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Lysis of Yeast Cell Walls: Glucanases from *Bacillus circulans* WL-12

GRAHAM H. FLEET[1] AND HERMAN J. PHAFF
Department of Food Science and Technology, University of California, Davis, California 95616

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Endo-β-(1 → 3)- and endo-β-(1 → 6)-glucanases are produced in high concentration in the culture fluid of *Bacillus circulans* WL-12 when grown in a mineral medium with bakers' yeast cell walls as the sole carbon source. Much lower enzyme levels were found when laminarin, pustulan, or mannitol was the substrate. The two enzyme activities were well separated during Sephadex G-100 chromatography. The endo-β-(1 → 3)-glucanase was further purified by diethylaminoethyl-cellulose and hydroxyapatite chromatography, whereas the endo-β-(1 → 6)-glucanase could be purified further by diethylamino-ethyl-cellulose and carboxymethyl cellulose chromatography. The endo-β-(1 → 3)-glucanase was specific for the β-(1 → 3)-glucosidic bond, but it did not hydrolyze laminaribiose; laminaritriose was split very slowly. β-(1 → 4)-Bonds in oat glucan in which the glucosyl moiety is substituted in the 3-position were also cleaved. The kinetics of laminarin hydrolysis (optimum pH 5.0) were complex but appeared to follow Michaelis-Menten theory, especially at the lower substrate concentrations. Glucono-δ-lactone was a noncompetitive inhibitor and Hg^{2+} inhibited strongly. The enzyme has no metal ion requirements or essential sulfhydryl groups. The purified β-(1 → 6)-glucanase has an optimum pH of 5.5, and its properties were studied in less detail. In contrast to the crude culture fluid, the two purified β-glucanases have only a very limited hydrolytic action on cell wall of either bakers' yeast or of *Schizosaccharomyces pombe*. Although our previous work had assumed that the two glucanases studied here are responsible for cell wall lysis, it now appears that the culture fluid contains in addition a specific lytic enzyme which is eliminated during the extensive purification process.

Polysaccharides of the glucan type constitute major structural components of the yeast cell wall. In particular, the rigidity and shape of the wall have been attributed to the presence of a predominantly β-(1 → 3)-linked glucan (35). Yeast wall glucans possessing β-(1 → 6)- and α-(1 → 3)-linkages are also known to occur in certain species (7, 27).

Enzymes of specific hydrolytic action represent valuable tools for the compositional and structural analyses of yeast cell walls, and over the past 10 years numerous sources of such enzymes have been described. (For reviews, see Phaff [35] and Villanueva and Acha [47]). In most cases, however, the major objective of the researchers was to apply the crude glucanase solutions to cell suspensions in order to prepare protoplasts rather than for the analysis of cell wall structure.

In the fine structural analyses of polysaccharides with the aid of specific and highly purified glucanases, it is of foremost importance that the substrate specificities and general properties of the purified enzymes employed be well understood (33, 36).

Tanaka and Phaff (43) reported earlier on the extracellular production of an endo-β-(1 → 3)- and an endo-β-(1 → 6)-glucanase by *Bacillus circulans* WL-12 when this bacterium was grown on bakers' yeast cell walls as the carbon substrate. They developed a method for the separation and partial purification of these two enzymes. Each enzyme was specific for its respective substrate (laminarin or pustulan), and both enzyme preparations were capable of hydrolyzing isolated bakers' yeast cell walls. The endohydrolases were subsequently used in

[1] Present address: Heriot-Watt University, Department of Brewing and Biological Sciences, Edinburgh EH1 1HX, Scotland.

screening the qualitative composition of cell walls from various yeast species (44).

The present paper describes an improved method for the separation and more thorough purification of these two glucanases from *B. circulans* WL-12, including a study of their properties. We consider the enzymes sufficiently pure for their use in the fine structural analysis of yeast cell wall polysaccharides. The hydrolytic actions of these enzymes on isolated yeast cell walls, which were found to be quite different from those previously observed (44), are also reported.

MATERIALS AND METHODS

Microorganisms and culture conditions. *B. circulans* WL-12 (43) and *Schizosaccharomyces pombe* C-277 were obtained from the culture collection of the Department of Food Science and Technology, University of California, Davis, Calif. Compressed commercial bakers' yeast (Red Star Yeast Co., Oakland, Calif.) was purchased in 1-lb (about 454-g) blocks at a local market.

B. circulans WL-12 was maintained on agar slants of yeast-nitrogen base (Difco) containing 0.1 M sodium phosphate buffer, pH 6.5, 0.5% dried bakers' yeast cell walls, and 0.1% glucose. For the production and purification of β-glucanases, *B. circulans* WL-12 was grown as liquid culture in yeast-nitrogen base containing 0.1 M sodium phosphate buffer, pH 6.5, and 0.4% bakers' yeast cell walls. The medium was dispensed in 1-liter volumes in Fernbach flasks. Flasks were inoculated with a 24-h starter culture (5% inoculum) grown in the same medium and then incubated for 3 days at 30 C on a rotary shaker (approximately 300 rpm).

Preparation and hydrolysis of yeast cell walls. Cultures of *S. pombe* were grown in 0.5% yeast autolysate-5% glucose medium, harvested, and prepared for cell breakage as described previously (16). Cell walls of *S. pombe* and bakers' yeast were prepared by mechanical disruption in a Braun (Bronwill Scientific) homogenizer and extensively washed by centrifugation (16). The purified walls were lyophilized and stored under a vacuum at 1 C until required.

For enzymatic hydrolysis, weighed portions of the lyophilized walls were uniformly suspended in distilled water by ultrasonic vibration for 1 min and then heated to 100 C for 5 min to inactivate endogenous glucanases (17). The walls were then centrifuged, resuspended in the desired enzyme solution, and incubated at 30 C with mild rotation on a Rollordrum. Samples were withdrawn for analysis as a function of time. The walls were sedimented by centrifugation, and the supernatant solutions were used for the determination of released reducing group equivalents (41) and total carbohydrate (15). Microbial contamination was controlled by the addition of sodium azide (0.01%) to the suspensions.

Enzymatic wall hydrolysis was qualitatively monitored by observing clearing or lysis in an agar plate containing suspended cell walls (43). Enzyme solutions (0.1 ml; 1 U/ml) were placed in wells in the agar, and clearing was recorded after 1 to 3 days.

Enzyme assays. In standard assays for glucanase activity, 0.5 ml of suitably diluted enzyme was incubated with 0.5 ml of glucan substrate (10 mg/ml) in 0.05 M sodium succinate buffer, pH 5.0, at 30 C for 15 min. Reactions, which showed linear rates with time, were stopped by the addition of 0.35 ml of 1.0 M sodium carbonate, and this was followed by the addition of 1.0 ml of distilled water. Samples (1-ml) (or dilutions if necessary) of the inactivated reaction mixtures were used for the determination of reducing sugars (41). One unit of glucanase is defined as that amount of enzyme which releases 1 μmol of reducing sugar equivalent, expressed as glucose, per min under the standard assay conditions.

Proteolytic (10) and phosphatase activities (4) were determined using Azocoll (Calbiochem) and *p*-nitrophenylphosphate (Calbiochem), respectively, as substrates. The substrates (10 mg/ml) were prepared in 0.05 M sodium succinate buffer, pH 5.0. Phosphomannanase activity was estimated as described by McLellan et al. (29).

Substrates. Laminarin (Nutritional Biochemical Co., Cleveland, Ohio) was routinely used as the substrate for β-(1 → 3)-glucanase assays. Pachyman, another primarily β-(1 → 3)-linked glucan (20), was isolated from the sclerotia of the fungus *Poria cocos* Wolf (42). Pustulan, previously prepared by Abd-El-Al and Phaff (1), was used for the estimation of β-(1 → 6)-glucanase activity. Pseudonigeran was used in the determination of α-(1 → 3)-glucanase activity; it was prepared from the mycelium of *Aspergillus niger* NRRL 326 by a modification of the procedures described by Johnston (22) and Hasegawa et al. (18). Detailed information on the properties of the above substrates has been described elsewhere (17).

Laminaribiose, laminaritriose, laminaritetraose, oat glucan, cellulose dextrins, bakers' yeast mannan, and phosphomannan (*Hansenula holstii*) were obtained from the laboratory collection (1, 2). A laminarin sample modified at both ends of the molecule was prepared by periodate oxidation (19). All other substrates and chemicals were obtained commercially.

Analytical measurements. Reducing sugars were determined by the Nelson-Somogyi method (41). Glucose was used for the construction of a standard curve. Protein was estimated by the method of Lowry et al. (24), with bovine serum albumin as standard. Total carbohydrate was determined by the phenol-sulfuric acid method (15).

Chromatography. Descending paper chromatography was done on Whatman no. 1 paper at 25 C with ethylacetate-pyridine-water (12:5:4 by volume). Sugar spots on chromatograms were detected by the alkaline silver nitrate reagent (46).

Column chromatography was done according to the following procedures. Diethylaminoethyl (DEAE)-cellulose was obtained from Eastman Organic Chemicals, pretreated, and packed into a column according to the recommendations of Peterson and Sober (34). Carboxymethyl cellulose (CM 52) was purchased from Whatman and prepared as described by this com-

pany. Gel filtration was performed with Sephadex G-100 according to the specifications of Pharmacia. Hydroxyapatite was a laboratory preparation, prepared according to the method of Tiselius et al. (45) and packed into columns as described by Bernardi (8). Protein in fractions eluted from the columns was estimated by using the Folin reagent (24).

RESULTS

Induction of the glucanases. Although β-(1 → 3)- and β-(1 → 6)-glucanases are produced when *B. circulans* WL-12 is grown on bakers' yeast cell walls (43), this substrate is not commercially or readily available in the quantities required for large-scale enzyme production. Some other, more available growth substrates (dissolved in yeast-nitrogen base medium) were therefore tested for their abilities to induce glucanase production (Table 1). The enzyme levels formed by growing the bacillus on yeast cell walls are included for comparison. Glucose or galactose induced negligible enzyme levels. When grown with laminarin or pustulan as the carbon source (0.1% or 0.2%), both β-(1 → 3)- and β-(1 → 6)-glucanases were induced. However, enzyme levels were much lower than those obtained when yeast cell walls served as the growth substrate. Interestingly, growth on laminarin induced β-(1 → 6)-glucanase activity as well as β-(1 → 3)-glucanase, a characteristic also noted when *Cytophaga johnsonii* was grown on laminarin (5). When mannitol served as carbon source, levels of both of the glucanases were of the same order of magnitude as those produced with laminarin or pustulan. *B. circulans* WL-12 did not grow on the following substrates: glycerol, L-arabinose, glucitol, and galactitol. Much higher levels of both glucanases were always obtained in a noncomplex medium (Table 1).

Since mannitol represented a potentially economical substrate for enzyme production, growth on this source was investigated further with the objective of increasing enzyme yields. Increasing the mannitol concentration in the medium from 0.1 to 0.5% gave approximately 1.5- and 3-fold increases in the levels of β-(1 → 3)- and β-(1 → 6)-glucanase, respectively. Cultures incubated at 30 C produced much higher levels of the glucanases than those grown at 20 or 37 C. Maximal levels of both enzymes in mannitol cultures were reached after 3 to 4 days of growth, whereafter decreases were noted. Under optimal growth conditions with mannitol as the substrate, however, glucanase levels were not nearly as high as those observed when bakers' yeast cell walls were the carbon source. Attempts to purify the glucanases formed during growth of *B. circulans* WL-12 on mannitol according to the procedure previously reported by Tanaka and Phaff (43) were not successful. On DEAE-cellulose chromatography, the culture fluid separated into two forms of endo-β-(1 → 3)-glucanase and into two forms of endo-β-(1 → 6)-glucanase. It was therefore considered more efficient for the present study to produce the glucanases by growing *B. circulans* WL-12 with yeast cell walls as the substrate.

TABLE 1. *Induction of endo-β-glucanases in Bacillus circulans WL-12 by various growth substrates*[a]

Growth substrate	β-(1 → 3)-Glucanase[b]	β-(→6)-Glucanase[b]
Glucose	0.002	0.000
Galactose	0.005	0.005
Mannitol	0.014	0.033
Laminarin	0.019	0.040
Pustulan	0.009	0.047
Bakers' yeast cell walls[c]	0.330	0.730
Bakers' yeast cell walls[c, d]	0.180	0.140

[a] Cultures were grown in 100 ml of yeast-nitrogen base medium containing 0.1 M sodium phosphate buffer, pH 6.5, and 0.1% (wt/vol) of the carbon substrate. The medium was dispensed in 1-liter conical flasks. The cultures were harvested after incubation for 3 days at 30 C on a shaker. The culture supernatants were dialyzed against 0.01 M sodium succinate buffer, pH 5.0, concentrated to 20 ml by rotary vacuum evaporation at 30 C and then used in enzyme assays. No losses in activities were encountered during concentration.

[b] Expressed as units per milliliter of concentrated culture fluid.

[c] Growth substrate concentration 0.25% (wt/vol).

[d] Yeast autolysate (0.1% wt/vol) was used in place of yeast-nitrogen base.

Separation of the endo-β-(1 → 3)- and endo-β-(1 → 6)-glucanases. A 10-liter culture of *B. circulans* WL-12 was grown as described in Materials and Methods, with 0.4% bakers' yeast cell walls as substrate. After 3 days of incubation at 30 C, the cells were separated from the culture by centrifugation and the supernatant fluid was used as the source of crude enzyme.

The enzyme solution was placed in dialysis sacs (500 ml per sac) and dialyzed for 48 h against 25 liters of 5 mM sodium succinate buffer, pH 5.0. The dialyzing buffer was changed every 12 h. The enzyme solution was then dispensed in a shallow layer (0.5 to 1 inch [about 1.3 to 2.5 cm] in depth) in glass or enamel trays, frozen, and lyophilized for 36 h in a commercial model Stokes freeze-dryer. The dried material was redissolved in approximately 100 ml of distilled water, dialyzed against 0.05 M sodium succinate buffer, pH 5.0, and cen-

trifuged at 10,000 × *g* to remove insoluble material. Both β-(1 → 3)- and β-(1 → 6)-glucanase activities remained in the supernatant. Resuspension of the lyophilized material in a smaller volume gave a solution too viscous for Sephadex G-100 chromatography. The viscosity was attributed to the undigested yeast cell wall mannan present in the culture fluid—an observation also made by Tanaka and Phaff (43).

A portion (56 ml) of the concentrated enzyme solution was applied to a Sephadex G-100 column equilibrated with 0.05 M sodium succinate buffer, pH 5.0. Elution was performed with the same buffer, and a typical elution pattern is shown in Fig. 1. The β-(1 → 3)- and β-(1 → 6)-glucanase activities were completely separated by this procedure. All of the active β-(1 → 3)-glucanase fractions were combined and dialyzed against 0.01 M sodium succinate buffer, pH 6.0. Fractions 350 to 480 representing β-(1 → 6)-glucanase activity were also pooled and dialyzed against 0.01 M sodium succinate buffer, pH 6.0.

Further purification of the β-(1 → 3)-glucanase. The enzyme from the Sephadex column was loaded onto a column of DEAE-cellulose (15 by 1.5 cm) equilibrated with 0.01 M sodium succinate buffer, pH 6.0. After the column was thoroughly washed with the same buffer, elution was carried out in two steps: (i) 200 ml of 0.01 M sodium succinate buffer, pH 6.0, containing 0.1 M NaCl; (ii) a linear gradient to 0.75 M NaCl (375 ml of 0.01 M sodium succinate buffer containing 0.1 M NaCl, pH 6.0, in the mixing chamber of the gradient apparatus and 375 ml of 0.01 M sodium succinate buffer containing 0.75 M NaCl, pH 6.0, in the reservoir). The β-(1 → 3)-glucanase activity eluted in a peak at 0.27 M NaCl. All of the active fractions were combined, dialyzed thoroughly against 5 mM sodium phosphate buffer, pH 6.5, and then loaded onto a column of hydroxyapatite equilibrated with the same buffer. Elution was done as shown in Fig. 2. The β-(1 → 3)-glucanase activity eluted as a sharp peak of protein. Fractions 65 to 110 were combined and dialyzed against 0.01 M sodium succinate buffer, pH 5.0. Enzyme of this purity was used in the study of its properties and action on yeast cell walls. Table 2 presents a summary of the steps involved in the purification.

Further purification of the β-(1 → 6)-glucanase. Chromatography of the crude enzyme solution on Sephadex G-100 (Fig. 1) gave an approximately 30-fold purification of β-(1 → 6)-glucanase activity. This enzyme fraction was next applied to a column of DEAE-cellulose equilibrated with 0.01 M sodium succinate buffer, pH 6.0. Under these conditions, β-(1 → 6)-glucanase did not adsorb to the column. However, additional protein and a contaminant giving the enzyme preparation a brownish color bound to the column and thus were removed.

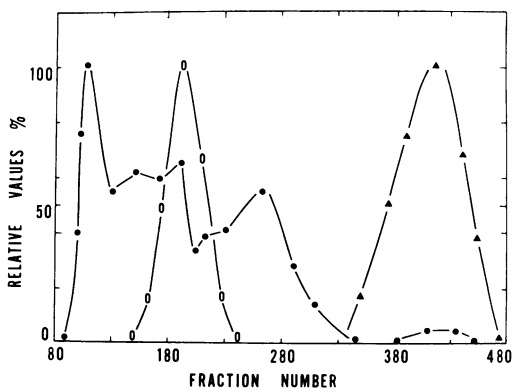

FIG. 1. *Sephadex G-100 chromatography of the β-(1 → 3)- and β-(1 → 6)-glucanases of Bacillus circulans WL-12. A 56-ml sample of the concentrated culture fluid (6.47 glucanase U/ml) was applied to a column (80 by 5 cm) of Sephadex G-100 equilibrated with 0.05 M sodium succinate buffer, pH 5.0. Elution was done with this same buffer at a flow rate of 1.0 ml/min, and fraction volumes of 5.2 ml were collected. Protein, ●; β-(1 → 3)-glucanase activity, O; β-(1 → 6)-glucanase activity, ▲.*

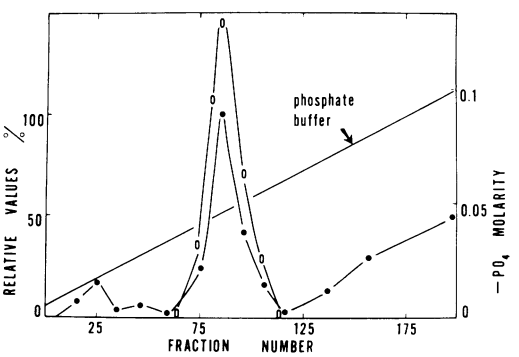

FIG. 2. *Hydroxyapatite chromatography of the β-(1 → 3)-glucanase from B. circulans WL-12. After DEAE-cellulose chromatography, the enzyme solution (450 ml with an activity of 1.21 U/ml) was applied to a column (15 by 1.5 cm) of hydroxyapatite equilibrated with 5 mM sodium phosphate buffer, pH 6.5. Elution was done with a linear gradient to 0.1 M sodium phosphate buffer, pH 6.5 (300 ml of 0.005 M sodium phosphate buffer in the mixing chamber; 300 ml of 0.1 M sodium phosphate buffer in the reservoir). Fraction volumes of 3.0 ml each were collected every 10 min. Protein, ●; β-(1 → 3)-glucanase activity, O.*

TABLE 2. *Summary of the steps involved in the purification of the extracellular β-(1 → 3)-glucanase from Bacillus circulans WL-12*

Purification step	Volume (ml)	Glucanase activity (U/ml)	Protein (mg/ml)	Sp act (U/mg of protein)	Purification (fold)	Yield (%)
Dialyzed culture supernatant	11,000	0.086	0.115	0.748		100
Lyophilization and redissolved	112.5	6.47	8.80	0.735		77
Sephadex G-100 chromatography	857.0[a]	0.70	0.360	1.94	2.6	63
DEAE-cellulose chromatography	450.0	1.21	0.175	6.91	9.2	57
Hydroxyapatite chromatography	396[a]	1.10	0.025	44.0	58.8	46

[a] Combined eluates from two column applications.

After thorough washing of the column with the same buffer, the enzyme solution was concentrated by rotary vacuum evaporation at 30 C. No loss in activity was encountered during concentration. β-(1 → 6)-glucanase of this purity was used in further studies of some of its properties and action on yeast cell walls. Table 3 gives a summary of the procedure for purifying the β-(1 → 6)-glucanase. It was later noted that the enzyme adsorbed on carboxymethyl cellulose equilibrated with 0.01 M sodium succinate buffer, pH 5.0, and could be further purified, if needed, by this procedure. For the present purpose, the enzyme was sufficiently pure after DEAE-cellulose chromatography and was not subjected to further purification. The β-(1 → 6)-glucanase did not adsorb to hydroxyapatite equilibrated with 5 mM sodium phosphate buffer, pH 6.5.

Properties of the β-(1 → 3)-glucanase: substrate specificity and action pattern. The enzyme specifically hydrolyzed molecules containing β-(1 → 3)-glucosidic linkages (Table 4). However, laminaribiose was not hydrolyzed and laminaritriose was only weakly cleaved. Proteolytic and phosphatase activities were absent from the purified preparation. In contrast, a partially purified preparation of the glucanase (the enzyme solution after DEAE-cellulose chromatography but before hydroxyapatite chromatography) was able to cleave the following substrates: cellulose dextrins, *p*-nitrophenyl-β-D-glucoside, Azocoll, and *p*-nitrophenylphosphate. All of these contaminating activities were removed by hydroxyapatite chromatography. The enzyme was not tested for its ability to hydrolyze chitin. Previous studies (43), however, have indicated that chitinase is not present in the crude culture fluid of *B. circulans* WL-12 when bakers' yeast cell walls are used as growth substrate.

Laminarin was hydrolyzed by random cleavage of the molecule (endo-mechanism) as determined by paper chromatography, giving a series of oligosaccharide products. Hydrolysis of a 2% laminarin solution with 1 U of enzyme per ml for 12 h yielded predominantly glucose, laminaribiose, and laminaritriose. Traces of the higher oligosaccharides were apparent along with gentiobiose. The appearance of the last product is consistent with the presence of a small percentage of β-(1 → 6)-glucosidic linkages in laminarin (9). When pachyman served as substrate under the above conditions, glucose, laminaribiose, and laminaritriose were again the major products, but no higher oligosaccharides were noted except for traces of a compound corresponding to laminarihexaose. Gentiobiose was absent.

Storage and stability. The endo-β-(1 → 3)-glucanase was stable in 0.01 M sodium succinate buffer, pH 5.0, for at least 3 months at 1 to 4 C or in the frozen state. Freeze-thawing twice did not cause any activity loss, but when this was done a third time, a 10% activity loss was noted. The enzyme was insensitive to sodium azide (0.01%) or merthiolate (0.01%). However, at concentrations slightly higher than 0.01%, merthiolate interferes with reducing sugar determinations.

Kinetics. Laminarin hydrolysis rate was linear for the first 40 min (0.1 U/ml of reaction mixture), after which a significant decrease was noted. Glucanase activity exhibited an optimum at pH 5.0, but the decrease in activity on the alkaline side of this value was not sharp. At pH 3.0 and 8.0, approximately 5 and 50% of maximal activity were exhibited, respectively.

The enzyme was saturated with substrate at a laminarin concentration between 2 and 3 mg/ml (Fig. 3). A K_m value of approximately 0.55 mg/ml was estimated from the figure. Calculation of the S_{90}/S_{10} ratio gave a value of around 60, suggesting an adherance to Michaelis-Menten kinetics (49). It should be stated that the S_{90}/S_{10} ratio (1.75/0.03) was obtained from a larger and more detailed graph, since the S_{10}

TABLE 3. *Summary of the steps involved in the purification of the extracellular β-(1 → 6)-glucanase from Bacillus circulans WL-12*

Purification step	Volume (ml)	Glucanase activity (U/ml)	Protein (mg/ml)	Sp act (U/mg of protein)	Purification (fold)	Yield (%)
Dialyzed culture supernatant	11,000	0.382	0.115	3.32		100
Lyophilization	112.5	34.10	8.80	3.87	1.16	91
Sephadex G-100 chromatography	2,000[a]	1.69	0.017	96.5	29.1	80
DEAE-cellulose chromatography	2,000	1.50	0.011	136.3	41.0	72
Concentration by vacuum evaporation	430	6.82	0.052	131.2	39.5	70

[a] Combined eluates from two Sephadex column applications.

TABLE 4. *Substrate specificity of the purified endo-β-(1 → 3)-glucanase from Bacillus circulans WL-12*[a]

Substrate	Main linkage type	Hydrolysis
Laminarin	β-(a → 3)	+
Laminaribiose[b]	β-(1 → 3)	–
Laminaritriose[b]	β-(1 → 3)	Weak
Periodate-oxidized laminarin	β-(1 → 3)	+
Pachyman	β-(1 → 3)	+
Oat glucan	β-(1 → 3); β-(1 → 4)	+
Pustulan	β-(1 → 6)	–
Gentiobiose	β-(1 → 6)	–
Pseudonigeran	α-(1 → 3)	–
Cellulose dextrins	β-(1 → 4)	–
Starch	α-(1 → 4)	–
Yeast mannan	α-(1 → 6); α-(1 → 2); α-(1 → 3) (mannose)	–
Phosphomannan (*Hansenula holstii*)	α-(1 → 2); α-(1 → 3) (mannose)	–
Methyl-β-D-glucoside		–
Phenyl-β-D-glucoside		–
p-Nitrophenyl-β-D-glucoside		–
Azocoll[c]		–
p-Nitrophenyl phosphate[c]		–

[a] Substrates (5 mg/ml, pH 5.0) were incubated at 30 C for 10 h in the presence of the enzyme (1.5 U/ml of reaction mixture), after which time samples were removed for the measurement of increase in sugar-reducing equivalents.

[b] Determined by paper chromatography only.

[c] See Materials and Methods for protease and phosphatase assays.

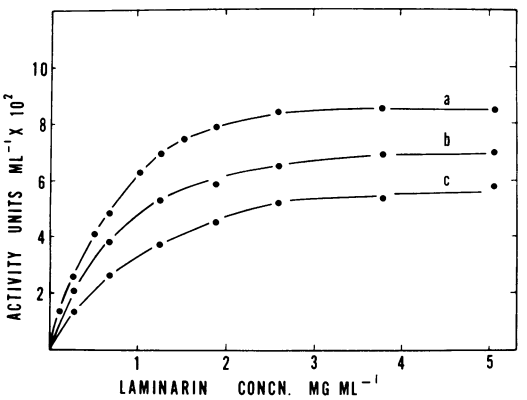

FIG. 3. *Effect of substrate concentration on the activity of the endo-β-(1 → 3)-glucanase from B. circulans WL-12. Initial reaction velocities were followed as a function of laminarin concentration in the absence and presence of the inhibitor, glucono-δ-lactone. (a) glucono-δ-lactone absent; (b) glucono-δ-lactone, 10 mM; (c) glucono-δ-lactone, 25 mM.*

value is especially difficult to determine from the curve as presented here. If the values of Fig. 3a were plotted according to the methods of Lineweaver and Burk or Hofstee, straight lines were obtained giving K_m values of around 0.9 mg/ml. If the values of Fig. 3a were plotted according to the Hill equation (49), the data shown in Fig. 4 were obtained. For laminarin concentrations up to 1 mg/ml, a straight line with a slope value of 1.2 (approximately) was recorded. For the higher laminarin concentrations, the slope of the line increased to a value near 2.5. This unusual behavior was reproducible. The substrate concentration yielding 50% of V_{max}, i.e., log $V/(V_{max} - V) = 0$, calculated

from Fig. 4, was found to be 0.52 mg/ml. This is consistent with the value obtained from Fig. 3. A slope value near 1 for Fig. 4 is characteristic of enzymes following Michaelis-Menten kinetics (49). However, it seems that at the higher substrate concentrations, deviations from this type of kinetics occur.

Activation and inhibition. Glucono-δ-lactone, a known inhibitor of glycosidase-glucanase type of reactions (38), inhibited β-(1 → 3)-glucanase activity. Figures 3 and 5 show the degree of inhibition with increasing concentrations of inhibitor. The shape of the curve in Fig. 5 is characteristic of enzymes following Michaelis-Menten kinetics (49). Identical curves and similar percentages of inhibition were obtained regardless of the substrate concentration used, indicating that the nature of the inhibition was noncompetitive (cf. Fig. 3).

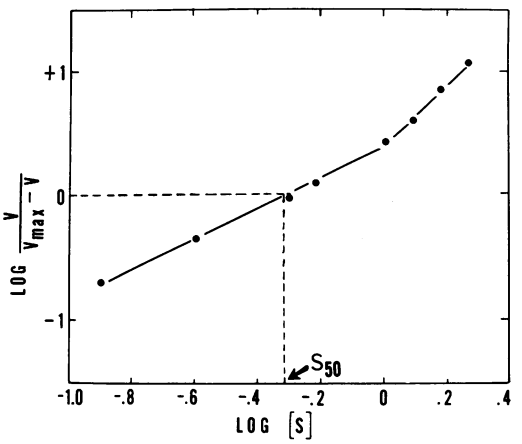

FIG. 4. *Plot of log $V/(V_{max} - V)$ versus log [S] according to the Hill equation (49) for the β-(1 → 3)-glucanase from B. circulans WL-12. The values for V, S, and V_{max} were taken from Fig. 3a.*

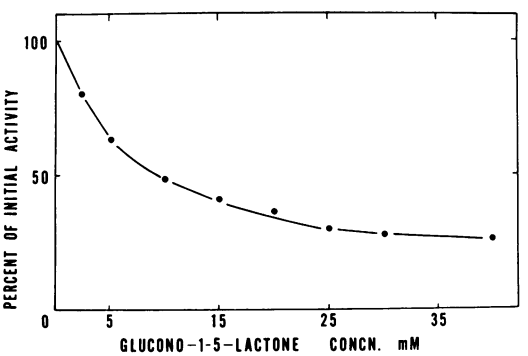

FIG. 5. *Inhibition of the endo-β-(1 → 3)-glucanase from B. circulans WL-12 by various concentrations of glucono-δ-lactone. Laminarin was used at a final concentration of 2.5 mg/ml in the assays.*

TABLE 5. *Effect of various agents on the activity of endo-β-(1 → 3)-glucanase from Bacillus circulans WL-12*[a]

Effector	Concn (M)			
	10^{-1}	10^{-2}	10^{-3}	10^{-5}
Co^{2+}	100	99	100	98
Ca^{2+}	100	102	100	99
Mg^{2+}	98	99	99	100
Zn^{2+}	88	85	100	95
Hg^{2+}	[b]	[b]	47	96
K^{+}	98	100	99	100
Ethylenediaminetetraacetic acid	[b]	96	100	98
Iodoacetamide	[b]	86	98	100

[a] Values represent activity relative to that of a control taken as 100%. The control activity was that measured in the absence of any effector. The cations were added as the chloride salt. Where necessary, corrections have been made for the effect of the agents on the reducing sugar determination.

[b] Extensive interference with reducing sugar determination.

TABLE 6. *Substrate specificity of the purified endo-β-(1 → 6)-glucanase from Bacillus circulans WL-12*[a]

Substrate	Main linkage type	Hydrolysis
Pustulan	β-(1 → 6)	+
Gentiobiose[b]	β-(1 → 6)	–
Laminarin	β-(1 → 3)	–
Pachyman	β-(1 → 3)	–
Pseudonigeran	α-(1 → 3)	–
Oat glucan	β-(1 → 3); β-(1 → 4)	–
Starch	α-(1 → 4)	–
Cellulose dextrins	β(1 → 4)	–
Bakers' yeast mannan	α-(1 → 6); α-(1 → 2); α-(1 → 3) (mannose)	–
Phosphomannan (*H. holstii*)	α-(1 → 2); α-(1 → 3) (mannose)	–
Methyl-β-D-glucoside		–
Phenyl-β-D-glucoside		–
p-Nitrophenyl-β-D-glucoside		–
Azocoll[c]		–
p-Nitrophenyl phosphate[c]		–

[a] See Table 6 for experimental details.

[b] Determined chromatographically.

[c] See Materials and Methods for details of protease and phosphatase assays.

Table 5 summarizes the response of β-(1 → 3)-glucanase activity to various reagents. Hg^{2+} ions were moderately to strongly inhibitory. Iodoacetamide and ethylenediaminetetraacetic acid were without effect, indicating an absence of free sulfhydryl groups in the active site and an absence of metal ion requirement for activity.

Properties of the β-(1 → 6)-glucanase. The properties of this enzyme were not determined in detail during this study, but the results of some exploratory studies are presented. Table 6 shows the substrate specificity of the enzyme. Only molecules with sequences of β-(1 → 6)-glucosidic linkages were hydrolyzed. Pustulan was hydrolyzed in a random fashion producing a series of oligosaccharides with R_{glc} values of 0.68, 0.41, and 0.23. The product with the value of 0.68 corresponded with authentic gentiobiose. Stability and storage characteristics of the enzyme were similar to those described above for the β-(1 → 3)-glucanase. The pH optimum for pustulan hydrolysis was 5.5, and the enzyme was saturated with substrate at approximately 15 mg/ml of pustulan. The metal ions Co^{2+}, Mg^{2+}, Zn^{2+}, Ca^{2+}, and K^{+} at concentrations up to 10^{-1} M did not show any effect on enzyme activity, but Hg^{2+} gave complete inhibition at 10^{-3} M. Iodoacetamide and ethylenediaminetetraacetic acid had no effect on the enyzme, and glycono-δ-lactone caused complete inhibition of activity at 25 mM.

Action of the enzymes on yeast cell walls. Initial studies were done with cell wall agar plates. In line with the earlier studies by Tanaka and Phaff (43, 44), the crude culture fluid from *B. circulans* WL-12 grown on bakers' yeast cell walls caused complete lysis or clearing of bakers' yeast cell walls and a partial or incomplete clearing of *S. pombe* cell walls incorporated in agar plates. The pure endo-β-(1 → 3)-glucanase, on the other hand, did not cause any lysis of bakers' yeast cell walls in plates. If a partially purified preparation of the β-(1 → 3)-glucanase was used—such as the enzyme obtained after DEAE-cellulose chromatography—lysis of bakers' yeast cell walls still took place, but in this case the lytic activity was lower than that found in crude enzyme preparations. On *S. pombe* cell wall plates, the pure β-(1 → 3)-glucanase caused a narrow margin of very weak clearing—appreciably less than with the crude enzyme preparation. The purified β-(1 → 6)-glucanase exhibited no lytic effect when applied to cell wall plates of *S. pombe* but gave the broad faint or weak zone of lysis on bakers' yeast wall plates as previously noted (43, 44). Application of a mixture of the two pure enzymes to bakers' yeast wall plates did not improve the lytic ability that was observed for the β-(1 → 6)-glucanase alone. Thus the inability of the β-(1 → 3)-glucanase to cause lysis was not due to the absence of the β-(1 → 6)-glucanase. Similarly, β-(1 → 6)-glucanase did not improve the lysis of *S. pombe* walls by the β-(1 → 3)-glucanase.

The action of the purified enzymes in cell wall suspensions was studied next. These enzymatic digestions were set up as discussed in Materials and Methods and in Tables 7 and 8. The enzymes remained active throughout incubation. A parallel series of digestions were performed on cell walls which had been pretreated with 0.02 M dithiothreitol for 12 h at 30 C and then washed free of this sulfhydryl reagent. Cell walls suspended in buffer without added enzyme (controls) gave no release of reducing sugars, provided the walls had been heated to inactivate endogenous wall-associated glucanases (17) (cf. also Materials and Methods).

The highly purified β-(1 → 3)-glucanase gave an almost negligible release of reducing sugars from the cell walls of bakers' yeast (Table 7). A slightly greater amount of wall hydrolysis was observed by digestion with β-(1 → 6)-glucanase. The two enzymes acting together caused a better digestion of the cell walls than the sum of the individual treatments, but the reducing sugar release was still very low as compared with that previously observed with another endo-β-(1 → 3)-glucanase isolated from the cell walls of *Schizosaccharomyces versatilis* (17). The latter results have been included in the table for comparison. There were no significant reductions in suspension turbidities by the bacillus glucanase treatments and no apparent light microscopic differences between treated and untreated cell walls. Pretreatment of the cell walls with DTT did not improve the ability of the enzymes to cause hydrolysis.

With walls of *S. pombe* (Table 8), the β-(1 → 3)-glucanase caused a steady but low release of reducing sugars. A lower, almost negligible hydrolysis was exhibited by the β-(1 → 6)-glucanase. In concerted action, the two enzymes gave a higher release of reducing sugars than when either acted alone. However, hydrolysis was still very limited in comparison to the action of the endo-β-(1 → 3)-glucanase from *S. versatilis*. Pretreatment of the cell walls with dithiothreitol did not improve wall hydrolysis. There were no noticeable decreases in suspension turbidities for any of the digestion mixtures, and under the light microscope the cell walls appeared the same before and after enzymatic treatment.

More significant extents of hydrolysis by the enzymes were noted for the two yeasts when total carbohydrate release was measured rather than only reducing group release. Total carbo-

TABLE 7. *Action of the purified endo-β-(1 → 3)- and endo-β-(1 → 6)-glucanases from B. circulans WL-12 on isolated bakers' yeast cell walls*[a]

Cell wall treatment	Incubation time (h)								
	0	1	4	7	17	23	30	45[b]	36
Walls + buffer (control)	0	0	0	0	0	0	0	0	0
Walls + β-(1 → 3)-glucanase	0	2	2	4	7	7	9	9 35[c]	4
Walls + β-(1 → 6)-glucanase	0	13		18	22	25	27	29 115[c]	9
Walls + β-(1 → 3)- and β-(1 → 6)-glucanase mixture	0	20	27	33	40	45	51	61 175[c]	15
Dithiothreitol-pretreated walls + β-(1 → 3)-glucanase	0		2			7		11	
Dithiothreitol-pretreated walls + β-(1 → 6)-glucanase	0		15			28		30	
Dithiothreitol-pretreated walls + β-(1 → 3)- and β-(1 → 6)-glucanase mixture	0		29			50		63	
Walls + β-(1 → 3)-glucanase from *S. versatilis*[d]	0	60	150		220		260		

[a] Experimental procedure is given in Materials and Methods. The values in the table represent reducing sugar release expressed as micrograms of glucose equivalents per milliliter. 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.

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

[c] Total carbohydrate (micrograms per milliliter) released from the walls.

[d] From Fleet and Phaff (17). Cell walls were used at a concentration of 5 mg/ml.

TABLE 8. *Action of the purified endo-β-(1 → 3)- and endo-β-(1 → 6)-glucanases of B. circulans WL-12 on isolated cell walls of S. pombe*[a]

Cell wall treatment	Incubation time (h)								
	0	1	4	7	17	23	30	45[b]	36
Walls + buffer (control)	0	0	0	0	0	0	0	0	0
Walls + β-(1 → 3)-glucanase	0	5	29	33	43	49	54	60 200[c]	13
Walls + β-(1 → 6)-glucanase	0	0	2	4	7	9	11	13 185[c]	0
Walls + β-(1 → 3)- and β-(1 → 6)-glucanase mixture[d]	0	31	38	50	67	81	100	103 335[c]	13
Dithiothreitol-pretreated walls + β-(1 → 3)-glucanase	0	3	21	24	30	35	40	45	
Dithiothreitol-pretreated walls + β-(1 → 6)-glucanase	0	0	2	3	6	8	10	12	
Walls + β-(1 → 3)-glucanase from *S. versatilis*[e]	0	230	547		623	628	625		

[a] Experimental procedure is given in Materials and Methods. The values in the table represent reducing sugar release expressed as micrograms of glucose equivalents per milliliter. 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.

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

[c] Total carbohydrate (micrograms per milliliter) released from the walls.

[d] Cell walls pretreated with dithiothreitol and incubated with a mixture of the two glucanases gave similar results.

[e] From Fleet and Phaff (17). Cell walls were used at a concentration of 5 mg/ml.

hydrate analysis gives a measure of the larger-molecular-weight polysaccharide species solubilized in addition to reducing sugars and is therefore a better parameter of enzyme action. Unfortunately, estimation of this property requires much larger sample volumes since all traces of residual undigested wall material must be completely removed before carbohydrate assay. Limitations of enzyme availability precluded a more detailed measurement of this parameter. For walls of *S. cerevisiae*, the total carbohydrate release after 45 h of digestion with a mixture of the two glucanases was 175 μg (cf. 61-μg reducing equivalents, Table 7). This represents approximately 7% of the wall material. In the equivalent case for *S. pombe* walls, 335 μg of carbohydrate material had become solubilized by hydrolysis with the enzyme mixture. This represents approximately 13% of the initial wall material.

DISCUSSION

Induction of the glucanases. Induction of enzyme formation by growth of *B. circulans* WL-12 on substrates other than yeast cell walls was economically desirable. Although laminarin and mannitol stimulated the production of both glucanases, the levels of the enzymes formed were consistently and considerably lower than those when yeast cell walls were the substrate for growth. Bacon et al. (5) made similar observations with *Cytophaga johnsonii* grown on laminarin and on yeast cell walls. Laboratory mutation of *B. circulans* WL-12, so that the glucanases would be constitutively produced in high yields, may be worthy of investigation.

Properties of the glucanases. Bacteria serve as convenient sources of β-(1 → 3)-glucanases. Although such enzymes have been well purified in a few cases (5, 11, 21, 23), their substrate specificities have not always been well characterized to the extent necessary for their use in yeast cell wall analyses. In such applications, traces of contaminating glucanases, mannanases, chitinases, phosphatases, or proteases would render results meaningless. The β-(1 → 3)- and β-(1 → 6)-glucanases purified in the present study revealed no known contaminating activities which might interfere with their use in the analysis of yeast cell walls. Insufficient supplies of enzymes were available for the determination of their electrophoretic homogeneities. The properties of the *B. circulans* WL-12 endo-β-(1 → 3)-glucanase are generally similar to those described for the endo-β-(1 → 3)-glucanases from another strain of *B. circulans* (21), from the plants *Nicotiana glutinosa* (31, 32) and *Phaseolus vulgaris* (3), and from fungi (37). All these enzymes hydrolyze laminarin randomly, producing a series of β-(1 → 3)-linked oligosaccharides, and exhibit weak affinity for laminaritriose. In contrast, the endo-β-(1 → 3)-glucanase from *S. versatilis* produces predominantly glucose and laminaribiose from laminarin and rapidly cleaves laminaritriose (17).

The β-(1 → 6)-glucanase from *B. circulans* WL-12 is an endoenzyme, thus confirming the preliminary studies by Tanaka and Phaff (43). The few properties studied of this enzyme were consistent with those reported for some fungal β-(1 → 6)-glucanases (39). Since *B. circulans* WL-12 produces this glucanase abundantly and a purification procedure has now been developed, it would be useful to characterize this enzyme more extensively. Only one other β-(1 → 6)-glucanase has been thoroughly studied (40).

Action of the glucanases on yeast cell walls. The purified bacterial glucanases caused only a very limited hydrolysis of bakers' yeast cell walls. This was unexpected, at least for the β-(1 → 3)-glucanase, since the major structural glucan component of the wall is predominantly β-(1 → 3) linked (26, 30). A minor component of β-(1 → 6)-linked glucan from bakers' yeast cell walls has recently been characterized (27), and this component may account for the limited wall hydrolysis observed with the β-(1 → 6)-glucanase. The ineffectiveness of the β-(1 → 3)-glucanase upon the walls might be attributed to a spatial inaccessibility of this enzyme to the glucan component(s). The wall matrix of mannan and protein could be responsible for such an impedance. However, objections to this explanation can be offered. In the case of isolated cell walls, an enzyme has access to both the inner and outer wall surfaces and the mannan complex is confined only to the outer layer (35). Pretreatment of the walls with dithiothreitol did not promote wall hydrolysis by the glucanases. Sulfhydryl reagents promote protoplast formation from intact yeast cells by crude glucanase preparations by permitting improved access of the lytic enzymes to the wall glucans (35). In a previous study (17), it was shown that an endo-β-(1 → 3)-glucanase isolated from the cell walls of the yeast *S. versatilis* caused extensive hydrolysis of bakers' yeast cell walls. This enzyme, with a molecular weight of 97,000 (17), certainly had access to the wall glucans and thus there is no reason to expect that this is not the case for the bacterial enzyme. Based on comparative behavior during Sephadex G-100 chromatography, the molecular weight of the bacterial glucanase was lower than this value.

The two enzymes, although both endo-β-(1 → 3)-glucanases, exhibited different patterns of laminarin hydrolysis as described above. In addition, these two glucanases showed different kinetics with respect to laminarin hydrolysis. Our data suggest that the bacterial glucanase hydrolyzes laminarin according to Michaelis-Menten kinetics, whereas the yeast glucanase follows sigmoidal kinetics (17). Thus, given accessibility of the bacterial glucanase to the wall glucans, some other property is required to cause wall hydrolysis.

Recent evidence suggests that unique lytic β-(1 → 3)-glucanases are produced by some microorganisms. In contrast to the highly purified glucanases used in this study, crude culture fluids of *B. circulans* WL-12 and partially purified preparations of these enzymes (43, 44) gave extensive hydrolysis of yeast cell walls. These observations indicate that some additional factor (normally present in the crude culture fluid) is required for wall lysis and was removed during enzyme purification. This factor was not a mannanase or phosphomannanase as suggested by McLellan et al. (28. 29) since these activities could not be demonstrated in the crude culture fluid. Bacon et al. (5, 6) first reported the presence of a lytic-type β-(1 → 3)-glucanase in the culture fluid of *Cytophaga johnsonii* along with the more predominant nonlytic β-(1 → 3)- and β-(1 → 6)-glucanases. Upon isolation, this new glucanase caused extensive hydrolysis of yeast cell walls but gave only a weak release of reducing groups from laminarin. This last property was consistent with the finding that laminarin was not degraded beyond the pentasaccharide stage. Similar lytic β-(1 → 3)-glucanases have now been reported in the culture fluids of *Arthrobacter* sp. (11, 12, 13, 23) and a species of the fungal genus *Rhizopus* (50). Doi et al. (12, 14) have shown that this enzyme produces predominantly laminaripentaose from the hydrolysis of isolated yeast cell wall glucan. The strongly lytic culture fluid of an *Oerskovia* sp. (25) probably contains this type of glucanase. It has now been established that *B. circulans* WL-12 also produces a lytic-type β-(1 → 3)-glucanase (F. M. Rombouts and H. J. Phaff, unpublished results), which is currently under investigation. Because of its very weak action on laminarin, this factor was overlooked in the present study.

Laminarin digestion by the fungus *Myrothecium verrucaria* requires the synergistic involvement of several types of β-(1 → 3)-glucanases (9). A similar situation probably exists for yeast cell wall degradation. Assuming the rigid glucan component of the wall to have the structure postulated by Misaki et al. (30), it was proposed that the lytic β-(1 → 3)-glucanase acts as a debranching enzyme cleaving laminaripentasaccharide side-chain units from the glucan (50). Such action could conceivably destroy the integrity of the cell wall and bring about lysis. A role of the nonlytic β-(1 → 3)-glucanase in further degradation of the released side chains could thus be envisaged. Not understood at present is how the lytic β-(1 → 3)-glucanases have access to the wall glucans. The action of these enzymes is not prevented by the outer mannan layer of the wall since they are capable of eliciting protoplast formation from intact yeast cells (23, 25, 50). Further information is required about the action and properties of the lytic β-(1 → 3)-glucanase. Used in conjunction with the β-(1 → 3) and β-(1 → 6)-glucanases described in this study, such an enzyme would be a most valuable tool in the structural analysis of yeast cell walls.

In contrast to bakers' yeast cell walls, walls of *S. pombe* were hydrolyzed to a slightly greater extent by the endo β-(1 → 3)-glucanase from *B. circulans* WL-12. This difference may reflect small differences in the β-(1 → 3)-glucan components of the two types of walls. Overall, cell walls of *S. pombe* differ from those of *S. cerevisiae* in that they contain galactomannan instead of mannan, and in addition they contain a considerable percentage of an α-(1 → 3)-linked glucan (7, 35). The latter component also may contribute to the rigid nature of the wall (17). As shown elsewhere (16), an α-(1 → 3)-glucanase is required to bring about complete wall hydrolysis of *S. pombe*.

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