

THE DIFFERENCE BETWEEN MALATE SYNTHASE SPECIFIC ACTIVITY OF LIGHT AND DARK SPORED AGARICS IS NOT DUE TO PHENOLIC CONTAMINATION

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ABSTRACT: The significant difference in specific activity of malate synthase between light and dark spored agarics is not due to phenolic contamination associated with dark spored wall pigments. First, the specific activity of malate synthase was determined for individual species. Then, the specific activity of malate synthase was measured for combined homogenates (i.e., a dark spored species combined with a light spored species). If no interfering phenolic compounds were present, the observed specific activity of malate synthase for the combined homogenate should equal the expected specific activity of malate synthase (i.e., the sum of the individual specific activity of malate synthase for the two species combined divided by two). Results of such assays found no significant difference between observed and expected specific activity of malate synthase for combined homogenates. The taxonomic implications of these findings are discussed.

KEY WORDS: Enzyme assay, gilled mushrooms, glyoxylate cycle, homobasidiomycetes, malate synthase.

INTRODUCTION

While conducting a survey to determine the ubiquity of the glyoxylate cycle in basidiospores of homobasidiomycetes, Ruch, *et al.* (1991) and Ruch and

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Table 1. Specimens used in this study and their Ball State University Herbarium Mycology numbers. Specimens are arranged by family (Miller, 1979).

Species	BSU Herbarium Number
Lepiotaceae (Spores primarily white)	
<i>Lepiota americana</i> Peck	18
Tricholomataceae (Spores primarily white)	
<i>Armillariella mellea</i> (Fr.) Karst	100
<i>Armillariella tabescens</i> (Scop. ex Fr.) Singer	1
<i>Flammulina velutipes</i> (Fr.) Singer	170
<i>Laccaria ochropurpurea</i> (Berk.) Peck	67, 88
<i>Lactarius psammicola</i> var. <i>glaba</i> Smith	79, 80
<i>Lentinellus ursinus</i> (Fr.) Kuehn.	129
<i>Leucoagaricus naucinus</i> (Fr.) Singer	87
<i>Mycena leaiana</i> (Beck.) Sacc.	21
<i>Oudemansiella radicata</i> (Fr.) Singer	25, 66
<i>Phyllotopsis nidulans</i> (Pers. ex Fr.) Singer	128
<i>Tricholomopsis platyphylla</i> (Fr.) Singer	19
Pluteaceae (Spores pink)	
<i>Pluteus granularis</i> Peck	154
Cortinariaceae (Spores shades of brown to bright rusty-orange)	
<i>Cortinarius</i> sp.	151
<i>Gymnopilus liquiritiae</i> (Pers.) Karst.	47
Agaricaceae (Spores chocolate-brown to blackish-brown in age)	
<i>Agaricus campestris</i> Fr.	50, 89
Bolbitiaceae (Spores rusty or earthy brown)	
<i>Agrocybe dura</i> (Fr.) Singer	82
Coprinaceae (Spores deep brown to black)	
<i>Coprinus insignis</i> Peck	94, 102
<i>Coprinus micaceus</i> (Bull. ex Fr.) Fries	9, 12
<i>Panaeolus foenisecii</i> (Fr.) Kuehn.	17
<i>Psathyrella velutina</i> (Fr.) Singer	62
Strophariaceae (Spores purple-brown to purple-black or black)	
<i>Stropharia ambigua</i> (Pk.) Zeller	15

Samuel (1992) reported some intriguing results concerning light spored and dark spored agarics. The dark spored agarics (Families Agaricaceae, Bolbitiaceae, and Coprinaceae) possessed much lower specific activity of malate synthase than did the white (Families Amanitaceae, Lepiotaceae, Hygrophoraceae, Russulaceae, and Tricholomataceae), pink (Families Volvariaceae and Rhodophyllaceae), and light brown (Family Cortinariaceae) spored agarics. By using the Mann-Whitney test (Zar, 1974), a nonparametric analog to the two-sample *t*-test, the dark spored agarics demonstrated significantly lower malate synthase specific activity than the lighter spored species (Mann-Whitney Test: $U = 280$, $P < 0.001$).

A possible explanation for the observed difference in malate synthase activity between light and dark spored agarics is the presence of contaminating phenolic compounds associated with the dark pigments of the wall. Since phenolic compounds can function as enzyme inhibitors, their presence might account for the low specific activity demonstrated in the dark spored species. Ruch, *et al.* (1991) and Ruch and Samuel (1992) did not test for the presence of such contaminants in homogenates of dark spored mushrooms. This study was undertaken to determine if the difference in malate synthase activity between light and dark spored agarics is due to phenolic interference or implies a taxonomic phenomenon.

MATERIAL AND METHODS

The mushrooms used in this study are listed in Table 1 along with their Ball State University Herbarium mycology numbers (BSUHM). Homogenates were prepared as previously described (Cooper and Beevers, 1969; Ruch, *et al.*, 1991). Spores were homogenized in a Braun MKS cell homogenizer for a total of 60 sec at 4° C. The homogenate was centrifuged for 10 min at 500x *g* at 4° C. After removing any lipids that had collected at the surface, the supernatant was used for enzyme assays. Malate synthase activity was assayed by measuring the formation of a yellow complex between DTNB (5,5-dithiobisnitrobenzoic acid) and the sulfhydryl group of coenzyme A at a wavelength of 412 nm at 25° C. Assays were initiated by addition of 0.02 ml enzyme extract and were done in triplicate. Specific activity (SA) of malate synthase equals units of enzyme activity per mg of protein. One enzyme unit is defined as the amount of enzyme that produced 1 μ mol of product per minute. Total protein concentration was determined by the method used by Lowry, *et al.* (1951).

To detect possible phenolic interference, malate synthase activity was first determined for the individual species. Next, the homogenate from a dark spored species was combined with the homogenate of a light spored species, and the specific activity of malate synthase (the observed SA) was determined for the combined homogenate. If no interfering phenolic compounds were present, the observed SA of malate synthase for the combined homogenate should equal the expected SA, which is the sum of the individual activities of the two species combined divided by two. (The sum is divided by two since mixing

two species dilutes the proteins (enzymes) by half, and assays were still initiated with 0.02 ml of homogenate.) If phenolic interference occurs, the observed specific activity of malate synthase for the combined homogenate should be significantly less than the expected SA.

The homogenates of different light spored species were also combined and assayed. These tests served as controls since the SA of malate synthase of light spored agarics was high and assumed not to be inhibited by phenolic contaminants. The homogenates of different dark spored species were also combined and assayed. These homogenates also served as controls. If the basis of the low SA of malate synthase in the dark spored species (Ruch, *et al.*, 1991; Ruch and Samuel, 1992) is due to phenolic contamination, then the observed SA for the combined dark spored homogenate is expected to be significantly lower than the expected SA due to the abundance of phenolics.

Statistical analyses were done using the Mann-Whitney *U*-test (Zar, 1974) and the chi square test (Sokal and Rohlf, 1981). For the Mann-Whitney *U*-test, the specific activity for all light and dark spored species from all experiments (Tables 2-5) were used. For species used in different assays, such as *Lepiota americana* (Tables 2, 3, 5), each assay was considered to be a separate sample.

RESULTS

The specific activities (SA) of malate synthase for individual and combined homogenates are summarized in Tables 2 through 5. Each table contains the results of all assays run at one time. For species used in assays run at different times, such as *Agaricus campestris* (Tables 2, 4, and 5), the results in each table represent the SA of malate synthase determined for that species during that particular assay; they do not represent an average SA for all assays run at different times in which the species was used. In all cases, the observed SA of malate synthase for combined homogenates (i.e., light plus light spored agarics, dark plus dark spored agarics, and light plus dark spored agarics) were practically identical to the expected SA (Tables 2-5).

Chi square analysis for the observed SA versus expected SA are summarized in Table 6. Chi squares were calculated for the combined results (i.e., light plus light spore agarics in Tables 2 through 5 combined; dark plus dark spored agarics in Tables 3 through 5 combined; and light plus dark spored agarics in Tables 2 through 5 combined). Chi square values were very low ($P > 0.995$), supporting the hypothesis that the observed SAs are equal to (or not significantly different from) the expected SAs.

DISCUSSION

The observed specific activity (SA) of malate synthase was not significantly different from the expected SA of malate synthase in all tests and was supported by a chi square analysis of the observed and expected SA ($P > 0.995$; Table 6). If the homogenization of dark agaric spores had released (or produced) significant amounts of phenolic contaminants, then these homogenates, when

Table 2. Malate synthase specific activity for individual and combined homogenates of agarics.

Species	Malate Synthase SA ^b	
Light Spored		
<i>Lepiota americana</i> ^a	8.83	
<i>Armillariella mellea</i>	6.69	
<i>Pluteus granularis</i> ^a	3.84	
<i>Tricholomopsis platyphylla</i> ^a	3.65	
<i>Cortinarius sp.</i> ^a	2.75	
Dark Spored		
<i>Agaricus campestris</i>	1.79	
Homogenates Combined	Malate Synthase SA Observed	Expected ^c
Light Spored x Light Spored		
<i>L. americana</i> x <i>A. mellea</i>	7.47	7.76
Dark Spored x Light Spored		
<i>A. campestris</i> x <i>L. americana</i>	5.15	5.31
<i>A. campestris</i> x <i>A. mellea</i>	4.07	4.24

^a Species that have not previously been assayed for malate synthase activity.

^b Specific activity (SA) of malate synthase equals units of enzyme activity per mg of protein. One enzyme unit is defined as the amount of enzyme that produced 1 μ mol of product per min.

^c Expected SA was determined by adding the SA of malate synthase for both species whose homogenates were combined and dividing by two (see Materials and Methods).

mixed with homogenates from light spored species, would have denatured the malate synthase in the mixture. Had this occurred, the observed SA of malate synthase for any of the light spored plus dark spored combined homogenates would have been significantly less than the expected SA (Smith and Berry, 1976). Since the experimental SA's were not reduced, no phenolic contamination occurred. This conclusion is supported by the findings for the two control groups (e.g. light plus light spored agarics and dark plus dark spored agarics). When homogenates of two different light spored species were mixed, the observed SA was not significantly different from the expected SA (Tables 2-5). This result was expected since the SA of malate synthase of light spored agarics was high and assumed not to be inhibited by phenolic contaminants. Likewise, when homogenates of two dark spored species were mixed, the observed SA was not significantly different from the expected SA (Tables 3-5). These findings were unexpected if the basis of the low SA of malate synthase in the dark spored species was due to phenolic contamination. If phenolic contamination was influencing maltate

Table 3. Malate synthase specific activity for individual and combined homogenates of agarics.

Species	Malate Synthase SA ^b	
Light Spored		
<i>Gymnopilus liquiritiae</i> ^a	8.15	
<i>Lactarius psammicola</i> var. <i>glaba</i> ^a	6.63	
<i>Lepiota americana</i> ^a	7.91	
<i>Mycena leaiana</i> ^a	4.35	
Dark Spored		
<i>Agrocybe dura</i> ^a	2.75	
<i>Coprinus micaceus</i>	1.41	
<i>Panaeolus foenisecii</i> ^a	2.06	
Homogenates Combined		
	Malate Synthase SA Observed	Expected ^c
Light Spored x Light Spored		
<i>M. leaiana</i> x <i>L. americana</i>	5.90	6.13
<i>L. psammicola</i> x <i>M. leaiana</i>	5.37	5.49
Dark Spored x Dark Spored		
<i>C. micaceus</i> x <i>P. foenisecii</i>	1.75	1.74
Dark Spored x Light Spored		
<i>C. micaceus</i> x <i>M. leaiana</i>	2.72	2.88
<i>C. micaceus</i> x <i>L. psammicola</i>	3.93	4.02
<i>P. foenisecii</i> x <i>L. psammicola</i>	4.20	4.35
<i>P. foenisecii</i> x <i>M. leaiana</i>	3.23	3.21

^a Species that have not previously been assayed for malate synthase activity.

^b Specific activity (SA) of malate synthase equals units of enzyme activity per mg of protein. One enzyme unit is defined as the amount of enzyme that produced 1 μ mol of product per min.

^c Expected SA was determined by adding the SA of malate synthase for both species whose homogenates were combined and dividing by two (see Material and Methods).

synthase activity, the observed SA for the combined dark spored homogenate would be predicted to be significantly lower than the expected SA due to the abundance of phenolic contaminants interfering with enzyme activity. Furthermore, Ruch, *et al.* (1991) and Ruch and Samuel (1992) did not observe a similar correlation between light and dark spored species of nongilled mushrooms. The dark spored Aphyllophorales and Gasteromycetes possessed high malate synthase activity, equal to that of the light spored species, suggesting the absence of phenolic contamination.

The absence of phenolic interference implies that the difference in specific activity of malate synthase between dark and light spored agarics is a taxo-

Table 4. Malate synthase specific activity for individual and combined homogenates of agarics.

Species	Malate Synthase SA ^b	
Light Spored		
<i>Armillariella tabescens</i>	5.83	
<i>Laccaria ochropurpurea</i>	5.10	
<i>Lentinellus ursinus</i> ^a	14.79	
<i>Leucoagaricus naucinus</i>	5.90	
<i>Oudemansiella radicata</i>	2.87	
<i>Phyllotopsis nidulans</i> ^a	11.61	
<i>Tricholomopsis platyphylla</i> ^a	4.35	
Dark Spored		
<i>Agaricus campestris</i>	0.77	
<i>Coprinus insignis</i> ^a	1.03	
<i>Psathyrella velutina</i> ^a	0.74	
<i>Stropharia ambigua</i> ^a	2.09	
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Homogenates Combined	Malate Synthase SA	
	Observed	Expected ^c
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Light Spored x Light Spored		
<i>A. tabescens</i> x <i>T. platyphylla</i>	4.89	5.09
<i>P. nidulans</i> x <i>L. ochropurpurea</i>	8.13	8.36
Dark Spored x Dark Spored		
<i>P. velutina</i> x <i>A. campestris</i>	0.75	0.76
<i>P. velutina</i> x <i>S. ambigua</i>	1.36	1.42
Dark Spored x Light Spored		
<i>C. insignis</i> x <i>L. naucinus</i>	3.33	3.47
<i>C. insignis</i> x <i>O. radicata</i>	1.76	1.95
<i>A. campestris</i> x <i>A. tabescens</i>	3.17	3.30
<i>A. campestris</i> x <i>L. ochropurpurea</i>	2.98	2.94
<i>S. ambigua</i> x <i>P. nidulans</i>	6.71	6.85
<i>P. velutina</i> x <i>L. ochropurpurea</i>	2.93	2.92

^a Species that have not previously been assayed for malate synthase activity.

^b Specific activity (SA) of malate synthase equals units of enzyme activity per mg of protein. One enzyme unit is defined as the amount of enzyme that produced 1 μ mol of product per min.

^c Expected SA was determined by adding the SA of malate synthase for both species whose homogenates were combined and dividing by two (see Material and Methods).

Table 5. Malate synthase specific activity for individual and combined homogenates of agarics.

Species	Malate Synthase SA ^b	
Light Spored		
<i>Flammulina velutipes</i> ^a	4.24	
<i>Laccaria ochropurpurea</i> ^a	6.21	
<i>Leucoagaricus naucinus</i>	7.92	
<i>Lepiota americana</i> ^a	6.72	
Dark Spored		
<i>Coprinus micaceus</i>	2.67	
<i>Agaricus campestris</i>	2.68	
Homogenates Combined	Malate Synthase SA Observed Expected ^c	
Light Spored x Light Spored		
<i>F. velutipes</i> x <i>L. ochropurpurea</i>	5.32	5.23
<i>L. naucinus</i> x <i>L. americana</i>	7.22	7.32
<i>F. velutipes</i> x <i>L. naucinus</i>	5.69	6.08
Dark Spored x Dark Spored		
<i>C. micaceus</i> x <i>A. campestris</i>	2.66	2.68
Dark Spored x Light Spored		
<i>C. micaceus</i> x <i>L. ochropurpurea</i>	4.31	4.44
<i>C. micaceus</i> x <i>L. naucinus</i>	5.03	5.30
<i>C. micaceus</i> x <i>L. americana</i>	4.46	4.70
<i>A. campestris</i> x <i>L. ochropurpurea</i>	4.48	4.45
<i>A. campestris</i> x <i>F. velutipes</i>	3.36	3.46
<i>A. campestris</i> x <i>L. americana</i>	4.47	4.70

^a Species that have not previously been assayed for malate synthase activity.

^b Specific activity (SA) of malate synthase equals units of enzyme activity per mg of protein. One enzyme unit is defined as the amount of enzyme that produced 1 μ mol of product per min.

^c Expected SA was determined by adding the SA of malate synthase for both species whose homogenates were combined and dividing by two (see Material and Methods).

Table 6. Chi squares for observed specific activity versus expected specific activity of malate synthase.

nomic phenomenon, a fact supported by the good correlation between the findings of this study and the current phylogenetic scheme for the gilled mushrooms. The classical approach to gill mushroom classification, based primarily on morphological, ultrastructural, and biochemical characteristics, separates the light spored agarics into one major lineage and the dark spored agarics into another lineage (Bruns, *et al.*, 1991; Cain, 1972; Moore-Landecker, 1996; Rayner, *et al.*,

Table 6. Chi squares for observed specific activity versus expected specific activity of malate synthase.

	<i>df</i> ^a	Chi Square	<i>P</i>
Dark x Dark	3	0.000	> 0.995
Light x Light	7	0.125	> 0.995
Light x Dark	17	0.400	> 0.995

^a *df* = Degrees of freedom.

1987; Singer, 1986; Tehler, 1988). Recent molecular evidence, using data derived from a comparison of 18S rRNA, 25S rDNA, and mitochondrial rDNA, clearly supports much of the classical view of classification (Bruns, *et al.*, 1991; Bruns, *et al.*, 1993; Johnson, 1997; Moncalvo, *et al.*, 1997; Tehler, 1988; Vilgalys, *et al.*, 1993). In fact Moncalvo, *et al.* (1997), using data derived from a comparison of 25S rDNA, reported that the split of light (or pale) spored agarics into one clade and dark spored agarics into a separate clade had a bootstrap value greater than 70.

A notable exception to the pale spored/dark spored split is *Coprinus comatus* S.F. Gray (Section *Coprinus*, subsection *Coprinus*; Smith, 1986). Although having black spores, *C. comatus*, based on 25S rDNA sequencing information, appears to be more closely related to species of the white spored family Lepiotaceae (Johnson, 1997; Moncalvo, *et al.*, 1997). *Coprinus comatus* was not available for the current study, but it would be interesting to ascertain whether its SA for malate synthase is high like light spored agarics or low like dark spored agarics.

Finally, as noted in previous reports (Ruch, *et al.*, 1991; Ruch and Samuel, 1992), the difference in malate synthase SA between the light and dark spored agarics used in this study was statistically significant as shown by the Mann-Whitney *U*-test ($U = 199.5$; $P < 0.001$; $n_1 = 20$, $n_2 = 10$).

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