

β -Glucosidase from *Phoma strasseri* and its Possible Role in a Disease of Peppermint

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ABSTRACT

Phoma strasseri produced β -glucosidase in a liquid medium with salicin as a carbon source. The culture filtrate contained salicyl alcohol and an unidentified compound that reacted with phenol-detecting reagents. The optimum pH for activity of β -glucosidase extracted from spores and mycelium was 4.5 - 5.5. Extracts of peppermint rhizomes inoculated with *P. strasseri* had

three times more β -glucosidase activity than noninoculated controls. Exudates that formed in inoculated peppermint rhizomes exhibited β -glucosidase activity, contained phenolics, and had no inhibitory effect on spore germination of *P. strasseri*.

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Additional key words: *Mentha*, tissue browning, polyphenol oxidase.

Phoma strasseri Moesz causes a root and rhizome rot of peppermint (*Mentha piperita* L. 'Mitcham'). In a preliminary experiment, we found that extracts of mycelium and spores exhibited β -glucosidase activity and that infected tissue contained higher β -glucosidase activity than healthy tissue. The possibility that this enzyme may play a role in pathogenesis was indicated by the formation of a brownish exudate at the point where peppermint rhizomes were inoculated with *P. strasseri*. We reasoned that the brownish material was formed by oxidation of phenolic compounds that were released by action of β -glucosidase on phenolic glucosides in host tissue. β -Glucosidases that release phenolics from glucosides have been shown to function in a number of plant diseases incited by fungi and bacteria (1,2,3,4,9,12). We therefore investigated the possible role of β -glucosidase in the *Phoma* disease of peppermint and we report here on: (i) a β -glucosidase from *P. strasseri*; (ii) β -glucosidase activity in healthy and inoculated peppermint rhizomes; and (iii) total phenolics and β -glucosidase present in the brown exudate produced on infected peppermint rhizomes. A preliminary report has been presented (7).

MATERIALS AND METHODS.—*Fungus culture.*—*P. strasseri* (ATCC No. 24146) was grown on a medium containing NaNO_3 , 3g; KH_2PO_4 , 1g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5g; KCl, 0.5 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01g; and 15g of salicin (Sigma Chemical Co., St. Louis) per liter at pH 7.0. Fifty ml of the medium were placed in each 250-ml Erlenmeyer flask, inoculated with disks of the fungus, and incubated for 7 days at 25 C. Culture filtrates were collected by filtration through fiberglass pads.

Enzyme preparations.— β -Glucosidase was extracted from the mycelium and spores of *P. strasseri* grown on 20 ml of Czapek-Dox agar for 2 weeks at 20 C. The fungus was scraped off the agar and homogenized for 2 min in 30 ml of 0.1 M Tris-acetate buffer (pH 6.8) containing 1 mM EDTA and 7% (w/v) insoluble polyvinylpyrrolidone (PVP, General Aniline and Film Corp.), in a Sorvall Omni-Mixer cooled in an ice bath. The resulting homogenate was centrifuged at 15,000 g for 10 min at 4 C. To the supernatant fraction, 10 volumes of cold acetone were added, and the mixture was kept overnight at -20 C. The precipitate was collected by centrifugation at 15,000 g for 10 min at 4 C, and dissolved in 0.1 M citrate-phosphate buffer (pH 5.0). To the resulting solution, solid ammonium sulfate was added to 70% saturation. The precipitate was collected by centrifugation at 15,000 g for 10 min at 4 C, and dissolved in 0.1 M citrate phosphate buffer (5.0). The resulting solution was dialyzed for 4 hr at 4 C to remove the ammonium sulfate. By the above procedures, β -glucosidase was purified about 17-fold.

β -Glucosidase, from *P. strasseri* cultures grown for 7 days in salicin medium, was obtained by adding 10 volumes of cold acetone to 40 ml of culture filtrate. The mixture was kept overnight at -20 C. The precipitate was collected by centrifugation at 15,000 g for 15 min at 2 C and dissolved in 10 ml of Tris-acetate buffer 0.1 M (pH 6.8). The resulting

solution was centrifuged at 15,000 g for 15 min at 2 C, and the supernatant liquid was assayed for β -glucosidase activity.

Peppermint rhizomes were inoculated with *P. strasseri* as reported previously (8). Five days after inoculation, β -glucosidase was extracted from diced healthy and inoculated peppermint rhizomes by the technique used for extraction of mycelia and spores, except that 1 g of PVP and 12 ml of extracting buffer were used for each 1 g of tissue. Insoluble PVP was used in the extraction process to prevent phenolic compounds from inactivating enzymes in the plant extract (5).

Lesion exudates were collected with a capillary tube from peppermint rhizomes 2 days after inoculation with *P. strasseri*. The collected exudates were diluted 1:5 (v/v) with sterile distilled water before β -glucosidase activity and total phenolics content were determined.

β -Glucosidase assay.—The formation of *p*-nitrophenol from *p*-nitrophenyl- β -D-glucoside (PNPG, Sigma Chemical Co.) was measured in a reaction mixture that consisted of 0.5 ml enzyme preparation, 1.0 ml of PNPG solution (1mg/ml), and 2.5 ml of 0.1 M citric acid-sodium phosphate buffer (pH 5.0) incubated for 30 to 60 min at 30 C. Controls were run with heat-inactivated enzyme preparation. The reaction was stopped by adding 1.0 ml of 0.2 M Tris. The absorbance of the *p*-nitrophenol in the reaction mixture was measured at 400 nm in a Spectronic 20 colorimeter. One unit of β -glucosidase activity is the amount of enzyme that catalyzes the formation of 1 μ mole of *p*-nitrophenol/min under the above conditions.

Analysis of phenolic compounds.—Total phenolic content in the lesion exudate was determined by Folin-Denis reagent (13) and was expressed in gallic acid equivalents.

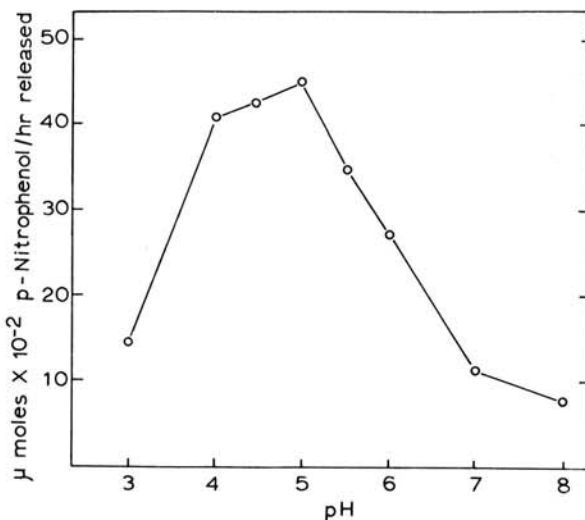


Fig. 1. pH optimum of β -glucosidase extracted from *Phoma strasseri* mycelium and spores, as determined by the release of *p*-nitrophenol from *p*-nitrophenyl- β -D-glucoside.

Phenolics in the ether extract of *P. strasserii* culture filtrate, grown on salicin medium, were detected by paper and thin-layer chromatography as described by Sherrod & Domsch (12), except that a spray reagent (10) was used in addition to ultraviolet light to locate the spots on the chromatograms.

RESULTS AND DISCUSSION.—When salicin was the sole carbon source, growth of *P. strasserii* was about equal to that previously observed for glucose (6). β -Glucosidase activity in the culture filtrate, after acetone precipitation, was 4.4×10^{-4} unit/ml.

Salicyl alcohol and an unidentified compound were detected in the ether extract of *P. strasserii* culture filtrate by paper and thin-layer chromatography. The presence of salicyl alcohol was also confirmed by the ultraviolet spectrum of the eluate from the spots. This suggests that salicin was broken down, presumably by β -glucosidase or some other mechanism, resulting in the release of salicyl alcohol.

Activity of the partially purified enzyme, extracted from mycelium and spores of *P. strasserii*, was optimum between pH 4.5 and 5.5 (Fig. 1). Similar results were obtained by Wilson & Niederpruem (14), who found that both intra- and extracellular forms of β -glucosidase from *Schizophyllum commune* exhibited a pH optimum of 5.4.

β -Glucosidase activity in healthy and diseased rhizomes was 2.2×10^{-3} and 6.1×10^{-3} unit/g fresh wt, respectively. Davis et al. (1) detected a β -glucosidase, capable of splitting salicin, in the vascular sap of tomato infected with *Fusarium oxysporum* f. *lycopersici*, but not in healthy plants. Also, Gubanov (2) found that β -glucosidase was more active in wood cells of cotton infected by *Verticillium dahliae* and *Fusarium vasinfectum* than in healthy plants.

The additional β -glucosidase activity in extracts of diseased peppermint rhizomes may be of fungal origin, although the fungus could also stimulate higher production by the host. Higher β -glucosidase activity may account for the release of excessive amounts of phenolic compounds from endogenous glucosides present in peppermint rhizomes. In this connection, both β -glucosidase (1.8×10^{-2} unit/ml) and phenolic compounds (1.36 mg gallic acid equivalent/ml) were detected in lesion exudates. Host polyphenol oxidases could convert such phenolics to quinones which would tan host tissues and contribute to the formation of black lesions.

The constituents of the lesion exudates do not appear to interfere with the development of *P. strasserii*, since spore germination of *P. strasserii*, tested by the depression slide technique (11), was not inhibited by the exudate.

LITERATURE CITED

1. DAVIS, D., P. E. WAGGONER, & A. E. DIMOND. 1953. Conjugated phenols in the Fusarium wilt syndrome. *Nature* 172:959-961.
2. GUBANOV, G. Y. 1962. The conversion of phenolic substances in wilt-infected cotton. *Fiziol. Rast.* 9:614-619.
3. HILDEBRAND, D. C., & M. N. SCHROTH. 1964. Antibiotic activity of pear leaves against *Erwinia amylovora* and its relation to β -glucosidase. *Phytopathology* 54:59-63.
4. HILDEBRAND, D. C., & M. N. SCHROTH. 1964. Arbutin-hydroquinone complex in pear as a factor in fire blight development. *Phytopathology* 54:640-645.
5. LOOMIS, W. D., & J. BATTAILE. 1966. Plant phenolic compounds and the isolation of plant enzymes. *Phytochemistry* 5:423-438.
6. MELOUK, H. A., & C. E. HORNER. 1972. Growth in culture and pathogenicity of *Phoma strasserii* to peppermint. *Phytopathology* 62:576-578.
7. MELOUK, H. A., & C. E. HORNER. 1972. β -Glucosidase from *Phoma strasserii*. *Phytopathology* 62:1104 (Abstr.).
8. MELOUK, H. A., & C. E. HORNER. 1972. Production of pectolytic and macerating enzyme by *Phoma strasserii*. *Can J. Microbiol.* 18:1065-1072.
9. SCHROTH, M. N., & D. C. HILDEBRAND. 1965. β -Glucosidase in *Erwinia amylovora* and *Pseudomonas syringae*. *Phytopathology* 55:31-33.
10. SEIKEL, M. K. 1964. Isolation and identification of phenolic compounds in biological materials. p. 33-76. *In* J. B. Harborne [ed.]. *Biochemistry of phenolic compounds*. Academic Press, Inc. N.Y.
11. SHARVELLE, E. G. 1961. *The nature and uses of modern fungicides*. Burgess Publishing Co., Minneapolis, Minn. 308 p.
12. SHERROD, L. L., & K. H. DOMSCH. 1970. The role of phenols and β -glycosidase in the pathogenicity mechanism of *Gliocladium catenulatum* to roots of peas (*Pisum sativum* L.). *Soil Biol. Biochem.* 2:197-201.
13. SWAIN, T., & W. E. HILLIS. 1959. The phenolic constituents of *Prunus domestica*. I. The quantitative analysis of phenolic constituents. *J. Sci. Food Agr.* 10:63-68.
14. WILSON, R. W., & D. J. NIEDERPRUEM. 1967. Control of β -glucosidases in *Schizophyllum commune*. *Can. J. Microbiol.* 13:1009-1020.