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Presence of a Major (Storage?) Protein in Dormant Spores of the Fungus *Botryodiplodia theobromae*†

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Approximately 23% of the protein isolated from dormant spores of *Botryodiplodia theobromae* consisted of a single polypeptide; the polypeptide is probably degraded during germination.

Fungal spore germination is a morphologically simple process of development in which a nongrowing, metabolically quiescent cell reactivates its physiological processes to produce a rapidly growing germ tube. In the course of our studies on the germination of *Botryodiplodia theobromae* pycnidiospores, protein was isolated from spores at various stages of germination and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The protein from dormant spores contained a prominent protein band which decreased with germination and was not present, or present in very low amounts, in the mycelium. Since, to our knowledge, a similar phenomenon has not been described for spores of any fungi, we have begun a series of experiments to examine the role and significance of this protein in germination and sporulation of *B. theobromae*. This report describes the identification, purification, amino acid composition, and some of the properties of this protein.

The techniques for the growth, harvest, and germination of *B. theobromae* spores have been described elsewhere (2). The fungal material was combined with equal volumes of buffer (0.08 M Tris, 0.001 M dithiothreitol, 0.01 M EDTA, 2% SDS [wt/vol], and 50 μg of phenylmethylsulfonyl fluoride per ml, pH 6.8) and 80% phenol containing 0.1 M ammonium acetate and 0.01 M dithiothreitol and homogenized with 1-mm glass beads in a Braun MSK mechanical homogenizer cooled with CO_2 (2). The homogenate was filtered through several layers of cheesecloth, and the phenol and aqueous layers were separated by centrifugation. The phenol layer, which contained 80 to 90% of the total cellular protein, was extracted three successive times with equal volumes of 0.08 M Tris, 0.001 M dithiothreitol, 0.01 M EDTA, and 0.1 M ammonium acetate, pH 6.8. Total protein was precipitated from the phenol phase by adding 5 volumes of methanol containing 0.1 M ammonium acetate and stored at −20°C for 2 h. Protein was collected by centrifugation, and washed three times with methanol-0.1 M ammonium acetate and finally one time with acetone.

Polyacrylamide gel electrophoresis of protein extracted from *B. theobromae* spores at various times during germination and from mycelium is shown in Fig. 1. The dormant spores contained two prominent protein bands, a major one (labeled A) as well as a minor one (labeled B), which have estimated molecular weights of 18,500 and 14,000, respectively. The amount of protein A present in the cells decreased with germination time (Fig. 1, slots 2 to 8) and was not present or present in very low concentrations in the mycelium (Fig. 1, slot 9). Likewise, protein band B was detected in extracts from dormant spores, and the amount of this protein decreased with germination. However, the mycelium contained a protein(s) which migrated similarly to protein B in polyacrylamide gels. At present it is not known whether the protein(s) present in the mycelium is identical to protein B. In separate experiments we estimate that protein A and protein B constituted about 23% (range of 18 to 27%) and 4%, respectively, of the total protein present in the dormant spore. The decrease in the amount of protein A was probably due to degradation since the total protein content in the spores only increases about 40% during the 6-h germination period (10). Very few additional changes in the protein patterns during germination were detected by these methods.

Protein A was purified to near homogeneity as judged by migration on SDS-polyacrylamide and isoelectric focusing gels (Fig. 2). The isoelectric point of the protein was about 7.85. The amino acid composition of protein A is reported in Table 1. The protein was rich in glutamic acid, aspartic acid, and arginine; because of the slightly basic isoelectric point, many of the aspartic and glutamic acid residues must exist in the amide form. The protein was deficient in

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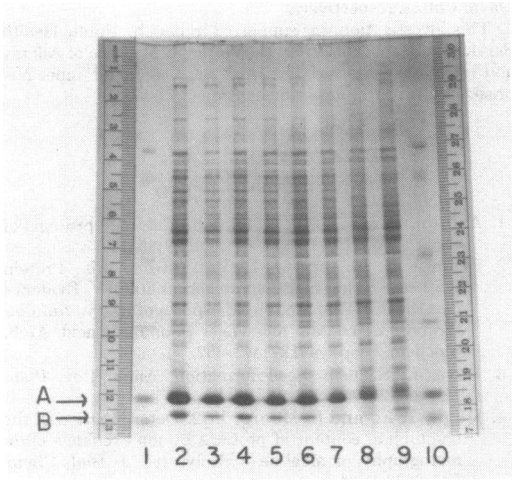

FIG. 1. *Polyacrylamide gel electrophoresis of protein isolated from B. theobromae spores, germinating spores, and mycelium. Slots 2 through 9 contain 40 μg each of protein isolated from dormant spores (slot 2), 1 h of germination (slot 3), 2 h of germination (slot 4), 3 h of germination (slot 5), 4 h of germination (slot 6), 5 h of germination (slot 7), 6 h of germination (slot 8), and mycelium (slot 9). Molecular weight standards of phosphorylase A (94,000), bovine serum albumin (67,000), alcohol dehydrogenase (41,000), chymotrypsinogen (25,700), myoglobin (17,200) and cytochrome c (11,700) were present in slots 1 and 10. Protein was taken up in 10% Ficoll, 2% SDS, 0.06 M Tris, 0.002 M EDTA, 0.005 M dithiothreitol, and 0.004% crystal violet (pH 8) and boiled for 120 s before layering onto a linear 8 to 20% polyacrylamide gel; the samples were subjected to electrophoresis in the buffer system of Laemmli (6) with the apparatus of Studier (14). At the conclusion of the run, the proteins were stained with Coomassie brilliant blue (7).*

sulfur amino acids; it had no cysteine and only two methionine residues. The minimum molecular weight of the protein as calculated from the amino acid composition was 10,000; since the molecular weight was estimated as 18,500 on SDS-polyacrylamide gels (Fig. 1), the actual molecular weight is 20,000. The protein was not a glycoprotein as judged by (i) an absence of staining with Schiff reagent (5), (ii) an absence of amino sugars on the amino acid analyzer, and (iii) the component amino acids that accounted for about 95 to 100% by weight of the purified protein.

Protein A is probably a single protein rather than a mixture of proteins since two-dimensional gels (11) of the spore protein revealed a single major band (data not shown). Protein A has been given the trivial name of muiridin.

Since we were surprised to find a major protein that decreased with germination in *B. theobromae* spores, protein extracted from six other diverse fungal spores was analyzed by SDS-polyacrylamide gels to determine whether this phenomenon was a general characteristic of fungal spores. Protein was isolated from sporangiospores of *Rhizopus stolonifer*, teliospores of *Ustilago maydis* and *Sphaceolotheca reilicena*, ascospores of *Neurospora crassa*, uredospores of *Uromyces phaseoli*, and spores of the slime mold *Dictyostelium discoideum*. However, none of these spores contained a prominent protein band like that observed in *B. theobromae*. Consequently, the phenomenon may occur in a limited number of fungi.

The function of muiridin in the germination of *B. theobromae* spores is unknown, although in some respects it resembles the storage proteins present in seeds of many higher plants (1, 3, 8). The resultant amino acids derived from the degradation of storage proteins during seed germination presumably serve as a source of amino acids for the synthesis of new proteins

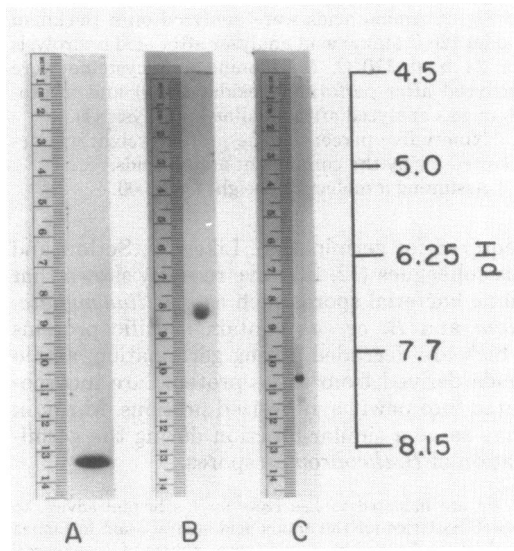

FIG. 2. *Analysis of purified muiridin (protein A) from dormant spores of B. theobromae on (A) a linear 8 to 20% polyacrylamide slab gel as described in the legend to Fig. 1, (B) a 20% polyacrylamide gel, and (C) an isoelectric focusing gel (2% ampholytes, pH 2 to 10) as described by O'Farrell (11). Muiridin was purified by extracting the isolated protein with 1.0 M acetic acid in 35% ethanol. The samples were centrifuged at 8,000 × g, and the pellet was re-extracted with 0.1 M acetic acid, pH 5.0. The combined supernatants were dialyzed against 0.1 M acetic acid, pH 5.0, and applied to a cellulose phosphate column (1.5 by 25 cm). Protein was eluted from the column with a linear 0.75 to 4 M NaCl gradient equilibrated with 0.1 M acetic acid, pH 5.0. Muiridin eluted at about 2 M NaCl and was concentrated by precipitating with methanol.*

TABLE 1. *Amino acid composition of muiridin isolated from dormant spores of B. theobromae*[a]

Amino acid	Protein[b] (nmol/mg)	No. of residues per molecule[c]
Asp	1,053	25
Thr	258	6
Ser	400	10
Glu	1,063	26
Pro	194	5
Gly	304	7
Ala	344	8
Val	306	7
Met	83	2
Ile	395	10
Leu	456	11
Tyr	174	4
Phe	231	6
Lys	381	9
His	246	6
Arg	701	17
Cys	0	0
Trp	139	3
NH_3	2,060	50

[a] The protein was purified as described in the legend to Fig. 2. Amino acids were analyzed on a Beckman model 120 C amino acid analyzer after acid hydrolysis for 24 h at 110°C. Methionine and cysteine were analyzed after performate oxidation (9) and tryptophan was analyzed after alkaline hydrolysis (4).

[b] Ninety-five percent of the protein weight was accounted for by the component amino acids.

[c] Assuming a molecular weight of 20,000.

required for germination. Likewise, Setlow and his colleagues (12, 13) have recently shown that some bacterial spores such as *Bacillus megaterium* and *B. cereus* contain specific proteins which are degraded during germination; amino acids derived from these proteins are incorporated into newly synthesized proteins. Muiridin may serve a similar function during the germination of *B. theobromae* spores.

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