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# Cell-Free Extraction of Oxaloacetase from White-Rot Fungi, Including *Coriolus versicolor*

Yasumi AKAMATSU*[1], Munezoh TAKAHASHI*[2]
and Mikio SHIMADA*[3]

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**Abstracts**——Oxaloacetase, which catalyzes production of oxalic acid from oxaloacetic acid, was cell-free extracted for the first time from a white-rot fungus, *Coriolus versicolor*. Several other wood destroying fungi were grown on glucose and their oxaloacetase activities in cell-free extracts were compared. It was shown that the amounts of oxalic acid accumulated in the culture media reached the maximal value on day 7 of the cultivation, and decreased thereafter. The enzyme activity gradually increased until day 7 and more rapidly increased thereafter until day 10.

*Keywords:* Oxalic acid, oxaloacetase, white rot fungi, *Phanerochaete chrysosporium*, *Coriolus versicolor*, wood decay, malate dehydrogenase.

## 1. Introduction

Elucidation of biochemical mechanisms for wood decay is of great importance to protect woods against the fungal attack. Since it was postulated that oxalic acid which is a metabolite of wood-rot fungi might be involved as an electron donor in Fenton's reaction causing oxidative degradation of cellulose[1–3] or as a proton donor in hydrolysis of wood carbohydrates such as cellulose and hemicellulose[4–6]. Thus, oxalic acid produced by brown-rot fungi has been receiving keen attention.

Although the enzymatic decomposition of oxalic acid catalyzed by oxalate decarboxylase[7,8] and lignin peroxidase system[9,10] was reported, biosynthesis of oxalic acid in wood-destroying fungi have little been investigated.

Recently, Akamatsu *et al.*[12] reported that oxalic acid is produced from oxaloacetic acid by the cell-free extracts from the brown-rot fungus *Tyromyces palustris* (Berkely et Curtis) Murrill. The experimental conditions for assay of oxaloacetase activities and various influencing factors on the enzyme activities have been reported[13,14].

As a chain of biochemical studies on oxalate metabolism in wood-destroying fungi[15], we attempted to survey the distribution of oxaloacetase among the wood-rot fungi, because

*[1] Present Address; The Fukui Prefectural General Green Center Maruoka, Fukui, Japan, 910-02.
*[2] Laboratory of Deterioration Control.
*[3] Laboratory of Biochemical Control.

the occurrence of this enzyme in white-rot fungi has not yet been reported. Here, we report the first example of the detection of oxaloacetase activity in the white-rot fungi such as *Coriolus versicolor* (Linnaeus ex Fries) Quéle and *Phanerochaete chrysosporium* Burds.

## 2. Materials and Methods

### 2.1. Chemicals and reagents

All chemicals, including oxaloacetic acid, were purchased from Nacalai Tesque and Biorad Laboratories. The commercial enzyme kit (Cat. No. 755,699) for the assay of oxalic acid was procured from Boehringer Mannheim Biochemicals.

### 2.2 Culture conditions

The following wood-rot fungi were grown at the temperatures shown on Fukinnbara's medium[16] as previously reported[13]; *Coriolus versicolor* (COV-1030) at 33°C, *Phanerochaete chrysosporium* (PHC BKM-1747) at 37°C, *Lentinula edodes* (Berkeley) Singer (L22) at 27°C, *Gloeophyllum trabeum* (Person ex Fries) Murrill (GLS-L4e), *Serpula lacrymans* (Wulfen ex Fries) Schroeter (SEL-8697) at 22°C, *Lentinus lepideus* Fries (LEL-8719) at 27°C. All fungi except for the fungus L22 have been maintained in this Institute, and the fungus L22 was from the stock culture of Fukui Prefectural General Green Center.

### 2.3 Assay of oxalic acid

The amounts of oxalic acid enzymatically formed from oxaloacetic acid or accumulated in the culture media of *C. versicolor* were determined by use of the enzyme kits as previously reported[13].

### 2.4 Enzyme preparation

The wood-rot fungi were grown for the different periods depending on their species until the mycelia reached a significant weight. The fungal mycelia were harvested and homogenized as described previously[13]. Since it was found that oxaloacetase activity was considerably lost after the overnight dialysis, the cell-free extracts were used without dialysis for the assay of the oxaloacetase activities.

### 2.5 Assay of oxaloacetase

Oxaloacetase activities were assayed by use of the commercial assay method throughout this experiment as previously reported[13]. However the procedure was slightly modified as follows. After the incubation of the oxaloacetase system, the mixtures were heated at 70°C for 10 min in order to stop the reaction as well as to inactivate malate dehydrogenase, which subsequently interferes the measurement of formate dehydrogenase. For this heat treatment, we also confirmed that oxalic acid is not decomposed by this heat treatment and that oxaloacetic acid is not decomposed either to form oxalic acid.

### 2.6 Assay of malate dehydrogenase

The reaction mixture (3.0 ml) contained 0.1 ml of the enzyme solution, 0.05 ml of 0.6 M oxaloacetate, 0.06 ml of 0.1 M NADH and 2.8 ml of 0.1 M phosphate buffer (pH 7.5).

The mixture was incubated at 30°C and the decrease in the absorbance at 340 nm was followed by Hitachi UV-Visible spectrophotometer Model U-3210.

## 3. Results

### 3.1 Assay of oxaloacetase in cell-free extracts of *C. versicolor*

We chose *C. versicolor* as a model fungus for this investigation, since it has been used as the test fugus of JIS (Japanese Industrial Standard) for wood preservation tests. As shown in Table 1, the cell-free homogenate of the mycelia of *C. versicolor* exhibited a considerable activity in the complete system. The results clearly show that production of oxalic acid from oxaloacetic acid substrate completely depends on the presence of both the enzyme preparation and the substrate, since neither of the reaction systems lacking the enzyme or the substrate yielded any significant amounts of oxalic acid. The heated enzyme gave only 7% of the amount of oxalic acid formed in the complete system. It is noteworthy that the crude enzyme preparation also contained strong activity of malate dehydrogenase which interfered the assay of formate dehydrogenase.

Table 1. Occurrence of oxaloacetase and malate dehydrogenase in the cell-free extracts of *C. versicolor*.

| Reaction system | Malate dehydrogenase activity[a)] | Oxaloacetase activity[b)] |
|---|---|---|
| Complete system | 1.41 | 3.34 |
| Complete substrate | 0 | trace |
| Complete enzyme | 0 | trace |
| Heated enzyme system[c)] | 0 | 0.24 |

a) The assay conditions are described in the Material and Method. The activity are expressed the amount (μmole) of NADH consumed/min/0.1 ml of the enzyme used.
b) The enzyme activity was assayed as described in the text; the values are the amounts of oxalic acid (μmole) produced/hr/0.5 ml of the enzyme used.
c) The heated enzyme was used instead of the untreated enzyme for the reaction.

### 3.2 Occurrence of oxaloacetase in wood-destroying fungi

Table 2 shows oxaloacetase activities of three white-rot fungi and three brown-rot fungi. The result shows that the homogenate of *C. versicolor* gave the greatest activity of oxaloacetase and that *P. chrysosporium* also exhibited a significant activity of this enzyme. However, GLS, SEL, LEL, and L22 gave no significant amounts of oxaloacetase activities.

### 3.3 Changes in oxaloacetase activities during the culture of *Coriolus versicolor*

Fig. 1 shows changes in oxaloacetase activities, pHs, and oxalic acid accumulated during the cultivation of *C. versicolor*. The results clearly show that activities of oxaloacetase

Table 2. Distribution of oxaloacetase among white-rot and brown-rot fungi tested.

| Fungi tested | Culture day | Total activity (nkat/culture) | Specific activity (pkat/mg protein) |
|---|---|---|---|
| White-rot fungi | | | |
| *C. versicolor* | 11 | 26.5 | 60 |
| *P. chrysosporium* | 8 | 1.5 | 32 |
| *L. edodes* | 30 | n.d[a)] | — |
| Brown-rot fungi | | | |
| *T. palustris*[b)] | 3 | 5.5 | 60 |
| *G. tarabeum* | 11 | n.d. | — |
| *S. lacrymans* | 31 | n.d. | — |
| *L. lepideus* | 21 | n.d. | — |

a) n.d.; not detected.
b) Data for *T. palustris*[14].

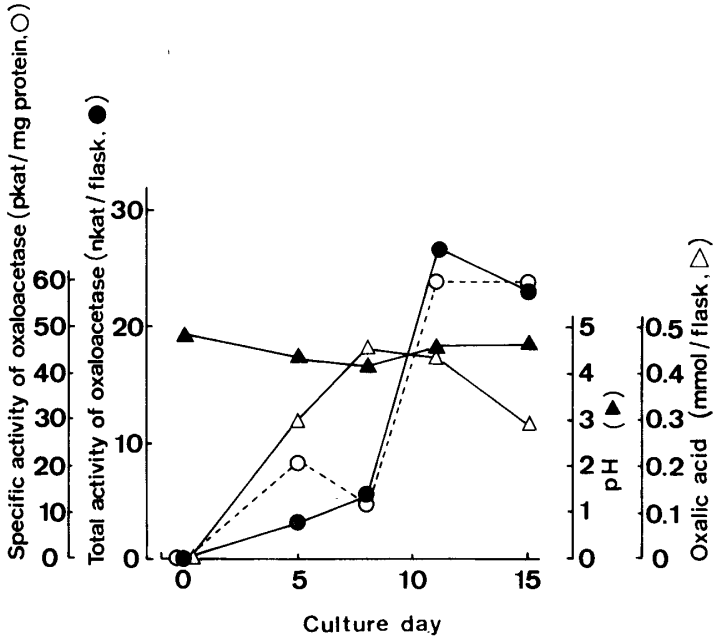


Fig. 1. Changes in activities of oxaloacetase during the cultivation of *Coriolus versicolor*.

appeared as a constitutive enzyme, which was also observed for the culture of *T. palustris*. The total activity/culture reached the maximum on day 11, and decreased gradualy thereafter. The specific activity also increased, reaching the maximum on the same day. This means that the proportion of this enzyme in total proteins is increasing with the cultivation period. It is noteworthy, however, that the moderate increase in the total activity found until day 8 and more intensively thereafter until day 11 correlated with the increase in amounts of oxalic acid produced, reaching the maximum (6 mM) on day 8.

The reason for decrease in the amounts of oxalic acid accumulated may be due to activation of oxalate decarboxylase and LiP systems.

It is interesting to note, however, that significant amounts of oxalic acid was accumulated in the culture of *C. versicolor* in contrast to the report that oxalic acid is not accumulated in the culture media[7].

## 4. Discussion

Oxaloacetic acid is a very important member of organic acids involved in TCA cycle which functions as the energy-producing device accompanied by production of $CO_2$ and also as the biochemical device to supply several aliphatic amino acids through the enzymatic transaminations of the keto-acids.

This investigation has shown that oxaloacetate, which is a precursor of aspartic acid, is alternatively converted to oxalate in white-rot fungi and consequently oxalate is decomposed to $CO_2$ by either the system of oxalate decarboxylase/formate dehydrogenase or the system of LiP/veratryl alcohol/$H_2O_2$[9–11]. Now that oxaloacetase was found to occur in white-rot fungi, this enzyme is commonly important for oxalate metabolism among the major three groups of wood-destroying fungi such as white-rot, brown-rot and soft-rot fungi[17]. Although accumulation of oxalic acid might be very important for degradation of wood carbohydrates in brown-rot decay[1,2], the role of oxalate metabolism in white-rot decay is not clear yet. We suspect one of the biochemical advantages of formation and decomposition of oxalate for white-rot fungi is that surplus organic acid metabolites from glucose are alternatively decomosed to $CO_2$ via the oxalate pathway, which may function as a regulatory valve for controlling the metabolic pool sizes of the overflooded organic acids whose metabolisms are beyond capacity of the TCA cycle in the manner similar to acetic

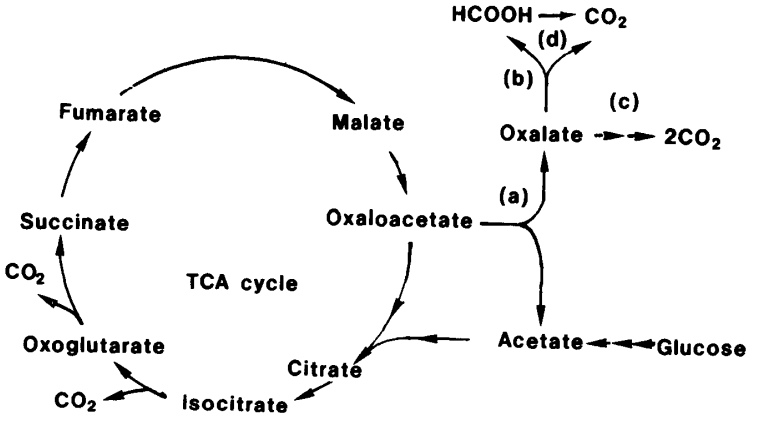


Fig. 2. A metabolic linkage of oxalate pathway with TCA cycle. The reaction steps (a), (b), (c) and (d) are catalyzed by oxaloacetase, oxalate decarboxylase, LiP/veratryl alcohol/$H_2O_2$ system, and formate dehydrogenase, respectively.

acid accumulation by Escherichia coli[18]. Thus, as shown in Fig. 2, oxalate pathway may play a role in maintaining rather low pool sizes of organic acids to keep the operation of TCA cycle normal even under the ammonium nitrogen limited condition in wood decay process. On the other hand, probably, under the ammonium-nitrogen-rich condition organic acid pool sizes are reduced since these acids flow into the amino acid pools required for protein synthesis. However, further research is required to explain the proposed role of the oxalate pathway in white-rot fungi.

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