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Localization of β-(1,3)-Glucanase in the Mycelium of *Sclerotium rolfsii*

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The role of the lytic enzyme β-(1,3)-glucanase in cell wall synthesis and its distribution in the mycelium of the fungus *Sclerotium rolfsii* were studied. Enzyme activity was determined after enzyme extraction with Triton X-100 from a cell wall preparation. Specific zones of immunofluorescence appeared in the hyphal tips, clamp connections, new septa, and lateral branching when a specific antiserum was used with the indirect method of the fluorescent antibody staining. Enzymatic activity in the cell wall preparation was inactivated by diethylpyrocarbonate. However, 69% of the total enzymatic activity was present in a latent form which was not affected by the ester. This result suggests that most of the β-(1,3)-glucanase was present along the hyphal cell walls in a "masked" form. An active enzyme appeared only in those regions which showed immunofluorescence. The activity of glucan synthetase, an enzyme essential for wall formation, was higher in the branching fungus grown on L-threonine-supplemented synthetic medium than in the synthetic medium-grown fungus.

Apical growth and branching in filamentous fungi are controlled by a delicate balance between cell wall synthesis and degradation (1, 4, 25). The role of lytic enzymes in cell wall growth of bacteria (12) and plants (17, 21) has also been demonstrated. Even though many fungi release extracellular β-(1,3)-glucanase (7, 8, 13, 22), its participation in apical growth has not yet been demonstrated (1, 25). Hyphal morphogenesis in filamentous fungi has been shown to be controlled by nutritional, chemical, and physical factors (5, 11, 15, 20, 24, 25). Recently, Kritzman et al. (14) found that in *Sclerotium rolfsii*, both growth rates of individual leading hyphae and the sequence of appearance of lateral hyphae are organized and predictable events which can be changed by the addition of L-threonine to the growth medium. No known model can explain such a phenomenon.

The current study was designed to look for regions of β-(1,3)-glucanase activity along the hyphal walls and to find the role of this enzyme in hyphal growth and branching of *S. rolfsii*.

MATERIALS AND METHODS

Growth conditions. *S. rolfsii* Sacc. type R (ATCC 26326) was grown at 30°C in petri dishes containing 15 ml of a synthetic medium (SM), prepared according to Okon et al. (19), supplemented with one of the following carbon sources: glucose, starch, laminarin, or cellulose. The plates were inoculated in the center with fungal mycelium cut from the edge of a 5-day-old colony. In specific experiments, solution of 10^{-1} M L-threonine, sterilized by passage though an 0.45-μm porosity membrane filter (Millipore Corp.), was added aseptically to the melted agar in a final concentration of 10^{-2} M.

The mycelium used for fluorescent antibody staining was grown on a cellophane membrane supported by glass beads in a petri plate containing liquid SM (15).

Cell fractionation. The mycelium was homogenized in an Ultra turrax homogenizer (Janke and Kunkel K.G., Germany) at 4°C for 3 min and then in an Omnimixer (Ivan Sorvall, Inc., Norwalk, Conn.) at 4°C for 5 min. The homogenate was then transferred for final homogenization to a tissue grinder (Elda, Israel) and homogenized at 4°C for 4 min. The cell-free extract was obtained from 3 g (wet weight) of mycelium homogenized in a 30-ml solution of 0.1 M sodium citrate buffer, pH 4.7. The homogenate was centrifuged (30 min at 20,000 × *g*), and the supernatant fluid was dialyzed overnight at 4°C against distilled water.

Fungal cell walls were separated from the homogenized mycelium by ultrasonic disintegration (using an MSE sonic oscillator, England) and further grinding according to Chet et al. (6). The walls were washed until no protein could be detected in the supernatant fraction. Folin phenol reagent was used to determine protein content (16). The membranes attached to the cell walls were solubilized for 15 min at 37°C by the detergent Triton X-100 (Packard Instrument Co., Inc., Rockville, Md.) at a concentration of 0.5 mg/ml (18). The solubilized material was separated from the insoluble residue of the walls by centrifugation at 20,000 × *g* for 1 h at 4°C, and its protein content was determined.

Determination of β-(1,3)-glucanase activity. β-(1,3)-glucanase activity was determined by following

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the release of glucose from soluble laminarin. Laminarin, obtained from the Institute of Seaweed Research (England), was solubilized in 0.1 M citrate buffer, pH 4.7, at 85°C for 10 min. Free glucose was determined by the glucose-oxidase method (3; Sigma technical bulletin no. 510). The incubation mixture contained the enzyme (200 μg of protein) and 2.5 mg of laminarin in a final volume of 3.0 ml of 0.1 M sodium citrate buffer (pH 4.7). The activity was found to be constant throughout the duration of the incubation (60 min at 45°C). The reaction was terminated by boiling the mixture for 5 min.

Enzyme fractionation. Triton X-100-solubilized membrane fraction was separated from the insoluble residues of walls by centrifugation and dialyzed for 2 days at 4°C against 5 liters of 1 mM sodium citrate buffer (pH 4.7), which was changed twice daily, and then freeze dried in 50-ml lots. The freeze-dried fraction was applied to diethylaminoethyl (DEAE)-cellulose columns (12 by 30 cm) which were washed first with 500 ml of 0.1 M phosphate buffer (pH 7.0) and then with an equal volume of 0.006 M phosphate buffer at the same pH level. After its adsorption, portions of the extract were eluted with phosphate buffer using a gradient from 0.006 to 0.3 M and a flow rate of 0.02 ml/min, and 5-ml fractions were collected. The fraction which showed enzymatic activity was lyophilized and stored at 15°C. It was further purified on a Sephadex G-100 column (1.5 by 80 cm) prewashed with 200 ml of sodium citrate buffer (0.1 M, pH 4.7). Five-milliliter portions of the citrate buffer freeze-dried enzyme preparations were applied to the column after centrifugation to remove insoluble material. The flow rate through the column was 0.9 ml/min; 4.5-ml fractions were collected. The two-column system separated the carbohydrates from the enzyme preparation.

Isoelectric focusing on 7.5% polyacrylamide gels (27) was employed for protein separation. An L.K.B. (Sweden) ampholine carrier, containing 40% ampholites with pH levels ranging from 3 to 10, was used. The gels (85 cm long, 7.0 mm in diameter) were chemically polymerized by potassium persulfate. Protein samples (containing 450 μg of protein each) of the collected enzyme were applied in 10% sucrose under a "protective layer" of ampholites. Electrophoresis was carried out at 4°C for 4 h, applying 1.5 mA/gel and gradually raising the voltage to 300 to 350 V. The pH gradient in the gels was determined by cutting gels without protein into 10-mm slices, soaking these in 2 ml of distilled water for 1 h, and measuring the pH with a radiometer M 62 pH meter (Copenhagen N.V.). β-(1,3)-Glucanase activity on the gel was localized by a modification of Goldberg's (9) tetrazolium technique. The gels were incubated in the dark with laminarin-buffered solution for 15 min and transferred to a solution containing 1 mg of tetrazolium chloride in a 0.1 M NaOH solution. The presence of reducing sugars which were previously released from laminarin by β-(1,3)-glucanase were detected as red bands after 1 h. Control gels which were incubated in the buffer without laminarin showed no staining. The gels were scanned at 475 mm with a Varian gel scanner at a scan of 2 cm/min.

Preparation of enzyme antisera. Samples of the purified enzyme solution (the 100-g eluate) containing 5 mg of protein were emulsified with Freund complete adjuvant and injected intramuscularly into rabbits. Four additional intravenous injections of 1, 2, 4, and 5 mg of protein were given, one per week, 28 days after the initial injection. Three weeks after the last injection, blood samples were obtained from the ear vein. Serum, prepared by centrifugation at 5,000 × *g* for 10 min, was stored until used in immunofluorescent antibody staining.

The procedure used in measuring the titer was as follows. The antiserum was diluted in saline to series of double multiplied dilutions. Amounts of 0.5 ml from the diluted antiserum were transferred to serological tubes containing 0.5 ml of an antigen suspension. These were incubated at 37°C for 10 h. The titer was determined according to the minimal concentration which showed agglutination.

Indirect fluorescent antibody staining. Mycelium or cell walls of *S. rolfsii* from cellophane-grown cultures were prepared for staining. One drop of cell walls or a square (0.5 cm^2) of mycelium cut from the cellophane was placed on a protein-coated (1.5% Difco gelatin solution containing 0.02% colorless merthiolate) glass slide and allowed to dry on a hot plate at 40°C. The slides were then washed in 0.01 M phosphate-buffered saline, pH 7.0, for 1 to 2 min, air dried, stained for 35 min in a humid chamber at 25°C with a 1:16 dilution of the antibody, and again washed with phosphate-buffered saline. Indirect staining was carried out by applying fluorescein isothiocyanate-labeled anti-rabbit immunoglobulin G goat (Miles-Yeda Ltd., Rehovot, Israel) to the antibody-stained slides. Staining and washing procedures were carried out as described.

Microscopy and photomicrography. Preparations were observed under a Zeiss UV microscope (West Germany). Photomicrographs were taken with Agfachrom 50 L professional 135-36, 3100 for artificial light.

Inactivation of β-(1,3)-glucanase by diethylpyrocarbonate. Diethylpyrocarbonate is a volatile ester which has been used by Solymosy et al. (26) to destroy ribonucleases. To test its ability to inactivate β-(1,3)-glucanase, 0.2 ml of diethylpyrocarbonate (K & K Laboratories, Inc., Plainview, N.J.) was applied under a nitrogenous atmosphere to a 1-ml suspension of cell walls, fungal hyphae or enzyme in 0.1 M citrate buffer, pH 4.7, for 60 s at 4°C. The suspension was then aerated in air for 20 min. Enzyme destruction efficiency of this treatment was also tested on glucose oxidase (Sigma EC 1.1.34) and cellulose (Sigma EC 3.2.14) as references.

Glucan synthetase. This enzyme was determined by a modification of the chitin-synthetase method of Bartnicki-Garcia and Lippman (1). Cell-free extracts were prepared in 0.1 M tris(hydroxymethyl)-aminomethane-hydrochloride buffer (pH 7.4) as described.

The reaction mixture contained: [*U*-^{14}C]UDP glucose (specific activity, 296 mCi/mmol; The Radiochemical Centre, Amersham, England), 0.15 μmol; $MgCl_2$, 0.5 μmol; cellobiose, 0.5 μmol; tris-(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.0), 1.0 μmol (0.25 ml); enzyme extracts, 200 μg of

protein in a total volume of 1.0 ml. After 60 min of incubation at 30°C, the reaction was stopped by boiling the mixture for 1 min. The reaction mixture, either containing boiled enzyme or without enzyme, was used as a control. The radioactive polymer was precipitated and collected on filter papers (Whatman no. 2) by rinsing with a 100-ml solution of 95% ethanol and 0.4 N NaOH (1:1, vol/vol). The radioactivity on the filter paper was proportional to the enzyme concentration. A more specific control was obtained by the application of β-(1,3)-glucanase to the produced glucan. This revealed that the polymer produced by the enzyme was indeed β-(1,3)-glucan which was specifically degraded by the glucanase. Net activity of the synthetase could therefore be determined by detecting the radioactivity released from the polymer by a specific corresponding glucanase.

RESULTS

Glucanase activity in *S. rolfsii*. Enzyme extractions were prepared from mycelium of *S. rolfsii* grown on different carbon sources. β-(1,3)-glucanase activity was significantly lower in the extract prepared from cellulose-grown fungus (Table 1). Addition of L-threonine (10^{-2} M) to the glucose-containing medium of *S. rolfsii* increased β-(1,3)-glucanase activity of the cell-free extract and Triton X-100-treated cell walls from 636 ± 6 and 275 ± 5 to 697 ± 4 and 305 ± 5 μg of glucose per mg of protein per h, respectively. These numbers represent the mean of at least five experiments.

Fractionation of the β-(1,3)-glucanase on DEAE-cellulose and Sephadex G-100 columns. A large part of the β-(1,3)-glucanase activity passed straight through the DEAE-cellulose column. Two peaks were obtained, one on fractions 6 to 7 and the second on fractions 15 to 17. The first peak showed a high lytic activity (a release of 1,600 μg of glucose per mg of protein per h), whereas the second peak showed lower activity (about 10%). The first peak was fractionated on Sephadex G-100 columns. Ninety-five percent of the activity applied to Sephadex was eluted in one peak in fraction 14 and showed a high specific activity (release of 2,600 μg of glucose per mg of protein per h). The enzyme in the second peak, which was found to be identical to the one in the first, was probably mixed with polysaccharides.

The isoelectric-focusing technique revealed the presence of two isoenzymes of β-(1,3)-glucanase after staining and scanning the gels at 575 nm (Fig. 1). Two peaks were obtained in the pH 6.2 and 8.5 regions at a distance of 5.7 and 7.8 cm, respectively, from the top of the gel (8.5 cm long). Optimal activity of β-(1,3)-glucanase from *S. rolfsii* was obtained with a soluble laminarin concentration of 0.8 mg/ml as substrate incubated at 45°C (Fig. 2). Optimal enzyme activity as found at pH 4.7 (sodium citrate buffer). A laminarin concentration of 2.1 mg/ml resulted in half of the maximum activity (Fig. 2).

Indirect fluorescent antibody staining. The titer of the serum obtained was at least 1:64. Using the fluorescent antibody technique, the indirect methods resulted in a specific yellow-green immunofluorescence at a maximum dilution of 1:32. Indirect labeling of the antibody to

TABLE 1. *β-(1,3)-glucanase activity in cell-free extracts and Triton X-100-treated cell walls of S. rolfsii grown on different carbon sources*

Carbon source	Enzyme activity (μg of glucose/mg of protein per h)			
	Cell-free extract[a]	SD[b]	Cell wall	SD
Glucose	640	3.54	280	3.33
Starch	570	3.47	255	3.71
Laminarin	560	3.66	250	3.38
Cellulose	48	1.97	157	3.27

[a] Each number represents the mean of 25 replicates.
[b] SD, Standard deviation.

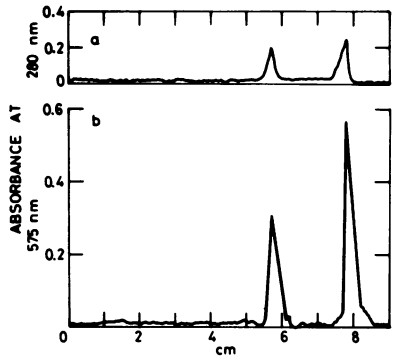

FIG. 1. *Identification of β-(1,3)-glucanase isoenzymes on polyacrylamide gels using the isoelectric-focusing technique. a, Proteins; b, enzyme activity.*

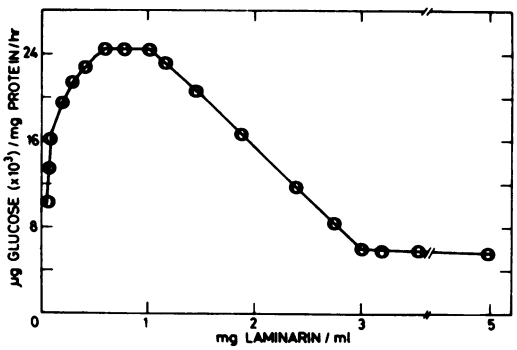

FIG. 2. *The effect of various concentrations of laminarin on the activity of β-(1,3)-glucanase from S. rolfsii.*

washed mycelial particles of *S. rolfsii* showed a sharp yellow-green immunofluorescence in the following specific points: hyphal tips, clamp connections, new septa, and branching area (Fig. 3 and 4). Very low fluorescence or none was found along the internal part of the cell walls.

The technique was verified by the absence of fluorescence in hyphae of *Rhizoctonia solani*, *Trichoderma* spp., *Fusarium oxysporum*, and *Pythium* spp. treated with the same antiglucanase antiserum. No precipitation could be observed when the anti-*S. rolfsii* glucanase antiserum was applied to a similar enzyme solution prepared from *Trichoderma* spp. However, enzyme precipitated by the antiserum lost only 10% of its activity. Mycelial particles washed with Triton X-100 before labeling with the antibody showed no fluorescence. The total activity of the β-(1,3)-glucanase removed from cell walls by Triton X-100 is shown in Table 1. However, only a few specific points showed its capability to bind the fluorescence antibodies (Fig. 3, 4). This problem was solved by detecting the active β-(1,3)-glucanase with diethylpyrocar-

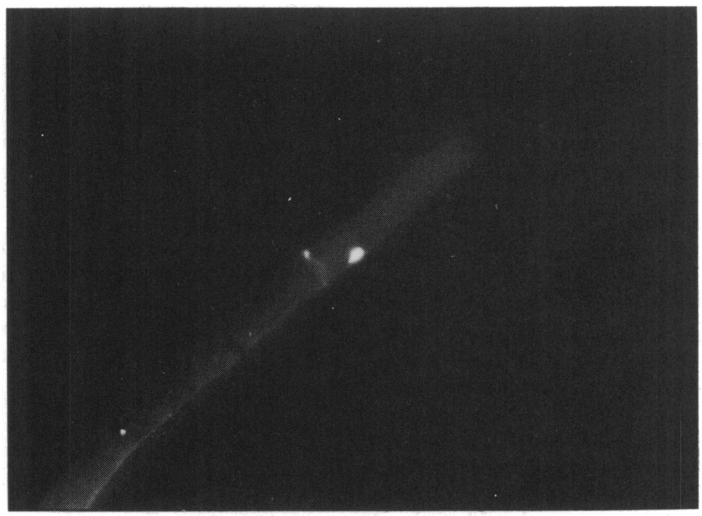

FIG. 3. *Localization of β-(1,3)-glucanase on mycelial particles of S. rolfsii labeled with a specific antibody using the indirect immunofluorescence antibody technique (×2000).*

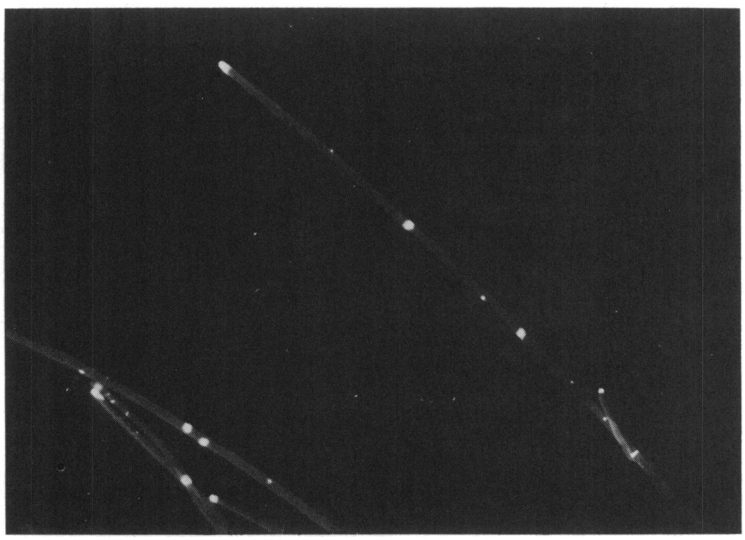

FIG. 4. *Localization of β-(1,3)-glucanase on a whole mycelium of S. rolfsii labeled with a specific antibody using the indirect immunofluorescence antibody technique (×800).*

bonate. The results (Table 2) show that the enzyme lost its activity after treatment with diethylpyrocarbonate. Similarly, a crude enzyme released from the cell wall by Triton X-100 was also completely and irreversibly inhibited by this chemical agent. On the other hand, when diethylpyrocarbonate was applied before enzyme extraction with Triton X-100, only 31% of the total enzyme activity in SM-grown fungal cell walls was lost. In the highly branched fungus, grown on L-threonine-supplemented SM, diethylpyrocarbonate was responsible for a higher percentage (48.7%) of lost activity (Table 2). The masked enzyme which was not inactivated by diethylpyrocarbonate did not react with the antibody either. However, treatment with diethylpyrocarbonate resulted in a total loss of antibody binding.

β-(1,3)-glucan synthetase. β-(1,3)-glucan synthetase activity was determined (Table 3). Results show that the activity in threonine-grown fungus was about 35% higher than that found in SM-grown fungus.

DISCUSSION

Growth and branching of filamentous fungi are regulated by both synthetase and lytic enzymes (1). *S. rolfsii* hyphal cell walls contain 65% laminarin (6), and one of the main autolytic enzymes in this fungus is β-(1,3)-glucanase. In the present study, this enzyme was partially purified by DEAE-cellulose, Sephadex columns, and isoelectric focusing in acrylamide gels. A high activity of β-(1,3)-glucanase was obtained after its release from the cell wall preparation by Triton X-100, which is capable of membrane solubilization (18). Labeling the mycelium with fluorescent antibodies specific for β-(1,3)-glucanases of *S. rolfsii* revealed specific points of fluorescence. The fluorescent regions always appeared in the hyphal tips, branching zones, clamp connections, and new septa. However, this fluorescence completely disappeared after treatment with Triton X-100. In no other place along the hyphae could such fluorescence be observed. Quantitative data of enzymatic activity points to a very high activity, which could not be explained by the few specific fluorescent points. A modification of the method described by Solymosy et al. (26) was used in this study. When diethylpyrocarbonate was applied to β-(1,3)-glucanase, the enzyme lost its activity. However, in cell walls which had been treated with this agent, the enzyme which was later released by Triton X-100 lost only 31% of its total activity. Assuming that this loss in enzymatic activity was caused by diethylpyrocarbonate, it was suggested that the other 69% of the enzyme was present in a latent form "masked" by a membrane. This idea was confirmed by the complete destruction of the enzyme which was first released by the detergent and only then exposed to diethylpyrocarbonate. It should, however, be noted that the detergent could also affect interaction between the enzyme and cell wall. Hyphal branching in *S. rolfsii* was induced by L-threonine as previously described (14). Indeed, in this fungus, many more fluorescent points were detected in comparison with SM-grown fungus. Grove and Bracker (10) suggested

TABLE 3. *The activity of β-(1,3)-glucan synthetase in cell-free extracts of S. rolfsii mycelium grown on SM or SM + L-threonine (10^{-2} M)*

Medium	Glucan synthetase activity[a] (cpm/mg of protein per h)	
	Total activity ± SD	Net activity[b] ± SD
SM	2,115 ± 95.8	1,882 ± 88.6
SM + L-threonine (10^{-2} M)	2,978 ± 103.1	2,467 ± 86.7

[a] Each number represents the mean of five independent experiments, five replicates each. Standard deviation (SD) is given.

[b] Net activity: Glucan synthetase activity determined by the release of [C^{14}]glucose from the synthetized glucans by β-(1,3)-glucanase.

TABLE 2. *The effect of diethylpyrocarbonate on the activity of β-(1,3)-glucanase*

Medium	Enzyme source	Diethylpyrocarbonate	μg of glucose/mg of protein per h	
			Enzyme activity	SD[a]
SM	Purified enzyme	−	3,152	6.85
SM	Purified enzyme	+	10.8	0.48
SM	Cell-bound enzyme[b]	−	283	3.51
SM	Cell-bound enzyme[b]	+	0.98	0.06
SM	Mycelium[c]	+	194.3	6.83
SM + L-threonine	Cell-bound enzyme[b]	−	296	5.17
SM + L-threonine	Cell-bound enzyme[b]	+	0.99	0.06
SM + L-threonine	Mycelium[c]	+	142.06	3.86

[a] SD, Standard deviation.

[b] Obtained by washing with Triton X-100.

[c] In this treatment, the mycelium was preincubated with diethylpyrocarbonate before enzyme extraction.

that the cell wall lytic enzymes are carried in vesicles from the production points to their sites of secretion into the wall polymers in the branching points. On the basis of our findings, it is suggested that lytic enzymes deposited along the hyphal cell walls are associated with membranes in an inactive form. It seems that at branching sites, the enzyme becomes exposed to the substrate and activated.

Lateral hyphal development involves not only lytic enzymes but also cell wall synthetases. β-(1,3)-glucan synthetase activity was higher in L-threonine-grown fungus, which indeed had many more branches and lateral hyphae. The *S. rolfsii* data confirms the basic concept of the model suggested by Bartnicki-Garcia (1) about the delicate balance between cell wall lytic and synthesizing enzymes. This equilibrium probably controls hyphal growth and branching.

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