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Lignin Biotransformations by an Aromatic Aldehyde Oxidase Produced by *Streptomyces viridosporus* T7A

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ABSTRACT

Streptomyces viridosporus produces an intracellular aromatic aldehyde oxidase that oxidizes aromatic and α, β-unsaturated aromatic aldehydes to their corresponding acids. It also produces extracellular oxidase as shown by zones of clearing when grown on agar containing insoluble dehydrodivanillin (DHDV). This extracellular form may be responsible for oxidizing aldehyde groups in lignin. The extracellular oxidase was expressed maximally after 3 d growth in medium containing only yeast extract. However, higher levels were produced when lignocellulose was in the medium. The enzyme was partially purified and its molecular weight was approximated to be about 80,000 daltons. Mutant cultures that had lost the ability to produce zones of clearing on DHDV-containing agar solubilized smaller quantities of lignin as compared to the wild type, except for one strain. A partially purified oxidase preparation was shown to oxidize a natural lignocellulose substrate.

Index Entries: *Streptomyces;* aldehyde oxidase; lignin; biotransformation; degradation.

INTRODUCTION

Streptomyces viridosporus strain T7A is known to mineralize the lignin component of lignocellulose (*1*). During this degradative process, a significant portion of the lignin is released as a water soluble polymer (2). We refer to this material as acid-precipitable polymeric lignin, or APPL, because it can be rendered insoluble by reducing the pH. It has also been

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found that this material is relatively resistant to further degradation by *Streptomyces* once produced (3). We are interested in identifying enzymes responsible for and the mechanism of its synthesis, since this material has potential commercial applications, such as for adjuvants (4), antixoidants (5), or as adhesives (5), and because it may clarify the mechanism of lignin degradation by *Streptomyces*.

We have hypothesized that aromatic aldehyde oxidase (Araldox) may be one enzyme that has a role in the release of APPL from lignocellulose. Araldox is capable of oxidizing a variety of aromatic aldehydes and α,β-unsaturated aromatic aldehydes to acids using oxygen as electron acceptor (6). It was originally thought that this enzyme was found only intracellularly, and its role in lignin degradation was the oxidation of monomeric lignin cleavage products. However, it was shown that zones of clearing were produced around *S. viridosporus* colonies growing on dehydrodivanillin (DHDV)-containing agar (7). Insoluble DHDV is oxidized to a soluble carboxylic acid. This indicated that aromatic aldehyde oxidase might be excreted into the extracellular environment where it could oxidize substrate groups in lignin. We believe this enzyme might be responsible for oxidizing aromatic aldehydes, such as coniferaldehyde end groups, which are known to be present in lignin (8), increasing its solubility and thereby aiding in the production of APPL. Additionally, it may produce extracellular hydrogen peroxide for use by lignin peroxidases (9).

There are examples of alcohol oxidases produced by organisms that have been suggested to be involved in lignin biotransformation. Iwahara et al. (10) identified an alcohol oxidase from a ligninolytic culture of *Fusarium solani* that oxidizes α,β-unsaturated aromatic alcohols in lignosulfonate. A similar enzyme was shown to be produced by *Polystictus versicolor* (11). Such extracellular oxidases are reasonably believed to be involved in lignin transformation because aerobic lignin degradation appears to be an oxidative process (12) and atmospheric oxygen could serve as a readily available oxidant.

We report here the demonstration of extracellular aromatic aldehyde oxidase produced by *Streptomyces viridosporus* T7A, and we have partially purified it from disrupted cells. This purified enzyme was tested for its ability to modify native lignin. We also tested mutants apparently lacking the enzyme for their ability to solubilize lignin relative to the wild type.

MATERIALS AND METHODS

Cultures

The lignocellulose-degrading organism, *Streptomyces viridosporus* strain T7A, was isolated as previously described (13). It grows optimally at 37°C and is maintained on slants of yeast extract-malt extract-dextrose agar (YEMED). Strains DHDV-D and DHDV-E are ultraviolet light generated aromatic aldehyde oxidase negative mutants of T7A that had lost the

ability to solubilize crystals of dehydrodivanillin (DHDV) by oxidizing it to either the mono- or di-acid. These mutant strains were isolated by Crawford et al. (*7*) and were stored lyophilized. After they were rehydrated, they were streaked on DHDV agar plates to ensure that they retained the phenotype for which they were isolated. Strains DV-1 and DV-9 are also aromatic aldehyde oxidase negative mutants that were isolated in the same way as those above. These mutant strains were also maintained on YEMED slants.

Culture Conditions

Streptomyces viridosporus was grown in broth culture containing 0.6% yeast extract (Difco), and 0.2% (w/v) 40 mesh, completely extracted corn lignocellulose (*2*), in phosphate mineral salts solution (*3*). The lignocellulose was autoclaved for 75 min separately from the yeast extract broth that was autoclaved for 20 min. 500 mL of medium in 1 L erlenmeyer flasks was inoculated with a loopful of spores from a slant. In some experiments, a suspension of spores in sterile water was used to provide a standard inoculum. The cultures were incubated shaking at 37°C at 150 rpm for various periods. An experiment was done to determine the optimum time of aromatic aldehyde expression in cell-free extracts. Three days was found to be optimum and so other cultures were incubated for this length of time.

The yield of soluble lignin produced by the aromatic aldehyde oxidase negative mutants after a 6-wk incubation was determined and was compared to that for the wild type strain. Growth of these cultures was in a dampened culture system, as described previously (*2*). After the incubation period, the soluble lignin was precipitated with acid and quantitated gravimetrically.

The inducibility of several aromatic aldehyde substrates was tested to see if insoluble substrates produced higher enzyme yields than soluble substrates. Flasks containing 0.6% (w/v) yeast extract were inoculated with T7A spores and were incubated shaking for 24 h. Various substrates were then added to the cultures, either as filter sterilized solutions or as autoclaved solids or solutions. After 2 additional d of shaking, cells were collected by filtration and cell-free extracts were prepared and assayed as described below. A similar experiment was done comparing the specific activity of cell-free extracts prepared from 3-d-old cultures initially containing either lignocellulose and yeast extract or only yeast extract.

Enzyme Sources

Three-d-old lignocellulose-containing broth cultures were filtered through Whatman #1 filter paper. The filtrate was concentrated fivefold or greater using an Amicon ultrafiltration cell and PM10 or PM30 membranes (Amicon Corp., Danvers, MA). The concentrated filtrate was brought to 90% saturation with ammonium sulfate (Schwarz/Mann) and

was then centrifuged at 20,000×g for 25 min. The pellet was redissolved in 20 m*M* Tris-HCl, pH 7.0, followed by centrifugation again at 20,000×g to remove insoluble, denatured material.

A cell-free extract was prepared from the residue retained on the filter paper above. The cells and undegraded lignocellulose were washed with cold 0.85% KCl solution and suspended in 20 m*M* Tris-HCl, pH 7.0. This suspension was sonicated (Bronwill Biosonik III) on high for 6 30-s periods with the suspension being allowed to cool on ice in between. The sonicated cells were centrifuged at 20,000×g for 25 min and the supernatent was used as the cell-free extract. It was stored frozen at −70°C with little or no loss in activity.

Enzyme Purification

Crude enzyme was partially purified by gel filtration chromatography and then by DEAE ion exchange chromatography. Sepherose CL-6B (Pharmacia) was used as packing in a 95 cm long×1.5 cm diameter column. Sample was loaded and eluted with 20 m*M* Tris-HCl at a flowrate of 0.25–0.27 mL/min. The absorbance at 280 nm of the eluent was followed using an Isco UA-5 column monitor and 2.5 mL fractions were collected. The most active fractions were pooled and were further purified on a DEAE Sepherose column that had been equilibrated with 20 m*M* Tris-HCl at pH 7.0. A sodium chloride gradient (0–2 *M*) was used to elute the enzyme. The eluent was monitored and fractionated as before.

Enzyme Assays

The activity of aromatic aldehyde oxidase containing solutions was determined by measuring the rate of disappearance of the vanillin (4-hydroxy-3-methoxybenzaldehyde) absorbance peak at 345 nm, as described previously (*15*). An assay to rapidly measure enzyme activity in chromatography fractions was also developed. It makes use of the ability of the enzyme to use 2,6-dichloroindophenol (DCIP) as an alternative electron acceptor. DCIP, which is normally blue, becomes colorless on reduction. Fifty μL of each fraction were pipetted into the wells of a 96-well microtiter plate (Costar #3590, Cambridge, MA). To the wells was added 100 μL of a mixture of 0.28 m*M* vanillin and 0.01% DCIP (Calbiochem, San Diego, CA) in 25 m*M* Tris-HCl, pH 7.5. Three drops of mineral oil were then applied to the top of each well to exclude air. The plates were incubated floating on the surface of a 37°C water bath for 10 min, after which the absorbance of the wells was measured at 620 nm with a reference at 690 nm in an SLT EAR 400 AT plate reader (SLT-Labinstruments, Austria). The most active fractions were pooled and the activity of the mixture was measured using the spectrophotmetric assay for vanillin disappearance.

Protein in samples was measured using the method of Bradford (*16*). The protein assay reagent was from Bio-Rad Laboratories (Richmond, CA)

and was used according the suppliers instructions. Bovine serum albumin (Sigma, Fraction V) was used in making standard curves.

Unpurified cell-free extract was electrophoresed in a 7.5% native polyacrylamide gel (*17*). A band of aromatic aldehyde oxidase was detected using an activity stain. Hydrogen peroxide detecting reagents (*see below*), along with 10 m*M* vanillin, in melted agar were poured over the gel. After the agar had solidified, the gel was incubated 37°C for 1.5 h, after which a red band developed.

Enzymic Oxidation of Lignin

Since our original hypothesis was that aromatic aldehyde oxidase from *Streptomyces viridosporus* oxidizes aromatic aldehydes in intact lignin, we determined whether the enzyme would produce expected reaction products from native lignin. Three methods were used in this determination. The first method used was the treatment of small squares of wood (pine, 1.5 cm×1.5 cm) with the active fractions (35 U/mL, where 1 U=1 μmole vanillin oxidized/min) from gel filtration chromatography of cell-free extract. The wood pieces were put in contact with the enzyme for 4 h at 37°C in a moist environment. After this period, the wood was rinsed with deionized water and allowed to air dry for 24 h. Control pieces were treated similarly but with water instead of enzyme solution. The dried surfaces of the wood pieces were then treated with phloroglucinol/HCl (*18*) to produce a color reaction with the lignin aromatic aldehydes (*8*). The ability of the enzyme to reduce DCIP using corn milled wood lignin (MWL) as substrate was also tested. One hundred μL volumes of the partially purified enzyme solution from above were added to 900 μL of a solution containing 1.3 mg of MWL in 50 m*M* Tris-HCl at pH 7.5 and dimethylformamide (5:2). The decrease in absorbance at 600 nm was followed. A sample of enzyme that had been heat treated with steam for 15 min was used as a control. The third method to determine if the enzyme attacks native lignin was to measure hydrogen peroxide released from corn lignocellulose incubated with active enzyme. In one experiment, unpurified cell-free extract (78 U/mL) was diluted 1 to 10 in 50 m*M* Tris-HCl buffer at pH 7.5. One mL of this dilution was added to 20 mg of 40 mesh, completely extracted corn lignocellulose in a 10×75 mm test tube. Tests were done in replicates of four, with the control being heat-treated enzyme. The tubes were incubated shaking at 37°C for 4 h, after which the tube contents were centrifuged and hydrogen peroxide was measured in the supernatant using 4-aminoantipyrene, phenol, and horseradish peroxidase according to the method of Frew et al. (*19*). A second, similar experiment was done using the pooled fractions from the gel filtration separation of concentrated culture filtrate, which had an activity of 25 U/mL. This experiment was also done in replicates of four and used heat treated enzyme as control, but it differed in that the enzyme solution was used undiluted.

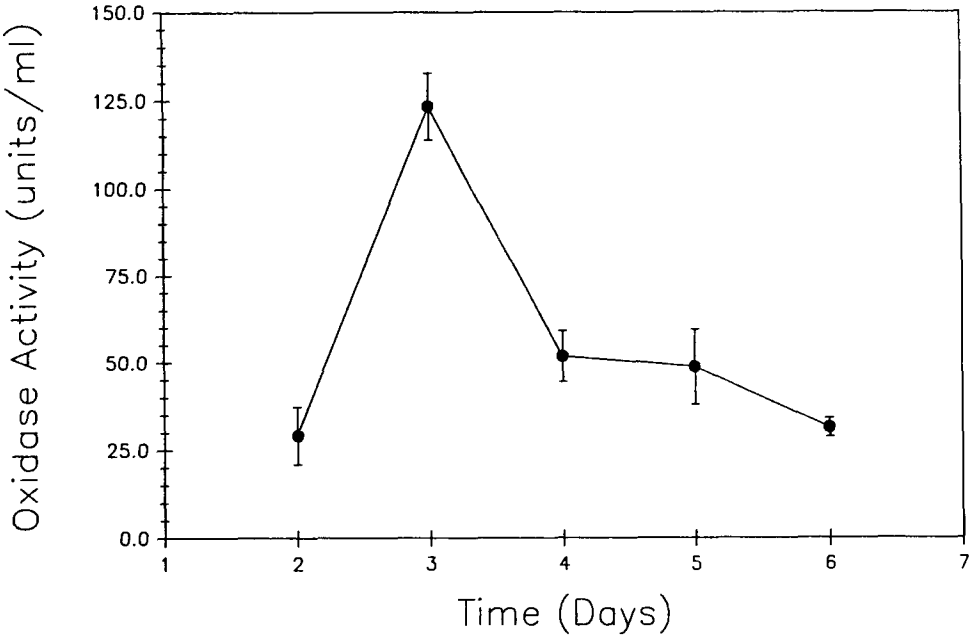

Fig. 1. Time for aromatic aldehyde oxidase expression.

RESULTS

Maximal yield of aromatic aldehyde oxidase in cell-free extracts from lignocellulose containing cultures was achieved after 3 d of growth. The yield gradually decreases after this period (Fig. 1).

If aromatic aldehyde oxidase has a role in oxidizing the aldehyde groups in lignin, releasing the lignin as soluble acid salts, it was thought that the yield of soluble lignin from oxidase negative mutants would be significantly lower than the yield for the wild type. Three of the mutants, DHDV-E, DHDV-D, and DV-9 did have significantly lower yields of soluble lignin. However, mutant DV-1 had a significantly higher yield of soluble lignin (Table 1).

Veratraldehyde and solubilized lignin (APPL), when added after 24 h of growth as inducers to yeast extract broth cultures of *S. viridosporus*, gave significantly higher levels of intracellular aromatic aldehyde oxidase than did the uninduced control culture and other simple aromatic aldehydes (Table 2). The yield of oxidase in the control culture, which had nothing added, was higher than all of the cultures that had additions made to them, except those induced with vanillin, veratraldehyde, and APPL. APPL was the only compound that induced significantly higher specific activity than the control. When aromatic aldehyde oxidase specific activity was measured in cell-free extracts produced from cultures grown in yeast extract broth in replicates of four, each either with or without lig-

Table 1
Soluble Lignin from Aromatic Aldehyde Oxidase Negative Mutants[a]

Strain	APPL yield mg, SD	Percent weight loss, SD
T7A	139.8 (3.3)	35.70 (.95)
DV-1	192.9 (16.3)	28.77 (.73)
DHDV-E	124.0 (3.0)	23.60 (.39)
DV-9	128.0 (4.9)	26.56 (.51)
DHDV-D	107.3 (9.2)	21.52 (.44)

[a] All APPL yields and weight losses are significantly different by Fisher's LSD at the 95% confidence level.

Table 2
Induction of Aromatic Aldehyde Oxidase by Various Substrates*

Substrate	Activity[a]	Specific activity[b]
Terephthalaldehyde[c]	49.5 (6.6)	5.65
Veratraldehyde[c]	75.5 (7.2)	7.44
APPL[d]	77.9 (4.2)	39.15
Syringaldehyde[c]	27.8 (3.6)	2.81
Vanillin	60.4 (3.0)	20.90
Dehydrodivanillin	33.8 (6.0)	12.90
Lignocellulose	51.9 (1.2)	16.53
Cinnamaldehyde[c]	3.0 (8.5)	1.52
Control (uninduced)	56.8 (9.1)	22.54
Lignocellulose[e]	118.9 (17)	87.3
Control[f]	73.0 (13)	48.7

* All were at 0.05% final concentration except lignocellulose, which was at 0.2% (w/v).
[a] Expressed as μ mole vanillin oxidized per min per mL enzyme solution.
[b] Expressed as μ mole vanillin oxidized per min per mg protein.
[c] Added as a solution in 30% ethanol.
[d] Added as a solution in 10 mL of a 50:50 mixture of 0.1 *M* NaOH and culture buffer.
[e] Lignocellulose present in the medium at time of inoculation and activity assayed after 3 d growth.
[f] Culture unsupplemented with lignocellulose and assayed after 3 d growth.

nocellulose present from time zero, it was found that the lignocellulose-containing cultures assayed after 3 d of growth had about twice as much activity as the cultures containing yeast extract only (Table 2).

In 3-d-old cultures, aromatic aldehyde oxidase activity could be found both intracellularly and extracellularly. The level of intracellular activity was much higher (data not shown) and provided a much more convenient source of enzyme for purification. A summary of a typical purification is shown in Table 3. The solution resulting from DEAE Sepharose ion exchange chromatography is not homogeneous, as shown by native gel electrophoresis. The enzyme is not very stable, thus making complete

Table 3
Summary of Aromatic Aldehyde Oxidase Purification

Purification step	Volume, mL	Protein, mg/mL	Activity, U/mL	Spec. act., U/mg	Purification, fold	Yield, percent
Cell-free extract	10	1.065	85.1	79.9	1	100
CL-6B gel filtration	21.6	0.090	30.8	342	4.3	78
DEAE sepharose	10.8	0.046	19.9	433	5.4	26

Table 4
DCIP Reduction Using Milled Wood Lignin as Substrate

Sample	Activity[a]
Gel filtration purified enzyme	0.023 (.001)
Heat treated enzyme	0.021 (.002)

[a]Expressed as the decrease in absorbance at 600 nm per min.

purification difficult. This is also the reason that some of the experiments used enzyme that had been only gel filtration purified or crude cell-free extract. The native gel electrophoresis followed by activity staining indicated the enzyme to have a molecular weight of approximately 80,000 daltons. Intracellular and extracellular forms of the enzyme appeared to have the same mobilities.

Experiments to determine if aromatic aldehyde oxidase attacks native lignin in wood suggests that it does not. Subjective visual examination of the wood blocks showed no obvious difference between the experimental and the control wood blocks. Reduction of DCIP by gel filtration purified enzymes using MWL as substrate indicated a slight difference between active enzyme and heat treated enzyme (Table 4). The measurement of hydrogen peroxide produced as a result of lignocellulose oxidation showed that the heat treated cell-free extract produced more hydrogen peroxide than the undenatured enzyme solution (Table 5). The gel filtration purified concentrated culture filtrate released more H_2O_2 than its corresponding control (Table 5).

DISCUSSION

These data do not totally support our hypothesis that aromatic aldehyde oxidase is important to the transformation of native lignin to soluble APPL, but they do provide some support for its involvement. Expression of the enzyme in response to addition of various substrates after 24 h of

Table 5
Hydrogen Peroxide Produced from Lignocellulose

Enzyme	μM H_2O_2	
	Experimental	Control
Cell-free extract	6.7 (3.8)[a]	20.3 (3.2)
Gel permeation purified culture filtrate	24.9 (8.3)	16.0 (0.3)

[a]Standard deviations enclosed in parentheses.

growth in yeast extract broth showed a clear pattern of induction by only APPL of the potential inducers examined. However, when lignocellulose was present in the growth medium from the beginning, oxidase specific activity was much higher than when it was absent. The experiment with the aromatic aldehyde oxidase negative mutants partially supports our hypothesis; however, the results with mutant DV-1 (Table 1) were the opposite of what was expected. Yet, this mutant, like the others, was impaired in lignocellulose-degrading ability. These mutants, since they were not characterized, may not have mutations in structural genes but may instead be regulatory mutants that may allow only low levels of or no enzyme expression. These mutants may also be debilitated in some other way that causes slower growth and substrate utilization, resulting in lower soluble lignin yields and lignocellulose weight losses for this reason.

There is strong evidence that extracellular aromatic aldehyde oxidase is present. We did not determine unequivocally whether this enzyme is the same or different from the intracellular form, but their similar electrophoretic mobilities suggest they are the same. This enzyme may be a result of cell lysis which probably occurs after 3 d of growth under these culture conditions, or it may result from excretion.

The data from experiments testing the lignin-oxidizing ability of the aromatic aldehyde oxidase suggests that the enzyme is at least slowly active on native lignin. The test using wood blocks and the phloroglucinol/HCl may not be reliable. The fact that there was no significant color difference between the blocks may be a result of factors other than the enzymes ability to oxidize the lignin. There may have been far more aldehyde groups present than could be oxidized during the period of incubation. The concentration of enzyme may also have been insufficient. The assays measuring DCIP reduction or H_2O_2 production during lignin oxidation should be more reliable, and indicate that the enzyme does not oxidize native lignin. However, there was a higher level of H_2O_2 measured in the incubation of gel filtration purified concentrated culture filtrate as compared to the control. For the cell-free extract, the control produced significantly more H_2O_2 than the experimental. This was as expected, since the crude extract contained catalase, which would degrade enzymatically

generated H_2O_2 as it was produced. Removal of catalase by partial purification of the oxidase allows detection of H_2O_2 resulting from lignocellulose oxidation. Thus, the experiments to measure oxidation of native lignin by the aldehyde oxidase support our hypothesis, although they also do not show unequivocally the enzyme is involved in lignin biotransformation. The fact that higher levels of aromatic aldehyde oxidase are produced in lignocellulose-containing cultures is circumstantial evidence that the enzyme has some involvement in lignocellulose degradation.

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