Purification and characteristics of a novel cytochrome *c* dependent glyoxylate dehydrogenase from a wood-destroying fungus *Tyromyces palustris*

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Abstract A new glyoxylate dehydrogenase which catalyzes dehydrogenation of glyoxylate to oxalate in the presence of cytochrome *c* has been purified as an electrophoretically homogeneous protein from the cell-free extracts of a wood-destroying basidiomycete *Tyromyces palustris*. The enzymatic reduction of cytochrome *c* was dependent on glyoxylate which was found to be the best substrate among the compounds tested. The K_m value for glyoxylate was determined to be 2.7 mM at the optimal pH (8.0). The UV-visible spectra of the enzyme in oxidized and reduced forms indicate that the enzyme belongs to a family of flavohemoproteins. The flavin nucleotide isolated from the native enzyme by heat denaturation was identified as FMN. The enzyme (M_r 331 000) consists of six identical homopolymers (M_r of subunit 59 000), which were found to constitute a symmetric octahedral shape by electron-microscopic observation with a negative staining method.

Key words: Glyoxylate dehydrogenase (cytochrome); Oxalic acid; Wood-rotting fungus; Flavohemoprotein; Glyoxylate metabolism

1. Introduction

Metabolism of oxalic acid, which widely occurs in higher plants, cultures of molds and wood-destroying fungi, and even in human urine, has long been investigated from a medicinal viewpoint [1]. It is a commonly known physiological trait that most brown-rot basidiomycetes, including *Tyromyces palustris* accumulate oxalic acid in culture media, whereas white-rot ones do not [2–5]. Recently, due to the unique multiple chemical nature of oxalic acid, it has been receiving much attention concerning lignin and cellulose biodegradations by wood-rotting basidiomycetes [6]. In white-rot fungi, oxalic acid acts as a reductant, scavenging the oxidative intermediates of the radical cations and manganic ions formed in the ligninolytic systems, and thus inhibiting lignin-degrading peroxidase (LiP) [7–9] and Mn-peroxidase (MnP) [10], respectively, or as a source of dioxygen reductant to produce hydrogen peroxide and other active oxygens [11,12], while in brown-rot fungi it serves either as an acid catalyst for the hydrolytic breakdown of cellulose and hemicellulose [13,14] and an iron-reducing agent for generating the Fenton type oxidation to break down cellulose [15]. In addition, oxalate serves as an excellent metal chelator and most copper tolerant brown-rot fungi, including *T. palustris* [16], detoxicate copper-containing wood preservatives to form an inactive cupric oxalate complex [17]. Furthermore, oxalate produced into the rhizosphere by symbiotic ectomycorrhizal basidiomycetes transforms fixed forms of phosphate into soluble phosphate, which is essential for the growth of woody plants [18].

In this context, it is important to investigate the enzymes involved in the biosynthesis of oxalic acid. In relation to the enzymatic formation of oxalate, some of the characteristics of glyoxylate dehydrogenases, such as NADP dependent glyoxylate dehydrogenase (CoA-acylating) from *Pseudomonas oxalaticus* [19], NAD dependent glyoxylate dehydrogenase from the plant pathogen *Sclerotium rolfsii* [20], and the membrane-bound glyoxylate oxidase from *Acetobacter dioxyacetonicus* [21], have been reported. Surprisingly, however, the oxalate producing enzymes from wood-destroying basidiomycetes have hardly been investigated, except in our previous reports on oxaloacetase [22,23] and glyoxylate oxidase [24,25] extracted from wood-rotting fungi.

Here, we report on the first example of the flavohemoprotein glyoxylate dehydrogenase which has successfully been purified from the mycelial homogenate of the brown-rot fungus *T. palustris*. This enzyme catalyzes dehydrogenation of glyoxylate to oxalate in the presence of cytochrome *c* and is distinguished from previously reported ones [19–21,25]. Some characteristics of the enzyme are described and discussed herein in relation to a possible physiological function of oxalate biosynthesis.

2. Materials and methods

2.1. Chemicals

All biochemicals such as cytochrome *c*, CoA, FMN, FAD, NAD, and NADP were purchased from Sigma-Aldrich and Nacalai Tesque (Kyoto). The specific enzyme assay kits for determination of oxalate were ordered from Boehringer Mannheim. All chemicals, including glyoxylic and oxalic acids, DCIP, and potassium ferricyanide, were obtained from Nacalai Tesque, Wako Pure Chemical Industries (Osaka), and Sigma-Aldrich. All chemicals used for this investigation were of reagent grades.

2.2. Organism and culture conditions

Stock culture of the brown-rot fungus *T. palustris* (Berkeley et Curtis) Murill was cultivated as previously reported [25].

2.3. Enzyme assays

The enzyme activity of glyoxylate dehydrogenase was assayed with cytochrome *c* as a natural electron acceptor as follows. The reaction mixture (1 ml) contained 20 μmol of glyoxylate, 0.1 μmol of cytochrome *c* and the enzyme solution (0.05–0.2 ml) and 35 mM potassium phosphate buffer (pH 8.0), unless otherwise stated. The enzymatic oxidation of the substrate was started by the addition of the substrate and followed by measurement of the increase in absorbance

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at 550 nm of cytochrome *c* reduced at 30°C. The enzyme activities were expressed as the international unit (kat) calculated from the amounts of cytochrome *c* reduced per second.

Alternatively, the formation of oxalate from glyoxylate with the purified enzyme in the presence of cytochrome *c* was confirmed by determining the product with oxalate assay kits with oxalate decarboxylase and formate dehydrogenase procured from Boehringer Mannheim.

2.4. Enzyme purification

All procedures were performed at 4°C. Protein extraction and ammonium sulfate fractionation were carried out in the same way as previously reported [25] except for the addition of 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM ethylenediaminetetraacetic acid (EDTA) in the extracting buffer. The ammonium sulfate precipitate was dissolved in a 20 mM K-Pi buffer (pH 7.6) containing 1 M ammonium sulfate. The enzyme solution was applied on a TSK gel Phenyl-Toyopearl 650M column which had been equilibrated with a 20 mM K-Pi buffer (pH 7.6) containing 1.8 M ammonium sulfate. The absorbed proteins were eluted with a linear gradient of ammonium sulfate (1.8–0 M) and were further eluted with 20 mM K-Pi buffer (pH 7.6). The eluted fractions containing the enzyme activity were combined and dialyzed against 20 mM K-Pi buffer (pH 6.4) containing 0.5 mM PMSF and 1 mM EDTA overnight. The dialyzate obtained was put on a Cosmogel DEAE column (8×75 mm) which had been equilibrated in a 20 mM K-Pi buffer (pH 6.4). The absorbed proteins were eluted with two steps of linear gradients (0–0.21 M NaCl in 5 ml K-Pi buffer, 0.21–0.3 M in 30 ml K-Pi buffer). The fractions containing enzyme activity were combined. The enzyme solution was passed through HiLoad 16/60 Superdex 200 pg (1.6×60 cm) which had been equilibrated with 50 mM K-Pi buffer (pH 7.6) containing 0.15 M NaCl. The fractions containing enzyme activity were combined and dialyzed against 20 mM K-Pi buffer (pH 7.6) containing 0.5 mM PMSF and 1 mM EDTA. The dialyzate was put on a Bio-Scale CHT2-I hydroxyapatite column (7×52 mm) which had been equilibrated with 20 mM K-Pi buffer (pH 7.6). The absorbed proteins were eluted with two steps of linear gradients (0.02–0.75 M K-Pi buffer (75 ml), 0.75–1 M K-Pi buffer (15 ml)). The eluted fractions containing the enzyme activity were combined and used for the characterization. Protein contents were determined by the method of Bradford with bovine serum albumin as the standard [26].

2.5. Estimation of the molecular weight of glyoxylate dehydrogenase

The standard proteins and the glyoxylate dehydrogenase were separately passed through TSK gel G3000 SW_{XL} column (0.1 M K-Pi buffer (pH 7.6), 0.2 ml/min). The molecular weight of this purified enzyme was estimated by use of the standard calibration. The standard proteins used were apoferritin (M_r 443 000), β-amylase (M_r 200 000), alcohol dehydrogenase (M_r 150 000), bovine serum albumin (M_r 66 000), carbonic anhydrase (M_r 29 000), and cytochrome *c* (M_r 12 400).

2.6. Gel electrophoresis

Native PAGE was conducted on a 2–15% polyacrylamide gradient gel by the method of Davis [27]. SDS-PAGE was done on a 10–20% polyacrylamide gradient gel by the method of Laemmli [28]. After electrophoresis the gels were treated with Coomassie brilliant blue for staining proteins. The subunit molecular weight of this purified enzyme was estimated by use of the calibration with the standard proteins (LMW Electrophoresis Calibration Kit (Pharmacia)). Alternatively, after the native PAGE of the enzyme was carried out, the enzyme activity was located on the electrophoretogram by the activity staining method with the solution containing both nitroblue tetrazolium as an electron acceptor and the substrate.

2.7. Electron microscope observation

The purified enzyme (0.65 mg protein/ml) in 0.3 M potassium phosphate buffer (pH 7.6) was diluted 10-fold and a drop of the enzyme solution was submitted to analysis of structures of the native form of the enzyme with the electron microscope of JEOL JEM100CX (100 keV) after working up with 0.2% uranium acetate.

3. Results

3.1. Purification of cytochrome c dependent glyoxylate dehydrogenase

Table 1 shows the purification of the cytochrome *c* dependent glyoxylate dehydrogenase extracted from the mycelium of *T. palustris*. The enzyme was purified 335-fold with 38% recovery as an electrophoretically homogeneous protein from the crude extracts. The molecular weight of the native enzyme was estimated to be ca. 331 000 on gel-filtration chromatography. The electrophoretogram obtained by the native PAGE of the purified enzyme (Fig. 1A) shows that the location of the single protein band observed is consistent with that of the enzyme activity which was visualized by staining with nitroblue tetrazolium. SDS-PAGE analysis of the enzyme indicates that the enzyme consists of six identical subunits with a molecular weight of 59 000 (Fig. 1B).

Alternatively, the shape of the native enzyme was analyzed by use of the electron microscope with the negative staining method. The enzyme was found to consist of six identical subunits in an octahedral shape, which supports the result obtained by chromatography and gel electrophoresis (Fig. 2).

3.2. Enzymatic formation of oxalate from glyoxylate

Since the enzyme was found to catalyze the reduction of cytochrome *c* in the presence of glyoxylate substrate, the enzymatic formation of oxalate from glyoxylate in the presence of cytochrome *c* was confirmed in turn by determining the product with the oxalate assay kits. The results are given in Table 2. No oxalate was formed in any of the control systems except for the control omitting cytochrome *c*, which gave less than 5% of the amount yielded in the complete system. The reason for this is probably because the electron was very slowly transferred from the reduced prosthetic groups of the enzyme to dioxygen in the absence of cytochrome *c*.

3.3. Optimum pH and K_m value for the enzyme

The purified glyoxylate dehydrogenase exhibited the maximal activity at pH 8 and the half maximal activities were found to be at pH 6 and 10 (data not shown). The Hanes-

Table 1
Purification of cytochrome *c* dependent glyoxylate dehydrogenase

Steps	Total protein (mg)	Total activity (nkat)	Yield (%)	Specific activity (nkat/mg protein)	Purification (-fold)
Crude extract	648.3	152	100	0.23	1
$(NH_4)_2SO_4$ ppt. (20–70%)	252.4	213	140	0.84	4
TSK gel Phenyl-Toyopearl 650M	38.9	273	180	7.0	30
Cosmogel DEAE	2.98	144	95	48.3	207
HiLoad 16/60 Superdex 200 pg	2.18	110	73	50.5	216
Bio-Scale CHT2-I	0.73	57	38	78.4	335

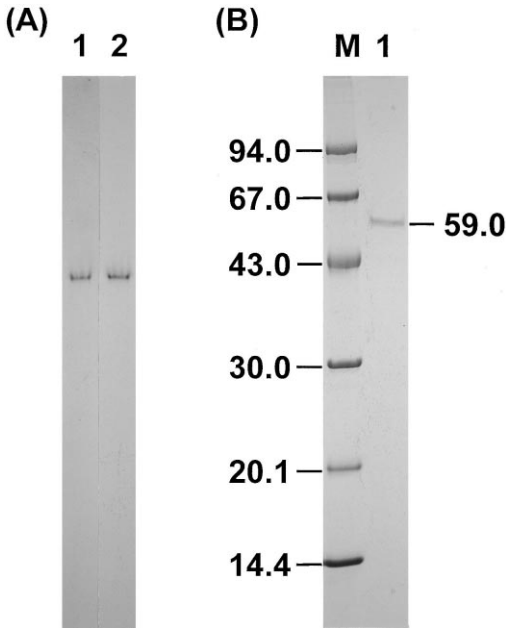

Fig. 1. Electrophoretograms of glyoxylate dehydrogenase. A: Native PAGE analysis was conducted on 2–15% gradient gels. The gels were stained with Coomassie brilliant blue (CBB; lane 1) and nitro-blue tetrazolium (activity staining; lane 2). Both lanes contain the purified glyoxylate dehydrogenase (1.8 μg). B: SDS-PAGE analysis was performed on 10–20% gradient gels. The gels were stained with CBB. Lane 1 contains the purified glyoxylate dehydrogenase (1.8 μg), and lane M contains molecular weight markers. The positions and molecular weights of standard proteins are indicated in the left lane.

Woolf plot obtained for the enzymatic dehydrogenation of glyoxylate showed that the K_m value for oxalate substrate was 2.7 mM under the conditions used.

3.4. Substrate specificity

The relative activities of glyoxylate dehydrogenase towards different substrates at optimum pH are shown in Table 3. Glyoxylate was the best substrate among the compounds tested. Only small amounts of glyoxal and glycolate were utilized, but none of the other compounds, such as glycolaldehyde, formate, oxalate, and L-malate significantly served as the substrate.

3.5. Spectral properties

Purified glyoxylate dehydrogenase in oxidized form shows a characteristic strong peak at 412 nm and a broader but much smaller absorption peak between 527 and 560 nm (Fig. 3). However, the addition of glyoxylate substrate to the enzyme solution resulted in the change of the original spectrum to the reduced form even under aerobic conditions (Fig. 3), which shows the maximal peak at 423 nm, and other characteristic smaller peaks at 330, 527 and 556 nm. These spectral data obtained revealed that the enzyme belongs to a group of flavohemoproteins [29–31].

Table 2
Enzymatic formation of oxalate from glyoxylate in the presence of cytochrome *c*

System	Oxalate (nmol/reaction mixture)
Complete	382
−Enzyme	0
−Glyoxylate	0
−Cytochrome *c*	17
Denatured enzyme	0

The reaction mixture (500 μl) contained 1.8 μg enzyme, 500 nmol cytochrome *c*, 10 μmol glyoxylate in 40 mM K-Pi buffer (pH 8.0). The mixture was incubated at 30°C, 30 min.

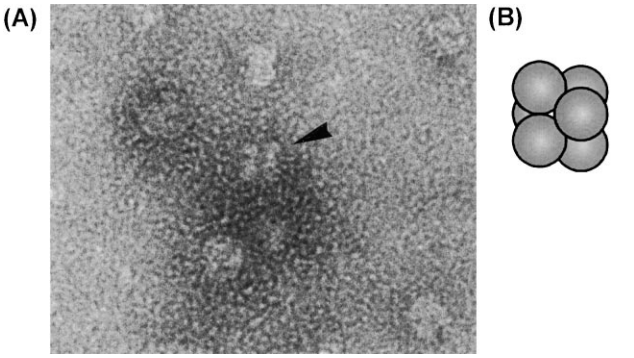

Fig. 2. The electron micrograph of *T. palustris* glyoxylate dehydrogenase molecules. This picture (A) indicates that the enzyme molecules have six identical subunits in a symmetric octahedral shape. One of the enzyme molecules indicated by an arrowhead corresponds to the simulated model (B).

3.6. Analysis of a prosthetic group of the enzyme

The absorption spectra suggested also that flavine nucleotide was involved in a prosthetic group of the enzyme. Purified enzyme was kept in boiling water for 5 min and was centrifuged to remove the denatured protein. The supernatant solution was examined, after concentration, by thin-layer chromatography (Silica Gel 60 F_{254} (0.25 mm thickness), Merck, Germany) with a solvent system, *tert*-amyl alcohol/formic acid/water (3:1:1). A single, yellow, and fluorescent spot ($R_f = 0.22$) which corresponded to authentic FMN was observed, and the R_f value of FAD was 0.13 under these conditions. Thus, the flavin moiety of the enzyme was identified as FMN.

3.7. Electron acceptors for glyoxylate dehydrogenation

Table 4 shows the effectiveness of electron acceptors for the enzymatic dehydrogenation of glyoxylate. In addition to natural cytochrome *c*, potassium ferricyanide and DCIP as artificial electron acceptors were efficiently utilized by glyoxylate dehydrogenase.

However, the dioxygen consumption rate was found to be negligible in the absence of cytochrome *c* (data not shown), as compared with the rate of the complete system with cyto-

Table 3
Substrate specificity of the glyoxylate dehydrogenase from *T. palustris*

Substrate used	Relative activity (%)
Glyoxylate	100
Glyoxal	10
Glycolate	6
Glycolaldehyde	3
Formate	2
Formaldehyde	1
L-Malate	1
Oxalate	0

The enzyme activities for various electron acceptors are expressed as the relative values (%) based on the specific activity of 78.4 nkat/mg protein for glyoxylate.

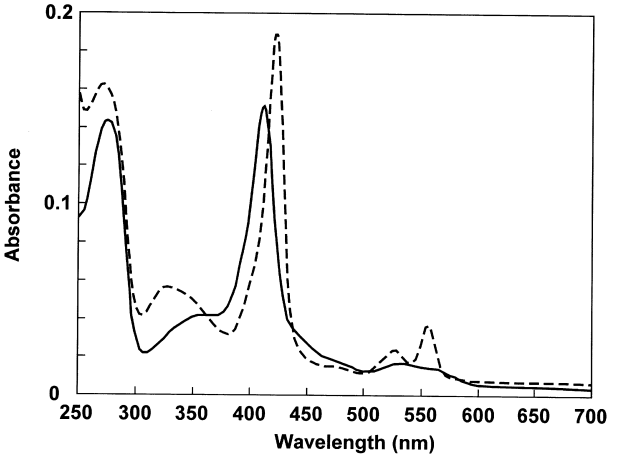

Fig. 3. UV-visible spectra of *T. palustris* glyoxylate dehydrogenase. Solid and dotted lines show the oxidized and reduced forms, respectively.

chrome *c*. It is noteworthy that in contrast to the other glyoxylate dehydrogenases, neither NAD nor NADP was utilized by this enzyme.

4. Discussion

Akamatsu and Shimada [25] have reported that the partially purified glyoxylate oxidase from the brown-rot fungus *T. palustris* did not utilize cytochrome *c* but dioxygen yielding H_2O_2, and artificial electron acceptors, such as DCIP and ferricyanide for the oxidation of glyoxylate. However, during the course of the experiment, we found out that the enzyme obtained from the cell-free extracts prepared in the presence of a protease inhibitor (PMSF) utilized cytochrome *c* as the electron acceptor and that dioxygen was not an effective electron acceptor any more. Thus, by use of the protease inhibitor throughout the purification procedures we could isolate the enzyme in an intact form as reported here. In fact, the intact enzyme treated with papain was spilt into flavin and heme domains (unpublished data). This finding is reminiscent of other flavohemoproteins such as L-lactate dehydrogenase (cytochrome) or cytochrome b_2 (EC 1.1.2.3) from baker's yeast [32] and cellobiose dehydrogenase (EC 1.1.3.25) from white-rot fungi [33], which have been demonstrated to consist of flavin and heme domains. Thus, the electrons derived from the substrate may be transferred to cytochrome *c* via these prosthetic groups during the oxidation of glyoxylate as proposed in Fig. 4.

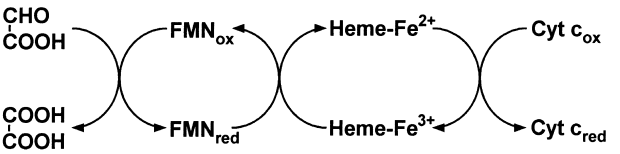

Fig. 4. A proposed scheme of electron-transferring function of the flavohemoprotein glyoxylate dehydrogenase during the oxidation of glyoxylate to form oxalate in the presence of cytochrome *c*.

The molecular mass and the shape of the native enzyme were analyzed by gel permeation chromatography, gel electrophoresis and electron-microscopic observation. The results indicate that the native enzyme was composed of six identical subunits in a symmetric octahedral shape (Fig. 2). Interestingly, the subunit composition of the glyoxylate dehydrogenase is quite different from the other flavohemoproteins reported (e.g. flavocytochrome b_2 (tetramer with 4-fold symmetry) [34], and monomeric cellobiose dehydrogenase [30]).

This investigation has first revealed that the glyoxylate dehydrogenase from *T. palustris* is clearly distinct from the previously reported glyoxylate dehydrogenases [19–21,25] based on the following crucial points. (i) Neither NAD nor NADP was utilized as an electron acceptor, in contrast to the case of the glyoxylate dehydrogenase reported [19,20]. (ii) Dioxygen is not prerequisite for oxidation of glyoxylate [25] because the enzymatic dehydrogenation of glyoxylate to form oxalate is dependent on cytochrome *c* even under aerobic conditions (Table 2) and the cytochrome *c* reduction in turn depends on glyoxylate substrate also under aerobic conditions. These findings are sharply contrasted with the previous findings that dioxygen is reduced to H_2O_2 or H_2O in the enzyme systems from *T. palustris* [25] or *Acetobacter acetonicus* [21], respectively.

To the extent that literature has been surveyed, the flavohemoprotein glyoxylate dehydrogenase has not yet been reported from any living organisms. Although a true physiological role of this enzyme in wood-rotting fungi has not yet been fully understood, this investigation indicates that, during enzymatic dehydrogenation of glyoxylate to oxalate, the flavohemoprotein enzyme may play a key role in transferring electrons from the substrate to cytochrome *c*. However, further characterization and biochemical roles of this enzyme remain to be investigated.

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Table 4
Effectiveness of electron acceptors for the enzymatic oxidation of glyoxylate

Acceptors (mM)	Wavelength (nm)	Relative activity (%)
Cytochrome *c* (0.1)	550	100
NAD (1)	340	0
NAD (1)+CoA (0.1)	340	0
NADP (1)	340	0
NADP (1)+CoA (0.1)	340	0
DCIP (0.1)	600	58
Potassium ferricyanide (1)	420	502
Nitroblue tetrazolium (0.33)	565	7.5

The enzyme activities for various electron acceptors are expressed as the relative values (%) based on the specific activity of 78.4 nkat/mg protein for cytochrome *c*.

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