after the partial digestion of nonfibrillar wall glucan. To answer this question, negative staining for electron microscopy was used to determine if the native cell walls would reveal the fibrillar component. Fig. 6 shows that after negative staining of untreated walls microfibrils are evident on the inner side of *S. cerevisiae* cell walls.

The hydrolysis of the cell walls also was followed by measuring the increase in reducing groups during treatment with these enzymes. The results are shown in Table I. It will be noted that β-(1 $\rightarrow$ 6)-glucanase was somewhat more effective than β-(1 $\rightarrow$ 3)-glucanase and that a combination of the two enzymes showed a synergistic effect. However, the total release of reducing groups was very limited, even though it resulted in release of significant amounts of total soluble carbohydrate and in a more or less complete removal of amorphous surface polysaccharide (Figs. 2, 3, 4).

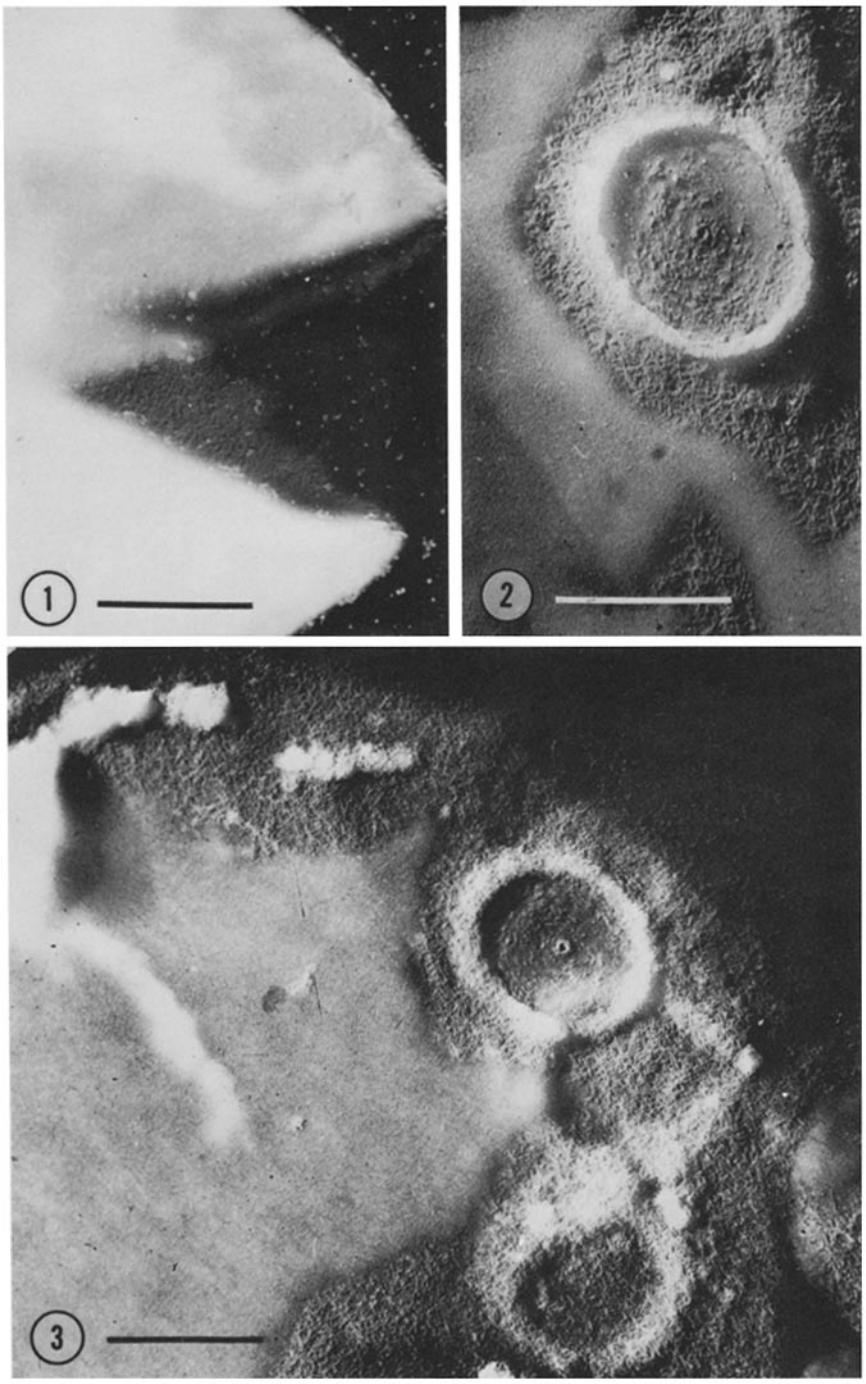
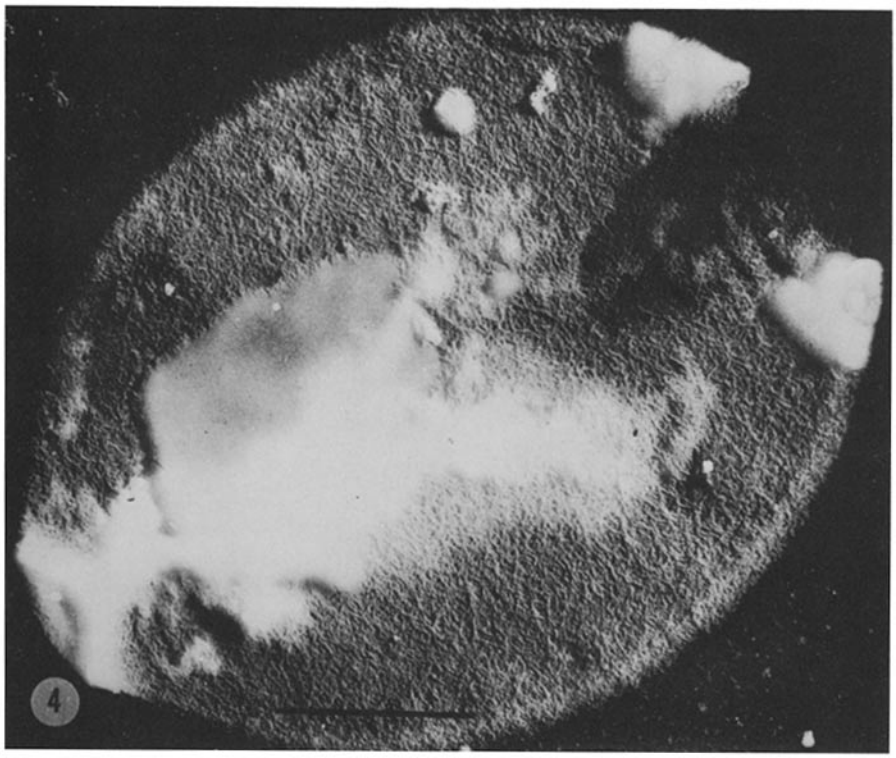


FIGURE 1 Cell wall of *S. cerevisiae* incubated in buffer and used as control. The external wall surface is smooth, but fine microfibrils are visible on the inner surface of the wall. Shadowed preparation. × 17,500.

FIGURE 2 Cell wall of *S. cerevisiae* treated for 48 h with endo-β-(1 → 3)-glucanase from *B. circulans*. Amorphous material of the outer wall surface is initially removed from the external margins of bud scars and from the region of mechanical breakage of the wall. Circularly oriented microfibrils are indistinctly visible in the bud scar plug. Shadowed preparation. × 25,000.

FIGURE 3 Cell wall of *S. cerevisiae* treated for 48 h with endo-β-(1 → 6)-glucanase of *B. circulans*. Amorphous material was partially removed from the external wall surface including the bud scars. Note the complete removal of the amorphous material from the lower scar and only a partial removal from the upper one, which shows circularly oriented microfibrils in the plug. Shadowed preparation. × 20,000.



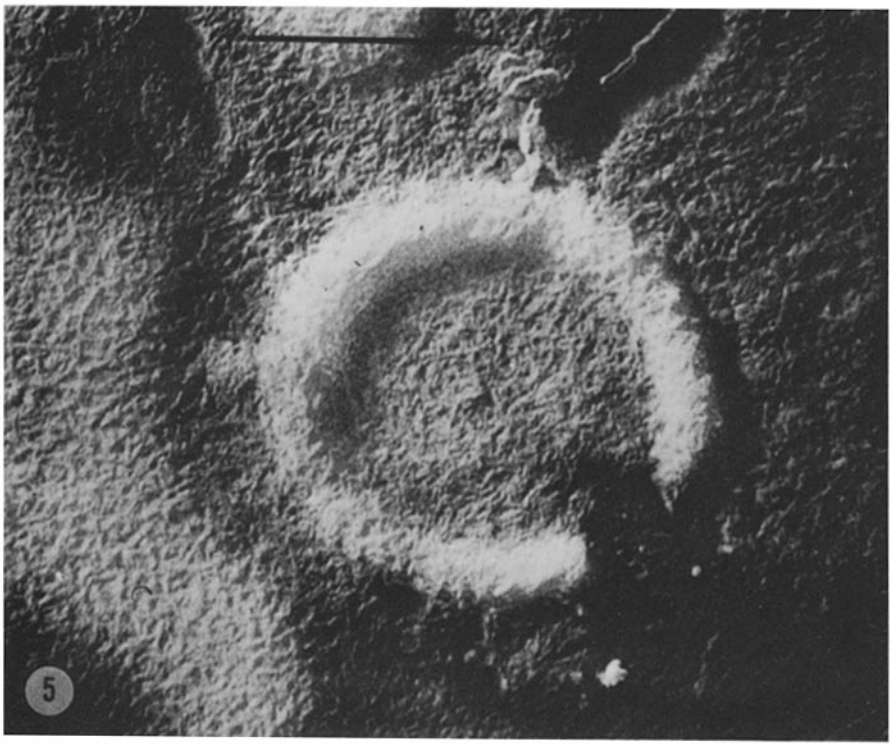


FIGURE 4 Cell wall of *S. cerevisiae* treated for 68 h with a mixture of endo-β-(1 → 3)- and endo-β-(1 → 6)-glucanases of *B. circulans*. Amorphous material of the external and internal wall surfaces is progressively (in this case partially) removed by the effect of the enzymes. Shadowed preparation. × 15,000.

FIGURE 5 Cell wall of *S. cerevisiae* treated for 92 h with the mixture of endo-β-(1 → 3)- and endo-β-(1 → 6)-glucanases of *B. circulans*. The fibrillar component present in the plug and ring of the bud scar has become visible; the fibrils have the same appearance as those in the cell wall. Shadowed preparation. × 35,000.

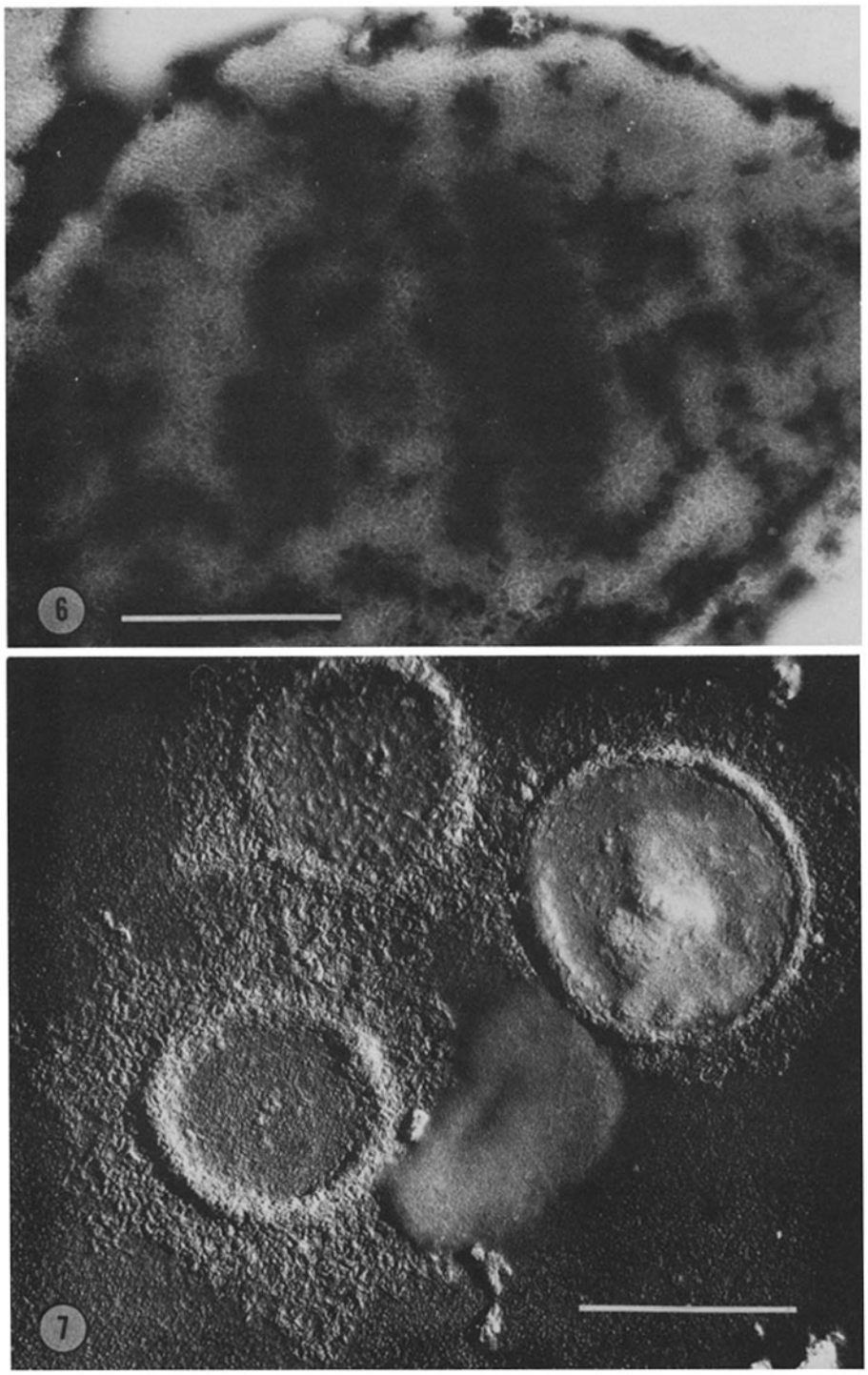


FIGURE 6 Cell wall of *S. cerevisiae*, not treated with enzymes. The walls were suspended in buffer and negatively stained by 1% phosphotungstic acid at pH 7.1. Fine microfibrils are visible on the inner wall surface. × 30,000.

FIGURE 7 Cell wall of *S. cerevisiae* treated for 48 h by endo-β-(1 → 3)-glucanase of *S. versatilis*. Bud scars remain from the digested walls. The fibrillarlike structures faintly visible on the periphery of the bud scars differ in morphology from the glucan fibrils of the walls. Shadowed preparation. × 30,000.

TABLE I

*Action of Purified Endo-β-(1 → 3)- and Endo-β-(1 → 6)-Glucanase from B. circulans WL-12 on Baker's Yeast Cell Walls**

Reaction mixtures	Time (hours)								
	0	1	4	7	17	23	30	45‡	36
Cell walls + β-(1 → 3)-glucanase	0	2	2	4	7	7	9	9 35§	4
Cell walls + β-(1 → 6)-glucanase	0	13	—	18	22	25	27	29 115§	9
Cell walls + β-(1 → 3)-and β-(1 → 6)-glucanase	0	20	27	33	40	45	51	61 175§	15
Cell walls pretreated with dithiothreitol + β-(1 → 3)-glucanase	0	—	2	—	—	7	—	11	—

* The values in the table represent reducing sugar release expressed as μg of glucose equivalents/ml. Wall substrates were used at a concentration of 2.5 mg/ml and the final concentration of each enzyme was 0.5 U/ml in 0.01 M sodium succinate buffer, pH 5.0. Sodium azide (0.01%) was added as an antimicrobial agent.

‡ At this time the walls were retrieved by centrifugation, washed in buffer and resuspended in fresh enzyme for further digestion.

§ Total carbohydrate (μg/ml) released from the walls.

Addition of a sulfhydryl compound did not stimulate action of the enzymes as is known to occur when snail digestive enzymes are used for this purpose (21).

The above experiments in which cell walls of *S. cerevisiae* were treated with purified preparations of endo-β-(1 → 3)- and endo-β-(1 → 6)-glucanase from *B. circulans* indicate that the fibrillar network existing in baker's yeast cell walls is resistant to hydrolysis by these two enzymes. Since we had available in our laboratory at Davis two additional glucanases in highly purified form, an exo-β-glucanase from cell homogenates of *S. versatilis* (7) and an endo-β-(1 → 3)-glucanase (8) from the cell walls of the same species of yeast (see Methods), cell walls of baker's yeast were incubated with these two enzymes. Table II shows the release of reducing groups as a result of these treatments. The effect of exo-β-glucanase was very limited and of the same order of magnitude as the bacterial enzymes. However, electron microscopically there was a difference in that exo-β-glucanase showed no detectable effect on cell walls of baker's yeast. Since with a high enzyme concentration (2 U/ml) the release of reducing groups came virtually to a halt after 24 h of incubation, we assume that this enzyme cannot penetrate the outer protective mannan layer of the walls and only acts to a limited extent on the glucan exposed at the fragmented periphery of the broken cells.

On the other hand, the endo-β-(1 → 3)-glucanase isolated from the cells walls of *S. versatilis* showed a very pronounced and continuing action on baker's yeast walls and was able to cause extensive digestion of the walls as judged by a visible decrease in turbidity of the suspension. Electron micrographs of cell walls treated with this enzyme showed complete digestion of the fibrillar wall component as well as amorphous glucan, leaving only fragments of cell walls as yet undigested (Fig. 8) or bud scars (Figs. 7, 9). Some of these bud scars resulting from the effect of the enzyme still showed microfibrils at their periphery (Fig. 9), although in others the microfibrils were completely digested by the enzyme. In those the remaining material revealed a different morphology of fibril-like structure (Fig. 7), probably consisting mainly of chitin (1).

Finally, since the outer layer of the cell wall is composed of a mannan-protein complex (21) susceptible to digestion by the broad-spectrum proteolytic enzyme Pronase (24, 27), cell walls were treated with this enzyme. Fig. 10 shows that this treatment, reported to hydrolyze and remove the mannan-protein complex, does not clearly reveal the fibrillar component as it is probably still embedded in a layer of amorphous glucan.

DISCUSSION

In the present paper we have demonstrated that a fibrillar component exists in the native wall of *S. cerevisiae*. Our conclusions are based on negative staining for electron microscopy together with

TABLE II

*Action of Purified Exo-β-Glucanase and of Endo-β-(1 → 3)-Glucanase from S. versatilis on Cell Walls of Baker's Yeast**

	Time (hours)							
	0	2	3	5	10	14	25	36
Baker's yeast walls control	0	0	0	0	0	0	0	0
Baker's yeast walls + 2 U/ml of exo-β-glucanase	0	17	17	19	24	33	44	44
Baker's yeast walls + 0.5 U/ml of endo-β-(1 → 3)-glucanase	0	120	145	165	188	209	247	280

* The values in the table represent reducing sugar released from the walls, expressed as μg of glucose per ml. The cell wall concentration was 5 mg/ml (dry weight basis) in 0.01 M sodium succinate buffer at pH 5.0. Sodium azide (0.01%) was used as an antimicrobial agent.

partial digestion of the cell walls with glucanases produced by *B. circulans*. These results contradict the conclusions of Bowden and Hodgson (4) about the nonexistence of a fibrillar component in yeast cell walls, whereas they are in general agreement with Lampen's concept (13) of the yeast cell wall structure.

It is now known that *Saccharomyces* cell walls contain at least three kinds of glucan (21, 14, 15). An alkali-soluble form of unknown structure is normally discarded during extraction and purification of the alkali-soluble mannan fraction. The alkali-insoluble glucan is composed of a major component (about 85%) consisting of a branched β-(1 → 3)-glucan of high molecular weight containing 3% of β-(1 → 6)--glucosidic interchain linkages (14). A minor component, which is tightly anchored to the major component, can be isolated by numerous extractions with hot 0.5 M acetic acid and has been shown to consist of a highly branched β-(1 → 6)-glucan with β-(1 → 3)-interchain and interresidue linkages (15). After extraction the latter polysaccharide is water soluble.

Complete degestion of the cell wall must therefore include hydrolysis of the insoluble β-(1 → 3)-glucan. During earlier work in the Davis laboratory (26) it was shown that the crude culture fluid of *B. circulans* grown on baker's yeast cell walls, as well as the β-(1 → 3)-glucanase (laminarinase) separated by DEAE-cellulose chromatography from the accompanying β-(1 → 6)-glucanase, were able to effect complete hydrolysis of *Saccharomyces* cell walls. In contrast, the very extensively purified β-(1 → 3)-glucanase (see Materials and Methods) used in the present study no longer had the ability to hydrolyze whole cell walls. This enzyme appears to hydrolyze only the amorphous glucan component which seems to be involved in connecting the outer mannan-protein layer to the underlying fibrillar component. This fortuitous circumstance enabled us to demonstrate the fibrillar layer embedded in the cell wall. We have recently shown (F. M. Rombouts and H. J. Phaff, unpublished results) that during the elaborate purification of endo-β-(1 → 3)-glucanase (laminarinase) from *B. circulans* a lytic factor, responsible for hydrolysis of alkali-insoluble glucan, is removed from the laminarinase, thus explaining the contradictory data from our early (26) and current experiments.

The digestion of the microfibrillar component by the highly purified endo-β-(1 → 3)-glucanase from the cell walls of *S. versatilis* shows that the glucan of the fibrillar component must be primarily made up of β-(1 → 3)-linked glucose units. This conclusion is in accordance with the recent structural studies by Manners et al. (14), and it is also in accordance with the nature of the fibrillar wall component synthesized by yeast protoplasts in liquid media (12).

The question arises why fibrils of β-(1 → 3)-glucan are resistant to hydrolysis by endo-β-(1 → 3)-glucanase from *B. circulans*. The same resistance was noted when the fibrillar component of yeast protoplasts regenerating in liquid media were treated with this glucanase (M. Kopecká and H. J. Phaff, unpublished data). The reasons for the differing hydrolytic activities of the yeast and bacterial endo-β-(1 → 3)-glucanases are not clear. Accessibility of the enzymes to the glucan fibrils within the cell wall does not seem to be a factor since the bacterial enzyme actually exposes the fibrils by removing amorphous glucan and mannan. The mol wt of the yeast endo-β-(1 → 3)-

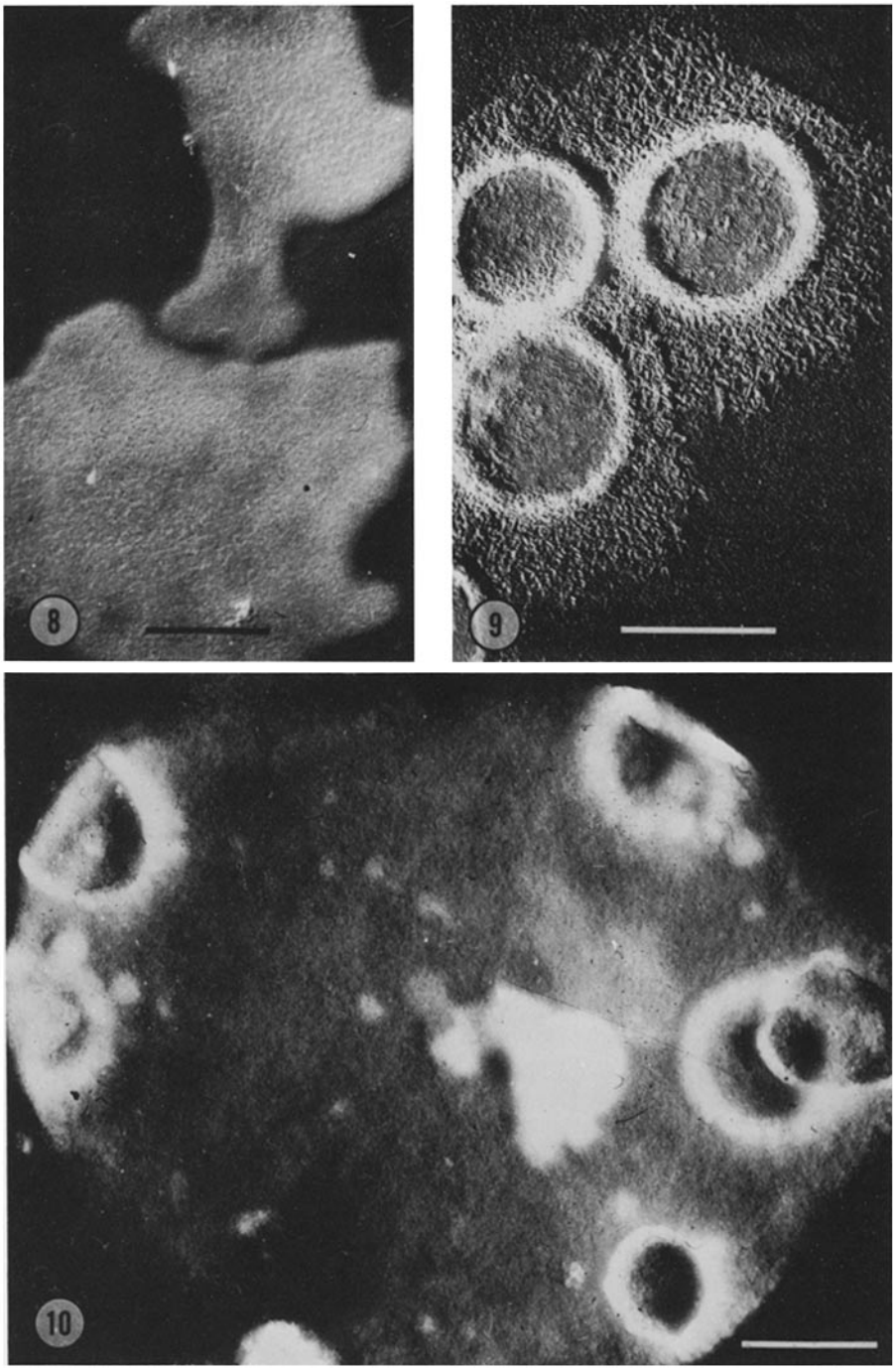


FIGURE 8 Remnants of partly digested walls treated by endo-β-(1 → 3)-glucanase of *S. versatilis* for 48 h. In this case as well as that of the bacterial enzymes (Fig. 4) the digestion is progressive from the edge. However, a major difference is that the yeast glucanase digests both the fibrillar and the amorphous glucan. Shadowed preparation. × 15,000.

FIGURE 9 Bud scars remaining from the walls of *S. cerevisiae* after the effect of endo-β-(1 → 3)-glucanase of *S. versatilis*. Fibrils still undigested are visible on the periphery of the bud scars. Shadowed preparation. × 20,000.

FIGURE 10 Cell wall of *S. cerevisiae* treated for 24 h by Pronase at 37°C. Mannan protein from the external wall surface was removed by the enzyme. Shadowed preparation. × 17,000.

glucanase is close to 100,000, and that of the bacterial enzyme (based on Sephadex gel chromatography) is considerably smaller (6). Thus the size of the enzyme molecule does not appear to be a factor.

One striking difference between the two endo-β-(1 → 3)-glucanases is that the yeast enzyme has two cooperating active sites per molecule whereas the bacterial glucanase appears to have only one such site (8). Based on its lower K_m value for laminarin, the yeast enzyme may have a much greater affinity for the fibrillar glucan of the cell wall than does the bacterial glucanase (6). It may be that these factors have in some way a decisive effect on the ability of the yeast endo-glucanase to hydrolyze glucan molecules which have arranged themselves through inter- and intramolecular forces (e.g., by hydrogen bonding) into glucan microfibrils. The highly crystalline nature of the glucan fibrillar network in regenerating protoplasts has been demonstrated (12).

Only one other enzyme of known purity has been shown to have the ability to hydrolyze the insoluble glucan of baker's yeast walls, viz. an exo-β-(1 → 3)-glucanase produced by Basidiomycete QM806 (2). Since the exo-β-(1 → 3)-glucanase from *S. versatilis* had no effect on the cell walls the detailed specificity and affinity of such β-glucanases for their substrates appears highly variable in spite of a superficial similarity in action pattern. One difference in hydrolytic behavior between the exo-β-glucanase from *Basidiomycete* QM806 and the endo-β-glucanase from *S. versatilis* (cf. Figs. 7 and 9) is that the former apparently does not remove the mannan layer from the bud scars (3).

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