Localization of Enzymes of Oxalate Biosynthesis in Microbodies of Sclerotium rolfsii

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ABSTRACT

ARMENTROUT, V. N., L. B. GRAVES, JR., and D. P. MAXWELL. 1978. Localization of enzymes of oxalate biosynthesis in microbodies of Sclerotium rolfsii. Phytopathology 68:1597-1599.

Sclerotium rolfsii secretes oxalic acid when grown on a medium containing 1% sodium polypectate. Mitochondria and microbodies were partially purified from hyphae grown under these conditions by centrifugation of mycelial homogenates on a sucrose density gradient in a zonal rotor. Peak activity of malate dehydrogenase, a mitochondrial marker, occurred at a buoyant density of 1.162 g/cm^3, whereas the peak activity of catalase, a microbody marker, occurred at 1.196 g/cm^3. The two key enzymes of oxalic acid biosynthesis, glyoxylate dehydrogenase and isocitrate lyase, had distributions similar to that of catalase, and hence appear to be localized in microbodies. No malate synthase activity was detectable. This is the first report of the involvement of microbodies in a process essential to the pathogenic capabilities of a fungus.

Sclerotium rolfsii Sacc. is a soilborne fungus that is pathogenic to a wide range of plant species throughout the tropical and warmer regions of the temperate zones of the world (11). Its extensive and varied host range includes many crop plants of great economic importance (11). Although *S. rolfsii* is more destructive on younger plants, it is a vigorous pathogen of older plants.

This ability to attack older plant tissues appears to be related to the organism's ability to break down calcium pectate, and hence to destroy rapidly the middle lamella of the cells (1). Studies by Bateman and Beer (1) indicate that both the production of exogenous oxalic acid and the secretion of pectic enzymes are involved in pathogenesis by *S. rolfsii*. The oxalic acid sequesters the calcium in the host cell walls and produces a more favorable pH range, thereby permitting the pectic enzymes secreted by the pathogen to hydrolyze the pectates in the middle lamella more readily (1).

Maxwell and Bateman (6,7) studied the conditions that influence oxalate accumulation and the pathway for its biosynthesis. They showed that glyoxylate was oxidized to oxalate by the action of a nicotinamide adenine dinucleotide (NAD)-requiring enzyme, glyoxylate dehydrogenase. They also showed a positive correlation between the rate of oxalate accumulation and the activity of glyoxylate dehydrogenase and found that the total enzyme activity was sufficient to account for the observed oxalate accumulation (7). Recently, Kritzman et al (4) showed that the addition of 1×10^{-2} M L-threonine to the medium significantly reduces both the levels of glyoxylate dehydrogenase in the hyphae and the amount of oxalic acid secreted by *S. rolfsii*. Hyphae grown under these conditions were unable to infect and destroy lima bean seedlings.

The glyoxylate necessary for oxalate biosynthesis is produced from isocitric acid, an intermediate in the tricarboxylic acid cycle, by the enzyme isocitrate lyase (7). This enzyme is localized in the microbodies of many organisms (10), including some fungi (5). Hyphae of *S. rolfsii* in infected tissue contain numerous microbodies (5,9), as do those grown on host wall-like polysaccharides (3,9). In these two situations, *S. rolfsii* secretes copious amounts of oxalic acid (1). When *S. rolfsii* is grown on glucose, however, its hyphae contain few microbodies (9), and the fungus secretes only traces of oxalic acid (6). The possibility exists, therefore, that glyoxylate dehydrogenase is also a microbody enzyme. Hence, this investigation was undertaken to determine the intracellular localization of the key enzymes responsible for oxalic acid biosynthesis in *S. rolfsii*, glyoxylate dehydrogenase and isocitrate lyase.

MATERIALS AND METHODS

Culture and fractionation techniques.—Stock cultures of *S. rolfsii* isolate 14 (6) were maintained on potato dextrose agar (PDA) slants at 24 C, and fresh cultures were prepared about every 2 mo by transferring sclerotia to fresh slants. Experimental cultures were grown in 500-ml Erlenmeyer flasks on a polypectate medium (PPM) consisting of basal salts (8), 0.05% yeast extract (Difco Labs, Detroit, MI 48232), and 1% sodium polypectate

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(Sunkist Growers, Ontario, CA 91764). Cultures to be used for experiments were started by placing several sclerotia in the center of standard 9-cm petri plates containing PDA. When the hyphal mat reached about 5 cm in diameter, plugs were removed from the margin of the mat with a No. 3 cork borer (0.6 cm diameter). Three such plugs were floated on the surface of 50 ml of PPM in each of 25 flasks; the flasks were incubated at 28 C until the hyphal mats just reached the edge of the flasks (4 days). The hyphal mats were transferred aseptically to a sterile Waring Blendor containing 100 ml of fresh PPM and homogenized at top speed for 45 sec. Final experimental cultures were then initiated by adding 7-ml portions of the homogenate to 100 ml of PPM in 30 flasks and incubated for 46 hr at 28 C on a rotary shaker.

Cell-free extracts of hyphae from shake cultures were prepared as previously described (2). The extracts were fractionated on sucrose density gradients in a zonal rotor essentially as described (2), except that the homogenizing medium contained 2 mM instead of 10 mM $MgCl_2$ and the gradient extended from 31.4 to 52.0% (w/w) sucrose. The sucrose concentration of each fraction was determined from its refractive index at 20 C.

Enzyme assays.—All spectrometric assays were performed at 25 C using a Beckman DU monochromator equipped with a Gilford 2220 adapter and light sources and a Gilford 410 digital absorbance meter. Malate dehydrogenase (EC 1.1.1.37) (2), malate synthase (EC 4.1.3.2) (2), isocitrate lyase (EC 4.1.3.1) (2), catalase (EC 1.11.1.6) (2), and glyoxylate dehydrogenase (EC 1.2.1) (7) were assayed essentially as described in the accompanying references.

RESULTS AND DISCUSSION

A large population of young hyphae of approximately the same age could be obtained by initiating shake cultures with hyphal fragments. Because *S. rolfsii* grew faster on potato dextrose broth (PDB) than on host wall-like polysaccharides (1), PDB was used in preliminary experiments as the culture medium for the still cultures from which these hyphal fragments were derived. Little separation of microbody from mitochondrial marker enzymes, however, could be obtained on sucrose density gradients from cell-free extracts of shake cultures initiated with hyphal fragments from cultures grown on PDB. The activity of catalase, the microbody marker enzyme (2,10), straddled that of malate dehydrogenase, the mitochondrial marker enzyme (2,5), with peaks of activity on either side of the malate dehydrogenase peak and separated from it by only one or two fractions.

Appreciable oxalate secretion did not occur until after the hyphae were transferred to the PPM. Also,

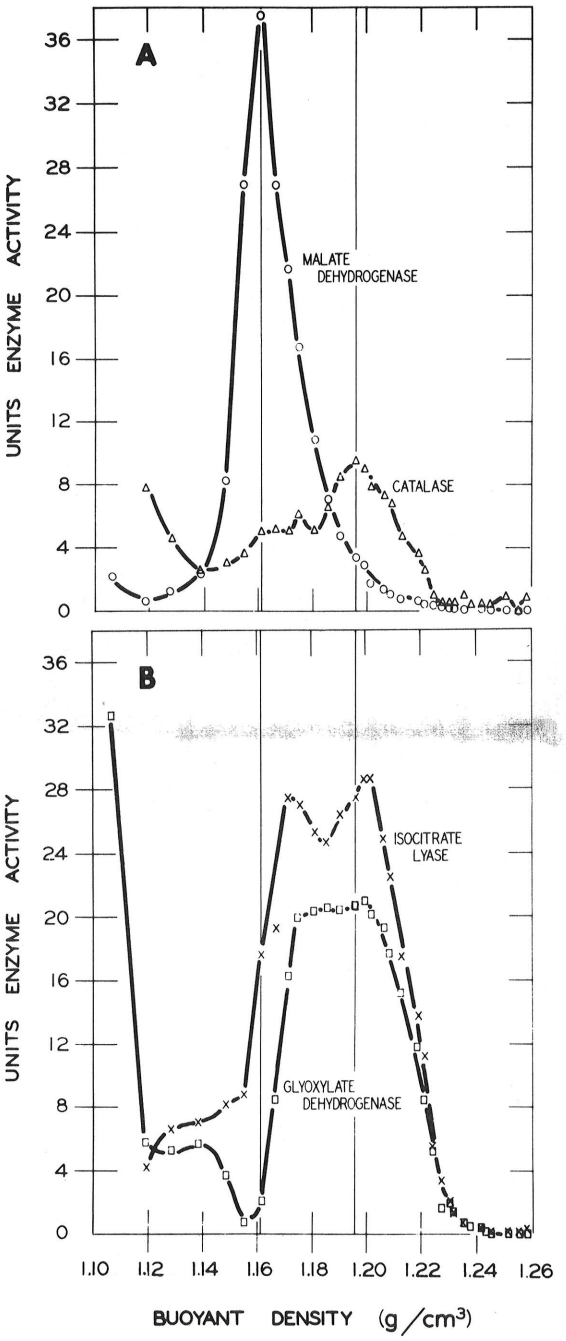

Fig. 1-(A, B). Enzyme distribution after centrifugation in zonal rotor of 3,000 × *g* supernatant from *S. rolfsii* homogenate on linear sucrose gradient. Vertical lines mark densities at which peak activities of malate dehydrogenase and catalase occur. Malate dehydrogenase activity is expressed as number of micromoles of substrate used per minute per 15-ml fraction. Glyoxylate dehydrogenase and isocitrate lyase activities are expressed as number of nanomoles of substrate used per min per 15-ml fraction. One unit of catalase is defined as 1/10 that amount of enzyme that will catalyze destruction of 50% of H_2O_2 present within 100 sec at 25 C under our assay conditions, and catalase is reported as units per 15-ml fractioin. **A)** Distribution of mitochondrial marker enzyme, malate dehydrogenase, and microbody marker enzyme, catalase. **B)** Distribution of key enzymes in oxalic acid biosynthesis, glyoxylate dehydrogenase biosynthesis, glyoxylate dehydrogenase and isocitrate lyase.

significantly fewer microbodies appear in hyphae grown on dextrose than in hyphae grown on polypectate (9). The assumption, therefore, was that the hyphae had to be grown on PPM much longer than 46–48 hr to insure maximum microbody development and optimum compartmentation of the enzymes of oxalate biosynthesis. Unfortunately, *S. rolfsii* produces large amounts of slime when grown on PPM in shake cultures for more than 48 hr, making the preparation of particulate organelle suspensions from such cultures impossible. Therefore, both the shake cultures and the still cultures from which they were derived were grown on PPM, as described in the materials and methods section. Under these conditions, a separation of microbodies from mitochondria was obtained (Fig. 1). The mitochondrial marker enzyme, malate dehydrogenase, had its peak of activity at a buoyant density of 1.162 g/cm^3, whereas the peak activity of catalase, the microbody marker, occurred at 1.196 g/cm^3. Glyoxylate dehydrogenase and isocitrate lyase had distributions on the gradient similar to that of catalase. Although the glyoxylate bypass enzyme, isocitrate lyase, had high activity in both the homogenate and the gradient, the other bypass enzyme, malate synthase, was not detectable in either the gradient fractions or the homogenate. Therefore, isocitrate lyase does not function as part of the glyoxylate cycle, but rather, under these conditions, as part of the pathway for oxalic acid synthesis.

Our results show that the key enzymes involved in the biosynthesis of oxalate in *S. rolfsii,* ie, glyoxylate dehydrogenase and isocitrate lyase, have the same distribution on a sucrose density gradient as does the microbody marker enzyme catalase. As catalase and isocitrate lyase previously have been shown to occur in the microbodies of other fungi (5), higher plants (10), and protozoa (10), a reasonable assumption is that they, as well as glyoxylate dehydrogenase, are localized in the microbodies of *S. rolfsii*. Although the presence of microbodies in fungi-parasitizing plants has been observed repeatedly (5), this is the first report that links microbody function with a process essential to the successful invasion of a host (1,4) by a plant pathogenic fungus.

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