JOURNAL OF BACTERIOLOGY, Aug. 1977, p. 702-706

Vol. 131, No. 2
Printed in U.S.A.

Survey for α-(1 → 3)-Glucanase Activity Among Yeasts

MELVIN T. MEYER AND H. J. PHAFF*
Department of Food Science and Technology, University of California, Davis, California 95616

Received for publication 8 February 1977

Activity of α-(1 → 3)-glucanase was found in species of *Cryptococcus*, *Rhodotorula*, and *Endomyces*. Observations on the expression and stability of this enzyme in *Rhodotorula minuta* var. *texensis* are presented.

Yeast cell walls contain a number of polysaccharides. The major structural component is an alkali- and acid-insoluble β-(1 → 3)-glucan (15, 19). An alkali-soluble β-(1 → 3)-glucan and an acid-soluble β-(1 → 6)-glucan of bakers' yeast have been characterized as well (9, 15). Additional wall glucans, consisting mainly of α-(1 → 3) linkages, occur in certain yeasts (3, 5, 21). α-(1 → 3)-Glucan also occurs in the cell wall of several higher fungi (12, 13, 17, 25). Except in one instance (25), where the polysaccharide serves as a storage carbohydrate, its function is unknown or presumed to be structural (10).

The presence of both intracellular and/or wall-associated endogenous enzymes capable of hydrolyzing the β-(1 → 3)-glucosidic linkage has been documented in several yeasts (1, 2, 4, 10, 18). Both endo- and exo-acting β-(1 → 3)-glucanases occur. The exoenzymes have the dual activity of hydrolyzing both β-(1 → 3) and β-(1 → 6) linkages (1, 11, 18). It has been postulated that these hydrolases are involved in various physiological functions requiring wall softening, such as wall expansion, budding, conjugation, and ascus lysis during sporulation. It is reasonable to postulate the occurrence of α-(1 → 3)-glucanases in yeast species that contain this particular wall polymer, especially if this component is a structural entity.

While a few yeasts are known to contain considerable proportions of α-(1 → 3)-glucan in the cell wall, there are no reports of α-(1 → 3)-glucanase activity associated with any yeast. *Aspergillus nidulans* synthesizes an α-(1 → 3)-glucan that serves as a reserve polysaccharide (25). During fructification, an exo-α-(1 → 3)-glucanase is synthesized for the utilization of this glucan (26). There are a limited number of other higher fungi (20) known to synthesize extracellular α-(1 → 3)-glucanases; these are thought to be involved in degrading exogenous glucan. Efforts to detect α-(1 → 3)-glucanase activity in species of the yeast genus *Schizosaccharomyces* (10), which contain a considerable proportion of α-(1 → 3)-glucan in their cell walls (5), by using water-insoluble α-(1 → 3)-glucan as the assay substrate, yielded negative results.

In the present work, several selected yeast species were investigated for α-(1 → 3)-glucanase activity by use of a soluble α-(1 → 3)-glucan assay substrate. β-(1 → 3)- and β-(1 → 6)-glucanase activities were determined at the same time for comparative purposes. *Rhodotorula minuta* var. *texensis*, which exhibited a relatively high level of α-(1 → 3)-glucanase activity, was examined further with respect to the expression and stability of the enzyme.

The organisms used for the study were obtained from the culture collection of the Department of Food Science and Technology, University of California, Davis, and are listed in Table 1. Cultures were maintained on agar slants of 0.5% yeast autolysate (Albimi Laboratories, Flushing, N.Y.) and 5% glucose. Cells were grown in liquid medium of this composition dispensed in 1-liter volumes in Fernbach flasks. The flasks were inoculated with exponential-phase cells (0.5%, vol/vol) and incubated on a rotary shaker (ca. 150 rpm) at room temperature (ca. 25°C). Cells were collected in late exponential phase of growth by refrigerated centrifugation. The culture supernatants were dialyzed for 24 h against distilled water at 4°C. Immediately after harvest, the cells were washed three times with 0.1 M sodium succinate buffer (pH 5.5) and resuspended as a thick slurry in the same buffer. This pH was chosen since an extract (see below) prepared of *R. minuta* in distilled water showed a pH of 5.6. The cells were then disrupted in a Braun MSK mechanical cell homogenizer (11). After the crude homogenate was decanted from the glass beads, the suspension was centrifuged at 16,000 × *g* (20 min at 4°C) followed by recentrifugation of the resulting supernatant fraction at 27,000 × *g* for 40 min. This second supernatant solution was used as the crude intracellular extract and assayed immediately for hydrolytic activities. *Cryptococcus* extracts were dialyzed for 24 h (as described for the culture supernatants) and then assayed. The cell wall pellets from the

TABLE 1. *Survey of glucanase levels in selected yeasts*[a]

Yeast	Culture	α-(1→3)-Glucanase			β-(1→3)-Glucanase			β-(1→6)-Glucanase		
		W[b]	IE[c]	CF[d]	W	IE	CF	W	IE	CF
Arthroascus javanensis	67-278	0.0	0.0	0.0	0.795	0.009	0.012	0.110	0.002	0.007
Cryptococcus albidus	68-150	0.134	0.004	0.0	2.63	0.113	0.023	1.50	0.009	<0.001
C. albidus	68-154	0.0	0.001	0.0	0.314	0.001	0.0	0.054	0.0	0.0
C. infirmo-miniatus	68-191	0.003	<0.001	0.0	0.131	0.005	0.0	0.0	<0.001	0.0
C. terreus	57-11	0.0	0.003	0.0	0.129	0.005	0.002	0.0	0.002	0.0
Endomyces reessii	60-21	0.005	<0.001	0.0	0.944	0.014	0.080	0.054	0.001	0.004
E. geotrichum	71-81	0.0	0.0	0.0	2.24	0.031	0.071	0.075	0.002	0.004
E. tetrasperma	68-122	0.035	0.0	0.0	2.88	0.446	0.035	0.214	0.007	0.0
Phaffia rhodozyma	67-210	0.0	0.0	0.0	3.61	0.176	0.001	0.196	0.0	0.0
Rhodotorula glutinis	68-259	0.083	0.001	<0.001	0.301	0.022	0.0	0.0	0.0	0.0
R. minuta var. *texensis*	68-288	0.092	0.001	<0.001	0.352	0.002	0.0	0.0	0.0	0.0
Schizosaccharomyces pombe	C-277	0.0	0.0	0.0						
S. malidevorans	70-49	0.0	0.0	0.0						
S. octosporus	C-103	0.0	0.0	0.0						

[a] Culture conditions, extracts, wall preparations, and method of activity assay are presented in text.
[b] Wall preparations; activity expressed as units per gram (dry weight).
[c] Intracellular extract; activity expressed as units per milligram of protein.
[d] Culture fluid; activity expressed as units per milliliter.

first centrifugation were washed with 0.1 M sodium succinate buffer (pH 5.0) and centrifuged at 5,000 × *g* (15 min at 4°C). This washing step was repeated five times, after which the cell walls were diluted sufficiently to enable preparation of an accurate volumetric measure for assay. *Cryptococcus* cell walls were dialyzed (as previously described for the culture filtrates) and then assayed. The standard assay consisted of 0.25 ml of a suitably diluted enzyme sample incubated for 15 to 60 min with 0.25 ml of substrate (10 mg/ml) at 30°C and with gentle agitation. All reported activities were corrected for endogenous release of reducing groups; depending on the yeast species, the reaction rates with walls were 1.3 to 1.9 times higher with than without added substrate. Enzyme concentrations were chosen so that reaction velocities were approximately linear during the reaction periods. β-(1 → 3)- and β-(1 → 6)-glucans were dissolved in 0.1 M sodium succinate (pH 5.0), whereas carboxymethyl-α-(1 → 3)-glucan was dissolved in 0.1 M sodium succinate buffer at pH 6.0. This pH was arbitrarily selected for the survey since it is close to the pH of a cell extract prepared in water. We realize that there may be variations in pH optimum of α-(1 → 3)-glucanases among the various species. Assays were done in triplicate and included a control (buffer without substrate). Reactions were terminated by heating for 5 min at 100°C, after which 0.5 ml of water was added. In the case of homogenates of *Rhodotorula*, heating appeared to be insufficient to stop all of the β-(1 → 3)-glucanase activity; consequently, 0.175 ml of 1 M sodium carbonate was added to terminate this reaction.

Reducing-sugar values were determined on 0.5-ml portions of the inactivated reaction mixtures (22). Before the determination, cell walls were removed by centrifugation and all tubes were centrifuged again immediately before spectrophotometric recording. One unit of activity is defined as the amount of enzyme that releases 1 μmol of reducing equivalents (as glucose) per min under the prescribed conditions. Laminarin (Nutritional Biochemicals Corp., Cleveland, Ohio) and pustulan (Calbiochem, San Diego, Calif.) were used for measuring β-(1 → 3)- and β-(1 → 6)-glucanase activities, respectively. α-(1 → 3)-Glucan, purified from *Aspergillus niger* NRRL 326 (10), was converted to the carboxymethyl derivative by established procedures (6, 23). The final product, being just soluble in the sodium succinate buffer at a concentration of 1% (wt/vol), had a degree of substitution of approximately 0.17 carboxymethyl group per glucose unit as determined by the acid-wash method (7). Reaction rates with this soluble, semisynthetic substrate were approximately 1.5 to 2 times as high as when insoluble α-(1 → 3)-glucan was used. Protein was estimated by the procedure of Lowry et al. (14). Reducing-sugar equivalents were determined by the Nelson-Somogyi method (22). Glucose was used as the standard sugar.

Descending paper chromatography was done on Whatman no. 1 paper at room temperature with the solvent system 1-propanol–ethyl acetate–water (6:1:3 by volume) for the α-linked series (13). Spots were detected by use of alkaline silver nitrate solution (24).

Estimations of hydrolytic activities associated with the cell wall, intracellular extract, and culture fluid of the yeasts examined are presented in Table 1. The species investigated were thought to contain α-(1 → 3)-glucan in their cell walls. The most significant activity

was detected in some strains of *Cryptococcus* and *Rhodotorula*, which exhibited good α-(1 → 3)-glucanase activities associated with the cell wall fractions but evidently at much lower levels than associated β-(1 → 3)-glucanase activities. *Cryptococcus* is known to contain a wall-associated α-(1 → 3)-glucan (3), but such a polymer has not been reported in *Rhodotorula*. Elinov and Vitovskaya (Abstr. VII Int. Symp. Carbohydrate Chem., Bratislava, Czechoslovakia, 1974, p. 194) found no evidence for the presence of α-(1 → 3)-glucan in a *Rhodotorula* species. Attempts by us to demonstrate the presence of this polysaccharide (10) in the α-(1 → 3)-glucanase-positive *R. minuta* var. *texensis* also were unsuccessful.

Both *Cryptococcus* and *Rhodotorula* species demonstrated significant β-(1 → 3)-glucanase activities associated with the cell walls; however, only *Cryptococcus* species exhibited low levels of β-(1 → 6)-glucanase activity.

Species of *Endomyces* were found to contain traces of α-(1 → 3)-glucanase activity, but, due to the low levels, the results are not conclusive. No α-(1 → 3)-glucanase activity was detected in the three *Schizosaccharomyces* species examined or in the recently described genus *Phaffia* (16). The presence of α-(1 → 3)-glucan in *Phaffia* was demonstrated by the positive action of a highly specific endo-α-(1 → 3)-glucanase from *Bacillus circulans* WL-12 on this polysaccharide extracted (10) from *P. rhodozyma* cell walls. Among the yeasts with positive activity, the major proportion of the α-(1 → 3)-glucanase was associated with cell wall fractions (Table 1). It is possible that the observed activities are the result of more than a single enzyme, as previously shown for endogenous β-glucanases (10).

Attempts to determine the pattern of action (i.e., endo- or exo-activity) of the crude α-(1 → 3)-glucanases in intracellular extracts and cell walls were inconclusive. This was most likely due to a combination of rapid inactivation (see below) and the simultaneous production of glucose and oligosaccharides from other carbohydrates, such as β-linked glucans.

When the cell wall fraction or the intracellular extract of *R. minuta* var. *texensis* was held at 4°C for 24 h without dialysis, the initially observed α-(1 → 3)-glucanase activity was lost. However, an intracellular extract dialyzed at 4°C for 24 h against 0.02 M sodium succinate buffer (pH 6.0) demonstrated a residual activity that was only slightly lower than that of the fresh extract. Next, the activity of fresh intracellular extract was monitored periodically against this buffer during dialysis at 4°C. The results (Fig. 1) show that activity rapidly increased, reaching a maximum value at 6 to 7 h, and then rapidly declined. When a portion of the extract was removed during the 24-h period of dialysis, while substantial activity remained (at 9 h), the rate of inactivation was initially high but soon decreased to a rate 10-fold lower (open circles in Fig. 1) than when dialysis was continuous. Redialysis of the removed fraction after all activity was lost (67 h) did not reestablish any observable activity. In another experiment, fresh intracellular extract was held for 39 h at 4°C in buffer plus 0.01% sodium azide as an antimicrobial compound. Continuous dialysis as before for 25 h was then initiated. As observed with the fresh extract, a considerable peak of highly unstable activity became apparent (solid triangles in Fig. 1).

During dialysis of the intracellular extracts, cytoplasmic materials, previously non-sedimentable at 27,000 × *g*, became insoluble. Fresh extract (20 ml) held at 4°C for 20 h was dialyzed for 7 h as described above. After the activated extract (0.03 U/ml) was centrifuged at 35,000 × *g* for 30 min, the clear supernatant was carefully removed, leaving a very small volume of solution remaining with the pellet. The pellet was resuspended in the small volume, and then both the suspension (1 ml) and the clear supernatant were assayed for activity. The result indicated that approximately 39% of the total observable α-(1 → 3)-glucanase activity was associated with the pellet fraction (ca. 5% of the original volume).

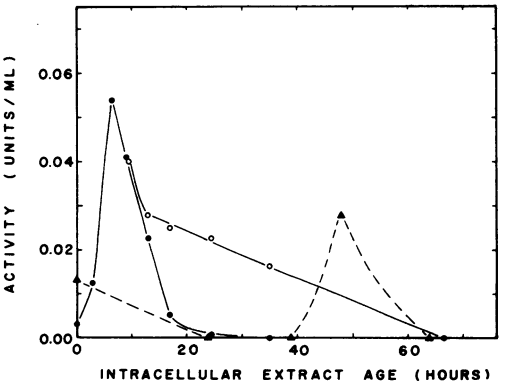

FIG. 1. *Effect of dialysis of the intracellular extracts of R. minuta var. texensis on α-(1 → 3)-glucanase activity level. Extracts were dialyzed at 4°C against 0.02 M sodium succinate at pH 6.0. Activity is expressed as units per milliliter, with carboxymethyl-α-(1 → 3)-glucan as substrate. Symbols: ●, continually dialyzed extract; ○, dialyzed extract removed from dialysis at 9 h and held at 4°C; ▲, extract held at 4°C for 39 h in the presence of buffer plus 0.01% sodium azide, and then dialyzed for 25 h.*

The optimum pH for fungal α-(1 → 3)-glucanase (20) has been reported to be 4.5 to 4.7. An assay at this pH was therefore made with a portion of activated extract of *R. minuta*. The pH was adjusted by adding an equal volume of 0.17 M succinate buffer (pH 4.0). A control was included in which 0.17 M succinate buffer (pH 6.0) was added to another portion of activated enzyme. After 15 min, the assay done at the low pH showed that only 16% of the original activity was measured, whereas the control at pH 6.0 showed 50% of the original activity, both adjusted for dilution. Thus, lowering of the pH and increasing the buffer concentration from 0.02 to 0.1 M both affected the enzyme activity adversely.

Addition of ethylenediaminetetraacetic acid (final concentration, 0.01 M) to an activated extract (6.5-h dialysis against the buffer) resulted in a loss of ca. 75% of the activity present in the control. Other portions of cell extract were dialyzed against 0.1 mM solutions of $CaCl_2$, $CoCl_2$, or iodoacetamide in 0.02 M succinate buffer (pH 6.0). As compared with that of the control, activity of the enzyme was about half with $CaCl_2$ and iodoacetamide, but dialysis against $CoCl_2$ resulted in a slight stimulation (ca. 20%). However, after 18 h, all activity in the presence of $CoCl_2$ and in the control was lost.

In an attempt to release the α-(1 → 3)-glucanase from the *Rhodotorula* cell walls, fresh preparations of cell walls were treated with 3 M lithium chloride (8) in 0.05 M sodium succinate at pH 5.0 or 6.0 for 4 h at 4°C. Although the LiCl and the succinate buffer extracts of the walls showed appreciable β-(1 → 3)-glucanase activities, none of the extracts contained α-(1 → 3)-glucanase activity. Autolysis of whole cells or isolated cell walls (10) also failed to yield active, soluble α-(1 → 3)-glucanase.

The detection of α-(1 → 3)-glucanase activity in the basidiomycetous genus *Rhodotorula* is somewhat surprising in that this yeast is not known to contain this glucan.

The very low intracellular activities relative to the higher wall-associated activities and the low activity of α-(1 → 3)-glucanase in the cell wall fraction in comparison with the β-(1 → 3)-glucanase levels are both compatible with an incomplete activation or instability.

The observations regarding the inactive and unstable intracellular α-(1 → 3)-glucanase of *Rhodotorula* (under the preparative conditions employed here) may perhaps apply also to the α-(1 → 3)-glucanases found in other species and genera.

In view of our results, it is most probable that the quantitative estimations of α-(1 → 3)-glucanase activity levels expressed here are not representative of their actual or potential cellular activity. More importantly, the apparent absence of α-(1 → 3)-glucanases in yeasts known to contain cell wall α-(1 → 3)-glucan must also be reinvestigated.

LITERATURE CITED

1. **Abd-El-Al, A. T. H., and H. J. Phaff.** 1968. Exo-β-glucanases in yeast. Biochem. J. 109:347-360.
2. **Abd-El-Al, A. T. H., and H. J. Phaff.** 1969. Purification and properties of endo-β-glucanase in *Hanseniaspora valbyensis*. Can. J. Microbiol. 15:697-701.
3. **Bacon, J. S. D., D. Jones, V. C. Farmer, and D. M. Webley.** 1968. The occurrence of α-(1 → 3)-glucan in *Cryptococcus, Schizosaccharomyces* and *Polyporus* species, and its hydrolysis by a *Streptomyces* culture filtrate lysing cell walls of *Cryptococcus*. Biochim. Biophys. Acta 158:313-315.
4. **Barras, D. R.** 1972. A β-glucan endo-hydrolase from *Schizosaccharomyces pombe* and its role in cell wall growth. Antonie van Leeuwenhoek; J. Microbiol. Serol. 38:65-80.
5. **Bush, D. A., M. Horisberger, I. Horman, and P. Wursch.** 1974. The wall structure of *Schizosaccharomyces pombe*. J. Gen. Microbiol. 81:199-206.
6. **Clarke, A. E., and B. A. Stone.** 1962. β-1,3-Glucan hydrolases from the grape vine (*Vitis vinifera*) and other plants. Phytochemistry 1:175-188.
7. **Eyler, R. W., E. D. Klug, and F. Diephuis.** 1947. Determination of degree of substitution of sodium carboxymethylcellulose. Anal. Chem. 19:24-29.
8. **Fan, D. P.** 1970. Cell wall binding properties of the *Bacillus subtilis* autolysin(s). J. Bacteriol. 103:488-493.
9. **Fleet, G. H., and D. J. Manners.** 1976. Isolation and composition of an alkali-soluble glucan from the cell walls of *Saccharomyces cerevisiae*. J. Gen. Microbiol. 94:180-192.
10. **Fleet, G. H., and H. J. Phaff.** 1974. Glucanases in *Schizosaccharomyces*. Isolation and properties of the cell wall-associated β-(1 → 3)-glucanases. J. Biol. Chem. 249:1717-1728.
11. **Fleet, G. H., and H. J. Phaff.** 1975. Glucanases in *Schizosaccharomyces*. Isolation and properties of an exo-β-glucanase from the cell extracts and culture fluid of *Schizosaccharomyces japonicus* var. *versatilis*. Biochim. Biophys. Acta 401:318-332.
12. **Johnston, I. R.** 1965. The composition of the cell wall of *Aspergillus niger*. Biochem. J. 96:651-658.
13. **Johnston, I. R.** 1965. The partial acid hydrolysis of a highly dextrorotational fragment of the cell wall of *Aspergillus niger*. Biochem. J. 96:659-664.
14. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol regent. J. Biol. Chem. 193:265-275.
15. **Manners, D. J., A. J. Masson, and J. C. Patterson.** 1973. The structure of a β-(1 → 3)-D-glucan from yeast cell walls. Biochem. J. 135:19-30.
16. **Miller, M. W., M. Yoneyama, and M. Soneda.** 1976. *Phaffia*, a new yeast genus in the *Deuteromycotina* (*Blastomycetes*). Int. J. Syst. Bacteriol. 26:286-291.
17. **Miyazaki, T., M. Yamada, and T. Ohno.** 1974. Isolation and structure of α-1,3-linked glucan from the hyphal wall of *Phytophthora infestans*. Chem. Pharm. Bull. 22:1666-1669.
18. **Notario, V., T. G. Villa, T. Benitez, and J. R. Villanueva.** 1976 β-Glucanases in the yeast *Cryptococcus albidus* var. *aerius*. Production and separation of β-glucanases in asynchronous cultures. Can. J. Microbiol. 22:261-268.

19. **Phaff, H. J.** 1971. Structure and biosynthesis of the yeast cell envelope, p. 135–210. *In* A. H. Rose and J. S. Harrison (ed.), The yeasts, vol. 2. Academic Press Inc., New York.
20. **Reese, E. T., A. Maguire, and F. W. Parrish.** 1972. α-1,3-Glucanases of fungi and their relationship to mycodextranase, p. 735–742. *In* G. Terui (ed.), Fermentation technology today. Society of Fermentation Technology, Osaka.
21. **Reid, I. D., and S. Bartnicki-Garcia.** 1976. Cell-wall composition and structure of yeast cells and conjugation tubes of *Tremella mesenterica*. J. Gen. Microbiol. **96**:35–50.
22. **Spiro, R. G.** 1966. Analysis of sugars found in glycoproteins. Methods Enzymol. 8:3–26.
23. **Stone, B. A.** 1971. O-carboxymethylpachyman, p. 384–385. *In* R. L. Whistler and M. L. Wolfrom (ed.), Methods in carbohydrate chemistry, vol. 6. Academic Press Inc., New York.
24. **Trevelyan, W. E., D. P. Procter, and J. S. Harrison.** 1954. Detection of sugars on paper chromatograms. Nature (London) **166**:444–445.
25. **Zonneveld, B. J. M.** 1972. Morphogenesis in *Aspergillus nidulans*. The significance of α-1,3-glucan of the cell wall and α-1,3-glucanase for cleistothecium development. Biochim. Biophys. Acta **273**:174–187.
26. **Zonneveld, B. J. M.** 1974. α-1,3-Glucan synthesis correlated with α-1,3-glucanase synthesis, conidiation and fructification in morphogenetic mutants of *Aspergillus nidulans*. J. Gen. Microbiol. **81**:445–451.