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Isolation, Properties, Function, and Regulation of Endo-(1 → 3)-β-Glucanases in *Schizosaccharomyces pombe*

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Cell-free extracts, membranous fractions, and cell wall preparations from *Schizosaccharomyces pombe* were examined for the presence of (1 → 3)-β-, (1 → 3)-α-, and (1 → 6)-β-glucanase activities. The various glucanases were assayed in cells at different growth stages. Only (1 → 3)-β-glucanase activity was found, and this was associated with the cell wall fraction. Chromatographic fractionation of the crude enzyme revealed two endo-(1 → 3)-β-glucanases, designated as glucanase I and glucanase II. Glucanase I consisted of two subunits of molecular weights 78,500 and 82,000, and glucanase II was a single polypeptide of 75,000. Although both enzymes had similar substrate specificities and similar hydrolytic action on laminarin, glucanase II had much higher hydrolytic activity on isolated cell walls of *S. pombe*. On the basis of differential lytic activity on cell walls, glucanase II was shown to be present in conjugating cells and highest in sporulating cells. Glucanase II appeared to be specifically involved in conjugation and sporulation since vegetative cells and nonconjugating and nonsporulating cells did not contain this enzyme. The appearance of glucanase II in conjugating cells may be due to de novo enzyme synthesis since no activation could be demonstrated by combining extracts from vegetative and conjugating cells. Increased glucanase activity occurred when walls from conjugating cells were combined with walls from sporulating cells. Studies with trypsin and proteolytic inhibitors suggest that glucanase II exists as a zymogen in conjugating cells. A temperature-sensitive mutant of *S. pombe* was isolated which lysed at 37°C. Glucanase activity was higher in vegetative cells held at 37°C than cells held at 25°C. Unlike the wild-type strain, this mutant contained glucanase II activity during vegetative growth and may be a regulatory mutant.

Bacteria and fungi possess endogenous enzymes capable of degrading their own cell walls. These autolytic enzymes are thought to be involved in the modification and limited degradation of the cell wall during the cell life cycle (27, 30).

In yeasts, (1 → 3)-β-glucanases have been postulated to be autolytic enzymes since (1 → 3)-β-glucan is the main structural component of the yeast cell wall (18, 24, 25). (1 → 3)-β-Glucanases having either an exo or endo mechanism of action have been found in all yeast species examined (25). Evidence for a physiological role of these enzymes in wall morphogenesis includes reports of higher levels of glucanase activity during bud initiation, cell expansion, cell conjugation, and sporulation (5, 15, 25).

In a number of yeasts, a complex of several different (1 → 3)-β-glucanases has been found (25). For example, at least three (1 → 3)-β-glucanases occur in *Saccharomyces cerevisiae* (9). It has been suggested that these enzymes have different roles in the cell cycle. An endo-(1 → 3)-β-glucanase in *S. cerevisiae* was shown to be active specifically during sporulation (6). The function of the exo-(1 → 3)-β-glucanase in this yeast, however, is unclear since mutants lacking this enzyme have a normal life cycle (29). Overall, evidence relating a particular glucanase activity to life cycle function is not strong, and further information is required. Regulation of these enzymes is largely unstudied.

In addition to (1 → 3)-β-glucan, the cell walls of yeasts contain (1 → 6)-β-glucan (19) and glucans of mixed (1 → 3)-β- and (1 → 6)-β-linkages (10). Some yeasts also contain (1 → 3)-α-glucan in their cell walls (24). Although evidence suggests that these linkages are important to cell wall integrity (11, 28), (1 → 6)-β-glucanases and (1 → 3)-α-glucanases have been demonstrated only in a limited number of yeast species, and relatively little work has been done on these enzymes (21).

Cell walls of yeasts in the genus *Schizosac-*

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charomyces contain up to 25% (1 → 3)-α-glucan as well as (1 → 3)-β- and (1 → 6)-β-glucans (3, 20). Although (1 → 3)-β-glucanases have been found in *Schizosaccharomyces pombe* and *Schizosaccharomyces versatilis* (2, 11, 12), no (1 → 3)-α-glucanases or (1 → 6)-β-glucanases have been found in these yeasts. However, these studies were carried out on cells in vegetative growth; the possible presence of other glucanases at different stages of the life cycle has not been examined.

The present investigation reports the glucanase activities found in *S. pombe* at different stages during the life cycle. Two types of endo-(1 → 3)-β-glucanases were isolated and characterized in order to understand their hydrolytic action on cell walls and their possible physiological roles. Enzyme activation studies and mutant isolations were also initiated to examine the question of glucanase function and regulation.

MATERIALS AND METHODS

Microorganisms and culture conditions. *S. pombe* (Linder) strains 7310 and 738 were obtained from the Department of Food Science and Technology, University of California, Davis. Strain 7310 was the original homothallic strain from which the heterothallic strain 738 was derived.

Yeast cultures were maintained on malt extract agar (Oxoid), and liquid cultures were grown in YEGS medium, which consisted of 10 g of yeast extract, 60 g of glucose, and 2.95 g of succinic acid per liter of medium, adjusted to pH 5.0. A mineral salts-glucose medium was prepared by the method of Mitchison (22). Cultures were incubated at 30°C, and liquid cultures were grown on a rotary shaker at 800 rpm.

Cultures for enzyme preparation were grown in 20-liter carboys containing YEGS. The carboys were inoculated with 1 liter of an exponentially growing culture and incubated at 28°C. Cultures were aerated by sparging with filtered air, and the cells were kept in suspension by magnetic stirring.

Preparation of yeast cell walls and cell extracts. Yeast cells were harvested by centrifugation and disrupted in a Braun homogenizer as described previously (11). Centrifugation of the homogenate at 1,000 × *g* gave a hard pellet of cell walls, while a lighter fraction consisting of membrane fragments sloughed off with the supernatant. This membranous fraction was separated from the intracellular extract by further centrifugation at 10,000 × *g* for 30 min. The cell walls were washed three times in 0.1 M succinate buffer (pH 5.0) and three times in 0.01 M succinate buffer (pH 5.0).

Autolysis of cell walls. Washed cell walls were suspended in 0.01 M succinate buffer (pH 5.0) containing 0.01% sodium azide as an antimicrobial agent. The cell walls were brought into fine suspension by using a Potter tissue grinder. Autolysis was carried out by incubation of the suspension at 30°C with gentle agitation either on a rotary shaker or with a magnetic stirrer. The enzymes solubilized by autolysis were recovered in the supernatant after centrifugation at 10,000 × *g* for 15 min.

Enzyme assays. Assays for (1 → 3)-β-, (1 → 6)-β-, and (1 → 3)-α-glucanase activities were done after release of reducing sugars, using, respectively, laminarin, pustulan, and carboxymethyl pseudonigeran as substrates (0.25% in 0.05 M succinate buffer, pH 5.0) according to the conditions described previously (11, 21). One unit of glucanase activity was defined as the amount of enzyme which released 1 μmol of reducing sugar equivalent, expressed as glucose, per min under the standard assay conditions.

Proteinase activity was determined by the method of Lenny and Dalbec (17), using the substrate 1% hemoglobin (Sigma) denatured with 6.6 M urea in 0.05 M phosphate buffer (pH 6.0).

Lytic activity was detected by zones of clearing in agar plates containing a suspension of *S. pombe* cell walls as described by Tanaka and Phaff (32).

Analytical procedures. Methods for the determination of reducing sugar, total carbohydrate, and protein and procedures for paper and column chromatography have been described previously (11). Column chromatography was carried out at 5°C, and fractions of 4.0 ml each were collected.

Polyacrylamide gel electrophoresis. Determination of protein homogeneity and its correlation with enzyme activity was done using slab gel electrophoresis (BioScientific Pty. Ltd., Sydney, New South Wales) with polyacrylamide concentrations increasing from 2.5 to 27% in a concave gradient (premade gels from Gradient Pty. Ltd., Sydney, New South Wales). Sodium phosphate buffer (0.1 M, pH 6.0) was used as the reservoir buffer, and 0.05 M phosphate buffer (pH 6.0) was used as the sample buffer. Samples containing 10 to 100 μg of protein were run at 5 to 10 mA for 24 to 36 h. Protein bands were detected by staining with Coomassie brilliant blue. Duplicate, unstained gels were sliced and assayed for glucanase activity by crushing the gel slices and incubating with laminarin for 12 h. Gels were stained for carbohydrate by using Schiff's reagent (8). Molecular weight determinations were made with sodium dodecyl sulfate-polyacrylamide gels and using a discontinuous buffer system as described by Laemmli (16).

Isolation of temperature-sensitive growth mutants. Mutants were isolated using nitrous acid, ethyl methane sulfonate, or UV light as a mutagen. Cells in logarithmic growth were harvested, washed once, and suspended to a final concentration of 10^8 cells per ml in buffer. For nitrous acid mutagenesis, citrate buffer (pH 4.5) was used. Sodium nitrite was added to a final concentration of 4 mg/ml, and the cells were incubated at 30°C for 15 min. The reaction was stopped by addition of cold 0.1 M KPO_4 buffer (pH 7.0). Ethyl methane sulfonate mutagenesis was carried out in 0.1 M KPO_4 buffer (pH 7.0); 0.1 ml of ethyl methane sulfonate (Eastman Organic Chemicals) was added to 1.9 ml of cell suspension, and the suspension was incubated at 30°C for 60 min. Thiosulfate was added to a final concentration of 5% (wt/vol) to stop the reaction. UV irradiation of cells was done on agar plates at 40 ergs per mm^2 for 1 to 2 min. Mutagenized cells were plated onto Malt Extract Agar and grown at 25°C. Temperature-sensitive growth mutants were

detected by replicating onto two fresh plates of Malt Extract Agar and incubating one set of replicates at 25°C and the other at 37°C. Colonies that failed to grow at 37°C were picked as presumptive mutants.

RESULTS

Glucanase activity in *S. pombe*. Intracellular extracts, membrane and cell wall preparations, and culture fluids prepared from exponential phase cultures of *S. pombe*, grown in either mineral salts or complex medium (YEGS), were assayed for (1 → 3)-β-, (1 → 6)-β-, and (1 → 3)-α-glucanase and lytic activities. In YEGS, the cells underwent conjugation and sporulation at the end of logarithmic growth; these cells were also examined for glucanase and lytic activity. Conjugation and sporulation did not occur in mineral salts medium.

Neither (1 → 6)-β-glucanase nor (1 → 3)-α-glucanase activity was detected in any of the subcellular fractions under the various conditions examined. (1 → 3)-β-Glucanase activity was found exclusively in the cell wall fractions. Lytic activity was detected in the cell walls of conjugating and sporulating cells, but not in the cell walls from vegetative cells. The zone of lysis produced by the walls of sporulating cells was more pronounced and larger than that produced by the walls from conjugating cells (Fig. 1). Wall preparations from the nonconjugating and nonsporulating heterothallic strain, *S. pombe* 738, did not produce lysis on cell wall plates (Fig. 1).

Table 1 compares the levels of (1 → 3)-β-glucanase activity in the walls of cells from different cultures. Cells grown in complex medium exhibited more glucanase activity than cells grown in mineral salts medium. The wall-associated activity was approximately five times higher in sporulating cells and two times higher in conjugating cells than in vegetative cells.

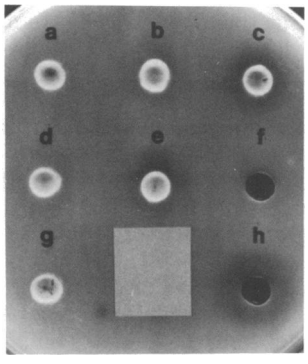

FIG. 1. *Lytic activity of cell wall preparations and isolated glucanases on cell wall plates. Isolated walls from vegetative cells (a), conjugating cells (b), sporulating cells (c), vegetative cells plus trypsin (d), and conjugating cells plus trypsin (e). Isolated cell walls from S. pombe heterothallic strain 738 (g). Glucanase I, 0.01 U (f). Glucanase II, 0.01 U (h).*

TABLE 1. *Levels of (1 → 3)-β-glucanase activity in S. pombe*

Culture	(1 → 3)-β-D-Glucanase activity (U/g [dry wt] of walls)	
	Initial cell wall preparation	Solubilized after 14 h of autolysis
Vegetative cells from mineral salts medium	0[a]	0.50
Vegetative cells from YEGS	0.29	0.54
Conjugating cells from YEGS	0.61	1.00
Sporulating cells from YEGS	1.52	2.70

[a] No activity could be detected.

Solubilization of wall-associated glucanase. When freshly isolated cell wall preparations were incubated at 30°C in buffer, a process of self-degradation or autolysis occurred. This process was characterized by a solubilization of cell wall polysaccharide material and wall-associated (1 → 3)-β-glucanase activity. Release of polysaccharide and enzyme was initially rapid, slowed at 10 h, and ceased after 12 to 14 h. At this time approximately 25% of the cell wall carbohydrates had been solubilized, and about 40 to 60% of the bound enzyme had been released. Further wall incubation did not increase wall degradation or glucanase release, and in some cases, solubilized enzyme activity decreased. Levels of (1 → 3)-β-glucanase activity found in the soluble fraction after 14 h of autolysis were approximately two times higher than the initial activity in the cell wall preparation (Table 1). (1 → 3)-β-Glucanase was not detected in fresh wall preparations of vegetative cells grown in minimal salts medium, but was detected after such walls had undergone autolysis.

The optimum pH for wall autolysis was 5.0, and the optimum temperature was 30°C. When autolysis was conducted at 37°C, the released glucanase underwent rapid inactivation. The addition of Mg^{2+} or Ca^{2+} at final concentrations of 50 mM did not improve enzyme release by autolysis, and, in fact, the presence of Mg^{2+} caused an inactivation of the released glucanase. Bovine serum albumin at 2.5 mg/ml was added to the autolysate to protect against possible proteolytic degradation of the released enzyme. However, enzyme recovery was not improved, and bovine serum albumin caused a delay in the onset of autolysis by as much as 6 h.

The rate of glucanase release was stimulated

by trypsin. When cell walls were autolyzed in the presence of 1 mg of trypsin per ml, the rate of glucanase release was approximately three times as fast as that in the absence of trypsin, and after 10 h, glucanase activity of the trypsin-treated autolysate was approximately three times as high as that of the untreated control (Fig. 2). The proteinase inhibitor phenylmethylsulfonyl fluoride (2 mM) had an inhibitory effect upon glucanase release during wall autolysis, and after 10 h, soluble glucanase activity was approximately 30% lower than that of the untreated control.

Attempts to extract the wall-associated (1 → 3)-β-glucanase activity with high concentrations of salts (5 M LiCl) or with the detergent sodium deoxycholate (20 mg/ml) were not successful. Autolysis as described above was the only effective means of obtaining solubilized enzyme.

Purification of wall-associated glucanase. Washed cell walls from 340 g (wet weight) of cells harvested from late-exponential to stationary-phase cultures were suspended in 500 ml of 0.1 M succinate buffer (pH 5.0) containing 0.1% sodium azide and were autolyzed at 30°C for 14 h. The supernatant, containing solubilized glucanase, was recovered by centrifugation at 10,000 × *g* for 20 min, dialyzed against 0.01 M succinate buffer (pH 5.5) at 4°C, and fractionated using DEAE-Bio-Gel A chromatography as described in the legend to Fig. 3. Approximately 30% of the total glucanase activity did not bind to the column under the conditions used. A minor peak of glucanase activity eluted at 0.12 M NaCl. The presence of this small peak, which was associated with high levels of carbohydrate, was not reproducible and was not studied further.

Two major peaks of (1 → 3)-β-glucanase

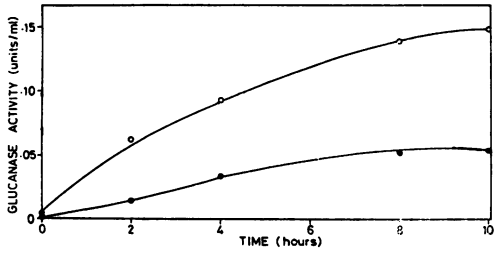

FIG. 2. *Effect of trypsin on (1 → 3)-β-glucanase release from cell walls during autolysis. Trypsin (1 mg/ml) was added to a suspension of S. pombe cell walls (40 mg/ml) in 0.01 M succinate buffer (pH 5.0). Autolysis was carried out at 30°C, and samples were taken over time. (1 → 3)-β-Glucanase activity was measured in the supernatants of centrifuged samples from the control wall suspension without trypsin* (●) *and from the suspension with trypsin* (○).

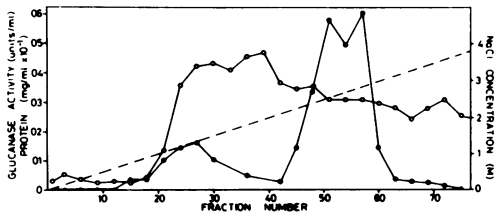

FIG. 3. *DEAE-Bio-Gel A chromatography of glucanase I. The dialyzed supernatant of a cell wall autolysate (565 ml) was loaded onto a column (18 by 2 cm) of DEAE-Bio-Gel A equilibrated with 0.01 M succinate buffer (pH 5.5). The column was washed with 200 ml of buffer, and the enzyme was eluted with a linear gradient of 0 to 0.5 M NaCl in 0.01 M succinate buffer (pH 5.5) (total volume, 400 ml). Fractions of 4.0 ml were collected at a rate of 0.3 ml/min. (1 → 3)-β-Glucanase activity* (●), *protein* (○), *and NaCl gradient* (- - -).

eluted from the DEAE-Bio-Gel A column at 0.25 and 0.28 M NaCl. Since the resolution was poor, the activity within these peaks (fractions 45 to 60) was combined, concentrated by ultrafiltration with an Amicon PM-10 membrane, and further fractionated on a column of Sephadex G-200. Two minor peaks and one major peak of activity were resolved on this column (Fig. 4). High levels of carbohydrate were found in all fractions containing glucanase activity. Fractions 36 to 48, comprising the major peak, were combined, dialyzed against 0.01 M sodium phosphate (pH 6.0), and subjected to hydroxyapatite chromatography (Fig. 5a). Glucanase activity eluted as a single band at 0.15 M $NaPO_4$. Fractions 37 to 41 were pooled, concentrated, and designated as glucanase I.

The unadsorbed effluent from the DEAE column was dialyzed against 0.1 M succinate buffer (pH 4.5) and fractionated on a column of carboxymethyl-Bio-Gel A. Glucanase activity eluted as a single peak at 0.15 M NaCl (Fig. 6). Fractions 25 to 43 were pooled, concentrated, and run on a Bio-Gel P-100 column. The enzyme eluted as a single peak soon after the void volume. Active fractions were combined, dialyzed against 0.01 M phosphate buffer (pH 6.0), and chromatographed on hydroxyapatite (Fig. 5b). Glucanase activity eluted as a single peak at 0.08 M $NaPO_4$. The active fractions were concentrated and designated as glucanase II. The purification steps for glucanase I and glucanase II are summarized in Table 2.

Electrophoretic properties of the glucanases. On native slab gel electrophoresis, glucanase I gave a single band of protein which corresponded to (1 → 3)-β-glucanase activity in the unstained, duplicate sample. A nonproteinaceous band of opaque, diffuse material, with a

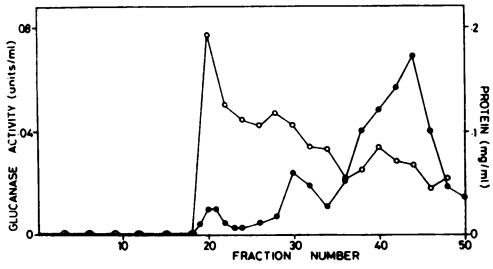

FIG. 4. *Sephadex G200 chromatography of glucanase I. Concentrated enzyme (fractions 45 to 60) from DEAE-Bio-Gel chromatography was applied to a Sephadex G200 column (78 by 2 cm) equilibrated with 0.05 M succinate buffer (pH 5.0) (bed volume, 245 ml; void volume, 90 ml). Fractions of 4.0 ml were collected at a rate of 0.1 ml/min. (1 → 3)-β-Glucanase activity (●), protein (○).*

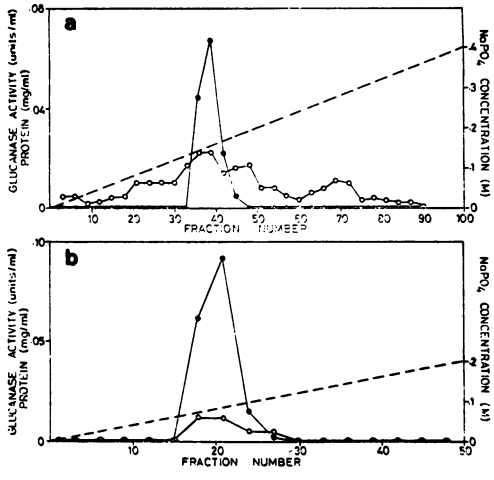

FIG. 5. *Hydroxyapatite chromatography of (a) glucanase I and (b) glucanase II. Enzyme was loaded onto a Bio-Gel HTP hydroxyapatite column (15 by 2 cm) equilibrated with 0.01 M $NaPO_4$ buffer (pH 6.0). The column was washed with 200 ml of buffer, and the enzymes were eluted with a linear gradient of 0.01 to 0.4 M $NaPO_4$ buffer (pH 6.0) (total volume, 400 ml). Fractions of 4.0 ml were collected at a rate of 0.3 ml/min. (1 → 3)-β-Glucanase (●), protein (○), and $NaPO_4$ gradient (- - -).*

relative mobility (R_m) value distinct from the protein band, was also seen. Since this enzyme preparation was found to contain 67% carbohydrate, duplicate samples were stained with Schiff reagent. Although the band did not stain positively for carbohydrate, the possibility remains that this band may consist of (1 → 3)-β- or -α-glucan, since periodate does not oxidize these linkages (13).

On sodium dodecyl sulfate-gel electrophoresis, glucanase I resolved into two bands of protein of approximately equal staining intensity. These protein bands corresponded to molecular weights of 78,500 and 82,000, suggesting a molecular weight of 160,500 for the native enzyme. This is consistent with the gel filtration behavior of this enzyme.

Resolution of glucanase II on native gels could not be assessed since a suitable buffer, in which the enzyme was negatively charged and remained active, was not found. Electrophoresis of glucanase II on sodium dodecyl sulfate gels gave a single protein band corresponding to a molecular weight of 75,000.

Substrate specificity and action pattern of the glucanases. Both glucanase I and glucanase II specifically hydrolyzed molecules containing (1 → 3)-β- linkages. Polysaccharides containing (1 → 4)-β-, (1 → 6)-β-, (1 → 3)-α-, or (1 → 4)-α- linkages were not degraded by these enzymes. The glycoside *p*-nitrophenyl-β-D-glucoside and the (1 → 3)-β-linked oligosaccharides laminaribiose and laminaritriose were not hydrolyzed by either enzyme. The products of laminarin hydrolysis were examined by paper chromatography as a function of reaction time. For both enzymes, oligosaccharides were the initial products of laminarin hydrolysis, and after 3 h, laminaribiose and laminaritriose were the predominant products, with minor amounts of glucose and higher oligosaccharides. These hydrolytic patterns are typical of enzymes acting by an endo-mechanism of action. Neither enzyme exhibited phosphatase, trehalase, or proteolytic activities.

Kinetic properties. Both enzymes exhibited Michaelis-Menten kinetics for laminarin hydrolysis, with a pH optimum of 5.0. For glucanase I,

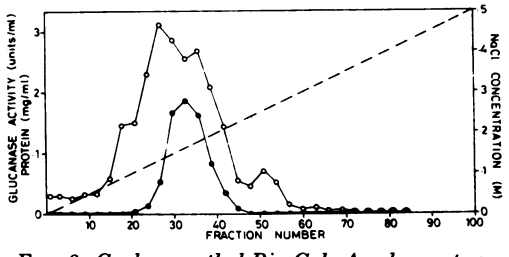

FIG. 6. *Carboxymethyl-Bio-Gel A chromatography of glucanase II. The unadsorbed enzyme (557 ml) from DEAE-Bio-Gel A chromatography was loaded onto a column (14 by 2 cm) of carboxymethyl-Bio-Gel A equilibrated with 0.01 M succinate buffer (pH 4.5). The column was washed with 200 ml of the same buffer and eluted with a linear gradient of 0 to 0.5 M NaCl in 0.01 M succinate buffer (pH 4.5) (total volume, 400 ml). Fractions of 4.0 ml were collected at a rate of 0.6 ml/min. (1 → 3)-β-Glucanase activity (●), protein (○), NaCl (----).*

TABLE 2. *Summary of steps involved in the purification of glucanase I and glucanase II*

Purification step	Vol (ml)	Glucanase activity (U/ml)	Protein concn (mg/ml)	Sp act (U/mg of protein)	Purification (fold)	Yield (%)
Initial autolysate	565	0.139	1.625	0.085	1.0	100
Glucanase I						
DEAE-Bio-Gel A	64	0.041	0.327	0.125	1.5	3.3
Sephadex G-200	52	0.045	0.248	0.181	2.1	3.0
Hydroxyapatite	48	0.034	0.018	1.888	22.2	2.1
Glucanase II						
Effluent of DEAE-Bio-Gel A	577	0.043	0.364	0.118	1.4	31.5
Carboxymethyl-Bio-Gel A	76	0.108	0.241	0.448	5.3	10.4
Bio-Gel P-100	32	0.154	0.086	1.790	21.1	6.2
Hydroxyapatite	36	0.053	0.009	5.888	69.3	2.4

a K_m value of 0.15 mg/ml and a V_{max} value of 8.8 μmol/min per mg of protein were obtained. Glucanase I activity was inhibited slightly by substrate concentrations over 1 mg/ml. Glucanase II had a K_m value of 0.69 mg/ml and a V_{max} value of 6.6 μmol/min per mg of protein. Substrate concentrations greater than 0.5 mg/ml were inhibitory to glucanase II.

Enzyme inhibition studies. Both enzymes were insensitive to the presence of EDTA (10 mM), suggesting an absence of any metal ion requirement. The lack of inhibition by hydroxymercuribenzoate (1 mM) or phenylmethylsulfonyl fluoride (1 mM) indicated the absence of sulfhydryl or serine residues in the active site. Calcium ions at 100 mM had no effect on glucanase II activity but inhibited glucanase I activity by about 10%. Magnesium ions at 100 mM inhibited both enzymes. Glucono-δ-lactone at 10 mM inhibited glucanase I activity by approximately 10% and glucanase II activity by 20%.

Action of glucanases on isolated cell walls of *S. pombe*. Glucanase I and glucanase II were incubated with suspensions of isolated *S. pombe* cell walls which had been heated to inactivate endogenous glucanase activity. Glucanase II exhibited much greater hydrolytic activity on cell walls than glucanase I, releasing a maximum of 0.37 mg of reducing sugar per ml and 1.37 mg of total carbohydrate per ml, whereas glucanase I released a maximum of 0.009 mg of reducing sugar per ml and 0.052 mg of total carbohydrate per ml (Fig. 7).

On cell wall plates, glucanase II produced a visible zone of lysis, whereas glucanase I did not (Fig. 1). Thus, glucanase II could be described as a lytic enzyme, and glucanase I could be termed a nonlytic enzyme.

Glucanase activation. The higher glucanase activities noted in cell walls from conjugating and sporulating cultures, as compared to those

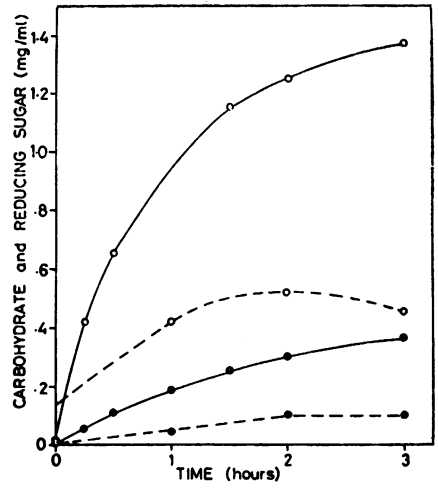

FIG. 7. *Hydrolysis of isolated cell walls of S. pombe by glucanase I (broken lines) and glucanase II (solid lines). A suspension (5 mg/ml) of S. pombe walls in 0.005 M succinate buffer (pH 5.0) was incubated at 30°C with the enzyme (0.01 U/ml). After appropriate time intervals, samples were removed and centrifuged, and the concentrations of reducing sugar (●) and total carbohydrate (○) solubilized in the supernatant were determined. Carbohydrate and reducing sugar release for glucanase I to be multiplied by 10^{-1}.*

from vegetative cultures (Table 1), may have been due to enzyme activation by factors that were produced only during conjugation and sporulation. To test such a possibility, cell homogenate (cell walls not separated from intracellular extracts) from a conjugating culture (0.020 glucanase unit per ml) was incubated for 1 h with an equal volume of cell homogenate from a vegetative culture (0.006 glucanase unit per ml) before assaying. The activity of the combined homogenate was 0.013 glucanase unit

per ml, which represents a simple additive effect rather than an activation of glucanase in the vegetative cell homogenate.

Cell wall preparations from cells at different growth stages were also combined and assayed for glucanase activity. Combination of cell walls from vegetative cells with walls from either conjugating or sporulating cells did not give glucanase activities different from that expected by addition (Table 3). However, the combination of walls from conjugating cells with those of sporulating cells resulted in glucanase activity that was 28% higher than that of an additive effect (Table 3).

The possibility of enzyme activation by proteolysis was tested by the addition of trypsin (1 mg/ml) to each of the cell wall preparations. Glucanase activity in walls from vegetative or sporulating cells was not significantly altered by trypsin treatment, but the glucanase activity of walls from conjugating cells was increased by 2.4-fold (Table 3). Trypsin-treated cell walls from conjugating cells also exhibited greater lytic activity on cell wall plates (Fig. 1). However, trypsin treatment did not alter the lytic activity of walls from vegetative or sporulating cells and trypsin alone did not have any lytic activity in cell wall plates (Fig. 1).

Endogenous proteinase activity was detected in cell wall preparations from vegetative, conjugating, and sporulating cells and was 0.107, 0.217, and 0.282 U/g (dry weight), respectively.

Glucanase activity in a temperature-sensitive growth mutant. Sixty mutants were isolated that were unable to grow at 37°C. Cell morphology at the restrictive temperature was studied by growing the mutant strains into the logarithmic phase at 25°C and then incubating the cultures at 37°C for 4 h before microscopic examination. At 37°C a number of mutants showed swelling and lysis in the polar region and in the area of the septum. The morphology of one such mutant, designated SPT-5, at both 25 and 37°C is shown in Fig. 8. Fifteen liters of this strain was grown at 25°C, and half of the culture was harvested in logarithmic growth. The remaining culture was shifted to 37°C and incubated for 4 h before harvesting. The cells were homogenized and fractionated as described earlier. As in the wild-type strain 7310, glucanase activity was found only in the cell wall fraction. The level of glucanase activity was 0.94 U/g (dry weight) of walls for the cells grown at 25°C but increased by about threefold to 2.5 U/g (dry weight) of walls for the cells held at 37°C for 4 h.

To test for possible enzyme activation at 37°C, separate samples of the cell homogenate of SPT-5 grown at 25°C were incubated at 25 and 37°C for 1 h before glucanase assay. Glucanase activity was found to be identical in both preparations, suggesting that the increased activity observed after incubation at 37°C was not due to activation but more likely due to new enzyme synthesis.

Cell walls from vegetative cells of SPT-5 grown at 25°C exhibited lysis on cell wall plates, indicating the presence of glucanase II. Similar lytic activity was seen for cell walls from SPT-5 held at 37°C.

TABLE 3. *Activation of (1 → 3)-β-glucanase activity in cell wall preparations of S. pombe*

Cell wall prepn[a]	Glucanase activity (U/ml)
Vegetative[b]	0.030
Conjugating[b]	0.008
Sporulating[b]	0.115
Vegetative and conjugating	0.018
Vegetative and sporulating	0.076
Conjugating and sporulating	0.078[c]
Vegetative and trypsin[d]	0.031
Conjugating and trypsin	0.019[c]
Sporulating and trypsin	0.120

[a] All combinations were incubated at 30°C for 1 h before assay.

[b] Equal volumes were used in the various combinations.

[c] Represents significant activation.

[d] Trypsin concentration, 1 mg/ml.

DISCUSSION

Although (1 → 3)-α- and (1 → 6)-β-glucans occur in the cell walls of *S. pombe*, no corre-

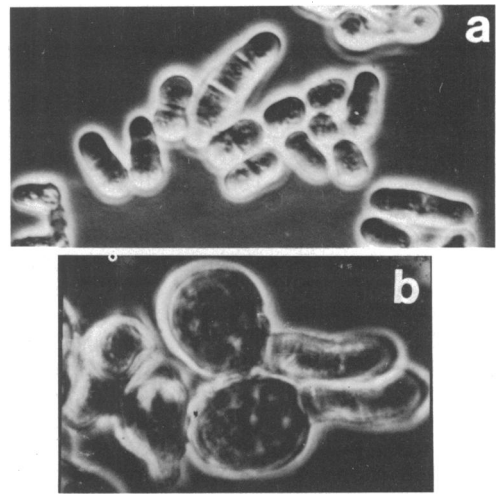

FIG. 8. *Cell morphology of S. pombe temperature-sensitive mutant SPT-5 at 25°C (a) and at the restrictive temperature of 37°C (b).*

sponding glucanase activities were detected in vegetative, conjugating, or sporulating cells of this yeast. Previous workers have also failed to detect these enzymes in *S. pombe* (2, 21). Although it is possible that these enzymes may be extremely unstable or may recognize only complex substrates, it seems from the present data that they are not required for cell wall modification during the life cycle of *S. pombe*.

Consistent with earlier reports on *S. pombe* (2) and the related species *S. versatilis* (11, 12), the endo-(1 → 3)-β-glucanase in *S. pombe* was associated with the cell wall fraction. In other yeasts, such as *S. cerevisiae*, glucanase activity has been found in intracellular extracts, membranous fractions, and the cell walls (25). The site of action for glucanases is postulated to be the cell wall, and evidence exists for their in vivo location in the periplasmic space (25). The presence of glucanases in the cytoplasm and in membranous vesicles is thought to reflect their origin of synthesis and mode of transport to the cell wall (9, 25). The absence of glucanase activity in the intracellular and membrane fractions of *S. pombe* suggests that the mode of glucanase synthesis may differ from that of other yeasts. Confirmation of the absence of intracellular glucanase in *S. pombe* would require further studies with protoplasts and examination for the possible presence of latent enzymes.

The endo-(1 → 3)-β-glucanases of *S. pombe* were not extracted or released from the cell wall by treatment with detergent or high salt concentration. Attempts to extract the wall-associated glucanases in other yeast species with salts, buffers, detergents, organic solvents, and thiol reagents have all been unsuccessful (1, 2, 11). In contrast, some (1 → 3)-β-glucanases associated with fungal cell walls have been released by these treatments (7, 14, 26, 30). The nature of the strong glucanase-wall affinity in yeasts is worthy of more detailed study because methods that would give a quantitative extraction or elution of the glucanase would facilitate enzyme isolation and purification.

To date, autolysis is the only effective means of solubilizing wall-associated glucanases for enzyme purification. In *S. pombe*, only 40 to 60% of the initial glucanase activity in walls was released by this procedure, and the possibility exists that the solubilized glucanases may be different from those remaining attached to the wall. After autolysis, the sum of soluble glucanase activity and the remaining wall-associated glucanase activity was often higher than the initial activity measured in the walls. Activation of enzymes during autolysis or greater access of the soluble enzyme to the substrate, laminarin, may be responsible for this observation.

Upon fractionation, cell wall autolysates yielded two predominant (1 → 3)-β-glucanases, termed glucanase I and glucanase II. Other minor peaks of activity were seen during fractionation, but low yields prevented further examination of these enzymes. Glucanases I and II were clearly distinct enzymes as indicated by their different chromatographic and electrophoretic properties and molecular weights. Glucanase I consisted of two nonidentical subunits and contained 67% carbohydrate. Further studies would be required to determine whether this enzyme is a true glycoprotein as has been reported for some other yeast glucanases (25). Glucanase II was a single polypeptide and did not contain any carbohydrate.

Although both glucanase I and glucanase II appeared to hydrolyze laminarin by a similar endo mechanism of action, they differed markedly in their hydrolytic action on isolated yeast cell walls. In cell wall suspensions, glucanase II solubilized approximately 30 times more wall carbohydrate than glucanase I, and on cell wall plates, glucanase II produced distinct zones of cell wall lysis. The endo-(1 → 3)-β-glucanase of *S. versatilis* is also lytic on yeast cell walls (11). The soil bacterium *Bacillus circulans* produces endo-glucanases that are either lytic or nonlytic towards yeast cell walls (28). The lytic capability of one of these enzymes, an endo-(1 → 6)-β-glucanase, has been attributed to its ability to cleave the bonds surrounding a branch point in a predominantly (1 → 3)-β-linked glucan (28). Although glucanase I and glucanase II show similar hydrolytic action patterns on laminarin, they may differ in their activity on branched or mixed-linkage polysaccharides such as occur in yeast cell walls. Further studies on the hydrolysis of mixed-linkage polysaccharides and isolated cell wall polysaccharides are necessary to understand the nonlytic and lytic natures of glucanase I and glucanase II. Such studies may also establish the importance of various wall components and linkages to the integrity of the cell wall and may lead to a better understanding of the functions of the two glucanases in cell wall modification.

Multiple (1 → 3)-β-glucanases have now been found in several yeasts, with some species possessing up to four enzymes (25). Both exo- and endo-(1 → 3)-β-glucanases have been found in well-studied species such as *S. cerevisiae* (6, 9), *Kluyveromyces phaseolosporus* (33), *Pichia polymorpha* (34), *Candida utilis* (35), and *Cryptococcus albidus* (23). Although no exo-(1 → 3)-β-glucanase could be detected in *S. pombe*, the related species *S. versatilis* contains one exo- and one endo-(1 → 3)-β-glucanase (12). The absence of exo-(1 → 3)-β-glucanase in *S. pombe*

supports the concept that this enzyme may not be essential for the growth cycle in some yeasts (29). Further studies are required to establish the importance of this enzyme in yeasts.

The total (1 → 3)-β-glucanase activity in some yeasts varies during the life cycle, suggesting the association of glucanases with specific cellular functions (6, 12, 23). Assignment of specific functions to individual glucanases has been prevented by the inability to distinguish the separate enzyme activities in a complex mixture. With *S. pombe*, the glucanase activity in sporulating cells was five times higher than that in vegetative cells. The lytic nature of glucanase II provided a differential assay for this enzyme during different stages of growth. Since lytic glucanase II activity was not present in vegetative cells, the (1 → 3)-β-glucanase activity in vegetative cells appeared to be due to glucanase I. Lytic activity was detected in conjugating cells and was highest in sporulating cells. Since conjugation is rapidly followed by sporulation in this yeast, it is possible that the lytic activity seen in conjugating cells may be due to a small percentage of sporulating cells in the population. Studies using vegetative diploids which are capable of azygotic meiosis, and mutants, which can conjugate but not sporulate, would be necessary to establish glucanase II involvement in conjugation. Glucanase II activity was not found in the heterothallic strain 738, which cannot undergo conjugation and sporulation without the opposite mating type. Thus, glucanase II may be involved in sporulation or the release of ascospores from the ascus. Kroning and Egel (15) previously suggested an involvement of cell wall autolytic enzymes in the conjugation and sporulation of *S. pombe*.

Since the lytic assay was not quantitative, it was not possible to establish whether the increased total glucanase activity (assayed with laminarin) seen in conjugating and sporulating cells of *S. pombe* was due to the appearance of glucanase II alone. The possibility that glucanase I may also be increasing at this time cannot be dismissed. The isolation of glucanase I from cells in these sexual phases demonstrated the presence of this enzyme under these conditions.

The increased glucanase activity in conjugating cells of *S. pombe* appeared to be due to de novo enzyme synthesis since no enzyme activation could be detected. Further studies are needed to confirm this. Enzyme activation was seen, however, in combined walls of conjugating and sporulating cells. Studies with trypsin confirmed the existence of a latent or less active form of glucanase II in conjugating cell walls. Trypsin did not increase glucanase activity in walls from vegetative or sporulating cells, presumably because glucanase II was absent in the former and already activated in the latter. The present evidence suggests that a zymogen form of glucanase II is synthesized during conjugation in *S. pombe* and is activated by proteolysis during sporulation. The fact that autolysis of cell walls from conjugating cells was stimulated by the addition of trypsin and inhibited by the presence of proteolytic inhibitors gives further support for proteinase activation of glucanases in *S. pombe*. The association of proteolytic activity with isolated cell walls may be significant in this context. Although there are no previous reports of activation of yeast glucanases, an autolytic enzyme in the bacterium *Streptococcus faecalis* is known to occur as a latent enzyme which is activated by proteinases (31). Chitin synthetase activity in yeasts is controlled by proteinase activation (4).

The lytic phenotype of the temperature-sensitive mutant SPT-5 was correlated with increased glucanase activity at 37°C. Unlike the wild-type strain, this mutant contained glucanase II activity during vegetative growth and may be a regulatory mutant, constitutive for glucanase II.

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