

Biosynthesis and Degradation of Storage Protein in Spores of the Fungus *Botryodiplodia theobromae*†

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Muiridin, a spore-specific protein of the fungus *Botryodiplodia theobromae*, comprises about 25% of the mature pycnidiospore protein. It has an apparent molecular weight of 16,000 to 17,000 and is rich in glutamine, asparagine, and arginine. Muiridin is synthesized in developing spores via a precursor with an apparent molecular weight of 24,000. Two other polypeptides present in young developing spores with apparent molecular weights of 18,000 and 15,000 are immunologically related to muiridin. We propose a pathway for muiridin synthesis. Muiridin is actively degraded during the germination of spores from 30-day-old cultures. This degradation is independent of exogenous amino acids in the germination medium. In contrast, glutamine and, to a lesser extent, asparagine partially inhibit the degradation of muiridin during germination of spores from 7-day-old cultures.

Fungal sporulation and spore germination are examples of morphological development in a simple eucaryote, and they have been investigated by a number of developmental biologists (e.g., see references 2 and 8 for reviews). One of the disappointments in many of these studies has been the inability to associate a specific protein(s) with development. Recently, we demonstrated that asexual pycnidiospores of the fungus *Botryodiplodia theobromae* Pat. contain a polypeptide which comprises about 25% of the total protein of the spore; the protein is undetectable in vegetative cells (9). This protein, given the trivial name muiridin, is apparently synthesized during sporulation and then disappears during spore germination. Muiridin, therefore, is one of only a few examples of a specifically amplified gene product associated with fungal development.

Muiridin has an apparent molecular weight on sodium dodecyl sulfate (SDS) polyacrylamide gels of 16,000 to 17,000. It is rich in glutamine, asparagine, and arginine and has an isoelectric point of 7.8 (9).

In the first section of this report, we describe investigations on the biosynthesis of muiridin during sporulation. We establish that muiridin is

produced in young spores as a larger precursor which is then posttranslationally modified to muiridin as the spores mature. In the second section of this report, we show that muiridin is actively degraded during germination and that under certain conditions, exogenous amino acids in the germination medium can influence the degradation.

MATERIALS AND METHODS

Fungal cultures. *B. theobromae* was grown at 25°C and under constant illumination in 1-liter Erlenmeyer flasks containing 300 ml of V-8 agar (1). Spores were harvested from flasks at 7, 9, 11, 15, 21, and 30 days after inoculation. Incorporation of ³H-labeled amino acids into sporulating cultures was accomplished by applying 1 mCi in 1 ml of water to the surface of 150 ml of V-8 agar in 500-ml flasks. This preparation was allowed to dry for several hours before the flasks were inoculated with the fungus. Growth and sporulation were normal in the presence of isotopic amino acids. The techniques for harvesting and germinating the spores on defined medium (no exogenous amino acids) have been described (1).

Spores were labeled during germination by adding a mixture of ³H-labeled amino acids (2 μCi/ml; New England Nuclear Corp.) to the germination medium at the times indicated below. In some cases, a mixture of unlabeled amino acids (Bacto-Casitone [1 mg/ml]; Difco Laboratories) or individual amino acids (each at 2 mM) were added to the defined medium.

Purification of muiridin. Extraction of total spore protein was described previously (9). This protein extract was suspended in 1 M acetic acid at 20 mg/ml and stirred at 25°C for 2 h; undissolved material was then removed by low-speed centrifugation. The supernatant was dialyzed extensively against 0.1 M acetic

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acid (pH 3.0) and applied to a phosphocellulose column previously equilibrated with 0.1 M acetic acid (pH 3.0). After eluting the unbound material, the column was eluted stepwise with 0.1 M acetic acid (adjusted to pH 5.0 with NaOH), 0.1 M acetic acid-0.75 M NaCl (pH 5.0), and 0.1 M acetic acid-4 M NaCl (pH 5.0). Muiridin, which eluted in 0.1 M acetic acid-4 M NaCl (pH 5.0), was collected, dialyzed against water, and lyophilized. The lyophilized muiridin was suspended in phosphate-buffered saline (PBS) at 2 mg/ml.

Serology. Antiserum was raised against muiridin purified from 30-day-old spores in randomly bred rabbits. The rabbits received four intramuscular injections in the thigh at 7-day intervals (1.0 mg muiridin plus 1.0 mg methylated bovine serum albumin in Freund incomplete adjuvant per injection). Injections were alternated between the right and left hind legs. At 11 days after the last intramuscular injection, 0.1 mg of muiridin in PBS was injected intraperitoneally. The rabbits were bled 1 week later by cardiac puncture. The serum immunoglobulin G (IgG) fraction was isolated by the method of Palmiter et al. (6), taken up in PBS (pH 7.4), and frozen in small portions.

Protein was immunoprecipitated by mixing equal volumes of sample protein (0.25 mg/ml in PBS) with IgG. The reaction mixture was adjusted to 1% Triton X-100 and incubated for 30 min at 37°C and then overnight at 4°C. Precipitated antibody-antigen complex was collected by centrifugation. The pellets were washed three times in PBS before suspension in 0.5% SDS. Normal serum prepared the same way as muiridin IgG was always run as a control in the experiments; it did not precipitate any of the fungal proteins.

Other procedures. Spore proteins were labeled *in vitro* by the method of Kumarasamy and Symons (3), using $^3\text{H-KBH}_4$ (35 Ci/mmol). One-dimensional polyacrylamide gel electrophoresis, densitometer scanning, and scan integration were as previously described (7). Two-dimensional acrylamide gel electrophoresis was done as described by O'Farrell (5).

RESULTS

Culture conditions. Under normal culture conditions mycelial growth covered the surface of V-8 agar 3 to 4 days after inoculation. Pycnidia appeared at 4 to 5 days, and spores could be harvested as early as 5 days; however, the yield was extremely low. These spores were hyaline (nonpigmented) and nonseptate and often contained remnants of the stalk which attached them to the pycnidium. The spore yield increased over the next few days, with the greatest increase occurring between days 8 and 9. At 12 to 13 days after inoculation, the production of spores appeared to be complete. No further changes in the culture or spores were detected after this point. Spores were routinely harvested at 30 days. About 20% of the 30-day spores were pigmented and septate. All of the spores, regardless of the age of the culture, germinated synchronously on the defined medium; germ tubes

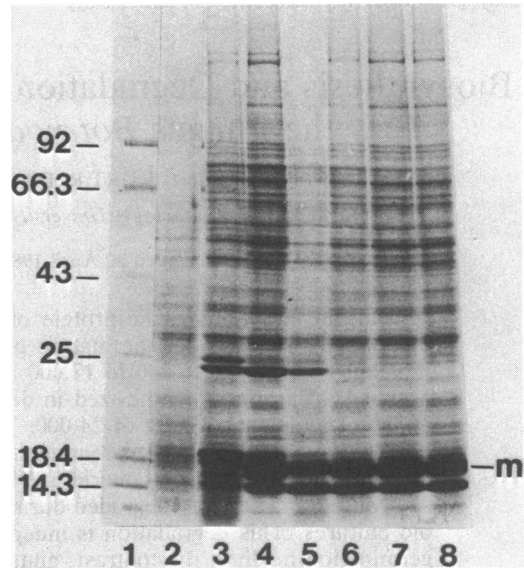


FIG. 1. Polyacrylamide gel electrophoresis of protein extracted from spores of various ages. Lane 1, Molecular weight reference proteins, phosphorylase A (92,000), bovine serum albumin (66,300), ovalbumin (43,000), α -chymotrypsinogen A (25,000), β -lactoglobulin subunit (18,400), and lysozyme (14,300). Lane 2, Protein from vegetative cells. Lanes 3 to 8, Protein from 7-, 9-, 11-, 15-, 21-, and 30-day spores, respectively. m, Muiridin.

were first observed after 2.5 to 3 h of germination, and by 5 to 6 h, 90 to 95% of the spores had germ tubes (1).

Evidence that muiridin is synthesized from a precursor. By polyacrylamide gel electrophoresis of protein extracted from spores harvested at various times during sporulation, muiridin first appeared in the spores at day 11 (Fig. 1). Although spores contained little muiridin at 7 and 9 days, they contained a number of other prominent proteins. These proteins decreased as muiridin increased in the spores, suggesting that one or more of them might be a precursor to muiridin.

As mentioned above, 7-day spores (although they contained no detectable muiridin) germinated normally. Electrophoresis of proteins extracted from germinating 7-day spores revealed that a protein with the same molecular weight and isoelectric point as muiridin appeared at 4 h. This protein was also precipitated with antibody to muiridin, and thus, we assume it is muiridin. The appearance of muiridin in these germinating spores was similar to, but more rapid than, the appearance of muiridin during normal sporulation. As in normal sporulation, the appearance of muiridin was associated with the disappearance of some of the major 7-day proteins (Fig. 2,

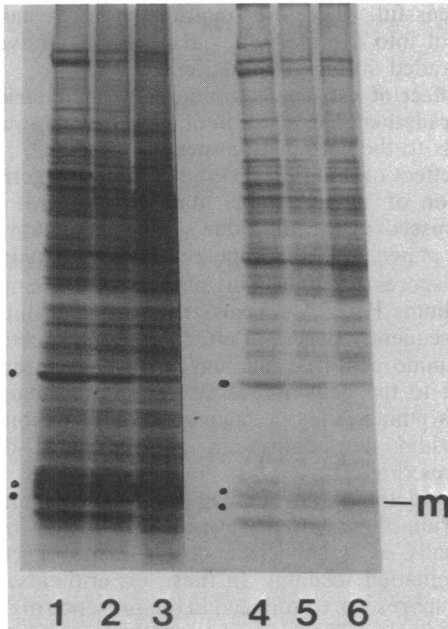


FIG. 2. Polyacrylamide gel electrophoresis of protein extracted from germinating 7-day spores. The spores were produced in the presence of ^3H -labeled amino acids as described in the text. Germination was in a defined medium containing unlabeled amino acids. Lanes 1 to 3, 0, 2, and 4 h of germination. Lanes 4 to 6, Fluorograph of lanes 1 to 3. The dots identify three proteins, relevant to this paper, which decrease in concentration with germination. m, Muiridin.

lanes 1–3). The appearance of muiridin in germinating 7-day spores provided a convenient system to investigate the relationship between the 7-day proteins and muiridin.

Radioactively labeled 7-day spores were produced by growing the fungus in the presence of a ^3H -labeled amino acid mixture as described above. These spores were then germinated in the presence of unlabeled amino acids. The germination rate of the spores was unaffected by the exogenous amino acids. Analysis of the proteins during germination (Fig. 2) indicated that the muiridin which appeared at 4 h was radioactively labeled. The converse experiment with unlabeled 7-day spores, but provided with ^3H -labeled amino acids during germination, labeled many proteins after 4 h but did not label muiridin (Fig. 3). This indicates that muiridin was synthesized from precursor(s) present in young 7-day spores.

To determine which sporulation proteins were muiridin precursors, antibody prepared against purified muiridin was incubated with *in vitro* ^3H -labeled protein from 7-, 11-, 15-, and 30-day spores. The precipitated protein was then ana-

lyzed by SDS-polyacrylamide gel electrophoresis (Fig. 4). Only muiridin was precipitated from 11-, 15-, and 30-day spore protein, confirming the specificity of the antibody. Several polypeptides from the 7-day spore protein were antigenically related to muiridin (Fig. 4, lane 2). Three of these, designated M1, M2, and M3, with apparent molecular weights of 24,000, 18,000, and 15,000, respectively, were major components of 7-day spore proteins; these proteins decreased during sporulation coincident with the appearance of muiridin (apparent molecular weight of 16,000).

Two-dimensional acrylamide gel analysis of 7- and 30-day spore protein (data not shown) revealed that the 24,000-dalton polypeptide had a pI of ca. 6.9. The 18,000- and 15,000-dalton polypeptide had similar pIs of ca. 8.0; muiridin had a pI of ca. 7.8.

Degradation of muiridin during germination. Electrophoretic analysis of protein extracted from 30-day spores at various times during germination revealed that muiridin virtually disappeared 4 to 6 h after the spores were placed in germination medium (Fig. 5). To determine whether the disappearance of muiridin reflected active degradation or resulted from dilution by

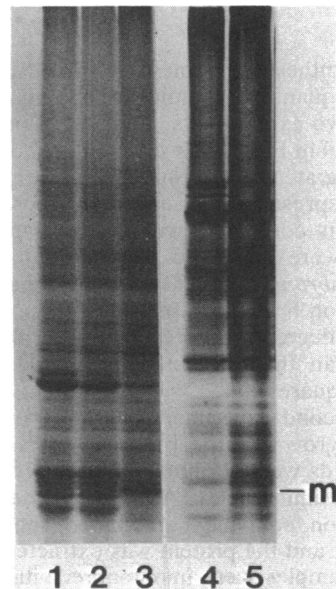


FIG. 3. Polyacrylamide gel electrophoresis of protein extracted from germinating 7-day spores. Unlabeled 7-day spores were germinated in a defined medium containing ^3H -labeled amino acids, as described in the text. Lanes 1 to 3, 0, 2, and 4 h of germination. Lanes 4 and 5, Fluorograph of lanes 2 and 3. Note: muiridin is formed during germination (lane 3), as detected by staining. However, no label appears in the region of muiridin (lane 5). m, Muiridin.

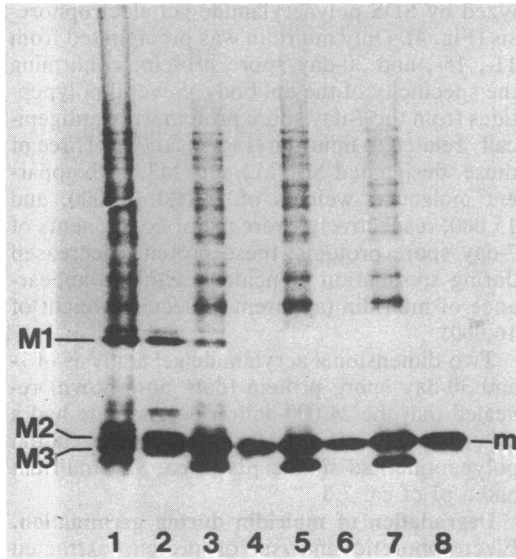


FIG. 4. Fluorograph of polyacrylamide gel electrophoresis of $^3\text{H-KBH}_4$ in vitro labeled protein from spores of various ages. Total protein from 7-day (lane 1), 11-day (lane 3), 15-day (lane 5), and 30-day (lane 7) spores was immunoprecipitated with antibody against purified muiridin. Ages of the immunoprecipitated materials in lane 2, 7-day; lane 4, 11-day; lane 6, 15-day; and lane 8, 30-day. m, Muiridin.

newly synthesized protein, we measured the absolute quantity of muiridin as a function of time in two experiments. First, the Coomassie-stained gel in Fig. 5 was scanned, and the peaks were integrated to determine the percent of total protein represented by muiridin. These values were then compared with those expected if muiridin were conserved. Total protein contents of *B. theobromae* spores at various times during germination have been previously reported (4). Muiridin decreased more rapidly during germination than that predicted by simple dilution (Fig. 6, squares).

In a second experiment, 30-day spores from cultures grown in the presence of ^3H -labeled amino acids were germinated in the presence of unlabeled amino acids. At various times during germination, equivalent amounts of spores were collected, and the protein was extracted. These protein samples were immunoprecipitated with muiridin antibody, and the antibody-precipitable radioactivity was determined and compared with the value from an identical number of ungerminated spores (Fig. 6, circles). If muiridin is conserved, the antibody-precipitable radioactivity would remain constant. In fact, muiridin decreased rapidly during germination.

Attempts to recover muiridin from medium in which mature spores had germinated were un-

successful. Therefore, muiridin is not released intact into the medium, but instead is actively degraded during germination.

Effect of exogenous amino acids on muiridin degradation. The addition of a mixture of amino acids to the germination medium (1 mg/ml) had no effect on muiridin degradation during germination of 30-day spores (data not shown). As discussed in the muiridin synthesis section of this paper, muiridin appeared in germinating 7-day spores 4 h after being placed in germination medium. Fig. 7A reveals that this muiridin is subsequently degraded after 6 h of germination in amino acid-free medium. However, in contrast to the behavior of 30-day spores, exogenous amino acids (1 mg/ml) partially inhibited muiridin degradation in germinating 7-day spores (Fig. 7B).

To determine which amino acids inhibited muiridin degradation, several were tested individually at a concentration of 2 mM in the germination medium. In these experiments, 7-day spores were produced in the presence of ^3H -amino acids. These spores were then germinated in medium containing one of each of 9 amino acids. Since muiridin arises from precursor(s) in

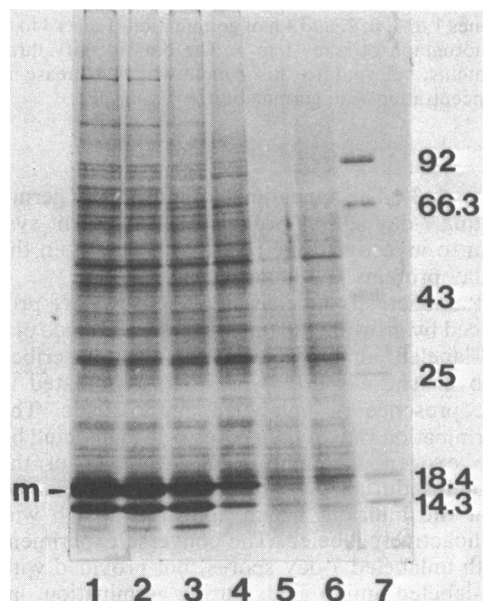


FIG. 5. Polyacrylamide gel electrophoresis of proteins extracted from spores at various times during germination. Thirty-day spores were germinated in a defined medium (lacking exogenous amino acids). Protein was extracted from ungerminated spores (lane 1), and at 0 h (lane 2), 2 h (lane 3), 4 h (lane 4), 6 h (lane 5), and 23 h (lane 6). The molecular weight marker proteins in lane 7 are the same as in Fig. 1. m, Muiridin.

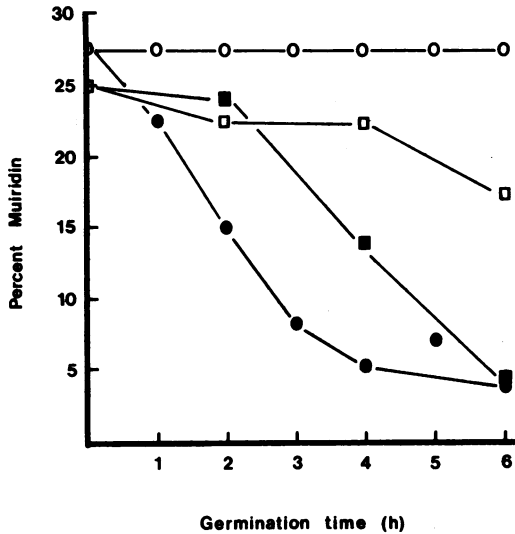


FIG. 6. The disappearance of muiridin during germination of 30-day spores. The gel in Fig. 5 was scanned with a densitometer and the percent of the total protein represented by muiridin determined (■). A hypothetical curve based upon spore protein content at various times during germination and assuming that muiridin was conserved is represented by □. In a separate experiment, spores produced in the presence of ^3H -labeled amino acids were germinated in the presence of exogenous unlabeled amino acids. At various times during germination, equal aliquots of spores were removed and protein was extracted and immunoprecipitated with muiridin antibody. If muiridin were conserved, the immunoprecipitated counts should have remained constant (○). The actual percentage of immunoprecipitable counts obtained are represented by ●.

these spores, any muiridin appearing at 4 h would be radioactively labeled, as would any muiridin remaining undegraded after 6 h. After 6 h of germination, total protein was extracted and suspended in PBS. An equal amount of total protein from each sample was immunoprecipitated with muiridin antibody and the amount of radioactivity in the immunoprecipitate determined. Glutamine was the amino acid most effective at inhibiting the degradation of muiridin in germinating 7-day spores (Table 1). Asparagine also inhibited muiridin degradation. The other amino acids tested had little or no effect.

DISCUSSION

Synthesis of muiridin. The results clearly indicate that 7-day spores contain a muiridin precursor. Three proteins designated M1, M2, and M3 present in 7-day spores are probable precursors of muiridin, owing to their antigenic relationships and because their concentrations decrease

coordinately with muiridin accumulation. Considering their antigenic similarities, as well as their molecular weights and isoelectric points, we propose that muiridin is first synthesized as a precursor (M1) with an apparent molecular weight of 24,000 and pI of 6.9. M1 is processed via limited site-specific proteolysis through two intermediates (M2 and M3) with apparent molecular weights of 18,000 and 15,000, respectively, and identical pIs of 8.0. M3 is then modified possibly by phosphorylation, glycosylation, ribosylation, acetylation, methylation, etc. This modification increases the M3 apparent molecular weight to 16,000 and lowers its isoelectric point to 7.8. Similar shifts have been reported in other systems. For example, J. H. Postlethwait, has observed a shift toward a higher apparent molecular weight and increased acidity when *Drosophila* spp. yolk protein precursors are modified by phosphorylation (personal communication). We labeled sporulating cultures with ^{32}P -orthophosphate, isolated protein from mature spores, and analyzed it on SDS-polyacrylamide gels, followed by fluorography. These experiments (not shown here) hint that muiridin might be phosphorylated to a limited extent. However, attempts to enzymatically or chemically dephosphorylate muiridin to M3 were unsuccessful. It, therefore, seems unlikely that phosphorylation alone explains the modification of M3. As reported earlier (9), muiridin contains

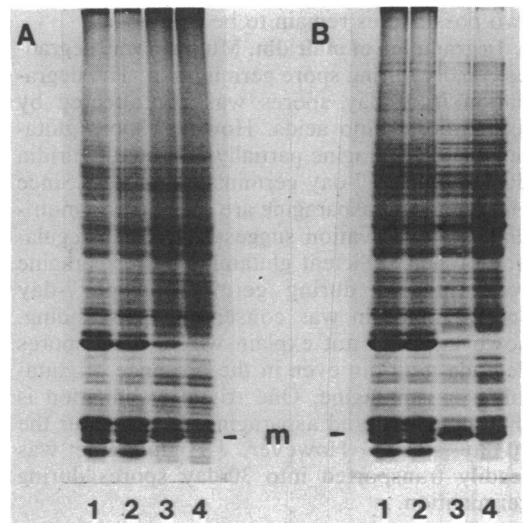


FIG. 7. The effect of exogenous amino acids on muiridin degradation during the germination of 7-day spores. The spores were germinated in the absence (A) and presence (B) of amino acids as described in the text. Protein was extracted from spores after 0, 2, 4, and 6 h (lanes 1 to 4, respectively) of germination. m, Muiridin.

TABLE 1. The effect of exogenous amino acids on muiridin degradation during germination of 7-day spores

| Germination conditions | % Muiridin remaining ^a |
|---|-----------------------------------|
| Ungerminated spores (control) | 100 |
| Spores germinated (6 h) with exogenous amino acids: | |
| None | 8 (13.5; 1.9; 8.7) ^b |
| Glutamine | 26.9 (36.8; 12.5; 31.5) |
| Asparagine | 20.8 (25.9; 8.5; 28) |
| Glutamic acid | 13.4 (14.7; 5.2; 20.2) |
| Aspartic acid | 8.9 (14; 3.9; —) |
| Arginine | 14.5 (20.6; 8.4; —) |
| Glycine | 8.1 (12; 5.6; 6.6) |
| Cysteine | 11.2 (18.8; 6.5; 8.3) |
| Tyrosine | 9.8 (20.3; 3; 6.2) |
| Threonine | 8.1 (13.3; 3; —) |

^a Muiridin was immunoprecipitated from protein extracted from an equal number of spores in each case.

^b The values in parentheses are percentages of muiridin remaining for three separate experiments. The amount of muiridin immunoprecipitated in the ungerminated spores (considered to be 100%) contained 2,079, 2,901, and 8,300 cpm, respectively, for the three separate experiments.

^c —, Not determined.

no detectable carbohydrates; therefore, glycosylation and ribosylation of M3 appears unlikely. This leaves acetylation and methylation. These two possibilities remain to be examined.

Degradation of muiridin. Muiridin was degraded rapidly during spore germination. The degradation in 30-day spores was not affected by exogenous amino acids. However, both glutamine and asparagine partially inhibited muiridin degradation in 7-day germinating spores. Since glutamine and asparagine are abundant in muiridin, our observation suggests feedback regulation. When sufficient glutamine and asparagine were present during germination of 7-day spores, muiridin was conserved. This finding, however, does not explain why 30-day spores degrade muiridin even in the presence of glutamine or asparagine. One trivial explanation is that glutamine and asparagine do not enter the 30-day spores. However, [³H]glutamine was readily transported into 30-day spores during germination.

A second possibility is that glutamine, asparagine, or both control the synthesis of a protease(s) which specifically degrades muiridin. This protease(s) is normally synthesized during sporulation between 7 and 30 days. In germinating 7-day spores, excess glutamine or asparagine inhibit the biosynthesis of this protease(s), whereas in 30-day spores, these amino acids have no effect, since the protease(s) has already been synthesized.

In summary, muiridin is a relatively rare example of a major fungal protein whose synthesis and degradation are developmentally regulated. Muiridin apparently serves as a storage protein or reservoir of amino acids which spores can utilize during germination in an amino acid-poor environment.

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