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New role for glyoxylate cycle enzymes in wood-rotting basidiomycetes in relation to biosynthesis of oxalic acid

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Abstract The key enzymes of the glyoxylate cycle, isocitrate lyase (ICL) and malate synthase (MS), were detected in varying amounts in the mycelia of the wood-rotting basidiomycetes tested, although they were grown in a glucose-rich medium. The highest specific activities of ICL (0.37 U/mg protein) and MS (0.63 U/mg protein) were measured for the brown-rot basidiomycetes *Laetiporus sulphureus* and *Fomitopsis palustris*, respectively. The results indicate that the glyoxylate cycle enzymes occur in wood-rotting basidiomycetes as the seemingly "constitutive" enzymes at varying levels. The glyoxylate cycle enzymes, including malate dehydrogenase (MDH), and the oxalate-producing enzymes glyoxylate dehydrogenase (GDH) and oxaloacetase (OXA) were found to have good correlation with biosynthesis of oxalic acid and fungal growth, which was also confirmed by use of an ICL inhibitor. A new role for the glyoxylate cycle is discussed in relation to oxalic acid biosynthesis in wood-rotting basidiomycetes.

Key words Glyoxylate cycle · Wood-rotting fungi · Isocitrate lyase · Malate synthase · Oxalic acid biosynthesis

Introduction

It is a commonly known physiological trait that most brown-rot basidiomycetes accumulate oxalic acid in culture media, whereas white-rot ones generally do not.[1–4] Recently, oxalic acid produced by wood-rotting fungi has been receiving much attention[5] because it may play an important role in their wood decay systems. It serves as a scavenger of active cation radicals formed in the lignin peroxidase system,[6,7] thus inhibiting the ligninolytic enzymes; and the concomitantly formed formate radicals reduce dioxygen to produce superoxide anion radicals, which may be utilized for bioremediation of pollutants.[8] The acid also serves as a proton source for hydrolytic destruction of wood carbohydrates,[9] including calcium pectinate.[10,11] Furthermore, because oxalate acts as a strong metal chelater, copper-containing wood preservatives suffer inactivation owing to the oxalate-producing fungi that are commonly known as copper-tolerant fungi.[12–14] Thus, protection of concrete or stone buildings and old wooden cultural heritage structures from acid-producing fungi have been an important research target in building mycology.[15,16]

Enzymatic investigations on the metabolism of oxalate in wood-rotting fungi are important from the biochemical aspects of wood preservation and the physiology of wood-rotting fungi. Akamatsu et al.[17,18] first reported the occurrence of two oxalate-producing enzymes, glyoxylate dehydrogenase (GDH) and oxaloacetase (OXA), in the brown-rot fungus *Tyromyces palustris* (presently called as *Fomitopsis palustris*); they catalyze production of oxalate by oxidation of glyoxylate and hydrolysis of oxaloacetate, respectively. Quite recently Tokimatsu et al.[19] reported that GDH, a new cytochrome *c*-dependent glyoxylate dehydrogenase, is one of the flavohemoprotein enzymes.

Glyoxylate and oxaloacetate are also important metabolic intermediates involved in the glyoxylate cycle, which may be linked with the biosynthesis of oxalic acid, as shown in Fig. 1. Almost nothing is known about the glyoxylate cycle marker enzymes isocitrate lyase (ICL) and malate synthase (MS) from the wood-rotting basidiomycetes, although MS was reported to occur in spores of saprophytic and symbiotic basidiomycetes.[20] Moreover, it has been believed as a paradigm that the glyoxylate cycle appears only when microorganisms are grown on a nonsugar substrate such as ethanol and acetate.[21,22]

In this context, we were motivated to investigate whether the glyoxylate cycle exists when the fungi are grown in a

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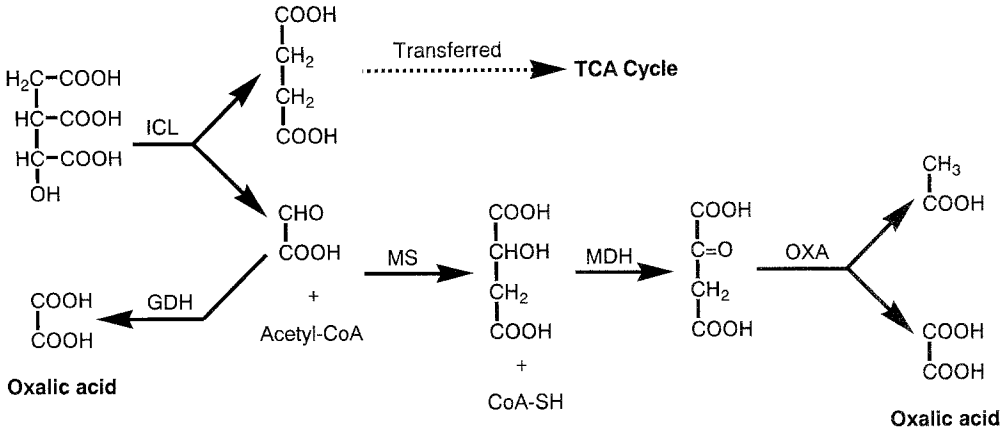

Fig. 1. Proposed metabolic linkage of the glyoxylate cycle enzymes with oxalic acid biosynthesis. *ICL*, isocitrate lyase; *MS*, malate synthase; *GDH*, glyoxylate dehydrogenase; *MDH*, malate dehydrogenase; *OXA*, oxaloacetase; *TCA*, tricarboxylic acid

glucose-rich medium. If it occurs, the key reactions catalyzed by ICL and MS may be coupled with the two oxalate-producing enzymes, GDH and OXA, via malate dehydrogenase (MDH), as shown in Fig. 1. To this end, we tested a number of important wood-rotting basidiomycetes, known as the ligninolytic fungi, edible mushrooms, standard test fungi for Japanese wood preservation, dry-rot and copper-tolerant fungi. Here we report new evidence for the occurrence of the glyoxylate cycle enzymes in mycelia of both white-rot and brown-rot fungi grown in a glucose-rich medium. The results are discussed in relation to a new role for the cycle in oxalate biosynthesis.

Materials and methods

Chemicals

All chemicals used in this study were of reagent grade. Acetyl-coenzyme A (CoA), DL-isocitric acid, phenylmethylsulfonyl fluoride (PMSF), and dithiothreitol (DTT) were obtained from Nakalai Tesque (Kyoto). Glyoxylic acid was purchased from Sigma Chemical (St. Louis, MO, USA), and the protein assay kit was from Bio-rad Laboratories (Hercules, CA, USA).

Organisms

The fungi (basidiomycetes, deuteromycetes, ascomycetes) used in this study are listed in Table 1. Most of the fungi used are from the stock cultures of the Wood Research Institute, except for *Ceriporiopsis subvermispora*, *Serpula lacrymans*, and *Trichoderma harzianum*, which were kind gifts from Prof. Rafael Vicuña (Departmento de Genetica Molecular y Microbiologia, Pontifica Universidad Catolitica, Chile), Prof. Shuichi Doi (Institute of Wood Technology, Akita Prefecture University), and Dr. Didiek H. Goenadi (Biotechnology Research Unit for Estate Crops, Indonesia), respectively. *Trichoderma reesei* and *Schizophyllum commune* were ordered from the Institute for Fermentation in Osaka.

Growth conditions

Inocula were prepared from cultures grown in potato dextrose agar plates. For preparation of the cell-free extracts, the fungi were grown in 1-l Erlenmeyer flasks with 200 ml of liquid medium containing 8 g peptone, 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 0.3 g $MgSO_4 \cdot 7H_2O$, and thiamine HCl 5 ppm/l. As a major carbon source, glucose was used at a final concentration of 2.5% (w/v) unless otherwise stated. The media were adjusted to pH 5.5 with 1 N HCl prior to sterilization. Fungal inocula were prepared from the fully grown colony using a cork borer (diameter 6 mm); as many as 10 agar plugs were used for each cultivation. In the case of *Aspergillus niger*, *Chaetomium globosum*, *Phanerochaete chrysosporium*, and *C. subvermispora*, spore suspensions (2 ml each) were used. All cultures were incubated statically in a dark place at 27°C, except for *S. lacrymans* and *S. commune*, which were incubated at 20° and 25°C, respectively. For the inhibition study, itaconic acid at a different concentration was added to the culture medium, which contained 1% (w/v) glucose.

Preparation of cell-free extracts

Fungal mycelia were harvested on different days depending on the fungal growth rate. The mycelia collected from two culture flasks were filtered through cheesecloth and rinsed thoroughly with cold 0.01 M potassium phosphate (K-Pi) buffer (pH 7.0). Mycelia were homogenized in a cold mortar with a pestle in 0.1 M K-Pi buffer (pH 7.0) containing 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM DTT, and 1 mM PMSF with a small amount of sea sand. The buffer solution was always freshly prepared. The homogenate was centrifuged at 14000*g* at 4°C for 30 min. This manipulation was performed twice, and the combined supernatant obtained was used as the crude enzyme solution.

Enzyme assays

The activity of ICL was spectrophotometrically assayed by the method described by Dixon and Kornberg[23] with slight

Table 1. Activities of the glyoxylate cycle enzymes detected from the glucose-grown mycelia

Fungus	Days	Total activity[a]		Specific activity[b]	
		ICL	MS	ICL	MS
White-rot fungi (basidiomycetes)					
Ceriporiopsis subvermispora	14	5	31	1	6
Coriolus versicolor	10	32	110	3	10
Flammulina velutipes	18	37	158	6	24
Ganoderma applanatum	10	86	78	5	4
Ganoderma lucidum	10	23	17	2	2
Grifora frondosa	10	27	53	5	10
Hygiptea sp.	13	11	67	1	8
Panus rudis	7	41	80	3	5
Phanerochaete crysosporium	5	45	76	4	7
Pleurotus cystidiosus	10	8	142	2	33
Pleurotus eringii	10	16	94	1	6
Pleurotus ostreatus	10	54	418	4	33
Schizophyllum commune (1)	9	126	96	10	8
Schizophyllum commune (2)	9	80	58	22	16
Stereum hirsutum	15	39	43	10	11
Brown-rot fungi (basidiomycetes)					
Coniophora puteana	18	40	64	8	13
Daedalea dickinsii	10	95	49	17	9
Fomitopsis palustris	7	212	418	32	63
Gloeophyllym trabeum	10	14	44	2	6
Laetiporus sulphureus	10	487	196	37	15
Lentinus lepideus	23	9	0	1	0
Serpula lacrymans (1)	20	66	0	20	0
Serpula lacrymans (2)	20	52	0	12	0
Soft-rot fungi (deuteromycetes and ascomycetes)					
Aspergillus niger	7	75	206	8	15
Chaetomium globosum	7	49	157	3	9
Trichoderma harzianum	5	0	0	0	0
Trichoderma reesei	5	0	0	0	0

ICL, isocitrate lyase; MS, malate synthase
[a] Total activity is expressed as 10 mU/culture of 200 ml
[b] Specific activity is expressed as 10 mU/mg protein

modification. The reaction mixture (3 ml) contained 40 mM K-Pi buffer (pH 7.0), 10 mM $MgCl_2$, 4 mM phenylhydrazine, 30 mM DL-isocitrate (pH 7.0), and the enzyme solution. The reaction was started by adding isocitrate. The reaction rate for the formation of glyoxylate, which was transformed instantly to phenylhydrazone, was determined by measuring the increase in absorbance at 324 nm (ε = 14626) at 30°C.

The MS activity was determined based on the consumption of acetyl-CoA with a decrease in absorbance at 232 nm.[23] The reaction mixture (3 ml) contained 1 mM glyoxylate, 30 μM acetyl-CoA, 50 mM Tris-HCl buffer (pH 8.0), 2 mM $MgCl_2$, and the enzyme solution. Absorbance at 232 nm (ε = 4450) was recorded for a few minutes to detect the possible presence of acetyl-CoA deacylase. The reaction was started by adding glyoxylate. OXA, GDH, and MDH were assayed according to the reported methods.[18,19,24]

The enzyme activities were expressed as units; 1 U of enzyme activity is defined as the amount of the enzyme that catalyzes the formation or consumption of 1 μmol of the product or substrate, respectively, per minute under the conditions described. Specific activities were given as units of enzyme activities per milligram of protein. Protein concentrations were determined by the Bradford method[25] using a protein assay kit with bovine serum albumin as a standard.

Results

Occurrence of glyoxylate cycle enzymes among wood-rotting fungi

Table 1 shows the total and specific activities of ICL and MS extracted from the mycelia of 15 white-rot and 8 brown-rot basidiomycetes grown in glucose-rich medium for different periods. Four soft-rot fungi that are not basidiomycetes were also studied for comparison.

As to the total activity of these basidiomycetes, *Laetiporus sulphureus* had the greatest ICL activity, followed by *Fomitopsis palustris*; and *F. palustris* and *Pleurotus ostreatus* yielded the greatest MS activity, followed by *L. sulphureus*. The competent fungi with MS activities of more than 1.00 U/culture were found to be in the order *Flammulina velutipes*, *Pleurotus cystidiosus*, and *Coriolus versicolor*. The others, including *P. chrysosporium*, *C. subvermispora*, *S. commune*, and *Gloeophyllum trabeum*, belong to a group

with lower production of MS enzyme. Interestingly, *Lentinus lepideus* and *S. lacrymans* did not produce MS but ICL. Among the soft-rot fungi tested, both *A. niger* and *C. globosum* yielded lower ICL activity but more MS activity, whereas *T. reesei* and *T. harzianum* produced neither ICL nor MS activity.

As to the specific enzyme activity, *L. sulphureus* also gave the highest ICL value (0.37 U/mg protein) followed by *F. palustris*, whereas the latter gave the highest MS value (0.63 U/mg protein) followed by *P. ostreatus* and *Pleurotus cystidious*. Importantly, among the 23 basidiomycetes tested, most produced higher specific ICL activity (average 0.09 U/mg protein) than previously reported for *Phanerochaete* sp. (0.025 U/mg protein)[26] or *Coprinus lagopus* (at most, 0.002 U/mg protein).[27] Furthermore, all of the basidiomycetes except *S. lacrymans* and *L. lepideus* also had much higher specific MS activity (average 0.14 U/mg protein) than the reported *Phanerochaete* sp. (0.003 U/mg protein).[26]

Taken together, most of the wood-rotting basidiomycetes tested in this investigation were found to produce the two key enzymes of the glyoxylate cycle at significantly higher levels as "constitutive" enzymes even when they were grown on glucose as a major carbon source.

Correlation between activities of the enzymes involved and oxalate production

Fomitopsis palustris, a Japanese wood-preservation test fungus, is known as an intense oxalate producer and copper-tolerant fungus,[28] and it has also been found to be a high producer of ICL and MS in this study. Therefore, we decided to use it as a model organism to examine if there is a correlation between the activities of the glyoxylate cycle enzymes (ICL, MS, MDH) and the oxalate-producing enzymes (GDH, OXA) during growth of the fungus. The results (Fig. 2) show that all the enzymes tested exhibited almost the same profiles of changes in activity, in a coordinated manner at an early stage of cultivation, although MDH had much higher activity than any other enzyme. Interestingly, Fig. 3 shows that the amounts of oxalate, starting to increase with a slight lag phase at an early stage of cultivation, continued to increase in parallel with the fungal growth. Taken together, these results (Figs. 2, 3) indicate that there is a good correlation of enzyme activity of the glyoxylate cycle and the oxalate-producing system with oxalate production and with fungal growth.

To examine if oxalate biosynthesis was also affected in the same manner as the glyoxylate cycle enzymes, we attempted to inhibit the initial step of the oxalate biosynthesis (Fig. 1) by use of the ICL-specific inhibitor itaconate.[29] The results in Fig. 4 clearly show that the higher the inhibitor concentration the lesser the amounts of oxalate that were produced. Surprisingly, growth and enzyme protein synthesis were significantly inhibited when 0.2 M itaconate was used. At this concentration, fungal growth was inhibited by 63%, and ICL and MS production was inhibited by 84% and 89%, respectively. Importantly, oxalate production was inhibited by 95% at this itaconate level. These results clearly show that there is a strong correlation between glyoxylate cycle enzyme activity and oxalic acid production.

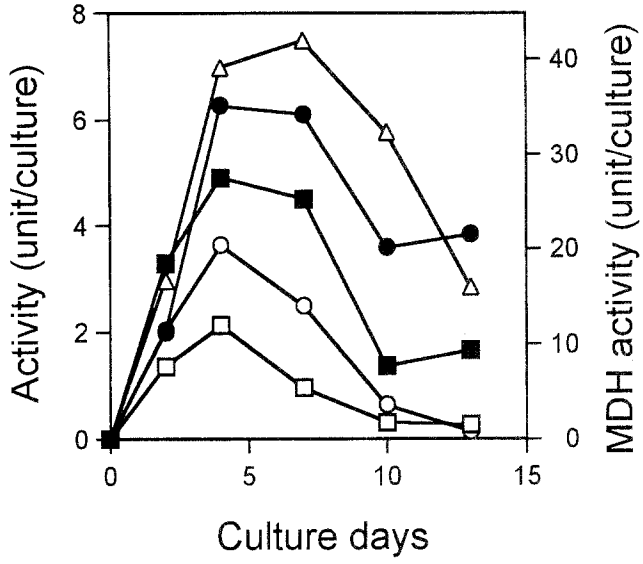

Fig. 2. Profiles of total enzyme activities during the cultivation of *Fomitopsis palustris*. The fungus was grown on 2% (w/v) glucose at 33°C. *Filled squares*, ICL; *filled circles*, MS; *open squares*, GDH; *open circles*, OXA; *open triangles*, MDH

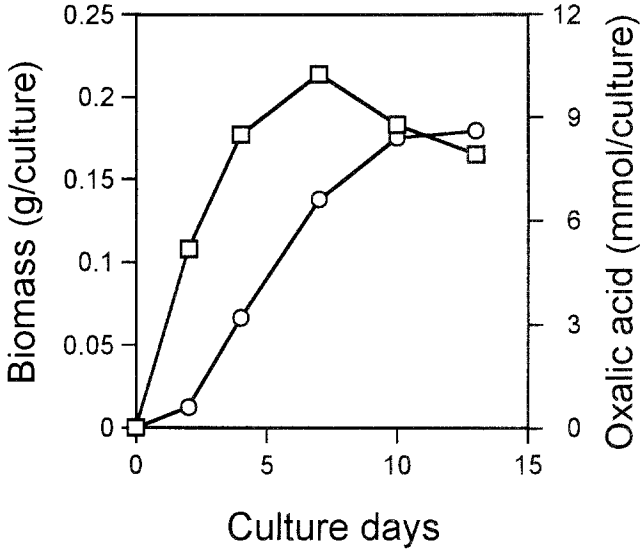

Fig. 3. Oxalic acid and biomass productions during the cultivation of *F. palustris*. The fungus was grown on 2% (w/v) glucose at 33°C. *Open squares*, biomass; *open circles*, oxalic acid

Discussion

The glyoxylate cycle, which was discovered from acetate-grown bacteria by Kornberg and Krebs in 1957,[30] has been known as an important biochemical device playing an

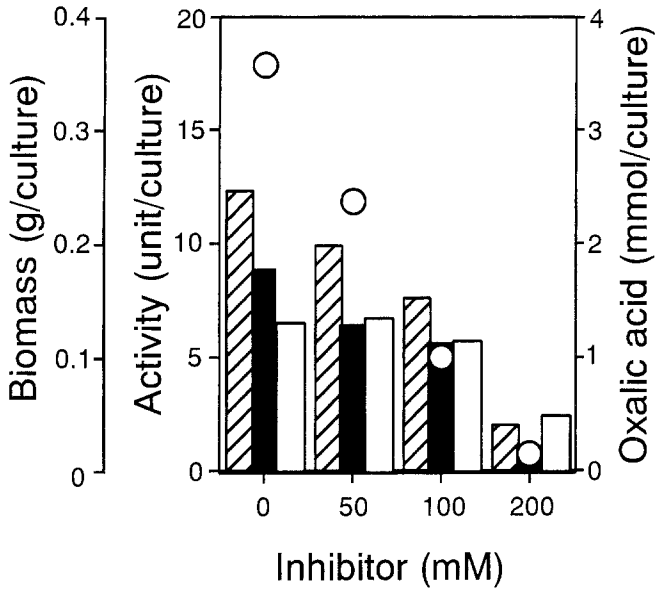

Fig. 4. Inhibition effect of itaconic acid on the production of oxalic acid, the enzyme, and the biomass. The fungus was grown on 1% (w/v) glucose at 33°C. All values were obtained from day 5 cultures. *Striped bars*, ICL; *filled bars*, MS; *open bars*, biomass; *open circles*, oxalic acid

anaplerotic function to support the tricarboxylic acid (TCA) cycle in coordination with gluconeogenesis (synthesis of glucose). In general, however, when microorganisms are grown in glucose-rich medium, they do not or little produce the glyoxylate cycle enzymes (ICL, MS) because of "catabolite repression" by glucose; they are induced as adaptive enzymes only when they are grown on nonsugar substrates.[21,22]

In contrast to the general concept of the function of the glyoxylate cycle, the results obtained (Table 1) show that the key enzymes occur seemingly as "constitutive" enzymes in the wood-rotting basidiomycetes grown on glucose, although the enzyme activities per culture and milligram of protein vary depending on the species and their cultivation time. Furthermore, these values are significantly higher than those previously reported for other fungi.[26,27,31,32]

At this point, one question arises about why the wood-rotting basidiomycetes "constitutively" produce the glyoxylate cycle enzymes despite the presence of glucose in the culture. We suspect that these "constitutive" enzymes may not coordinate with gluconeogenesis but possibly with oxalate biosynthesis, because the wood-rotting fungi commonly produce oxalate in significant amounts.[1–4] Using the brown-rot basidiomycete *F. palustris* as a model fungus, we found that the activities of the major glyoxylate cycle enzymes (ICL, MS, MDH) increased with increases in the activity of the oxalate-producing enzymes (GDH, OXA). Moreover, all five enzyme activities increased along with increasing amounts of oxalate produced (Figs. 2, 3). It is noteworthy that much more activity of MDH was detected from the same enzyme preparation (Fig. 2). This finding indicates that MDH plays an important role not only in bridging between MS and OXA to produce oxalic acid but also in generating NADH as an energy source. This investigation provides the first evidence of a strong correlation between glyoxylate cycle enzyme activities and oxalic acid production in wood-rotting basidiomycetes. It is still difficult to generalize this correlation for all basidiomycete species owing to their different physiological traits. In white-rot basidiomycetes alone there are four oxalate-decomposing enzymes: oxalate decarboxylase,[1] oxalate oxidase,[33] lignin peroxidase,[6] and manganese peroxidase.[34] Moreover, some brown-rot fungi were reported to have oxalate decarboxylase.[35,36] Therefore, it is highly possible that wood-rotting basidiomycetes with high glyoxylate cycle enzyme activity have little accumulation of oxalate.

The glyoxylate cycle enzymes have been reported to contribute to protein synthesis in nitrogen-fixing bacteria,[37] riboflavin biosynthesis in *Ashbya gossypii*,[38] and oxalate biosynthesis in a plant pathogen *Sclerotium rolfsii*.[39] These reports indicate that the glyoxylate cycle may function differently in different microorganisms not only for gluconeogenesis but also for other biosynthetic processes.

Conclusions

In wood-rotting basidiomycetes, the glyoxylate cycle enzymes, occurring as "constitutive" enzymes, may play an important anaplerotic role in supporting the biosynthesis of oxalic acid, as shown in Fig. 1. Thus, the glyoxylate cycle enzymes appear not only during the germination of basidiospores but also during vegetative growth of the mycelia. The reason wood-rotting basidiomycetes produce oxalic acid physiologically remains to be investigated in relation to fungal growth.

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