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Oxalate decarboxylase in the brown-rot wood decay fungus *Postia placenta*

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Oxalate decarboxylase in the brown-rot wood decay fungus Postia placenta

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Keywords: Wood decay, brown rot, oxalic acid, oxalate decarboxylase, *Postia placenta*

1. Introduction

It has long been known that most brown-rot fungi accumulate large quantities of oxalic acid in certain culture media (T. SHIBAMOTO et al., 1952; H. SHIMAZONO, 1955). Under similar conditions, white-rot fungi fail to accumulate oxalic acid because of the activity of oxalate decarboxylase (ODC), the enzyme that converts oxalic acid into formic acid and carbon dioxide (H. SHIMAZONO, 1955). This idea was confirmed by S. TAKAO (1965) and is routinely repeated in most papers involved with the oxalic acid metabolism of wood decay fungi. Although most brown-rot fungi initially accumulate large quantities of oxalic acid, the compound is broken down in later stages of decay, both in wood (E. ESPEJO and E. AGOSIN, 1991; F. GREEN et al., 1991; C. J. SCHMIDT et al., 1981) and artificial culture media (J. A. MICALES, 1994; J. A. MICALES and T. L. HIGHLEY, 1989). The mechanism of this decline has not been explained, but oxidative mechanisms have been proposed (E. ESPEJO and E. AGOSIN, 1991; C. J. SCHMIDT et al., 1981). In the study reported here, ODC was identified and partially purified from high- and low-decay isolates of the brown-rot fungus *Postia placenta* (Fr.) M. Larsen & Lombard. This is the first report of ODC in brown-rot fungi and confirms the production of this important enzyme by both major groups of wood decay fungi.

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2. Materials and methods

2.1 Isolate storage and cultural conditions

All isolates were maintained on 2 % malt-extract agar tubes at 4°C for the duration of the study. The fungi were grown at room temperature (27°C) on a rotary shaker in 0.055 M (= 1 % w/v) glucose in a basal salts solution (T. L. HIGHLEY, 1973) with 0.05 M ammonium phosphate as the nitrogen source. The pH of the medium was adjusted to 5.5 by the addition of 1.0 M NaOH before autoclaving. The media were inoculated with 50 ml of a mycelial suspension made from 7- to 14-day-old cultures of the fungi growing in the same medium.

2.2 Oxalic and formic acid assays

Oxalic acid concentrations were determined using a microtiter plate adaptation of a diagnostic test kit for oxalic acid (Sigma Chemical Company[3], St. Louis, MO), as previously described (J. A. MICALES, 1994). Oxalic acid concentration was measured in millimoles based on a standard curve that was constructed using known concentrations of oxalic acid (Sigma oxalic acid standards). Formic acid was detected by high performance liquid chromatography (HPLC) using a 1050 Ti solvent delivery system and autosampler (HewlettPackard, Avandale, PA) and a HPX-87H$^+$ cation exchange column (Bio-Rad, Hercules, CA). Formic acid was eluted from the column with 5 mM sulfuric acid at a flow rate of 0.5 ml/min and a temperature of 65°C. Detection was achieved by monitoring effluent flow for absorbance at 205 nm (bandwidth 25 nm) using an LC-235 C diode array detector (Perkin Elmer, Norwalk, CT). Quantitation was by reference to an external standard. An enzymatic test kit for the detection of formic acid (Boehringer Mannheim, Indianapolis, IN) was also employed. Formic acid was measured in millimoles based on a standard curve that was formed using known concentrations of formic acid.

2.3 Detection and quantification of carbon dioxide

The amount of carbon dioxide released from oxalic acid in the presence of ODC was determined by a carbon dioxide detection kit (Sigma Diagnostics, St. Louis, MO).

Carbonate/chloride combined standards (Sigma) were used for construction of the standard curve.

2.4 Oxalate decarboxylase assay

Oxalate decarboxylase activity was quantified by determining the rate of oxalic acid decomposition. Samples were combined with an oxalic acid stock solution to give a final concentration of 2 mM oxalic acid. Water or an appropriate buffer was used in place of culture filtrate for a negative control. The solutions were incu-

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3 The use of trade or firm names in this publication is for reader information and does not imply endorsement by the U.S. Department of Agriculture of any product or service.

bated at 40°C for 1 min to 2 h depending upon the activity of the preparation. The oxalic acid concentration of the sample following incubation was determined using the Sigma oxalic acid assay. One unit of ODC activity was the amount of enzyme needed to degrade 1 mg oxalic acid per minute at 40°C.

2.5 Partial purification of oxalate decarboxylase

Oxalate decarboxylase was at least partially purified using a variation of the technique of M. V. DUTTON et al. (1993). Fungal mycelium was collected by vacuum filtration through Miracloth. Excess moisture was removed from the mycelium by blotting it with filter paper. The mycelium was then wrapped in aluminum foil and rapidly frozen over liquid nitrogen; it could be stored this way for several days when necessary. The frozen mycelia were ground to a fine powder in a pre-chilled mortar and pestle using liquid nitrogen to keep the preparation frozen. Citrate-phosphate buffer, 0.1 M, pH 2.2, was added to the mycelial powder until a thick paste formed (usually 1 ml buffer: 3 g fungal biomass). The preparation was centrifuged for 20 minutes at 10,500 × g. The supernatant was collected and dialyzed overnight against 0.1 M citrate-phosphate buffer, pH 2.2, at 4°C. The sample was applied to a 7.5 ml CM Sepharose CL6B cation exchange column and eluted with a stepwise gradient (15 ml aliquots) of 0.1 M citrate-phosphate buffer containing 0, 0.1, 0.25, and 0.50 M NaC1. The relative protein concentration of the eluted fractions was determined by measuring absorbance with a UV detector (Isco, Lincoln, NE) at 280 nm. The fractions were assayed for ODC. Further concentration was done by filtration with Amicon Centricon-30 concentrators or ammonium sulphate (70 % w/v) precipitation.

2.6 SDS-polyacrylamide gel electrophoresis

Oxalate decarboxylase was prepared from ground mycelia of MAD 698 (18-day-old cultures) and ME 20 (9-day-old cultures) as described. The column-purified, concentrated material was resuspended in 0.5 M Tris-Cl buffer, pH 6.8, diluted 1:4 with sample buffer, and subjected to SDS-polyacrylamide gel electrophoresis using the Mini-Protein II electrophoretic system (Bio-Rad, Richmond, CA). The sample was boiled for 5 min and applied to precast BioRad 10 % SDS acrylamide Tris-HCl gels. Protein bands were visualized by silver staining (BioRad silver stain kit).

2.7 Effect of pH on ODC activity

The effect of pH on enzyme activity was determined by overnight dialysis of the partially purified enzyme in 0.2M HCl/KCl buffer (pH 1.00, 1.25, 1.50, 1.75, 2.00, and 2.20) and 0.l M citrate-phosphate buffer (pH 2.2, 2.5, 3.0, 3.5, 4.0, 5.0, and 6.0) at 4°C. Oxalate decarboxylase activity and actual pH of the sample were determined.

3. Results

Oxalate decarboxylase was at least partially purified from mycelial extracts of three isolates of *P. placenta:* MAD698, a dikaryotic high-

decay isolate; ME20, a monokaryotic low decay isolate; and Hybrid, a low-decay hybrid of ME20 and a high-decay monokaryon. The specific activities of the enzyme from these strains are shown in Table 1. Oxalate decarboxylase activity was detected only erratically, usually in low concentrations (0.02 - 0.14 units/ml), in the culture filtrate. Much higher activities were associated with the cell-free mycelial extracts.

Table 1: Specific activities of ODC from mycelial extracts of MAD698 and ME20 before and after chromatography.

Strain	Harvest (days)	Biomass (g)	Protein (mg)	ODC activity units	ODC activity (units/g biomass) before test[b]	Specific activity ODC (units/mg protein)[b,c] before test	after test
MAD698	11	4.7	0.146	0	0	0	ND
	15	4.6	0.266	4.4	1.0	16.5	181
	22	3.2	0.230	11.7	3.7	50.9	700
ME20	11	3.9	0.240	1.0	0.3	4	ND
	15	12.0	0.607	215.0	17.9	354	4,447
	22	3.2	0.176	33.0	10.3	188	2,660

[a] Mycelia obtained from shake culture of three replicate 500-ml flasks containing 300 ml of 1% glucose + 0.05 M ammonium phosphate + basal salts (T. L. HIGHLEY, 1973), initial pH 5.5. Cultures incubated at room temperature (27 °C).

[b] Before or after chromatography.

[c] ND is not determined. Samples with low ODC activity were not subjected to chromatography.

The activity of ODC derived from mycelial extracts varied with time (Table 1). Mycelial extracts of ME20 displayed highest activity (per gram of mycelial weight or milligrams of protein) on day 15. Mycelial extracts of MAD698 exhibited their highest activity on day 22, after fungal biomass and protein concentration had started to decline. The mycelial extracts of ME20 contained much higher levels of enzyme activity than MAD698 on all three harvest dates. This increased activity was *more* than could be explained by the production of more mycelium or by increased levels of protein concentration. Oxalate decarboxylase activity was detected in cultures of ME20 (data not shown) as early as day 9. No activity was detected in extracts of MAD698 before day 15.

Oxalate decarboxylase was partially purified using CM-Sepharose CL-6B (Fig. 1). This material is usually a cation exchanger. At the low pH used in this study, the carboxynethyl groups of the column matrix were uncharged, thereby preventing cation exchange. Most of the cellular

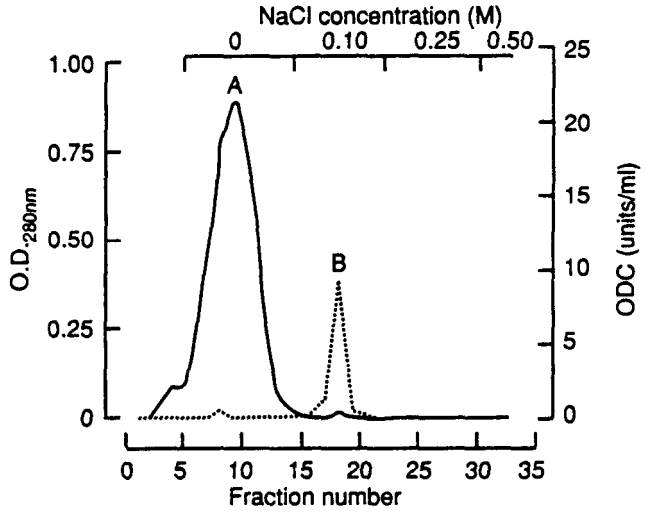

Figure 1: Cation exchange chromatography of mycelial extract of MAD698 in 0.1M citrate phosphate buffer, pH 2.2. (A) Relative absorbance at 280nm (elution of general proteins); (B) oxalate decarboxylase (ODC) activity (units/ml).

material did not bind to the uncharged column. Oxalate decarboxylase consistently bound to the column, perhaps by an affinity mechanism, and was eluted by the addition of 0.1 M sodium chloride in 0.1 M citrate-phosphate buffer, pH 2.2. Single protein bands were detected on SDS-polyacrylamide electrophoretic gels from column-prepared samples of moderate activity (approximately 4 units/ml). Four to five additional minor bands were detected when the column fractions were further concentrated approximately ten-fold by membrane filtration.

The ability of the partially purified enzyme to convert oxalic acid to formic acid was demonstrated by HPLC (Fig. 2) and was confirmed by enzymatic assays for formic acid and carbon dioxide. Enzyme preparations from both MAD698 and ME20 were able to convert oxalic acid into formic acid and carbon dioxide in a 1:1:1 stoichiometric ratio.

The effect of pH on ODC activity from strains MAD698 and ME20 is shown in Figure 3. In both strains, ODC was most active under extremely acidic conditions with an optimum pH of 2.0 -2.2 in HCl/KCl or citrate/phosphate buffer. Enzyme activity was greatly diminished at pH 3.0 and was totally inhibited at pH 4.0. The enzyme from ME20 appeared to be slightly more pH-sensitive than the enzyme from MAD698.

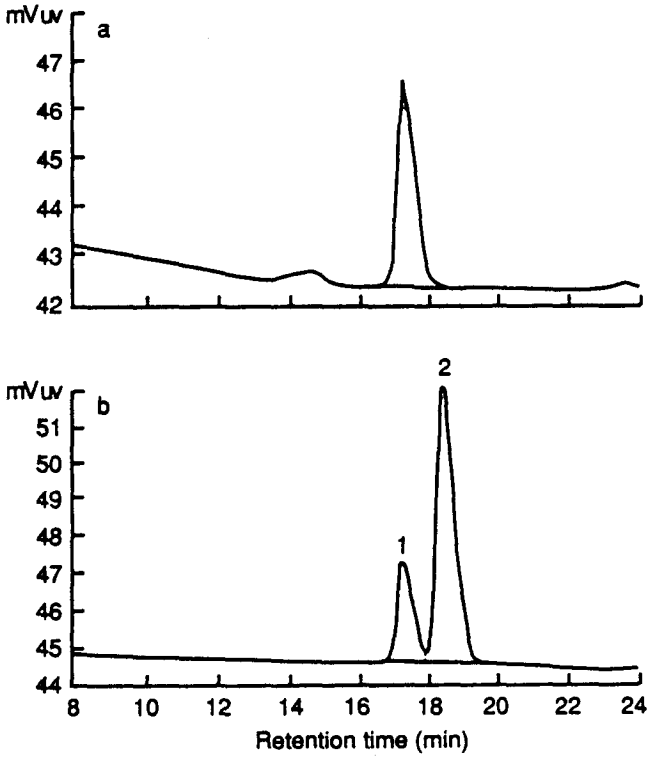

Figure 2: Identification of formic acid as product of enzyme reaction. (A) High performance liquid chromatography of 2 mM oxalic acid incubated with column-purified ODC from ground mycelia of ME20; (B) Peak 1, formic acid standard; Peak 2, fumaric acid standard (an internal standard).

4. Discussion

Oxalate decarboxylase activity was detected in high- and low-decay isolates of *P. placenta*, a brown-rot fungus that accumulates oxalic acid through most of its life cycle. The enzyme required extremely acidic conditions for activity. The acid requirements of ODC from white-rot decay fungi and other fungi are not as low. H. SHIMAZONO (1955), working with 12 different white-rot fungi, found the optimum pH of the reaction to be 2.5-3.0. This was confirmed by M. V. DUTTON et al. (1994), who concluded that ODC isolated from the white-rot fungus *Trametes versicolor* (L:Fr.) Pilát had a pH optimum of 3.0. The actual optimum may have been lower since 3.0 was the lowest pH tested. The enzyme from *T. versicolor* retained low levels of activity at pH 4.0 and 5.0 (M. V. DUTTON et al., 1994), unlike ODC from *P. placenta*. Oxalate decarboxylase from the white-rot fungus

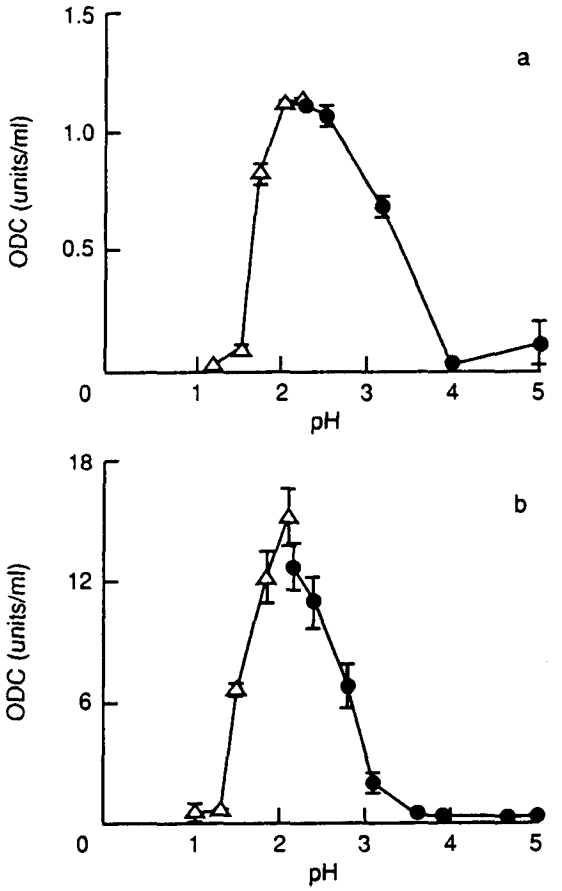

Figure 3: Effect of pH on activity of ODC from mycelial extracts of MAD698 (a) and ME20 (b). Mycelial extracts were dialyzed in 0.2 M HCl/KCl buffer, pH 1.0 - 2.2 (△) or 0.1 M citrate phosphate buffer, pH 2.2 - 5.0 (●). Each reading represents the average of four replications Standard error bars are shown or are negligible. Note the difference in scale of enzyme activity between the two isolates.

Flammulina velutipes (Curtis:Fr.) P. Kumm was stable over a wide pH range but showed optimal activity at pH 3.0 (A. Mehta and A. Datta, 1991). Maximal activities of ODC from *Myrothecium verrucaria* (Albertini & Schwein.) Ditmar:Fr., *Aspergillus niger* Tiegh., and *Sclerotinia sclerotiorum* (Lib.) de Bary were obtained at pH 4.0 (E. B. Lillehoj and R. G. Smith, 1965), 4.7 (E. Emiliani and P. Bekes, 1964), and 3.5 (P. Magro et al., 1988), respectively. Brown-rot fungi create very acidic environments (F. Green et al., 1991), so it is not surprising that an enzyme probably involved in pH regulation would have such a low pH optimum.

Strain ME20, a low-decay isolate of *P. placenta*, is unable to depolymerize cellulose or cause weight loss in wood (J. A. MICALES and T. L. HIGHLEY, 1989; F. GREEN et al., 1991). In the study reported here, the low-decay isolate appeared to produce ODC earlier than did the high-decay isolate. It also displayed much higher enzyme activity. The inability of the low decay isolate to accumulate oxalic acid over a specific period of time, which results from its overproduction of ODC early in the decay process, may prevent it from effectively decaying wood.

Oxalate decarboxylase may be used to help regulate and buffer the pH in the immediate environment of the fungus, thus maintaining the wood at a pH low enough to allow decay (E. AGOSIN et al., 1989) but not cause hyphal damage. J. H. CONNOLLY and J. JELLISON (1994) hypothesized that brown-rot fungi actively regulate the pH of their environment. J. BECH-ANDERSON (1989) hypothesized that the presence of two organic acids, in this case oxalic and formic acids, would form a better buffer system than the presence of a single acid. In culture, ODC appears to be expressed rather late in the growth cycle of the „normal, " high-decay isolate of *P. placenta* and may be induced only after the fungus has created such acidic conditions that it threatens its own survival. Such conditions may occur earlier in wood than in liquid culture. F. GREEN et al. (1991) demonstrated that the pH of wood inoculated with *P. placenta* rapidly decreased to a pH of 1.7 -2.0 and then increased slightly and stabilized concurrent with a decrease in oxalic acid concentration. The lowest pH reached by the wood corresponded to the acidity at which ODC is most active. The initial production of oxalic acid by the fungus probably triggered the activity of ODC by lowering the pH of the microenvironment around the fungus. Oxalic acid may also be an inducer of ODC, as shown for white-rot fungi (M. V. DUTTON *et al.*, 1993). Such an induction mechanism would cause increased ODC production under conditions of low pH so that the enzyme is produced only when it would be active and a substrate is present.

The accumulation and decomposition of oxalic acid in liquid culture can also be explained by the activity of ODC. Oxalic acid only accumulates in cultures of *P. placenta* that have an initial pH of 5.0 or higher (J. A. MICALES, in press). The initial production of small quantities of oxalic acid by the fungus may rapidly lower the pH of more acidic media into the range in which the enzyme is active. Oxalic acid would then be broken down rapidly by the enzyme and would never accumulate to measurable levels.

5. Summary

Oxalate decarboxylase, the enzyme associated with the decomposition of oxalic acid in white-rot fungi, was identified and partially purified from low- and high-decay cultures of the brown-rot fungus *Postia placenta*. The enzyme was most active under conditions of extreme acidity (pH 2.0 - 2.2) and was severely inhibited at pH levels greater than 2.6. A low-decay isolate of *P. placenta* produced large quantities of this enzyme and formed it earlier than did a standard high-decay isolate. The possible role of the enzyme in the regulation of pH during decay is discussed.

Zusammenfassung

Oxalat-Decarboxylase in dem Braunfäulepilz *Postia placenta*

Oxalat-Decarboxylase, das Enzym, das beim Abbau von Oxalsäure durch Weißfäulepilze eine Rolle spielt, wurde aus Braunfäulekulturen von *Postia placenta* mit niedrigem und hohem Abbauvermögen gewonnen und teilweise gereinigt. Das Enzym war bei extrem hohen pH-Werten (pH 2,0 - 2,2) am aktivsten und wurde durch pH-Werte über 2,6 stark gehemmt. Eine *P. placenta*-Kultur mit niedrigem Abbauvermögen produzierte große Mengen dieses Enzyms und außerdem früher als eine Standard-Kultur mit hohem Abbauvermögen. Die mögliche Rolle dieses Enzyms bei der Regulierung des pH-Wertes während des Abbauprozesses wird diskutiert.

Résumé

Oxalate décarboxylase dans le champignon *Postia placenta*, une pourriture brune du bois

L'oxalate décarboxylase, une enzyme associée à la décomposition de l'acide oxalique dans les champignons à pourriture blanche, a été identifiée et partiellement purifiée à partir de cultures de *Postia placenta* provoquant de faible ou fort pourrissements. L'enzyme était plus active dans des conditions d'acidité extrêmes (pH 2.0 - 2.2), et était inhibé à des pHs supérieurs à 2.6. Une souche de *P. placenta* isolée de faible pourriture, a produit plus d'enzyme et ce plutôt qu'une souche standard à pourriture élevée. Le rôle de l'enzyme dans la régulation du pH durant le pourrissement est discuté.

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