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# Ligninases from *Chrysonilia sitophila* (TFB-27441 Strain)

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## ABSTRACT

Ligninase found in the extracellular medium of cultures of *Chrysonilia sitophila* was purifieded by ion exchange chromatography. Sodium dodecylsulfate/polyacrylamide gel electrophoresis allowed the determination of 68,000, 48,300, and 48,000 daltons for the molecular weights of ligninase I, II, and III, respectively. The absorption spectrum of the enzymes indicated the presence of a heme prosthetic group. The absorption maximum of the native enzyme II at 400 nm decreased in the presence of one equivalent of hydrogen peroxide. With an additional equivalent of phenol the maximum at 400 nm shifted to 417 nm. This spectrum is similar to horseradish peroxidase compound II. The pyridine hemochromogen absorption spectrum and iron content indicated that ligninases I, II, and III contained a Fe/heme ratio values of 0.8, 1.3, and 1.2 by a molecule of protein, respectively. These enzymes oxidize lignin efficiently, followed by the fluorescence technique and by the photon emission method.

**Index Entries:** Ligninase; chrysonilia silophila; lignin biodegradation; photon emission.

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## INTRODUCTION

Recently it has been reported that one type of ligninase has a molecular weight of 42,000 daltons and contains one heme group (*1*). It is a glycoprotein containing about 13% carbohydrate. It needs H2O2 for activity, but takes oxygen from the air for oxidation reactions. Kuwahara et al. (*2*) reported the characterization of two extracellular H2O2-dependent oxidases from *P. chrysosporium* (MM 446 strain). One was indentical to the one described by Tien et al. (*1*), but the other was a peroxidase that catalyzed the oxidation of a variety of dyes and the decarboxylation of vanillic acid in the presence of $Mn^{+2}$ ions. Evans et al. (*3*) have purified an extracellular haem-containing glycoprotein from the culture of *Coriolus versicolor* (28A PRL strain) with a molecular weight of 53,700 d similar to that described by Kuwahara et al. (*2*). Recently, evidence furnished by Harvey et al. (*4*), Kuila et al. (*5*), and Renganathan et al. (*6*) showed that ligninase has peroxidase behavior. Duran et al. (*7–10*) have recently shown that dioxane-lignin (in dioxane-$H_2O$ system) is rapidly degraded in the presence of horseradish peroxidase, and that the most probable intermediate of the peroxidase could be compound-III (Fe-III-$O_2^-$) (*8*). Very recently, Dordick et al. (*11*) also showed that in other organic solvents the same degradation occurs. Lignin peroxidase has been purified to homogeneity from the extracellular medium of large agitated (*12*) and small stationary cultures (*1*) of *P. chrysosporium*. Three different molecular forms of lignin peroxidase have recently been identified (*13*). Physical chemistry properties (*5,14*) and action on a lignin model by several ligninases were studied (*15–22*). Mechanistic studies were also performed (*23–25*).

Recently, we have isolated a strain of *Chrysonilia sitophila* (TFB-27441), which has low cellulolytic activity and high ligninolytic activity (*26,27*). This fungus grows in *Pinus radiata* wood efficiently suggesting that this fungus has potential for a biopulping process (*28*). The crude extract from the fungal cultures degrade untreated (*10*) and irradiated (λ300 nm) lignins efficiently (*29*).

In this report we describe the purification and properties of novel ligninases from *Chrysonilia sitophila* (TFB-27441 strain).

## EXPERIMENTAL PROCEDURES

### Culture Conditions

The strain of *Chrysonilia sitophila* (TFB-27441) and culture conditions used for production of the enzyme were described previously (*26–28*). The 200 mL culture in a 2 L Erlenmeyer flask was grown as described before, in a modified Fries medium: Ammonium tartrate (2 g/L), $NH_4NO_3$ (0.4 g/L), $KH_2PO_4$ (0.4 g/L), $MgSO_4$ (0.2 g/L), NaCl (0.04 g/L), $CaCl_2$ (0.04 g/L), $CuSO_4.5H_2O$ (0.1 mg/L), $FeSO_4$ .$7H_2O$ (0.2 mg/L), $MnSO_4$ (0.02

mg/L), $ZnCl_2$ (0.15 mg/L), and glucose (.10 g/L). Enzyme activity appeared 2–3 d after culture initiation and was maximal in 5-d-old cultures.

## Purification

Cultures were filtered through a sintered funnel and to minimize proteolysis, *p*-methylphenylsulfonyl fluoride (0.2 m*M*) (SIGMA) was added to the filtrate. The filtrate was concentrated under vacuum to 80% of the original volume. After overnight dialysis against a 5 m*M* sodium tartrate buffer, pH 4.5, the sample was applied to the DEAE-Sephadex A-50-120 column (2×12 cm). The column was washed with 100 mL of sodium tartrate buffer (pH 4.5) and a salt gradient was then applied (0-0.1 M NaCl in 5 m*M* sodium tartrate buffer, pH 4.5, total volume 400 mL) (*1*). The fractions containing hemoproteins were dialyzed separately against distilled deionized water and stored as stable liophilized powder at −20°C.

## SDS Electrophoresis and Isoelectric Focusing

Purity of the enzymes was assessed by isoelectric focusing (*30*) and SDS-polyacrylamide gel electrophoresis, as described by Weber and Osborn (*31*). Protein bands were stained with Coomassie blue. Molecular weight markers (SIGMA) were lysozyme (14.2 K), horseradish peroxidase (40 K), and albumin (66 K).

## Protein and Carbohydrate Determination

Protein content was routinely determined with Coomassie blue (*32*). The carbohydrate determination was carried out by the phenol sulfuric acid procedure (*33*).

## Enzyme Assays

Enzyme activity was measured with two assays: Quantification by UV spectroscopy of veratraldehyde (λ310 nm, 9300 M cm) formed on the oxidation of veratryl alcohol either at pH 2.5 (1 U = μmol of veratryl alcohol oxidized/min/mL) (*1*), or at pH 5.0, and by chemiluminescence of alcohol oxidation in the presence of the enzyme (*34*). By the UV method, we measured the ligninase activity following the absorption band at 310 nm: 10–20 μg of protein/mL was incubated with 0.4 m*M* $H_2O_2$ and 2 m*M* veratryl alcohol in 0.1 M sodium tartrate buffer pH 2.5 or 5.0 at 32°C. Addition of $H_2O_2$ started the reaction. The veratryl alcohol in the spent reaction mixture was characterized by NMR and TLC techniques. The chemiluminescence method was carried out under the same conditions as above and measured as counts per min in a liquid scintillation counter with a chemiluminescence computer program in a LKB-Model 1211 Rackbeta (*29*). By this method, it was possible to measure the oxidation of lignin under the same conditions. The values were normalized in function of horseradish peroxidase activity (*29*).

### Metal Analysis of the Enzyme

Transition metals were determined by Atomic Absorption Spectroscopy with the previous elimination of extraneous metals by dialysis against twice distilled deionized water.

### Pyridine Hemochromogen

The heme was quantified by the absorption of the pyridine hemochromogen complex after extraction of the heme from the enzyme as described previously (*35*).

### Spectroscopic Procedures

Absorption spectra were recorded on a DMS-100 Intralab Instruments (Brazil) Spectrophotometer. Measurements were taken on the protein dissolved in 0.1 M sodium tartrate buffer pH 4.5. Fluorescence spectra were recorded on a Perkin-Elmer Spectrofluoremeter MPF-44B. Measurements were taken on a lignin dissolved in dioxane-$H_2O$ (1:1) and then diluted in 0.1 M sodium tartrate buffer with pH of 4.5 ($\lambda$exc. 360 nm, $\lambda$em. 400 nm).

### Chemicals

All chemicals were reagent grade. Veratryl alcohol was synthetized from vanillin and purified with vacuum distillation (*36*). Dioxane-lignin was obtained from rice hulls as described before (*34*).

## RESULTS AND DISCUSSION

### Fungal Growth

*C. sitophila* grew satisfactorily in a large nonagitated flask (200 mL modified Fries medium into 2 L Erlenmeyer flask) (*28*), buffered with 50 m*M* potasium biphthalate pH 6.0. The veratryl alcohol oxidation activity of the enzyme from 5-d-old cultures was 91 U/L at pH 2.5. These measurements were carried out at pH 2.5 in order to compare with those of Kirk et al. (*22*). The ligninase activity for *P. chrysosporium* special strains as SC-26 and BKM-F-1767 were 34 and 23 U/L, respectively (*22*). Then a higher activity on *C. sitophilia* (TFB-27441 strain) than in those of *P. chrysosporium* strains were observed.

### Photon Emission of the Extracellular Culture Medium/$H_2O_2$/Lignin System

The oxidation of dioxane-lignin by extracellular culture medium/$H_2O_2$ initially showed a very rapid decrease of the emission band at 400 nm ($\lambda$exc. 360 nm). This is related to the chromophore in alpha-carbonyl to phenyl

moiety degradation in the complex structure of lignin (*29,34*). This rapid degradation is accompanied by a chemiluminescence process. As in the lignin oxidation by horseradish peroxidase/$H_2O_2$ system, this chemiluminescence is produced by unspecific radical recombination (*8,9*). Figure 1 shows the photon emission from the ligninase crude extract acting on both lignin and veratryl alcohol. The photon emission correlates with the ligninase activity, using horseradish peroxidase as a references standard (*29*). A very pronounced effect on the photo emission in the presence of carbonate ions was observed. This was an indication of unspecific radical recombination (*29*). This method is exhibited as an excellent procedure to measure the ligninase activity acting on lignin.

## Purification

The elution profile of the lignolytic enzymes from the DEAE-Sephadex A-50-120 (SIGMA) is shown in Fig. 2. From the protein elution three fractions were isolated: fraction I (corresponding to fractions n°54–65), fraction II (corresponding to fractions n° 66–68), and fraction III (corresponding to fractions n° 165–180). These fractions containing hemoprotein were dialyzed separately against deionized water and stock as stable

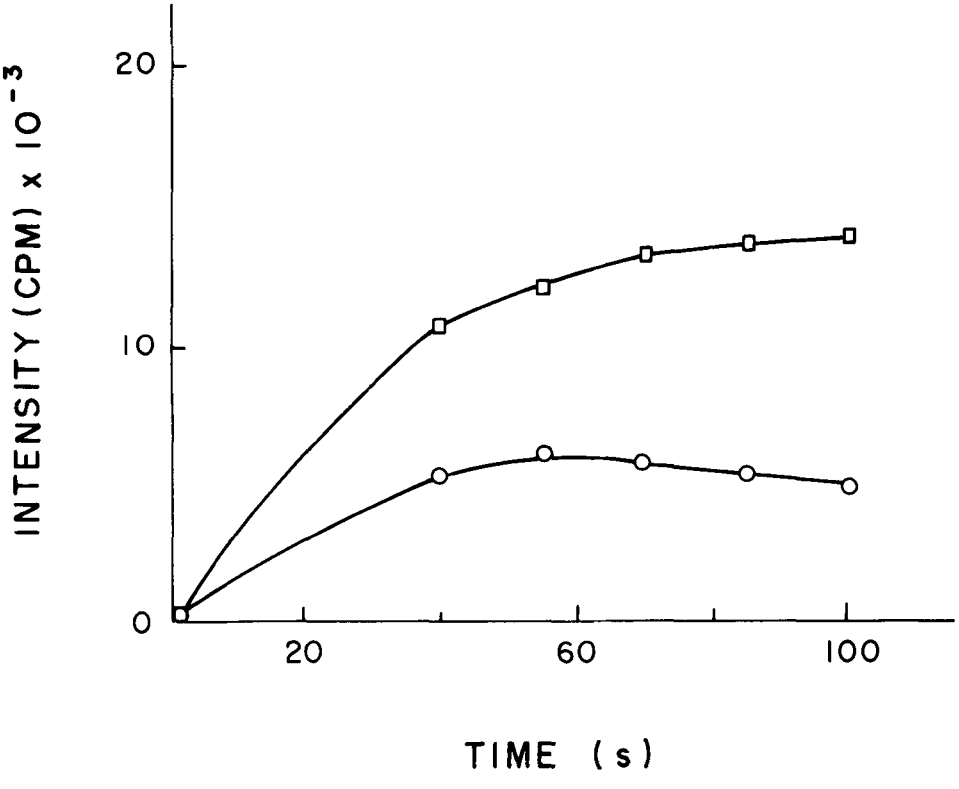


Fig. 1. Temporal behavior of photon emission of ligninase acting on lignin (— ○ —) and veratryl alcohol (— □ —) (*see* Experimental section).

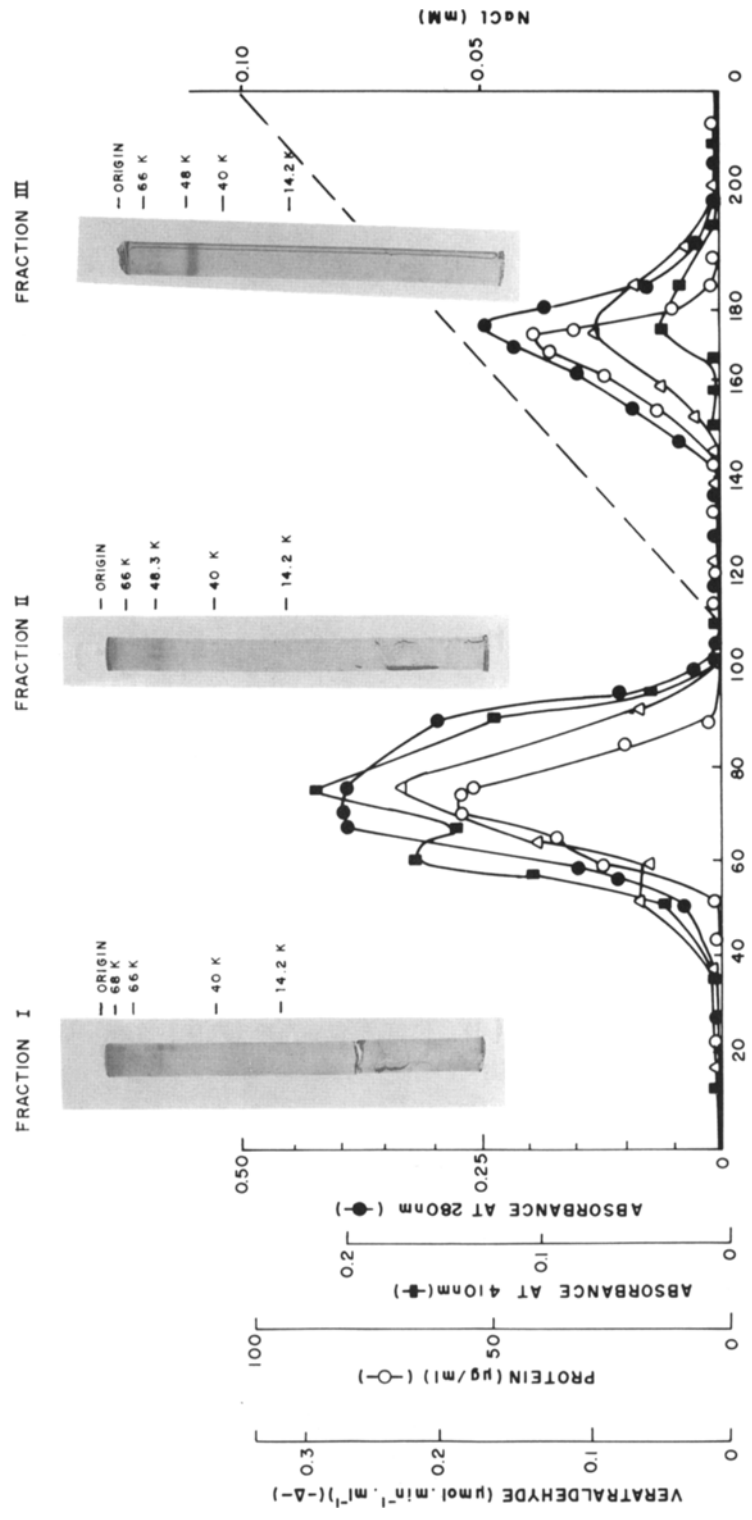


Fig. 2. Elution profile of the ligninase from a DEAE Sephadex A-50-120 column. The column was eluted with a 5 m*M* sodium tartrate (pH 4.5), then, after fraction number 110, a salt gradient was applied. Fractions (2 mL) were assayed for $H_2O_2$-requiring activity for the oxidation of veratryl alcohol (— △ ---), protein content (— ○ —), absorption at 280 nm (— ● —), and absorption at 410 nm (— ■ —). Purity is assessed by NaDodsulfate/polyacrylamide gel electro-phoresis (inset) (fraction I = fractions 54–65; fraction II = fractions 66–88; fraction III = fractions 165–180). Numbers adjacent to gel are $Mr \times 10^{-3}$).

liophilized powder at −20°C. The three protein bands were eluted with $H_2O_2$-requirement oxidative activity against veratryl alcohol, and hemoprotein absorption band (λ410 nm). Isoelectric focusing and SDS-polyacrylamide gel electrophoresis (inset, Fig. 2) confirmed the purity of fractions I, II, and III. Fraction I revealed the existence of a protein with an isoelectric point of 9.1, and molecular weight of 68,000 d (ligninase I). Fraction II showed a protein with an isoelectric point of 7.8 and a molecular weight of 48,300 d (ligninase II), whereas fraction III revealed a protein with an isoelectric point of 4.5 and a molecular weight of 48,000 d (ligninase III). The protein distribution calculated as hemoprotein (relative area under the 410 nm absorption band), was around 33%, 62%, and 5% for ligninase I, II, and III, respectively.

## Carbohydrate Content

The neutral carbohydrates content of ligninase I, II, and III were determined to be 26.0, 14.5, and 18.0%, respectively, in a similar manner as reported by other researchers (*1,12,13,20*).

## Metal Analysis and Hemochromogen

Based on pyridine hemochromogen at 557 nm (*32*) and atomic absorption spectroscopy of the heme content indicated that ligninase I, II, and III contain a Fe/heme ratio values of 0.8, 1.3, and 1.2 by molecule of protein, respectively. Isoelectric focusing and SDS-polyacrylamide gel electrophoresis confirmed the ligninases purities.

## Spectral Properties

The absorption spectrum of the ligninase II was studied in more detail because of the higher content of this protein (*see* Fig. 3). Addition of 1–1.5 equivalent of $H_2O_2$ to the enzyme results in a decrease of the absorption band at 400 nm. Similar behavior is observed with horseradish peroxidase, forming HRP-I. Veratryl alcohol, lignin (not shown), or phenol (0.5–1.0 equivalents) shift the relatively stable spectrum at 400 nm to 417 nm in a similar manner to horseradish peroxide compound I, transforming to compound II (*8,9*). In the ligninase cycle in the presence of $H_2O_2$, there was a partial denaturation of the enzyme (not shown). The spectra of these enzymes suggest that it is a hemoprotein, and this was verified by the formation of the pyridine hemochromogen complex.

## pH Optima

Activities for veratryl alcohol oxidation by ligninase II and III are shown in Fig. 4. For the case of ligninase II and III the pH optima were around 5.0 and 4.0, respectively, showing that the enzymes were of different nature. No Mn(II) dependent peroxidase was found in this fungus at the rage of 2.5–5.5 pH.

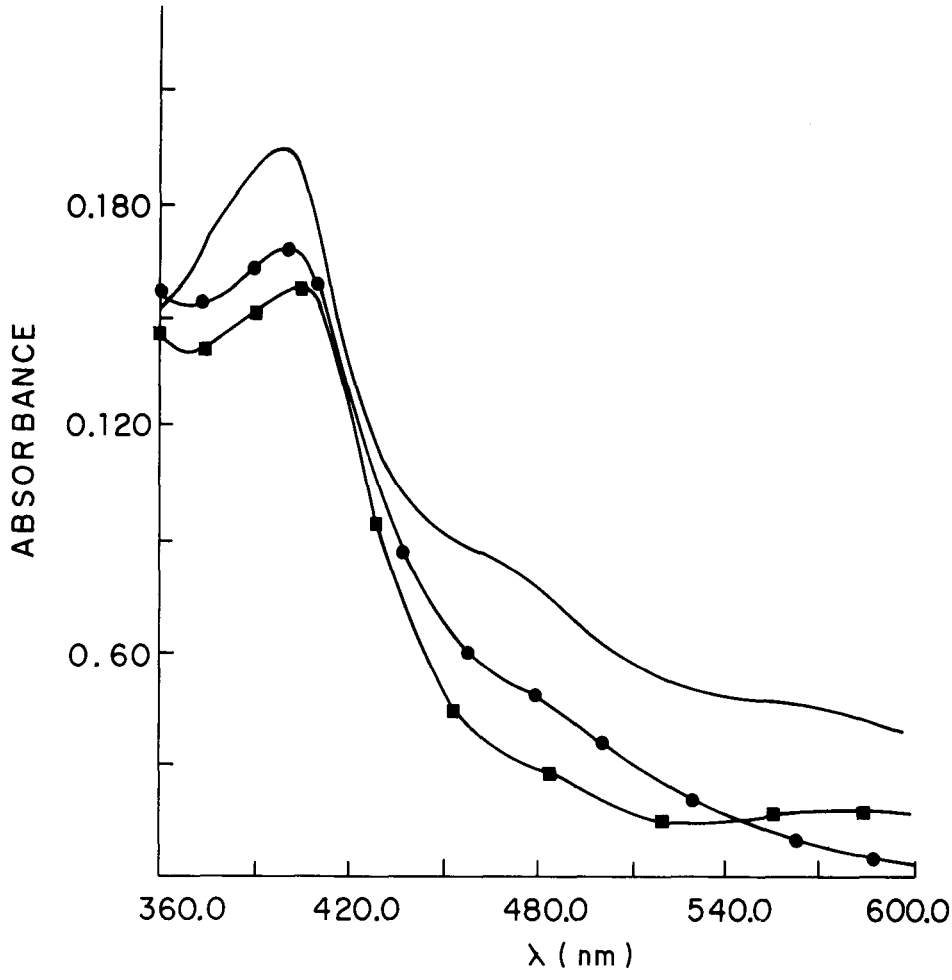


Fig. 3. Absorbance spectra of the native ligninase II (——) and after addition of 1.5 equivalents of $H_2O_2$ (— ● —). Spectrum of ligninase II after addition of 1.5 equivalents of $H_2O_2$ and 1.0 equivalent of phenol (— ■ —). All the spectra were carried out in a 0.1 M sodium tartrate buffer pH 4.5.

## Kinetics of Veratryl Alcohol Oxidation

Varying the concentration of either veratryl alcohol or $H_2O_2$ resulted in saturation kinetics. Apparent Kms for veratryl alcohol and $H_2O_2$ were calculated from double reciprocal plots (Table I). The Km approx values for veratryl alcohol for ligninase II and III were 0.3 and 0.12 m*M*, respectively.

Hydrogen peroxide is required for the oxidation of the veratryl alcohol. The optimal concentration of $H_2O_2$ is around 0.1 m*M* with a Km app of 37 $\mu$M for ligninase II and a 33 $\mu$M for ligninase III.

The specific activities of the three enzymes to veratryl alcohol were different. Ligninase I, II, and III have values of 6.0, 4.4, and 9.1 $\mu$mol $min^{-1}$ $mg^{-1}$, respectively. All three appeared to be glycoprotein similar to that reported by Renganathan et al. (*13*) or by Leisola et al. (*37*) in *P. chrysosporium*.

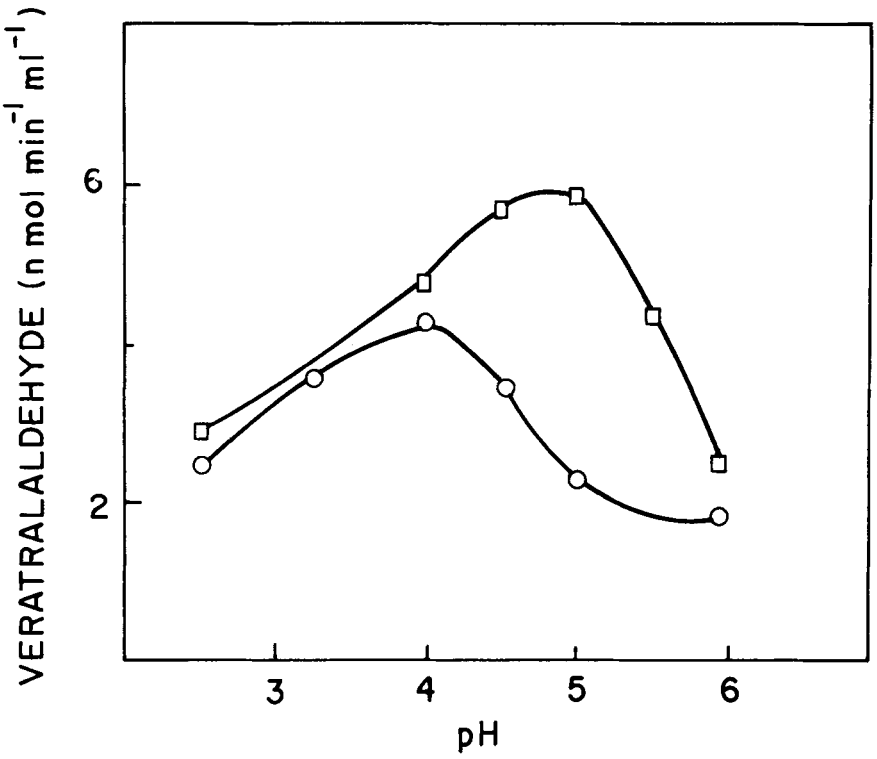


Fig. 4. ph Profile at ionic strength of 0.22 M on the veratryl alcohol oxidation catalyzed by ligninase II (53 μg/mL) (— □ —), and ligninase III (54 μg/mL) (— ○ —) in 0.4 m*M* $H_2O_2$, 2 m*M* veratryl alcohol, and 0.1 M sodium tartrate buffered solutions.

Table 1
Kinetic Constants of Ligninases Purified from *Chrysonilia sitophilia* (TFB 27441 strain) Cultures[a]

| | Veratryl aldehyde | | Hydrogen peroxide | |
|---|---|---|---|---|
| | Km app., mM | V max., nmol min$^{-1}$ mL$^{-1}$ | Km app., μM | V max., nmol min$^{-1}$ mL$^{-1}$ |
| Ligninase II | 0.3 | 8.3 | 37 | 7.2 |
| Ligninase III | 0.12[b] | 2.5[b] | 33[c] | 4.8[c] |

[a]The kinetic constants were calculated from Linewaver-Burk plots.
[b]V max (veratryl alcohol) and Km (veratryl alcohol) (*P. chrysosporium*) were ~1.7 nmol min$^{-1}$ mL$^{-1}$ and 55 μM, respectively (*13*).
[c]V max ($H_2O_2$) and Km ($H_2O_2$) (*P. chrysosporium*) were ~12.2 nmol min$^{-1}$ mL$^{-1}$ and 30 μM, respectively, at pH 3.0 (*1*).

## CONCLUSION

Although ligninase III (Ip 4.5; Mr 48,000; 18.0% carbohydrates) of the *Chrysonilia sitophilia* is similar in many respects to that isolated by Tien and Kirk (*1*) (Ip 3.5; Mr 42,000; 13% carbohydrates), Renganathan et al.

(*13*) (Mr 43,000; 6% carbohydrates), or that of Paszczynski et al. (*20*) (Mr 43,000; 21% carbohydrates) from *P. chrysosporium,* ligninase II (Ip 7.8; Mr 48,300; 14.5% carbohydrates) the major component of the crude extract is different in certain aspects. First it was not retained by the ion exchange column, as was ligninase III, although the molecular weight is similar to that of ligninase III the isoelectric point is different.

Everything indicates that we have different kinds of fungi producing ligninase with a high activity in the crude extract. This high activity in the crude extract (91 U/L) leads us to predict its high potential for biopulping processes, although we were aware that large quantities of the enzymes must be produced inexpensively concomitantly with the development of large scale production facilities to make biopulping economically and technologically feasible. Preliminary experiments in this direction have been performed in our laboratories. Mechanistic studies with ligninase II and a more detailed analysis of the ligninase I are underway.

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