昭和36年8月5日第3種郵便物認可 毎月1回23日発行 第750号 平成14年3月23日発行
ISSN 0916-8451

Vol.66, March, 2002

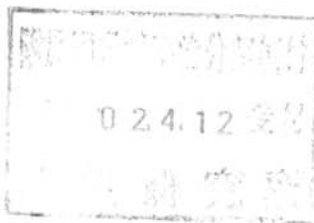
*Analytical Chemistry*

*Organic Chemistry*

*Biochemistry & Molecular Biology*

*Food & Nutrition Science*

*Microbiology & Fermentation Technology*



JSBA

Published by
Japan Society for Bioscience, Biotechnology, and Agrochemistry
BBBIEJ 66(3)489-704(2002)
http://www.jsbba.or.jp/

*Biosci. Biotechnol. Biochem.*, **66** (3), 576–581, 2002

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# Purification and Characterization of Malate Synthase from the Glucose-grown Wood-rotting Basidiomycete *Fomitopsis palustris*

Erman MUNIR, Takefumi HATTORI, and Mikio SHIMADA†

*Wood Research Institute, Kyoto University, Uji, Kyoto 611-0011, Japan*

Received September 25, 2001; Accepted November 13, 2001

**Malate synthase (EC 4.1.3.2), the key enzyme of the glyoxylate cycle, was purified to a homogeneous protein from the wood-rotting basidiomycete *Fomitopsis palustris* grown on glucose. The purified enzyme, with a molecular mass of 520 kDa, was found to consist of eight 65-kDa subunits, and to have $K_m$ of 45 and 2.2 μM for glyoxylate and acetyl-CoA, respectively. The enzyme activity was competitively inhibited by oxalate ($K_i$, 8.5 μM) and glycolate ($K_i$, 17 μM), and uncompetitively by coenzyme A ($K_i$, 100 μM). The potent inhibition of the activity by *p*-chloromercuribenzoate suggests that the enzyme has a sulfhydryl group at the active center. However, the enzyme was inhibited moderately by adenine nucleotides and weakly by some of the metabolic intermediates of glycolysis and tricarboxylic acid cycle. The enzyme was completely inactive in the absence of metal ions and was maximally activated by $Mg^{2+}$ ($K_m$, 0.4 μM), which also served to significantly prevent enzyme inactivation during storage.**

**Key words:** malate synthase; glyoxylate cycle; wood-rotting basidiomycetes; oxalate biosynthesis; *Fomitopsis palustris*

Malate synthase (MS) (EC 4.1.3.2), catalyzing the condensation of glyoxylate with acetyl-CoA to form malate and coenzyme A,[1,2] is a key enzyme of the glyoxylate (GLOX) cycle to support anaplerotically the tricarboxylic acid (TCA) cycle. Together with isocitrate lyase (ICL), as another key enzyme of the GLOX cycle that yields succinate and glyoxylate from isocitrate, MS bypasses the decarboxylation steps of the TCA cycle to permit gluconeogenesis from non-sugar substrates such as two-carbon ($C_2$) compounds.[3] These key enzymes have been reported to be induced in a wide variety of bacteria, fungi, protozoa, and nematodes grown on acetate and ethanol, and appear transiently in germinating plant seeds (for a review, see ref.[4]). However, recent studies have shown that the GLOX cycle enzymes are also present even in some vertebrates: in adipose tissues of black bears in hibernation[5] and livers of rats with alloxan-induced diabetes,[6] although there was a belief that the glyoxylate cycle did not occur in mammalian tissues.

Quite recently, we have found that both ICL and MS occur constitutively in wood-rotting basidiomycetes that were grown on glucose.[7] This finding sharply contrasts with the previously reported ones that either little or no activity of ICL and MS was detected from microorganisms grown in glucose-containing media,[8-11] because of catabolite repression by glucose.[3,12] Then, we have also demonstrated that MS, which coordinates with malate dehydrogenase generating NADH by oxidation of malate to oxaloacetate, plays a pivotal role in producing oxalate from glucose in the brown-rot fungus *Fomitopsis palustris*;[13] the oxalate biosynthesis has been found for the first time to metabolically link with the energy-producing processes of the TCA and the GLOX cycles,[13] whereby the fungus can grow.

Although MS has been purified and characterized from a number of plant species,[14-16] bacteria,[17-19] and yeasts,[20,21] no report has described the purification and characterization of MS from any basidiomycetous fungi. Because the GLOX cycle enzymes have been shown to play an important role in oxalate biosynthesis in wood-rotting basidiomycetes, we have been motivated to investigate characteristics of the purified enzyme. Furthermore, purification of MS from the wood-rotting fungi may contribute to not only fully understanding its regulatory role in the carbon metabolism of basidiomycetes but also elucidation of the molecular mechanism of the expression of the enzyme. Here we report the first example of the purification of MS from the wood-rotting basidiomycete *F. palustris*. The results are discussed in comparison with the characteristics of MS isolated from other organisms.

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† To whom correspondence should be addressed. Mikio SHIMADA, Phone: 81-774-38-3624; Fax: 81-774-38-3682; E-mail: mshimada@kuwri.kyoto-u.ac.jp

*Abbreviations*: MS, malate synthase; ICL, isocitrate lyase; GLOX, glyoxylate; TCA, tricarboxylic acid; DTT, dithiothreitol

## Materials and Methods

*Chemicals.* Biochemical and chemical reagents, such as acetyl coenzyme A, oxalic acid, glycolic acid, ethylenediaminetetraacetate (EDTA), and dithiothreitol (DTT), were purchased from Nacalai Tesque (Kyoto) and glyoxylic acid from Sigma Chemicals (St. Luis, MO, USA). Coenzyme A and SDS-PAGE gels were obtained from Oriental Yeast (Tokyo) and Daiichi Pure Chemicals (Tokyo), respectively. The protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA, USA).

*Organism and growth conditions.* The oxalic acid-producing wood-rotting basidiomycete, *Fomitopsis palustris* (Berkeley et Curtis) Murill (formerly called *Tyromyces palustris*), was used in this study, because this fungus is an important copper-tolerant fungus, which has been used as a Japanese Industrial Standard fungus for testing wood preservatives efficacy. The fungus was grown on glucose medium as previously reported.[7]

*Enzyme assays.* The enzyme activity was measured by a slight modification of the Dixon and Kornberg method[22] based on the consumption of acetyl-CoA with a decrease in the absorbance at 232 nm. The reaction mixture (3 ml), containing 2 mM glyoxylate, 20 $\mu$M acetyl-CoA, 50 mM Tris-HCl buffer (pH 8.0), 5 mM $MgCl_2$, and the enzyme solution, was incubated at 30°C. The reaction was started by the addition of glyoxylate. One unit of the enzyme activity was defined as the amount of enzyme that catalyzes the consumption of 1 $\mu$mol of acetyl-CoA per minute under the conditions described. Specific activities were given as units of enzyme activities per mg of protein ($\mu$mol $min^{-1}$ $mg^{-1}$ protein). Protein concentrations were measured by the Bradford method using a protein assay kit with bovine serum albumin as a standard.[23]

*Enzyme purification.* One-week-old mycelia grown on glucose were used as the enzyme source. Cell-free extract was prepared as previously reported,[7] and all manipulations were done at 4°C. All buffer solutions contained 2 mM $MgCl_2$, 1 mM EDTA, and 1 mM DTT, except for the buffer used for mycelial homogenization, which contained 2 mM additional glyoxylate. The homogenate was centrifuged and the supernatant was precipitated by 20% $(NH_4)_2$ $SO_4$. After centrifugation, solid additional ammonium sulfate was slowly added to the supernatant to 70% saturation with continuous stirring, and the pH was adjusted to 7.5 with $NH_4OH$. The solution was left for 3 hours and centrifuged at 10,000 rpm for 30 minutes. The pellet thus obtained was dissolved in 20 mM potassium phosphate (KPi) buffer (pH 7.2) containing 1 M ammonium sulfate, and the undissolved matter was removed by centrifugation. The supernatant was put on a TSK gel phenyl-Toyopearl 650 M (1.5 × 20 cm) which had been equilibrated with 20 mM KPi buffer (pH 7.2) containing 1.8 M ammonium sulfate. The adsorbed proteins were eluted with a linear gradient of ammonium sulfate (from 1.8 M to 0 M). Fractions containing enzyme activity were collected, combined, and dialyzed overnight against 20 mM Tris-HCl buffer (pH 8.0). The dialyzate obtained was passed through the Protein-Pak DEAE 15HR (1 × 10 cm) which had been equilibrated with 20 mM Tris-HCl buffer (pH 8.0). The adsorbed proteins were eluted with a linear gradient of NaCl (from 0 M to 0.3 M) in 20 mM Tris-HCl buffer (pH 8.0). The fractions containing enzyme activity were combined and concentrated by using concentrators (Centriprep 10, Amicon, Inc. Beverly, MA, USA). The concentrated enzyme solution was passed through HiLoad Superdex 200 (1.6 × 60 cm) which had been equilibrated with 20 mM KPi-buffer (pH 7.2) containing 0.15 M NaCl. After we collected fractions with enzyme activity, the purity of each fraction was examined by SDS-PAGE. The thoroughly purified enzyme fractions were combined and used for the characterization.

*Measurement of molecular masses of the native enzyme and the subunit.* The molecular mass of the purified enzyme was measured by passing it through a TSK gel G3000$SW_{XL}$ (100 mM KPi buffer (pH 7.2), 0.2 ml/min). The standard proteins (Sigma Chemicals) used were thyroglobulin (669 kDa), apoferritin (443 kDa), $\beta$-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa). The molecular mass of the subunit of the purified enzyme was measured by SDS-PAGE by the method of Laemmli[24] in a vertical slab gel instrument using a 10–20% gradient gel. Electrophoresis was done with constant current at 40 mA, and the gel was treated with Coomassie brilliant blue R-250. The subunit molecular mass of MS was estimated by using the standard proteins (LMW Electrophoresis Calibration Kit, Pharmacia).

## Results and Discussion

*Purification of the enzyme*

Table 1 summarizes the purification of MS extracted from the mycelia of *F. palustris*. The enzyme was purified 235-fold in 10% yield after gel filtration chromatography (HiLoad Superdex 200). Figure 1 shows the electrophoretogram of SDS-PAGE analysis of the enzyme protein from each purification step. The homogeneously purified enzyme (lane 5) was found to have a subunit molecular mass of 65 kDa. Alternatively, the enzyme was also recognized as a single protein band on the electrophoretogram of the native PAGE (data not shown).

Table 1. Purification of MS from *F. palustris*

| Purification steps | Total protein (mg) | Total activity (units) | Yield (%) | Specific activity (units/mg) | Purification (fold) |
|---|---|---|---|---|---|
| Crude extract† | 368 | 125.9 | 100 | 0.34 | 1 |
| $(NH_4)_2SO_4$ precipitation (20–70%) | 226 | 108.1 | 86 | 0.48 | 1 |
| TSK gel phenyl-Toyopearl 650 M | 25.0 | 62.1 | 49 | 2.48 | 7 |
| Protein-Pak DEAE 15HR | 8.95 | 40.9 | 32 | 4.57 | 13 |
| HiLoad Superdex 200 | 0.16 | 12.8 | 10 | 80.00 | 235 |

† Crude extract was prepared from one-week old mycelia collected from 100 culture flasks.

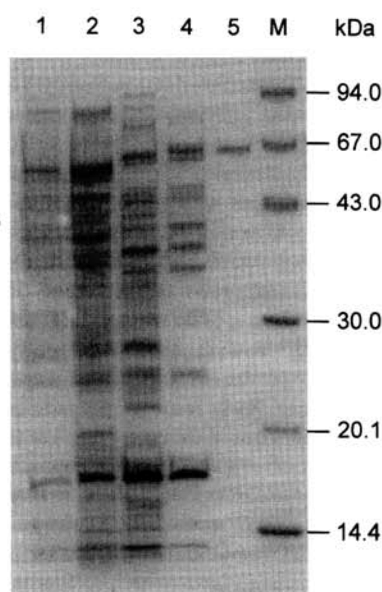


Fig. 1. SDS-PAGE Analysis of the Enzyme Protein during the Course of Purification.

Lane 1, crude extract; lane 2, ammonium sulfate precipitation (20–70%); lane 3, after TSK gel phenyl-Toyopearl 650 M; lane 4, after Protein-Pak DEAE 15HR; lane 5, purified MS (65 kDa) after HiLoad Superdex 200; lane M, molecular mass markers.

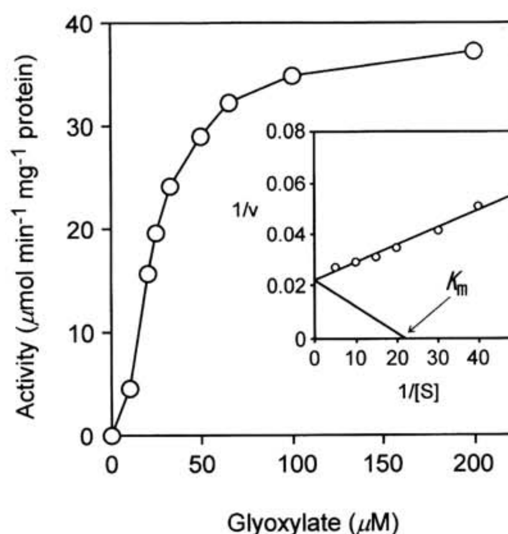


Fig. 2. Dependence of the MS Activity on Glyoxylate Concentrations.

Insets, the double reciprocal plots of 1/v versus 1/[S], showing $K_m$ of 45 μM for glyoxylate.

By gel filtration on a TSK gel G3000SW$_{XL}$ column, the purified enzyme was estimated to have a molecular mass of 520 kDa, consisting apparently of eight identical subunits (65 kDa) as an octamer, which is very close in molecular size to the enzymes from higher plants, such as *Ricinus communis* and *Pinus taeda* with the molecular masses of 575 kDa (64 kDa subunit)[25] and 520 kDa (62 kDa subunit),[16] respectively. However, the enzymes from *Pinus densiflora* and *Gossypium hirsutum* have slightly higher molecular masses of 630 kDa (62 kDa subunit)[15] and 750 kDa (63 kDa subunit),[26] respectively. Interestingly, there is no significant difference in the subunit molecular mass, whereas the enzymes from *Acinetobacter calcoaceticus* and *Corynebacterium glutamicum* are in a monomeric form with a molecular mass of 75[18] and 82 kDa,[19] respectively.

### *Substrate specificity and $K_m$ values*

The enzyme has shown a high substrate specificity for glyoxylate, which is consistent with the result reported for MS from *Pseudomonas ovalis*.[27] No C-S bond cleavage of acetyl-CoA was observed in the presence of oxaloacetate, pyruvate, 2-oxoglutarate, or glyoxal. Kinetic studies have shown that the *F. palustris* MS has high affinities for both glyoxylate and acetyl-CoA. With the increase in glyoxylate concentrations at the fixed concentration of acetyl-CoA and $Mg^{2+}$, the reaction rates follow the typical substrate saturation pattern (Fig. 2). The double reciprocal plot (inset in Fig. 2) shows that the $K_m$ for glyoxylate is 45 μM, which is in the same order as reported for the enzymes from *A. calcoaceticus* (35 μM)[18] and *C. glutamicum* (30 μM).[19] The affinity of the enzyme for acetyl-CoA is significantly high; 30% of the maximal enzyme activity was observed at the lowest concentration (1 μM) of acetyl-CoA tested. An inhibitory effect was observed when acetyl-CoA concentration was over 20 μM (Fig. 3). The double reciprocal plot (inset in Fig. 3) shows that the $K_m$ for acetyl-CoA is 2.2 μM. This $K_m$ is the lowest one ever reported for

**Table 2.** Comparison of $K_m$ Values of MS for Glyoxylate and Acetyl-CoA from Different Sources

| Sources | $K_m$ (μM) | | Reference No. |
|---|---|---|---|
| | Glyoxylate | Acetyl-CoA | |
| Glucose-grown *Fomitopsis palustris* | 45 | 2.2 | This study |
| Alkane-grown *Candida tropicalis* | 1000 | 80 | 21) |
| Malonate-grown *Acinetobacter calcoaceticus* | 35 | 15 | 18) |
| Acetate-grown *Corynebacterium glutamicum* | 30 | 12 | 19) |
| Acetate-grown *Pseudomonas ovalis* | 93 | 63 | 27) |
| Acetate-grown *Euglena gracilis* | 50 | 80 | 28) |
| Pollen of *Pinus densiflora* | 76 | 52 | 15) |
| Endosperm of *Ricinus communis* | 2000 | 10 | 25) |
| Seedlings of *Ricinus communis* | 85 | 67 | 29) |

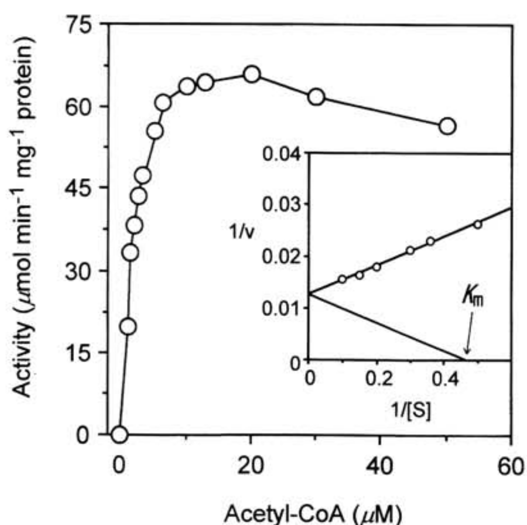


**Fig. 3.** Dependence of the MS Activity on Acetyl-CoA Concentrations.

Inset, the double reciprocal plots of 1/v versus 1/[S], showing $K_m$ of 2.2 μM for acetyl-CoA.

MS. Table 2 shows the $K_m$ for glyoxylate and acetyl-CoA of the enzymes from different sources, for comparison.

*pH optimum*

Greater enzyme activities were obtained in the Tris-HCl buffer than in the KPi buffer. The maximum enzyme activity in the Tris-HCl buffer was observed at pH 8.5, which is slightly higher than the optimum pHs of the reported MSs from *Zea mays* (pH 7.6),[14] *P. densiflora* (pH 8.0),[15] and *A. calcoaceticus* (pH 8.0)[18] in the same buffer.

*Requirement of $Mg^{2+}$ for the full enzyme activity and the enzyme stability*

The dependence of the MS activity on $Mg^{2+}$ concentrations was examined with the dialyzed $Mg^{2+}$-free enzyme solution. The maximal enzyme activity was obtained at 5 to 10 mM $Mg^{2+}$. Kinetic studies have shown that the $K_m$ for $Mg^{2+}$ is 0.4 mM, which is comparable to that of the enzyme from *P. ovalis* (0.5 mM),[27] but is much lower than that of the enzyme from *C. tropicalis* (4.7 mM).[21] Dependence of the MS activity on $Mg^{2+}$ was also reported for the enzyme from *R. communis*.[29] Table 3 shows that no activity was detected without a metal ion added and the maximal enzyme activity was obtained by the addition of $Mg^{2+}$. Furthermore, $Ca^{2+}$ and $Mn^{2+}$ at 5 mM activated the enzyme by 19% and 16% as compared with $Mg^{2+}$, respectively. Little activity was observed in the presence of $Na^+$ and $K^+$. However, $Ca^{2+}$ or $Mn^2$ coexisting with $Mg^{2+}$ inhibited the enzyme activity by 60%. A recent study has demonstrated that $Mg^{2+}$-glyoxylate-enzyme complex is important to polarize glyoxylate for nucleophilic attack by the enolate intermediate of acetyl-CoA to form malate.[30]

**Table 3.** Dependence of the MS Activity on Metal Ions

| Metal ions added | Concentration (mM) | Relative activity (%) |
|---|---|---|
| None | 0 | 0 |
| $Mg^{2+}$ | 5 | 100 |
| $Ca^{2+}$ | 5 | 19 |
| $Mn^{2+}$ | 5 | 16 |
| $Na^+$ | 5 | 10 |
| $K^+$ | 5 | 5 |
| $Ca^{2+}+Mg^{2+}$ | 5+5 | 38 |
| $Mn^{2+}+Mg^{2+}$ | 5+5 | 34 |
| $Na^{+}+Mg^{2+}$ | 5+5 | 92 |
| $K^{+}+Mg^{2+}$ | 5+5 | 93 |

A higher $MgCl_2$ concentration (8 mM) was used during the purification procedure[16,26] to prevent the enzyme inactivation. In our experiment also, $Mg^{2+}$ was found to have a strong effect on retention of the enzyme activity; no loss of the activity was observed after one-month storage at 0°C in the presence of 2 mM $MgCl_2$, in contrast to the 100% loss in its absence. Thus, $Mg^{2+}$ played an important role in protecting the enzyme from inactivation during storage.

*Inhibitory effects of metabolites and compounds*

Table 4 shows effects of metabolites of the glycoly-

**Table 4.** Inhibitory Effects of Compounds on the MS Activity

| Compounds added | Concentration (mM) | Relative activity (%) |
|---|---|---|
| No addition | | 100 |
| Oxalate | 1 | 43 |
| Glycolate | 1 | 77 |
| Coenzyme A | 0.1 | 56 |
| Fructose 1,6-bisphosphate | 10 | 60 |
| Phosphoenolpyruvate | 1 | 66 |
| Citrate | 1 | 82 |
| Pyruvate | 1 | 89 |
| Oxaloacetate | 1 | 92 |
| Glyoxal | 10 | 70 |
| ATP | 1 | 50 |
| ADP | 1 | 50 |
| AMP | 1 | 54 |
| PCMB | 0.05 | 9 |
| Iodoacetate | 5 | 6 |

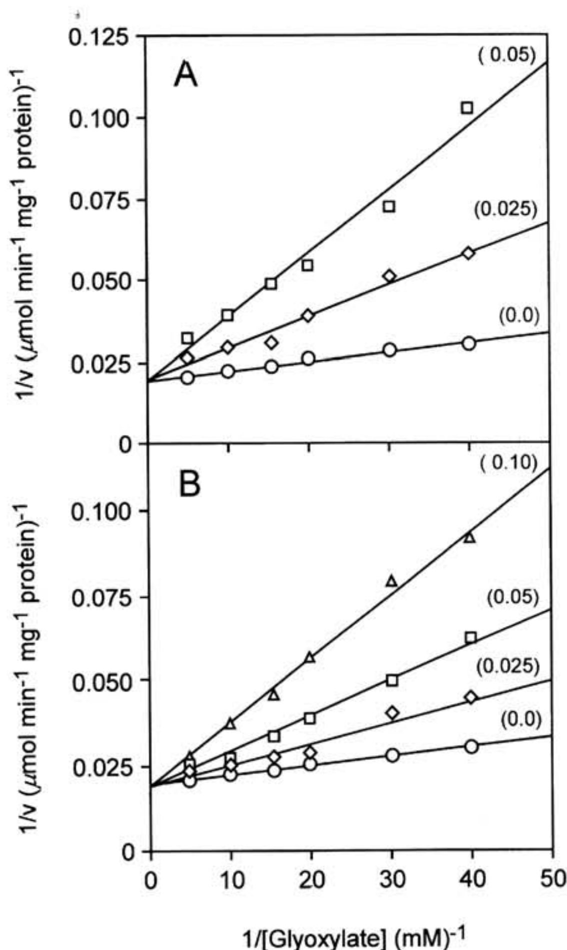


**Fig. 4.** The Lineweaver-Burk Plots for the Competitive Inhibitions of the MS Activity by Oxalate (A) and Glycolate (B).

The concentrations of inhibitors (mM) are indicated in each plot.

sis and the TCA cycle, and several other compounds on the enzyme activity. The enzyme activity was found to be strongly inhibited by glyoxylate analogues, such as oxalate and glycolate. Kinetic inhibition analyses have shown that oxalate ($K_i$, 8.5 μM) and glycolate ($K_i$, 17 μM) are the competitive inhibitors as indicated in Fig. 4A and 4B, respectively. These results are consistent with the inhibition patterns previously reported for the enzymes from *P. densiflora*,[15] *A. calcoaceticus*,[18] and *R. communis*.[25] Furthermore, coenzyme A, which has never been reported to inhibit the MS activity in previous investigations, was found to be an uncompetitive inhibitor ($K_i$, 100 μM) for acetyl-CoA.

Although the glycolysis metabolites (fructose 1,6-bisphosphate, phosphoenolpyruvate, and pyruvate) and the TCA cycle metabolites (citrate and oxaloacetate) weakly inhibited the enzyme activity, adenine nucleotides, such as ATP, ADP, and AMP, were found to be moderate inhibitors, as previously reported for the enzyme from maize scutella.[31] However, none of the other metabolites, including isocitrate, succinate, malate, acetate, formate, γ-amino butyrate (GABA), glutamate, and lactate, inhibited the MS activity at 10 mM (not shown in Table 4). Nevertheless, the enzyme activity was potently inhibited by SH reagents such as *p*-chloromercuribenzoate (PCMB) and iodoacetate, suggesting that MS contains a sulfhydryl group at the active site.

Importantly, oxalate, the major organic acid that was detected (about 70% yield of the total consumption of glucose) as an end product in the culture fluid of this fungus,[13] has been found to be a potential inhibitor to control the GLOX cycle. Then, even a slight increase in the level of oxalate within the cell may be strong enough to inhibit the metabolic coupling of the GLOX and TCA cycles that are an important energy-generating device of the fungus. Therefore, the oxalate-producing fungi are hypothesized to have an elaborate system to transport oxalate from the intracellular sites to the extracellular sites. However, an oxalate transportation mechanism remains to be investigated.

## Acknowledgments

This work has been supported by a Grant-in-Aid (13556024) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, which has also provided scholarship for the first author E. M., a student from Indonesia, through the Japanese Society for the Promotion of Science Core University Program.

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