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Distribution of Glyoxylate Pathway in different parts of fruit bodies of Gasteromycetous fungi

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Five Gasteromycetous fungi *Rhizopogon rubescens, Lycoperdon perlatum, Geastrum fornicatum, Dictyophora indusiata and Cyathus striatus* were collected from Santiniketan and adjoining areas. Specific activities of the key enzymes of the glyoxylate pathway were determined from different vegetative and reproductive parts of each fungus. Enzymes were obtained from cell-free extracts (CFE) of fungal tissues after homogenization followed by centrifugation at 15,000 x g. Isocitrate lyase (ICL), the first key enzyme of glyoxylate pathway, was detected with much higher activity in the reproductive tissue i.e., gleba of all the five members tested. Activity of this enzyme was significantly low in vegetative tissues of the fungi except for purse of *C. striatus*. This indicated that the distribution of ICL was organ specific and it was particularly important in reproductive tissues. Higher specific activities of the anaplerotic enzyme fructose-1, 6-bis-phosphatase and the β-oxidation enzyme crotonase were also found in the glebal tissues. However, the activity of the second key enzyme of the glyoxylate pathway, malate synthase, was almost uniformly distributed in all parts with only a slight increase in the glebal tissue.

Key words: *Anaplerotic enzymes, fruit body, Gasteromycetous fungi, Glyoxylate pathway*

INTRODUCTION

In plants and some microorganisms, acetyl groups are the most usual source of intermediates required to synthesize the carbon skeletons of carbohydrates. In such organisms the citric acid cycle operates in a specialized and modified way called the glyoxylate cycle. The pathway is regarded as an anaplerotic one (Lehninger, 1982). In the glyoxylate cycle acetyl CoA condenses with oxalacetate to form citrate. The breakdown of isocitrate does not occur via the usual isocitrate dehydrogenase (ICDH), but through a cleavage, catalysed by isocitrate lyase (ICL) to form succinate and glyoxylate. The glyoxylate then condenses with acetyl CoA to yield malate by the action of malate synthase (MS). The malate is then dehydrogenated to oxaloacetate, which can condense with another molecule of acetyl CoA to repeat the cycle.

Glyoxylate by-pass is important for carbon conservation for survival when readily metabolizable carbon sources are not available. This pathway is also induced when acetate is provided as a sole source of carbon in medium. In fungi this pathway has been reported in *Phycomyces* blakesleeanus (Rua *et al.*, 1989), *Neurospora crassa* (Thomas and Baxter, 1987) arbascular mycorrhizal fungus *Glomus* sp. (Bago *et al.,* 1999) and *Aspergillus* sp. (Delucas *et al.,* 1997) when the organisms were grown in media containing acetate as sole carbon source. This pathway is required for conferring fungal virulence by *Cryptococcus neoformans* (Lorenz and Fink, 2002; Rude *et al.,* 2002). This pathway only operates naturally under certain conditions such as in the fruit bodies of some members of the Homobasidiomycetes (Ruch *et al.,* 1991) and in wood-rotting fungi (Munir *et al.,* 2002). Although higher fungi are a large and ecologically very important group, their biochemical aspects, and particularly the availability of different metabolic pathways are not widely studied. Information on the

occurence of this anaplerotic pathway is scarce, particularly for higher Gasteromycetous fungi. In this communication the importance of two key anaplerotic enzyme of the glyoxylate pathway, and fructose 1, 6-bis phosphatase and crotonase of β-oxidation in Gasteromycetes are assessed.

MATERIALS AND METHODS

Fungal specimens were collected from the forest floor at Santiniketan and adjoining area.

Preparation of cell free extract (CFE)
Individual parts of fruit bodies of each specimen were carefully manually separated. During separation of peridium, capillitium or columella great care was taken to avoid any contamination with glebal tissue. This was done initially by the manual removal of respective tissue followed by washing in phosphate buffer at pH 7.0 three times and then centrifugation at 4000 rpm. The pellets were initially homogenized in a mortar and pestle and then subjected to sonication at 4°C in a 0.05 M phosphate buffer solution pH 7.0 with 5 mM β-mercape to ethanol. Sonication was done by an ultrasonic needle probe at 100 W for a total 5 min. in a Braun Sonicator allowing the probe to rest for one minute intermittently after every 30 sec. pulse. Cell debris and intact hyphal remnants were removed from the crude extract by centrifugation at 15,000×g for 10 min. A supernatant of the crude cell free extract (CFE) was kept in an ice bath and used as source of enzymes.

Enzyme assays Isocitrate lyase (ICL), E.C.4.1.3.1 was assayed following the method of Mc Fadden (1969) as follows : a mixture containing Tris buffer (0.1M) pH 7.6, reduced glutathione (0.125 M) and 0.1 ml CFE containing approx. 300-500 μg protein was preincubated at 30°C for 10 min. The reaction was initiated by the addition of 40 mM isocitrate solution followed by thorough shaking and incubation for 10 min at 30°C, after which 10% TCA was added. One ml of this mixture was then reacted with 6 ml of oxalic acid plus phenyl hydrazine HCL (5 parts of 10 mM oxalic acid + 1 part freshly prepared 1% phenyl hydrazine HCl) in a small beaker on a hot plate. Beakers were removed when the solutions started boiling and rapidly cooled. To it 4 ml concentrated HCl was then added, followed by 1% potassium ferricyanide and the preparation was mixed thoroughly. The OD of the mixture was recorded at 520 nm against a control. One unit of enzyme was defined as the amount that catalysed I μ mol of isocitrate per minute under the conditions of the assay. The amount of isocitrate that disappeared is equivalent to the amount of glyoxylate produced. Specific activity was defined in units/mg of protein.

Malate synthase (MS), E.C. 4.1.3.2 was assayed according to the procedure described by Cooper and Beevers (1969). The reaction mixture contained phosphate buffer pH 6.5, 100 μmol; acetly CoA, 0.5 μmol; glyoxylate, 3 μmol; $MgCl_2$ 10 μmol; 5,5′ dithiobis–2 nitro benzoic acid (DTNB), 0.1 μmol and CFE of approximate 300-500 μg protein equivalent. The reaction was initiated by the addition of glyoxylate for 10 min. in a cuvette and the OD was measured at 412 nm. Molar activity was calculated from the glyoxylate consumed during the reaction as measured from Δ OD/min and by taking the molar extinction coefficient value of the DTNB (1.36×10^4) into consideration. Specific activity was subsequently expressed as n-moles of substrate consumed per min per mg of protein. All enzyme assays were done in triplicate and the data obtained were averaged.

Fructose 1, 6-bis phosphatase (FBPase), E.C. 3.1.3.11 was assayed following the method of Stowers and Elkan, (1983) with the reaction mixture which contained tris HCl buffer, pH 8.0, 40 μ mol; $MgSO_4$ $7H_2O$ 12 μmol; fructose 1, 6-biphosphate 3 μmol, glucose 6-P dehydrogenase, NADP + 0.5 units and CFE 300-500 μg equivalent protein.

Crotonase, E.C.4.2.1.17 was assayed following the method of Stern (1955). The reaction was started by addition of 0.01 ml of CFE to the reaction mixture and the decrease in OD was recorded at 263 nm in time scan mode in a double beam spectrophotometer. The specific activity was calculated based on the molar extinction coefficient of crotonylus-CoA at 263 nm. Protein was estimated following the procedure of Lowry *et al.* (1951)

RESULTS AND DISCUSSION

Characteristic features of each fungal specimen are detailed below :

Rhizopogon rubescens var. *rubescens* Tul.–These were collected from forest floor of *Shorea robusta* at Ballavpur. Fruit bodies contained fleshy gleba and were purely hypogeous in nature. The stipe was highly reduced. Almost globose fruit bodies were about 2.5-3.5 cm in diameter. The thickness of peridium was 0.2 to 0.4 cm. Collection time : July.

Lycoperdon perlatum Pers.: These were collected from forest floor of *Shora robusta* and *Tectona grandis* at Santiniketan. The fruit bodies were pear shaped and were surrunded by a two-layered rind or peridium. On maturity the outer layer cracked into numerous scales and warts. Within the lower side of the peridium there was a non-sporing mass of threads — known as capillitium and on the upper side gleba were present. The fruit bodies were borne on a sterile stipe of 1 cm. The total length of the fruit body was about 3.0 cm. Collection time : July and August.

Geastrum fornicatum (Huds.) Hook : These earth star fruit bodies were collected from the leaf litters of shady trees such as *Artocarpus heterophyllus* and *Mangifera indica* at both domestic localities and in the forest belt of Santiniketan. The exoperidium was composed of three layers, an outer mycelial layer, a middle fibrillose layer and an inner fleshy layer. At first the exoperidium closely covered the endoperidium, but finally split into several rays that turned outwards to give the earth star appearance. The endoperidium was thin and membranous. Mature gleba were powdery. The capillitium threads were long, simple and attached to the inner side of the endoperidium. Some capillitum threads were also attached and projected inside from a spherical, head-like columella. Collection time : August and September.

Dictyophora indusiata f. *indusiata* : –These were collected both from domestic shade trees as well as the forest floor, on sandy and gravel soils. A prominent stipe was encircled at the base with a volva and a glanduler cup-like receptacle at the top. An indusium developed from the base of the receptacle which gave the impression of a skirt. Collection time : mid July to September.

Cyathus striatus (Huds.) Hoffm :–These were collected from the cut ends of decaying wood and bamboo. Fruit bodies were recognized by the furrowed inner wall of their cups. In young fruit bodies, the mouth of the funnel was closed over by a thin papery epiphragm which ruptured at maturity and peridioles were exposed. these were lens shaped, slate-blue in colour and attached to the peridium by a complex funiculus. The peridioles were surrounded by a thick tunica and cortex. The inner part of the peridiolum was made up of thin walled hyphae where basidia and basidiospores developed. The funiculus consisted of sheath, middle piece, purse, funicular cord and hapteron. Collection time : August and September.

Assays of ICL and MS of the glyoxylate pathway, crotonase of the β-oxidation pathway and FB Pase of the gluconeogenic pathway were made and the results are presented in Table 1. ICL levels were variable between different parts of the fruit bodies, but the specific activity of ICL was always highest in the glebal tissues of the mature fruit bodies. ICL activity also varied between the glebal tissues of different species. The highest activity (45 nmol min^{-1} mg^{-1} protein) was recorded in glebal tissue of *C. striatus,* whereas the lowest was in *L. perlatum.* The tissues innately involved or associated with the glebal tissue, such as capillitium threads and columella, also contained high amounts of ICL, whereas the tissues immediately surrounding the gleba did not contain significant amounts of the enzyme. Surprisingly the stipes of every specimen showed higher ICL activity in comparison to the peridium. It may be assumed from this data that the presence of high amounts of ICL is required for the development as well as the maintenance of glebal tissues. Induction of glyoxylate pathway was also reported earlier for development and differentiation of ascocarp of *Tuber borchii I*(Lacourt *at al.,* 2002)

Of the vegetative tissues, the purse of *C. striatus* showed the highest specific activity of ICL (41 μmol min^{-1} mg^{-1} protein). Higher specific activity of ICL in glebal tissues might be a positive correlation of higher levels of lipid and fatty acid content in glebal tissues (our unpublished observation). A positive correlation between the fat contents of basidiospores and the malic enzyme of the glyoxylate pathway had also been reported earlier (Ruch *et al.,* 1991). This may also be true for the purse of *C. striatus* where the lipid content was substantially high. Induction of ICL by fatty acid as

well as lipid had also been observed in *Candida.* To confirm the importance of fatty acid in glebal tissues or other parts, crotonase – an enzyme for β - oxidation of fatty acid was assayed from the CFE of different parts of fruit body of these fungi. Crotonase results were almost identical to those seen from ICL (Table 1). Fructose-1,6-bis phosphatase (FBPase), a gluconeogenic enzyme, was also assayed from the same CFEs and a similar pattern of result was obtained (Table 1). The comparatively low level of ICL in glebal tissues of *Lycoperdon* might also be indicative of its better spore dispersal mechanism as well as the low fat content of spore walls (Ruch *et al.,*1991). On the other hand, where the spore dispersal mechanism is poor viz., *C. striatus.* ICL leval is high in glebal tissues of this species. Thus in the species where the spore dispersal mechanism is not well developed, these gluconeogenic enzyme might play an indirect role in the maintenance of the basidiospores as well as the glebal tissues. In mature glebal tissue, the availability of ready-made carbon source decreases with time and so simulates a starvation like process. During this situation conservation of energy for better survival is necessary. Decrease in activities of catabolic enzymes and increase in activities of anabolic enzymes have been reported during carbon starvation in fungus (Sonavaria *et al.,*1986). Lower activity of catabolic enzymes like phospho-fructokinase from glebal tissue of *D. indusiata* and *G. fornicatum* has also been reported (Bakshi and Mandal, 2006). Therefore, higher activities of ICL, FBPase or crotonase, which are directly or indirectly involved in gluconeogenic process or energy conservation are consistent with these requirements.

Table 1 : Specific activities of key enzymes of glyoxylate pathway in different parts of the fruit bodies of Gasteromycetes.

Parts of fruit body	Enzyme activities*							
	1CL	SE	MS	SE	Croto-nase	SE	FBPase	SE
Rhizopogon rubescens								
Reduced stipe	15	0.94	62	1.53	21	1.24	18	0.47
Peridium	8	0.43	51	1.53	10	0.47	6	0.40
Gleba	37	0.70	75	1.06	75	1.77	53	0.47
Lycoperdon perlatum								
Stipe	12	0.47	53	0.89	10	0	09	0.61
Capillitium	17	0.94	64	1.41	18	0.47	09	0.47
Peridium	10	0.94	55	0.94	9	0.61	8	0.94
Gleba	35	2.35	81	1.06	91	2.76	62	1.41
Geastrum fornicaturn								
Exoperidium (rays)	5	0.23	36	1.88	07	0.23	11	0.47
Inner peridium (capsule wall)	9	0.47	42	0.89	08	0.47	09	0.63
Columella	18	1.88	52	0.47	27	0.94	29	0.89
Capillitium threads	20	0.94	59	1.88	25	0.47	23	0.47
Gleba	39	0.94	69	2.35	95	1.88	67	1.41
Dictyophora indusiata								
Volva	7	0.47	32	0.94	10	0.23	13	0.94
Stipe	8	0.47	40	1.41	11	0.46	10	0.47
Indusium	13	0.89	57	0.89	18	0.94	19	0.47
Receptacle with gleba	42	1.88	68	1.88	92	1.26	72	1.09
Cyalhus striatus								
Stipe	12	0.47	35	0.94	17	0.94	21	0.47
Peridium	7	0.94	34	1.82	13	0.47	17	0.94
Peridiolum (Tunica)	11	0.94	42	0.94	21	0.47	20	0.94
Purse	41	1.41	81	1.88	95	1.44	70	1.41
Gleba	45	0.47	86	2.35	106	2.82	92	1.88

*Specific activities of enzymes were expressed as n moles of substrate consumed min^{-1} mg^{-1} of protein.
ICL–Isocitrate lyase; MS–Malate synthase; FBPase–Frutose 1, 6-bis-phosphatase; SE–Standard error.

The on the key enzyme MS was detected in high levels in almost all the parts of the basidiocarps and showed a 30-40% increase in activity in glebal tissues (Table 1). High activity of this enzyme indicated that MS is almost equally important throughout the fruit body and it is constitutive in nature.

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