Biochimica et Biophysica Acta, 401 (1975) 318—332

BBA 67662

GLUCANASES IN *SCHIZOSACCHAROMYCES*. ISOLATION AND PROPERTIES OF AN EXO-β-GLUCANASE FROM THE CELL EXTRACTS AND CULTURE FLUID OF *SCHIZOSACCHAROMYCES JAPONICUS* VAR. *VERSATILIS*

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(Received August 25th, 1975)

Summary

(1) Cell extracts and extracellular culture fluids of species of the yeast genus *Schizosaccharomyces* exhibited exo-β-(1 → 3)- and exo-β-(1 → 6)-glucanase (EC 3.2.1.-) activities.

(2) Using a combination of Sephadex G-100 and DEAE-cellulose chromatography, the exo-β-(1 → 3)-glucanases from the cell extracts and culture fluid of *Schizosaccharomyces japonicus* var. *versatilis* were purified extensively. The enzymes from either location exhibited similar purification and other properties.

(3) The purified enzymes hydrolysed the β-(1 → 6)-glucosidic linkage in addition to the β-(1 → 3) linkage. Heat denaturation, inhibition and electrophoretic studies indicated that both hydrolytic activities were the properties of a single protein. Laminarin and pustulan hydrolysis followed Michaelis-Menten kinetics. The K_m and V for laminarin hydrolysis were 6.25 mg/ml and 350 μmol of glucose released/min/mg protein, and for pustulan they were 166 mg/ml and 52 μmol of glucose released/min/mg protein.

(4) The exo-β-glucanase was assigned a molecular weight of 43 000.

(5) The purified enzyme failed to hydrolyse isolated cell walls from either baker's yeast or *Schizosaccharomyces pombe* or to induce protoplast formation from intact cells of *S. japonicus* var. *versatilis* or *Saccharomyces cerevisiae*.

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Introduction

The component responsible for the rigidity and shape of the yeast cell wall is primarily a β-(1 → 3)-linked glucan [1]. Enzymes capable of hydrolysing the β-(1 → 3)-glucosidic linkage have been found in yeasts. This occurrence has stimulated suggestions that these glucanases (EC 3.2.1.-) function as cell wall hydrolytic agents (plasticizers) during yeast growth activities such as budding [2,3], conjugation [4,5], ascus lysis [6,7], cell wall extension and expansion [8,9] and autolysis [10,11].

The presence of an exo-β-glucanase in *Saccharomyces cerevisiae* was first reported by Brock [5]. This enzyme hydrolysed both the β-(1 → 3)-linked glucan, laminarin, and the β-(1 → 6)-linked glucan, pustulan. Abd-El-Al and Phaff [6] confirmed this observation and found similar enzymes in *Kluyveromyces fragilis* and *Hansenula anomala. Sacc. cerevisiae* was recently shown to contain endo-β-(1 → 3)-glucanase activity in addition to the exo-β-glucanase [2,3,11,12]. So far, only endo-β(1 → 3)-glucanases have been found in the bipolarly binding yeasts *Hanseniaspora valbyensis* and *Hanseniaspora uvarum* [7].

The present paper describes the isolation and properties of an exo-β-glucanase from the cell extracts and the extracellular culture medium of the fission yeast *S. japonicus* var. *versatilis*. A preliminary report of this work has been made [13] and the isolation and properties of an endo-β-(1 → 3)- and an exo-β-glucanase from the cell walls of *S. japonicus* var. *versatilis* have been described [14]. Bearing in mind the possible function of glucanases as cell wall plasticizers, the action of the enzyme on isolated yeast cell walls was also investigated.

Materials and Methods

Microorganisms and culture conditions. The following cultures were obtained from the culture collection of the Department of Food Science and Technology, University of California, Davis, under the numbers indicated. *Schizosaccharomyces pombe* C-277; *Schizosaccharomyces japonicus* var. *versatilis* 60-255 (referred to in the text as *S. versatilis*); *Schizosaccharomyces octosporus* C-103; *Schizosaccharomyces malidevorans* 70-49. Compressed commercial baker's yeast (Red Star Co., Oakland, Calif.) was purchased from a local market.

Yeast cultures were maintained on 0.5% yeast autolysate (Albimi, Pfizer and Co., New York) 5% glucose agar slants. For general enzyme production and cell wall preparations, the yeasts were grown in a liquid medium of the above composition dispensed as one-l volumes in Fernbach flasks. These were incubated on a rotary shaker (200 rev./min) at room temperature. The cells were harvested by centrifugation. For large scale enzyme and cell wall preparations, the yeasts were grown as 50—60-l batches in an 80-l fermentor as described previously [14].

Preparation of cell extracts and culture fluids. Immediately after harvesting, the yeast cells were washed with 0.05 M sodium succinate buffer, pH 5.0 and disrupted by shaking with glass beads in a Braun homogenizer for 3 min at

4000 oscillations/min with liquid CO_2 as coolant [14]. Glass beads and cell walls were removed from the crude homogenate by centrifugation at 1000—2000 × *g*. The supernatant fraction was recentrifuged at 20 000 × *g* for 30 min to remove particulate material, and this supernatant was used as the cell free extract. All of the purification operations were carried out between 0 and 4°C.

The culture fluid represented the supernatant after removal of the yeast cells from the culture medium by centrifugation.

Enzymatic hydrolysis of cell walls. The isolation and purification of cell walls from *S. pombe* and from commercial baker's yeast have been described previously [13,14]. The walls were lyophylized and stored at 1°C under a vacuum. Alkali-extracted walls of *S. pombe* were prepared as described by Fleet [15].

Weighed portions of dried wall material were suspended in distilled water by mild ultrasonic vibration and then heated to 100°C for 5 min to inactivate endogenous glucanases. The walls were sedimented by centrifugation, resuspended in buffer and 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. Microbial contamination was controlled by the addition of sodium azide (0.01%).

Enzyme assays. Enzyme solution (0.5 ml) 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 were terminated by the addition of sodium carbonate [14] and 1.0 ml samples of the inactivated reaction mixtures were used for the determination of reducing sugars [16]. One unit of glucanase is defined as that amount of enzyme which hydrolyses 1 μmol of reducing sugar equivalent, expressed as glucose, per min under the standard assay conditions.

Proteolytic [17] and phosphatase [11] activities were determined using Azocoll (Calbiochem) and *p*-nitrophenylphosphate (Calbiochem) as substrates, respectively. The substrates, 10 mg/ml, were prepared in 0.05 M sodium succinate buffer, pH 5.0.

Substrates. Laminarin (Nutritional Biochemical Co., Cleveland, Ohio, U.S.A.) was routinely used as the substrate for β-(1 → 3)-glucanase assays. Pustulan [6] was used for the estimation of β-(1 → 6)-glucanase activity. Pseudonigeran was used in the determination of α-(1 → 3)-glucanase activity and was prepared from the mycellium of *Aspergillus niger* NRRL 326 by a modification [15] of the procedures described by Johnston [18] and Hasegawa et al. [19]. Detailed information on the properties of the above substrates has been described previously [14]. Pachyman [20], periodate oxidized laminarin [21], laminaribiose, laminaritriose, oat glucan, cellulose dextrins, baker's yeast mannan, and phosphomannan (*Hansenula holstii*) were obtained from the laboratory collection [6,7,14]. All other substrates were obtained commercially.

Analytical measurements. Reducing sugars were determined by the Nelson-Somogyi method [16]. Glucose was used for the construction of a standard curve. Glucose concentrations were also measured using Glucostat special reagent as recommended by the manufacturer (Worthington Biochemical Corporation). Protein was estimated by the method of Lowry et al. [22] using bovine serum albumin as standard. Total carbohydrate was determined by the phenolsulphuric acid method [23].

Chromatography. The products of carbohydrase reactions were identified using descending paper chromatography on Whatman No. 1 paper at 25°C and the following solvent system: ethylacetate/pyridine/water in the ratio 12 : 5 : 4 by vol. Sugar spots on the chromatograms were detected by the alkaline silver nitrate reagent [24].

Details of column chromatographic procedures have been given elsewhere [14]. In addition, hydroxyapatite was prepared according to the method of Tiselius et al. [25] and packed into columns as described by Bernardi [26].

Gel electrophoresis. Protein molecular weight was determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to the procedure described by Weber and Osborn [27]. Conventional gel electrophoresis whereby enzyme activity was preserved was done as described previously [14].

Results

Survey of glucanases in the fission yeasts

As seen from Table I, very low levels of β-(1 → 3)- and β-(1 → 6)-glucanase activities were found in both cell extracts and culture fluids of *S. pombe*, *S. octosporus* and *S. malidevorans*. Extracts of *S. versatilis* showed by far the highest levels of β-(1 → 3)-glucanase activity and significant amounts of this enzyme were also excreted into the culture medium. No α-(1 → 3)-glucanase activity was noted in any of the preparations. Enzyme activities were measured immediately after extract preparation and again after 24 h of dialysis of the extract. Similar results were found at both times, except with cell extracts from *S. pombe* where activity losses were encountered on storage. Similar enzyme instability was reported with extracts of *H. anomala* [6]. Because of the high levels of β-(1 → 3)-glucanase in both the extracts and culture fluid of *S. versatilis*, the enzymes of this yeast were chosen for detailed study.

TABLE I

A COMPARISON OF THE GLUCANASE ACTIVITIES IN THE SPECIES OF THE GENUS *SCHIZOSACCHAROMYCES*

The different species were grown and prepared under conditions as described in Materials and Methods except that *S. octosporus* was grown in a medium of 20% glucose rather than 5%. All cultures were harvested in the late exponential phase of growth. In the case of *S. pombe*, *S. malidevorans* and *S. octosporus* enzyme assays were continued for up to 5 h.

Yeast species	Cell extracts*			Culture fluids**		
	β-(1→3)-glucanase	β-(1→6)-glucanase	α-(1→3)-glucanase	β-(1→3)-glucanase	β-(1→6)-glucanase	α-(1→3)-glucanase
S. pombe	2	2	0	***	***	0
S. versatilis	630	8	0	150	2	0
S. octosporus	2	0.3	0	2	2	0
S. malidevorans	1	0.5	0	2	1	0

* Units/mg of protein × 10^3. Protein concentrations for the cell extracts in descending order for species were: 1.0, 1.7, 2.4 and 5.0 mg/ml.

** Units/ml.

*** Low levels of activity detectable only after concentration of the culture fluid.

Variation of β-(1 → 3)-glucanase activity with culture age

When grown with agitation in yeast autolysate-glucose medium, *S. versatilis* passes through the following morphogenic stages (i) normal vegetative growth during which the cells divide by fission; (ii) conjugation and zygote formation which is accompanied by extensive agglutination of the culture; (iii) ascus development and sporulation; and (iv) lysis of the asci and liberation of the spores (cf. ref. 28). Analyses of cell extracts from cultures at the different stages of growth showed β-(1 → 3)-glucanase activity to be highest during the conjugation phase. At this stage the enzyme activity was 14.3 units (expressed as units per g dry wt of cells) while in the vegetative and sporulating cultures, the activities were 4.2 and 6.7 units respectively.

Purification of the β-(1 → 3)-glucanase from cell extracts

Quantitative evidence was previously given showing that the β-(1 → 3)-glucanase in crude cell extracts of *S. versatilis* was an exo-enzyme i.e. hydrolysed terminal glucose units from laminarin [13]. Endo-β-(1 → 3)-glucanase activity was not detectable in such extracts since activity measurements by specific glucose determination and reducing group formation were similar and periodate oxidized laminarin was not hydrolysed [29,30].

Cell-free extracts were prepared as described under Methods from the growth of a 50-l fermentor culture of the yeast harvested during the conjugation phase. The extract was dialysed against 5 mM sodium succinate buffer, pH 5.5 at 1°C and then freeze dried. The dried material was reconstituted in distilled water and centrifuged at 20 000 × *g* for 20 min to remove insoluble matter. Glucanase activity remained in the supernatant fraction and no loss was noted after these treatments.

Samples of the concentrated enzyme were subjected to gel filtration on Sephadex G-100 as described in Fig. 1. Fractions 59 to 81 containing β-(1 → 3)-glucanase activity were combined and dialysed against 0.01 M sodium succi-

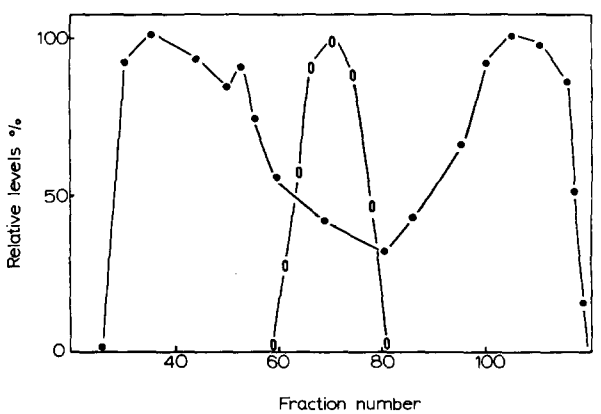

Fig. 1. Sephadex G-100 chromatography of the exo-β-glucanase. A 12-ml sample of enzyme was applied to a column (100 × 2 cm) equilibrated with 0.05 M sodium succinate buffer, pH 5.5. The bed volume of the column was 330 ml and the void volume was 125 ml. Elution was done with 0.05 M sodium succinate buffer, pH 5.5. Fraction volumes of 4.0 ml were collected and the flow rate was 0.3 ml per min. Protein (●), β-(1 → 3)-glucanase activity (○).

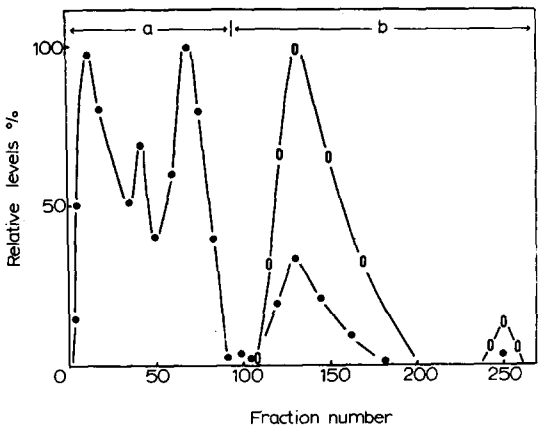

Fig. 2. DEAE-cellulose chromatography of the exo-β-glucanase. The enzyme was applied to a column (25 X 1.5 cm) of DEAE cellulose equilibrated with 0.01 M sodium succinate buffer, pH 5.5. After washing with the equilibrating buffer, the column was eluted with (a) a linear gradient to 0.02 M sodium succinate buffer containing 0.2 M NaCl pH 5.5 (total volume 500 ml), and (b) a linear gradient to 0.02 M sodium succinate buffer containing 0.75 M NaCl pH 5.5 (total volume 900 ml). The flow rate was 0.4 ml per min and fraction volumes of 5.2 ml were collected. Protein (●) β-(1 → 3)-glucanase activity (○).

nate buffer, pH 5.5. The enzyme was then loaded onto a DEAE—cellulose column equilibrated with the same buffer and eluted in a two-step procedure as described in Fig. 2. Two peaks of activity were obtained. The major peak I eluted at 0.33 M NaCl while the minor peak II eluted later at 0.7 M NaCl. Fractions 105—109 of peak I were discarded because of potential contamination with inert protein. The remaining fractions of Peak I and all those of Peak II were combined separately and dialysed against 0.05 M sodium succinate buffer, pH 5.5.

A sample of the purified enzyme (Peak I) after concentration by ultrafiltration through a PM-10 membrane (Amicon Corp.) and rechromatography on Sephadex G-100 eluted as a single protein band. A summary of the purification procedure is presented in Table II.

Purification of the extracellular β-(1 → 3)-glucanase

The essential details of this procedure have been described previously [13]. Elution behaviour of the enzyme of Sephadex G-100 and DEAE-cellulose was identical to that described above for the glucanase from cell extracts.

Properties of the purified glucanases

Unless indicated otherwise, the following results apply only to the enzymes represented by Peak I of the DEAE-cellulose columns. The extracellular and intracellular glucanases exhibited similar properties.

(i) Substrate specificity and action pattern. The following carbohydrate substrates were not hydrolysed by samples of the purified enzyme: cellulose dextrins, oat glucan, pseudonigeran, starch, baker's yeast mannan, phosphomannan, trehalose. As expected, the β-(1 → 3)-glucans, laminarin and pachyman, along with their oligosaccharides, laminaribiose and laminaritriose, were readily hydrolysed. Periodate-oxidized laminarin [30] was not cleaved. The

β-(1 → 6)-glucan pustulan, and its disaccharide gentiobiose were hydrolysed by the enzymes although at a much lower rate than the β-(1 → 3)-glucans. *p*-Nitrophenyl-β-D-glucoside was readily cleaved. Proteolytic and phosphatase activities were not detectable in the purified enzyme preparations.

The products of laminarin and pustulan hydrolysis were monitored paper chromatographically as a function of reaction time. Enzyme concentrations of 0.25 and 1.0 units/ml were used in these reactions. With laminarin (5 mg/ml), glucose was the only product detectable in the early stages (up to 1 h) of hydrolysis. In the later stages (12 h) traces of laminaribiose and laminaritriose were noted in addition to glucose. Glucose was the only product observed at any time during the hydrolysis of pustulan. Traces of gentiosaccharides were not found, presumably because of the very weak affinity of the enzyme for pustulan (see later). These results are consistent with the action pattern of other exo-glucanases [29,31].

(ii) Stability and heat denaturation. The β-(1 → 3)-glucanase activity was stable in 0.05 M sodium succinate buffer, pH 5.0—5.5 for at least 3 months at 1—4° C. Sodium azide (0.01%) was effective in controlling microbial contamination without affecting enzyme activity. Merthiolate (a mercuric thiol preservative) at a concentration of 0.01% caused a 70% inhibition of β-(1 → 3)-glucanase activity. Twice freezing and thawing of the enzyme did not cause any activity loss, but storage in the frozen state after eight months gave only a 50% activity recovery. Freeze drying of the purified enzyme gave a 50% activity loss. Heat inactivation curves for both β-(1 → 3)- and β-(1 → 6)-glucanase activities are presented in Fig. 3. The activities were stable at 50° C for up to 30 min, but at higher temperatures, the two activities underwent inactivation at similar rates.

(iii) Kinetics. Laminarin hydrolysis was linear for up to 40 min (0.1 unit/ml of reaction mixture, pH 5.0). Both laminarin and pustulan hydrolyses ex-

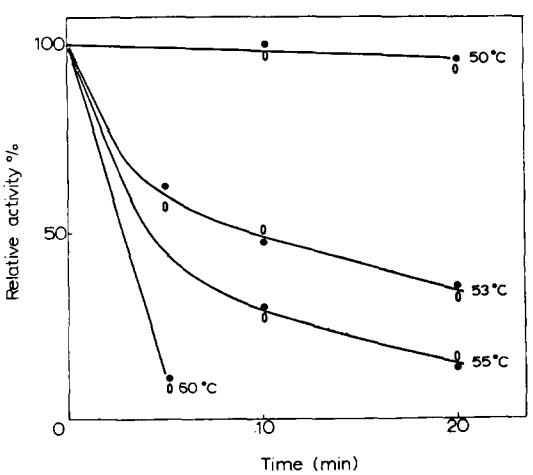

Fig. 3. Heat inactivation of the purified exo-β-glucanase. The enzyme (0.5 unit) was heated in the presence of 0.5 M sodium succinate buffer, pH 5.0. Samples were taken and activities determined as described in Methods. Laminarin substrate (●) pustulan substrate (○).

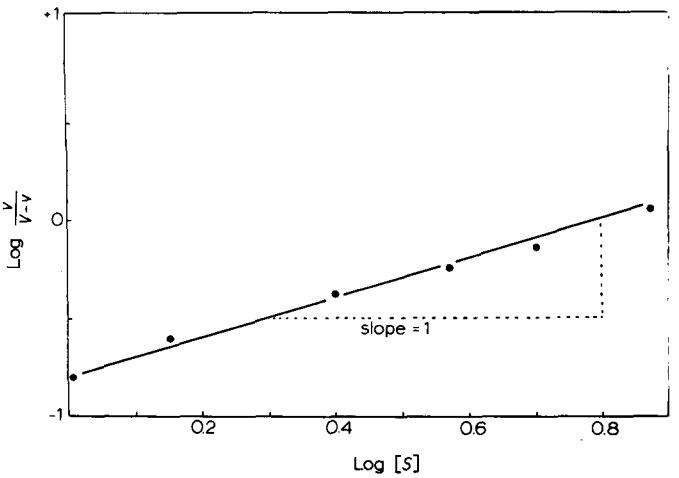

Fig. 4. A plot of log $v/(V-v)$ versus log S according to the Hill equation for the exo-β-glucanase (laminarin substrate).

hibited a pH optimum of 5.0 and followed Michaelis-Menten kinetics. Fig. 4 shows a plot of the data for laminarin hydrolysis according to the Hill equation [32]. A straight line of unity slope was obtained and is characteristic of enzymes following Michaelis-Menten kinetics. This form of representation is provided here as a comparison with a similar plot for the endo-β-(1 → 3)-glucanase from *S. versatilis* which showed deviations from Michaelis-Menten kinetics [14]. Both intracellular and extracellular enzymes exhibited similar kinetic constants. A K_m value of 6.25 mg/ml and a V of 350 μmol of glucose released/min/mg protein were found for laminarin hydrolysis. Pustulan hydrolysis gave a K_m value of 166.6 mg/ml and a V of 52 μmol of glucose released/min/mg of protein.

Activation and inhibition

Metal ion effects on β-(1 → 3)-glucanase activity were determined as described previously [14]. The enzyme activity was not stimulated by any of the following ions: Ca^{2+}, Mg^{2+}, Co^{2+}, Zn^{2+}, Hg^{2+}, K^+, Na^+. However, Zn^{2+} and Hg^{2+} at concentrations up to 10^{-3} M were inhibitory to enzyme activity. The plant hormone, auxin (indole-3-acetic acid) at 20 mg/l and 2-deoxy-D-glucose (12.5 mM and 25.0 mM) gave slight inhibitions (less than 10%) of laminarin hydrolysis.

Glucono-δ-lactone inhibited both β-(1 → 3)- and β-(1 → 6)-glucanase activities. Fig. 5 shows the degree of inhibition of β-(1 → 3)-glucanase activity with increasing concentration of inhibitor. Identical curves were obtained using varying laminarin concentrations indicating the non-competitive nature of the inhibition. The shape of the inhibition curve is also characteristic of enzymes conforming to Michaelis-Menten kinetics [32] (cf. endo-β-(1 → 3)-glucanase from *S. versatilis*, [14]). The non-competitive nature of glucono-δ-lactone inhibition was confirmed by plotting reaction velocity versus substrate concentra-

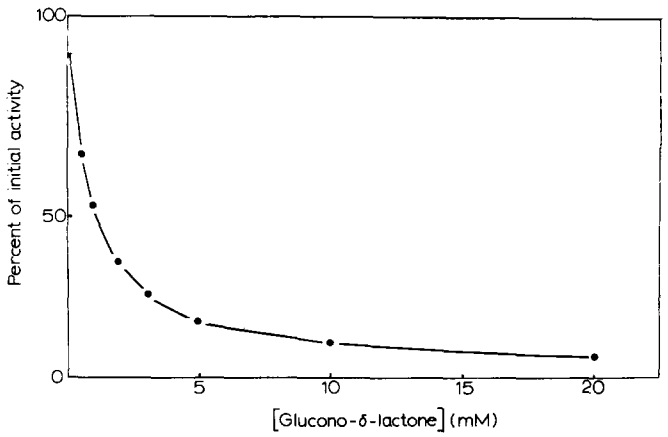

Fig. 5. The effect of glucono-δ-lactone concentration on exo-β-(1 → 3)-glucanase activity. The inhibitor was prepared immediately before use.

tion in the presence and absence of inhibitor [32] and showing an alteration of V. Both laminarin hydrolysis and pustulan hydrolysis were inhibited to the same extent by a given concentration of inhibitor. The inhibitor constant for both activities was 0.9 mM glucono-δ-lactone (Fig. 6).

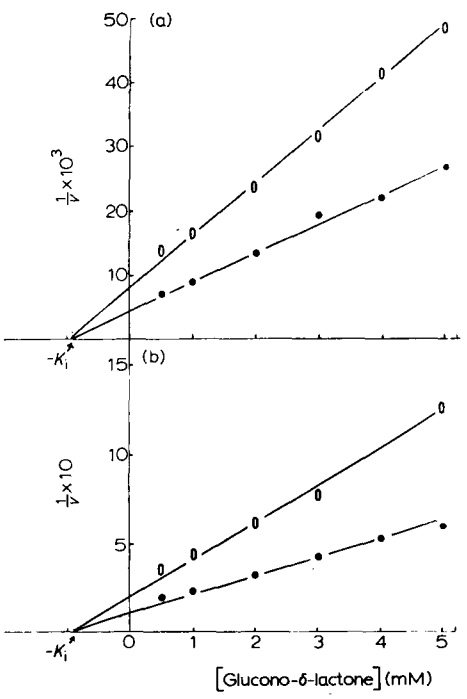

Fig. 6. Determination of the inhibitor constant, K_i, for glucono-δ-lactone. Various levels of glucono-δ-lactone were incubated with the exo-β-glucanase and substrate. Enzyme activities are expressed as μmol of reducing equivalents released/min/mg protein. Laminarin substrate, (a) pustulan substrate (b). Substrate concentration, 5 mg/ml, (●) 2.5 mg/ml (○). The intercept of the two lines was used to calculate K_i [32].

TABLE II

SUMMARY OF VARIOUS STEPS APPLIED IN THE PURIFICATION OF INTRACELLULAR EXO-β-GLUCANASE FROM *S. VERSATILIS*

Activity units were determined using 0.4% laminarin, pH 5.5, as the substrate. The total eluate from the Sephadex column was the result of nine separate applications.

Step	Volume (ml)	Concentration of protein (mg/ml)	Activity (units/ml)	Specific activity (units/mg of protein)	Purification	Recovery (%)
Dialyzed intracellular preparation	3000	1.71	0.73	0.43		100
Lyophilization, reconstitution and centrifugation	91	47.6	24.74	0.52	1.2	103
After Sephadex G-100	810	0.17	2.03	11.95	27.8	75
After DEAE-cellulose						
Peak (I)	700	0.006	1.08	180.0	418.6	34.4
Peak (II)	100	—	0.088	—	—	0.4

Evidence for a single protein possessing both β-(1 → 3)- and β-(1 → 6)-glucanase activities

The heat-denaturation curves and glucono-δ-lactone inhibition studies presented above suggested that a single protein might be responsible for both glucanase activities. The ratio of β-(1 → 3)-glucanase activity to β-(1 → 6)-glucanase activity was 100 : 1 for both the purified and crude preparations of the intracellular and extracellular enzymes. This value remained constant throughout the various purification steps (Table II) including passage of the enzymes over CM-cellulose and hydroxyapatite columns on which they did not adsorb (CM-cellulose equilibrated with 0.01 M sodium succinate buffer, pH 5.0; hydroxyapatite equilibrated with 0.01 M sodium phosphate buffer, pH 6.8). Some variation in the ratio of the two activities would be expected during these various purification steps if two separate proteins were involved.

Further evidence for the dual activity of an apparently single protein was provided by its electrophoretic behaviour. Analysis of polyacrylamide gels run at pH 5.0 (sodium succinate buffer), pH 6.0 and 7.0 (sodium phosphate buffer) gave only one major band of protein. The corresponding region in an unstained identically treated gel exhibited both β-(1 → 3)- and β-(1 → 6)-glucanase activities in the expected ratio of 100 : 1. Only one band of protein was noted on sodium dodecyl sulfate-gel electrophoresis and on the basis of its migration a molecular weight of 43 000 was calculated for the enzyme.

The mixed substrate method [6,33] for ascribing multiple activities to a single protein was not applicable to the present enzyme because of the very poor affinity for one of its substrates (pustulan).

DEAE-cellulose peak II glucanase

Insufficient amounts of this protein were obtained for a detailed analysis. However, this enzyme was an exo-β-(1 → 3)-glucanase and it also hydrolysed pustulan.

Action of the exo-β-glucanase (peak I) on isolated yeast cell walls and intact cells

The pure exo-β-glucanase was studied for its ability to hydrolyse isolated cell walls of *S. pombe* and of baker's yeast. Walls of *S. pombe* were chosen for this study rather than *S. versatilis* walls for reasons explained elsewhere [14]. Initial studies with cell wall agar plates [34] showed no hydrolysis of baker's yeast or *S. pombe* walls with enzyme concentrations up to 75 units/ml.

Cell wall hydrolysis experiments were set up as described under Methods and in Table III. As seen from the table, the exo-β-glucanase caused negligible hydrolysis of either *S. pombe* or baker's yeast cell walls. The small initial hydrolysis was possibly due to the digestion of extensively fragmented cell wall material or to the hydrolysis of the more complete walls at the points of rupture where the wall glucan may be exposed. Electron microscopic observations of untreated and enzyme-treated walls revealed no overall differences [35]. Pretreatment of the cell walls with dithiothreitol did not promote further hydrolysis. However, alkali extracted walls of *S. pombe* underwent approx. 10% hydrolysis (Table III) in the presence of the exo-β-glucanase.

Findings similar to those shown in Table III were obtained when cell wall digestion was followed by the release of total carbohydrate [23].

Intact cells

Exponential-phase cells of *S. versatilis* and *Sacc. cerevisiae* were prepared for protoplast formation as described previously [14]. Incubation of these yeast cells in the presence of up to 100 units/ml of the exo-β-glucanase failed to result in any protoplast formation or cell lysis upon dilution of the osmotic stabilizer.

TABLE III

ACTION OF PURIFIED EXO-β-GLUCANASE FROM *S. VERSATILIS* ON CELL WALLS OF *S. POMBE* AND BAKER'S YEAST

The values in the table represent reducing sugar release expressed as μg of glucose/ml. Wall substrates were used at a concentration of 5 mg/ml and final enzyme concentration in the suspensions was 2 units/ml in 0.01 M sodium succinate buffer, pH 5.0. The control suspensions contained buffer only. The enzyme remained active throughout incubation.

Wall materials	Time (h)								
	0	2	3	5	10	14	25	36*	12
S. pombe (control)	0	0	0	0	0	0	0	0	0
S. pombe + enzyme	0	56	65	—	66	68	65	68	3
S. pombe, pretreated with dithiothreitol; + enzyme	0	54	61	—	63	65	65	63	2
S. pombe, alkali extracted; + enzyme	0	195	228	251	308	325	405	405	56
Baker's yeast (control)	0	0	0	0	0	0	0	0	—
Baker's yeast + enzyme**	0	17	17	19	24	33	44	44	—

* A this time the walls were retrieved by centrifugation, washed in buffer, and resuspended in fresh enzyme for further incubation.

** Similar results were obtained after pretreatment of the walls with dithiothreitol.

Discussion

Nature of the glucanases in fission yeasts

In addition to β-(1 → 3)-glucan, the cell walls of *Schizosaccharomyces* species contain around 20% of an α-(1 → 3)-linked glucan [1]. It was reasonable therefore to check these yeasts for the presence of an α-(1 → 3)-glucanase. However, in line with previous studies by Barras [36], no such activity was found. Interestingly, α-(1 → 3)-glucanase activity has been found in the extracts of *Aspergillus nidulans* which, like *Schizosaccharomyces* species, has cell walls containing α-(1 → 3)-linked glucan [37]. In this case, α-(1 → 3)-glucanase production was a function of cultural conditions [38] and it is possible that in the present study with *Schizosaccharomyces*, the necessary conditions for enzyme production may not have been used.

In contrast to the high levels of exo-β-(1 → 3)-glucanase, cell extracts of *S. versatilis* exhibited no endo-β-(1 → 3)-glucanase activity. This was unexpected since the cell walls of this yeast contain endo-β-(1 → 3)-glucanase activity which is readily released from the cell walls by autolysis immediately on cell disruption [14]. It is possible therefore that the detection of endo-activity in the cell extracts might be supressed by some inhibitory substance such as occurs with the chitin synthetase system [39]. Both exo- and endo-β-(1 → 3)-glucanase activities have been demonstrated in extracts of *Sacc. cerevisiae* [3,12] while extracts of the bipolarly budding yeasts *Hanseniaspora valbyensis* and *Hanseniaspora uvarum* exhibit only endo-β-(1 → 3)-glucanase activity [7]. At present, these differences cannot be interpreted.

Properties of the exo-β-glucanase

The exo-β-glucanase from *S. versatilis* exhibited substrate specificities similar to those described for the enzymes from *Sacc. cerevisiae*, *Kluyveromyces fragilis*, and *H. anomala* [5,6] in that it hydrolysed both laminarin and pustulan. All evidence suggests that a single protein is responsible for both hydrolytic activities and this may be a general property of all yeast exo-β-glucanases. The kinetic parameters K_m and V for these two substrates vary with the different yeast species (cf. ref. 6), but a physiological interpretation of these differences is not yet possible. The other well-studied exo-β-(1 → 3)-glucanases from Basidiomycete QM806 [40,29] and from *Euglena gracilis* [41] do not hydrolyse pustulan or laminaribiose. The inhibitory action of Hg^{2+} and the lack of effects of EDTA and iodoacetamide on the *S. versatilis* enzyme are consistent with the properties of exo-β-(1 → 3)-glucanases in general [42,43].

Auxin causes cell wall expansion in some yeasts and it was suggested that this hormone acts by stimulating endogenous exo-β-(1 → 3)-glucanase action [9]. Auxin did not stimulate the in vitro exo-β-(1 → 3)-glucanase activity of *S. versatilis* suggesting that this hormone acts at the level of enzyme synthesis rather than on the enzyme itself. The metabolic analogue, 2-deoxy-D-glucose, had no stimulatory effect on *S. versatilis* exo-β-(1 → 3)-glucanase activity, supporting the view that this compound induces yeast cell lysis [8] by interference with cell wall glucan synthesis rather than by an activation of lytic enzymes.

Glucono-δ-lactone is an inhibitor of glycosidase- and glucanase-catalysed reactions [44,45,42]. Both the β-(1 → 3)- and β-(1 → 6)-glucanase activity of

the *S. versatilis* enzyme were sharply inhibited by this compound. Although aldonolactone inhibition of glycosidase activities is competitive [46], differing results have been obtained on the nature of glucanase inhibition by glucono-δ-lactone. Farkas et al. [12] have found that exo-β-(1 → 3)-glucanase from *Sacch. cerevisiae* was competitively inhibited by glucono-δ-lactone. In contrast, the action of this inhibitor on the exo-β-(1 → 3)-glucanase activity of *S. versatilis* was non-competitive.

Action of the exo-β-glucanase on yeast cell walls

In contrast to the inactivity of the exo-β-glucanase on yeast cell walls, the endo-β-(1 → 3)-glucanase from *S. versatilis* causes extensive degradation [14]. On consideration of the limited action of an exo-enzyme as opposed to an endo-enzyme, this difference is not unexpected. The β-(1 → 3)-glucan of yeast cell walls is a branched molecule with side chain branches terminating in non reducing glucose residues [47,48]. The inability of the exo-β-glucanase to hydrolyse intact cell walls suggests that the terminals of the glucan side chains may be buried or masked in the amorphous matrix of the wall. Alternatively, they may be involved in linkages with other molecules and thus be unavailable for enzyme hydrolysis. Side chains of the wall mannan molecule which are substituted with phosphate or *N*-acetylglucosamine are resistant to hydrolytic cleavage by an exo-mannanase [49,50]. In the case of *S. pombe* cells walls, we have shown limited hydrolysis by the exo-enzyme (Table III) after removal of the α-(1 → 3)-glucan and galactomannan components by alkali extraction [51, 52].

The marked inactivity of the yeast exo-β-glucanase on yeast cell walls or intact cells is in sharp contrast with the action of the exo-β-(1 → 3)-glucanase from Basidiomycete QM806. Bush and coworkers have demonstrated extensive hydrolysis of *S. pombe* and *Sacc. cerevisiae* cell walls by this fungal enzyme [53,54]. Although it is difficult to reconcile these results with the limited action of an exo-enzyme, some understanding of the effectiveness of this enzyme may be found in (i) its much higher affinity for laminarin compared to the yeast enzyme [40] (ii) its ability to bypass β-(1 → 6)-glucosidic linkages when they occur in β-(1 → 3)-glucans (see ref. 29 for details). Since no evidence was provided as to the homogeneity of the fungal enzyme preparation used in these studies [53,54], the possibility of contamination with a minor proportion of endo-β-(1 → 3)-glucanase [55] cannot be overlooked.

Location and function of the yeast exo-β-glucanase

Although the exo-β-glucanase of *S. versatilis* can be isolated from cell extracts, the extra-cellular fluid and isolated cell wall fractions [14], the in vivo location(s) of this enzyme has not been established. With *Sacc. cerevisiae* the bulk of the exo-β-glucanase activity occurs in vivo external to the plasmalemma and is entrapped within the periplasmic space. Only a small percentage of the activity is truly intracellular and this is confined to membranous vesicles [3]. As mentioned in the introduction, yeast exo-β-glucanases are thought to function as cell wall plasticizers and consequently it was reasonable to expect hydrolytic action of these enzymes on isolated yeast cell walls. Such activity was not observed in the present studies. On general principles of hydrolytic

cleavage and as shown elsewhere [14], an endo-β-(1 → 3)-glucanase would be a more effective wall plasticizer than an exo-β-(1 → 3)-glucanase. The exo-enzyme could play a secondary role of rapidly degrading the short oligosaccharide fragments generated by the endo-enzyme action. The glucose so produced could then be reutilized by the cells metabolic machinery.

Although the present in vitro experiments do not suggest a wall hydrolytic role in the exo-β-glucanase, there are other observations which suggest a definite function of this enzyme in that capacity. As noted in the present studies with *S. versatilis* and also by Brock [4] with *Hansenula wingeii*, exo-β-glucanase activity is significantly increased in conjugating cultures where localized cell wall dissolution would be required. Abd-El-Al and Phaff [6] have noted a correlation between the rapidity of ascus lysis in some yeasts and exo-β-glucanase level, thereby implying a role of the enzyme in ascus wall hydrolysis. In the present investigation, *S. versatilis* exhibited abundant sporulation and rapid ascus lysis under the growth conditions employed, and possessed high exo-β-glucanase activity. In contrast, the other three species of Schizosaccharomyces (Table I) did not sporulate and exhibited only very low exo-β-glucanase activity. As found in *Sacc. cerevisiae* exo-β-glucanase activity increasing immediately before the onset of budding thereby suggesting an involvement of this enzyme in the wall hydrolytic activities of cell division [3].

It is evident from these contradictory observations that a clearer assessment of the role of exo-β-(1 → 3)-glucanases in yeasts must await further study. In this respect it would be advantageous to have mutants lacking in this enzyme.

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