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Production and Purification of the

Ligninases

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Major professor

Date 3/3/88

PRODUCTION AND PURIFICATION OF THE LIGNINASES

By

William A. Ashbaugh

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ABSTRACT

PRODUCTION AND PURIFICATION OF THE LIGNINASES

By

William A. Ashbaugh

The white rot fungus, Phanerochaete chrysosporium produces extracellular peroxidases which catalyze the degradation of lignin. These ligninases have also been shown to degrade recalcitrant organopollutants. Their production and purification has been a challenge to laborartories, since the enzymes are only produced in relatively small amounts under nutrient starvation conditions.

Proteins from the extracellular fluid of agitated fungal cultures were purified by DEAE-sepharose and Fast Protein Liquid chromatographies (FPLC). FPLC detected 19 proteins, and subsequently 6 ligninases were purified. Their specific activities ranged from 20 to 70 micromoles of veratryl alcohol oxidized/min-mg. Their apparent molecular weights were either 42,000 or 47,000, and all cross-reacted with antibodies made to one of the proteins. The amino acid composition of two of the isozymes showed considerable homology. Isoelectric focusing gels separated 11 acidic proteins in different crude preparations, having denatured pI's between 4.00 and 5.10, but the amounts of the various ligninases varied between crude samples.

Dedication

To God who made life

Acknowledgements

I would like to thank Dr. Schubert for his grandfatherly concern and insight, Steve Aust and John Bumpus for their guidance, Skip Mileski for his patience, Barry Brock for all his help with the experimental methods, Tudor Fernando for his friendship, and Dennis Miller and Dave Reif for cleaning the chicken fryer.

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Abbreviations

VAO -Veratryl Alcohol Oxidase Activity

FPLC -Fast Protein Liquid Chromatography

HSB -High Salt Buffer

SDS -Sodium Dodecyl Sulfate

BSA -Bovine Serum Albumin

ECF -Extracellular Fluid

ISF -Isoelectric Focusing

PAGE -Polyacrylamide Gel Electrophoresis

ELISA -Enzyme Linked Immunoabsorbant Assay

DDT -1,1-bis(4-chlorophenyl)-2,2,2-trichloroethane

Introduction

Wood is the most abundant biomass on earth (1). It is comprised of cellulose, hemicellulose, and lignin, a cementing material which bonds the wood cellulose fibers together. Lignin gives wood its rigidity, and is estimated to represent one quarter of the weight of dry wood (2). Therefore, next to cellulose, lignin is the second most abundant biopolymer on earth. Yet, until recently, only slow progress was being made in determining the mechanism of its turnover in the environment. One reason for the slow progress was undoubtedly the very complex structure of lignin.

Lignin is a random, sterically irregular polymer made by free radical polymerization of three dehydrogenated p-hydroxycinnamyl alcohols: coniferyl, sinapyl,and coumaryl as shown in Figure 1 (3-5,32). The free radical mechanism responsible for the synthesis of lignin explains the randomness of the biopolymer which is shown in Figure 2. The predominate linkages (shown in the dashed boxes) are covalent bonds between the beta-carbon on a propyl side chain from one subunit and the oxygen from ring carbon #4 on adjacent subunits. Many other

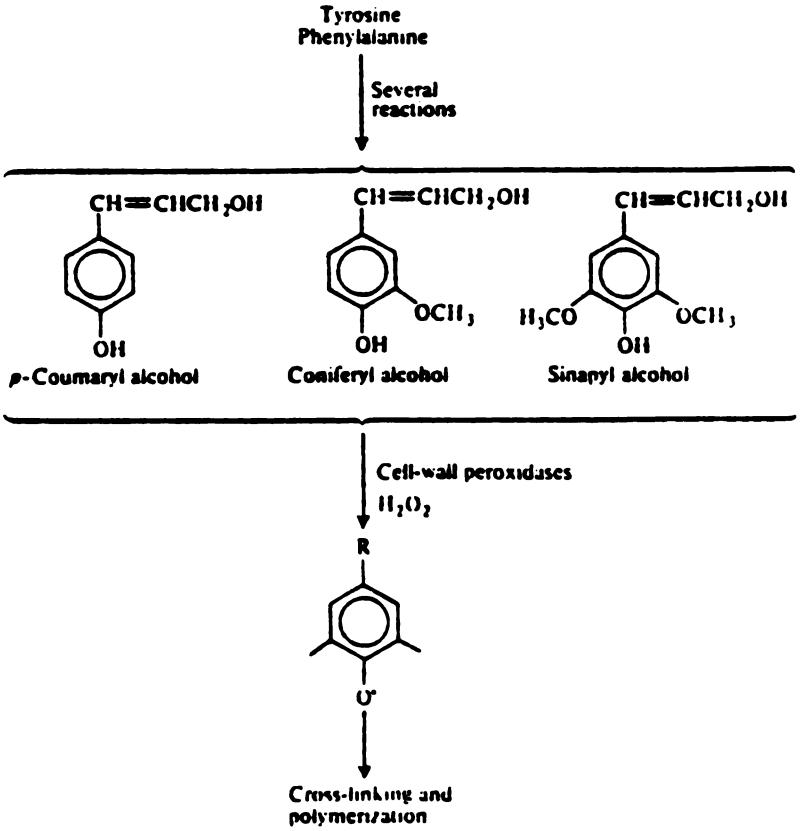


Figure 1 Synthesis of Lignin (33)

Outline of the synthesis of lignin found in plant cell walls. Lignin is derived by the oxidation of various phenols into phenoxy radicals which then polymerize. The phenols are derived by adding hydroxyl and methoxyl groups onto the benzene ring of the amino acids tyrosine or phenylalanine. Oxidation of phenols to phenoxy radicals is probably achieved by one or more peroxidase enzymes bound to the plant cell walls.

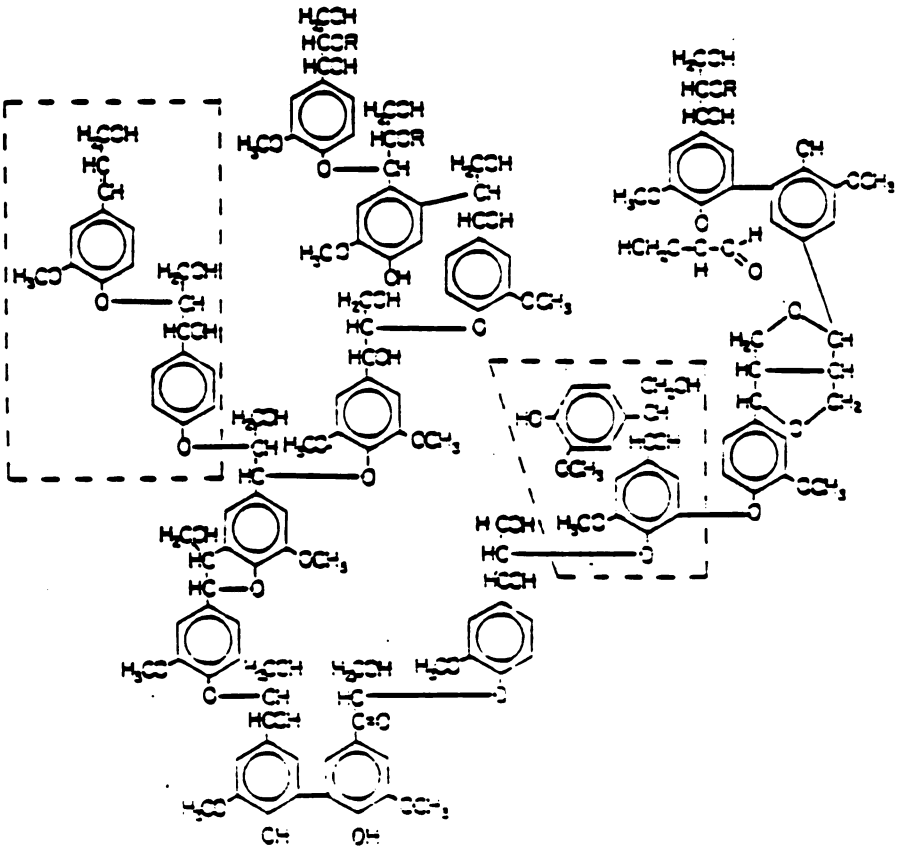
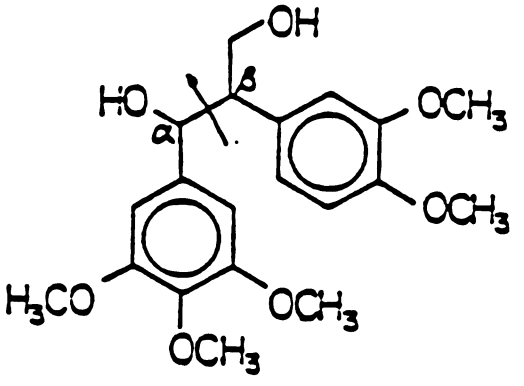


Figure 2 Proposed Structure of Lignin (32)

linkages are found in lignin due to the free radical coupling of different resonance forms of the p-hydroxycinnamyl alcohol free radicals.

The many different bonds in this irregular biopolymer may explain why it is resistant to attack from most microbes. Only certain white rot fungi, and possibly a few other fungi and select strains of bacteria, can completely decompose lignin to carbon dioxide and water (4). Progress in understanding the mechanism of lignin biodegradation was greatly aided by the use of substructural model compounds, two of which are shown in Figure 3. These compounds were degraded in a fashion analogous to degradation of the lignin polymer. That is alpha-beta carbon bond cleavage and alpha carbon oxidation of the propyl side chain (7-9). In most of the degradation studies, the white rot fungus, Phanerochaete chrysosporium was used because of its high rate of lignin degradation compared to other white rot fungi, its rapid growth in liquid cultures, and its rapid sporulation (10). Further work established that the fungus would degrade lignin only when nutrient carbon, nitrogen, or sulfur became exhausted in the growth media (11,12). Researchers also showed that hydrogen peroxide was produced by the fungus during lignin degradation (13,14). This led researchers to incubate the model compounds with various cellular fractions and hydrogen peroxide.



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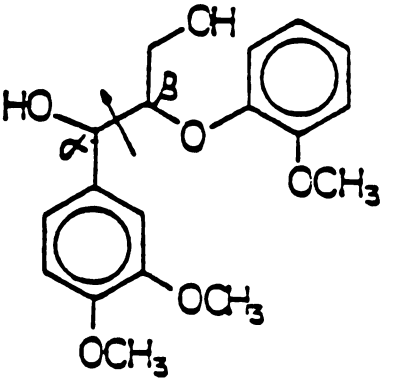


B

Figure 3 Lignin Model Compounds (20)

Compound A - 1-(3,4,5-trimethoxyphenyl)-2-(3',4'-dimethoxyphenyl) propane-1,3-diol

Compound B - 1-(3,4-dimethoxyphenyl)-2-(o-methoxyphenoxy) propane-1,3-diol

Enzymatic activity was found in the extracellular fluid. The enzyme, discovered four years ago, was called ligninase due to its ability to catalyze a peroxide dependent cleavage of both the model compounds and the lignin polymer (15,16).

The initial literature reported the ligninase as one enzyme (15,16). Subsequent work, however, showed that there were possibly multiple enzymes (16-19), which were glycosylated, heme-containing proteins (19,20).

A major roadblock in studying the ligninases has been the difficulty of producing them in sufficient quantity. Numerous researchers in the past few years have attempted to increase enzyme production in a number of ways. Nutrient levels were adjusted (11), trace metals were adjusted (21), and various compounds were added (22) to increase levels of ligninase production. Besides altering media conditions, fungal cultures have been grown in stationary cultures (20), agitated cultures (17), and even in rotating disk fermentation units (18) in an attempt to scale up production.

While this work was going on, other laboratories took an interest in the ligninase and its possible use in degrading environmental pollutants. The facts that stimulated reseachers to consider this possibility were: 1) the ligninases were able to catalyze numerous, non-specific cleavage reactions in the lignin lattice; 2)

the fungus was shown to be able to degrade lignin and chlorinated lignin (obtained as by-products of the Kraft pulping process) to carbon dioxide; 3) many of the carbon skeletons of recalcitrant organohalides, such as DDT, polychlorinated biphenyls, and polychlorinated dibenzo(p)dioxins could be found within the proposed structure of the lignin polymer (22). The researchers used ^{14}C-labeled organopollutants, and showed that carbon dioxide evolved during the degradation experiment. Current research in this laboratory is focused on exploring the fungus's ability to degrade other organopollutants and determining what role the ligninases have in their degradation.

One aspect of that research must include the production and purification of the ligninases. Because industrialization of a detoxification system using the ligninases would be more economical with larger quantities of the enzymes than the fungus is making under present experimental conditions, efforts are being focused toward increasing enzyme production. Unfortunately, large scale production of the enzyme has still not met with much success. It is hoped therefore, that isolation of purified ligninases will lead to the cloning of the ligninase genes and eventually the overproduction of the active enzyme. One of the ligninases has been cloned (34) and sequenced (23), but

at this time, it has not been expressed in active form.

Besides the need of pure enzymes for future cloning, much enzymology needs to be done on their role in xenobiotic and lignin degradation. In all past studies, crude ligninase preparations were used in the degradation studies. Future work with purified ligninase isozymes may further elucidate the substrate specificity and the complete enzymatic mechanism of this class of extracellular peroxidases. This thesis will discuss the purification of the ligninases and report some of their physical properties.

Materials and Methods

Materials

Bacto agar, malt extract, and Freund's adjuvant were obtained from Difco Lab, Detroit, Michigan. $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, $CuSO_4 \cdot H_2O$ were obtained from Fisher Scientific, Fairlawn, New Jersey. $FeSO_4 \cdot 7H_2O$, ammonium tartrate, H_2O_2, sodium tartrate, trichloroacetic acid, urea, and glycerol were obtained from Mallinckrodt Inc., Paris, Kentucky. NaCl, $ZnSO_4 \cdot 7H_2O$, H_3BO_3, KH_2PO_4, glucose, absolute ethanol, absolute methanol, and glacial acetic acid were obtained from J.T. Baker Chemical Co., Phillipsburg, New Jersey. $MnSO_4 \cdot H_2O$, $CoSO_4$, $NaMoO_4 2H_2O$, O-phenylenediamine, benzyl alcohol, Tween 20, thiamine, Coomassie Blue G-250, acrylamide, N,N'-methlenebisacrylamide, N,N,N',N'-tetramethylenebisacrylamide (TEMED), triton x-100, sodium azide, bovine serum albumin (BSA), SDS-molecular weight markers 70L, isoelectric focusing protein markers, and DEAE-sepharose C1-6B beads were obtained from Sigma Chemical Co., St. Louis, Missouri. $AlK(SO_4)_2 \cdot 12H_2O$, nitriloacetic acid, and 2,2-dimethylsuccinate were obtained from Aldrich Chemical Co., Milwaukee, Wisconsin. Veratryl alcohol was obtained

from Fluka Chemical Corp., Rowtonkoma, New York. BCA Protein Assay Reagents were purchased from Pierce Chemical Co., Rockford, Illinois. Ammonium persulfate was obtained MC/B Manufacturing, Chemist Inc., Cincinnati, Ohio. Sodium dodecyl sulfate (SDS) was purchased from Boehringer Mannheim Biochemicals, Indianapolis, Indiana.

Fungi and Inoculum

Spore inoculum of Phanerochaete chrysosporium BKM-F 1767 (ATCC 24725) maintained on 2% malt agar slants at room temperature was prepared under sterile conditions according to Kirk et al (1) by filtering conidial suspensions through glass wool. The concentration of the collected spore suspension was adjusted so as to have between $4x10^6$ spores/mL to $8x10^6$ spores/mL (Abs at 650nm=0.5/cm for a concentration of $2.5x10^6$ spores/mL).

Preparation of Agitated Fungal Cultures

Cultures were prepared under sterile conditions according to Kirk et al (24). Filter-sterilized basal media (45 mL of 1.47 $x10^{-2}$ M KH_2PO_4, 1.08 mM ammonium tartrate, 2.03 mM $MgSO_4 \cdot 7H_2O$, $6.8x10^{-1}$ mM $CaCl_2 2H_2O$, 2.97 micromolar thiamine HCl, and 10 mL/L of a trace element solution) were inoculated with 5 mL of Phanerochaete chrysosporium spore inoculum (Abs 650nm between 0.8 and 1.6) and placed in a 37°C incubator (Napco model 3512)

for 48hrs. The trace element solution consisted of : 7.8 mM nitriloacetic acid, $1.2x10^{-2}$ M $MgSO_4 \cdot 7H_2O$, 2.9 mM $MnSO_4 \cdot H_2O$, $1.7x10^{-2}$ M NaCl, $3.59x10^{-4}$ M $FeSO_4 \cdot 7H_2O$, $7.75x10^{-4}$ M CoCl, $9.0x10^{-4}$ M $CaCl_2$, $3.48x10^{-4}$ M $ZnSO_4 \cdot 7H_2O$, $4x10^{-5}$ M $CuSO_4 \cdot 5H_2O$, $2.1x10^{-5}$ M $AlK(SO_4)_2 \cdot 12H_2O$, $1.6x10^{-5}$ M H_3BO_3, and $4.1x10^{-5}$ M $NaMoO_4 \cdot 2H_2O$. The resulting fungal mat was homogenized in a total of 100 mL of filter-sterilized growth media (100 mL/L basal Media, 100 mL/L of 10 mM 2,2-dimethylsuccinate (DMS) pH 4.5, 1% glucose, 0.1% Tween 20, 0.4 mM veratryl alcohol, 0.6 mM benzyl alcohol, 60 mL/L of trace mineral elements, and 1 mg/L thiamine HCl) for ten seconds in a sterilized waring blender. Sterile growth media (900 mL) was inoculated with the 100 mL of fungal mat homogenate in 2800 mL Fernbach flasks and agitated at 200 RPM on a G-10 Gyrotory Shaker (New Brunswick Scientific Co. Inc., Edison, N.J., USA) at 37°C. One milliliter aliquots were taken each day and assayed for veratryl alcohol oxidase activity.

Inoculation Using Preformed Fungal Pellets

Fungal pellets formed in the agitated shake cultures were isolated by filtering the suspension through Miracloth (Chicopee Mills Inc., Milltown, N.J., USA) after 5 to 7 days of growth. The fungal pellets were then used to inoculate the same volume of fresh sterile growth media.

Purification of the Ligninases

A. Collection and Concentration of Extracellular Fluid

Extracellular fluid (10 liters) containing veratryl alcohol oxidase activity was separated from the fungal pellets by filtering the suspension through Miracloth. The filtrate was then concentrated using a Minitan Concentrator (Millipore Corp., Bedford, Mass.,USA) to approximately 500 mL. The material was frozen and thawed to precipitate the mucopolysaccharide material (slime). After centrifugation at 10,000 RPM for 10 minutes, the supernatant was further concentrated using Amicon stirred cell concentrators (400 mL and 50 mL, Amicon Corp.,Danvers, Mass.,USA) to between 5 mL and 30 mL.

B. DEAE-Sepharose Chromatography

The concentrated extracellular fluid was dialized (12 KD -14 KD molecular weight cutoff,Spectrum Medical Industries Inc., Los Angeles, Ca., USA) against 20 mM sodium succinate, pH 6.0, and applied to a DEAE-Sepharose column which had been equilibrated with 20 mM sodium succinate, pH 6.0. After sample application, the column was washed with two column volumes of equilibration buffer and the protein was eluted with a linear 0 to 0.4M NaCl gradient in four column volumes of the equilibration buffer. An elution profile was obtained on the collected

fractions by measuring the absorbances at 410nm (for heme group) and 280nm (protein), as well as assaying each fraction for veratryl alcohol oxidase activity. Peak fractions were pooled, concentrated using the Amicon stirred cell concentrators, and dialized against 10 mM sodium acetate, pH 6.0, in preparation of further purification.

C. Purification by Fast Protein Liquid Chromatography

A Mono Q HR5/5 column (Pharmacia Fine Chemical, Uppsala, Sweden) was inverted and connected to the FPLC system to wash the column. Sodium acetate, 10 mM, pH 6.0 buffer was used as the rinse. The column was washed in the following way: 1) 1 mL of 2 M NaCl followed by 2 mL rinse, 2) 5x 1 mL of 2 M NaOH followed by 2 mL rinse, 3) 1 mL NaCl follwed by 1 mL rinse, 4) 4x 500 µL 0f 75% acetic acid followed by 1 mL rinse, 5) 1 mL of 1 M sodium acetate pH 6.0, 6) equilibrate column with 5-10 mL of rinse.

The column was reconnected in its upright position and rinsed with another 5 mL of 10 mM sodium acetate, pH 6.0. Protein (a maximum of 8 mg) was then loaded on the column and eluted with at least 20 mL of a linear gradient of 10 mM to 1 M sodium acetate, pH 6.0. Fractions were collected and assayed for veratryl alcohol oxidase activity. The column was washed extensively

according to the above procedure after every second or third run depending on the amount of protein loaded on the column.

Preparation of Antibody

Two New Zealand White rabbits received subcutaneous injections of 0.5 mg of protein suspended in Freund's adjuvent. The rabbits were boosted with 0.3 mg of protein suspended in Freund's adjuvent one month later, and were bled 10 days following the boost. Octerlony double diffusion analyses were performed on the serum from both rabbits. Antigen (0.5 micrograms) was placed in the center well, with a serial dilution of the serum in the outer wells.

SDS-Polyacrylamide Gel Electrophoresis

A 10% acrylamide SDS gel with a 4.75 % stacking gel was prepared and run according to a modification of the method of Laemmli (25) at room temperature. The protein bands were stained after the completion of the electrophoresis by 0.1% Coomassie Blue for three hours and gels were destained in 7% acetic acid, 40% methanol until clear. Prestained molecular weight markers (180,000, 116,000, 84,000, 58,000, 48,500, 36,500, 26,600) and unstained molecular weight markers (66,000, 45,000, 36,000, 29,000, 24,000, 20,100, 14,200) were used to

establish the apparent molecular weights of the proteins.

Isoelectric Focusing Gel Electrophoresis

Isoelectric focusing gels were run according to a modification of the method of Gilbert et al (26). The gel was composed of 5% acrylamide, 9.13M urea, 2% Triton x-100, 10% glycerol, and 2% ampholines. Ampholines, pH 3-10 and 2.5-5 were added in a 1:2 ratio. An SE-600 Vertical Slab Unit (Hoefer Scientific Instruments, San Francisco, Ca.,USA) was used along with 0.70mm gel spacers. NaOH and H_3PO_4 (0.02 M) were the cathodic and anodic buffers respectively. The protein samples were loaded cathodically in 20% sucrose, 0.01 M NaOH, and 1% ampholines. The gel was run at 100 volts for 1hr, at 300 volts for 30 minutes, and finally at 400 volts for 20 hrs at room temperature. Focusing could be visibly followed by the presence of methyl red dye which focuses at pI= 3.75.

Following focusing, the gel was carefully removed and fixed in 100 mL of 20% TCA for 10 minutes followed by further fixing in 100 mL of 40% ethanol, 10 % acetic acid, and 0.25% SDS for 10 minutes. The gel was washed for 30 minutes with 100 mL of 40% ethanol, 10% acetic acid solution twice to remove any remaining ampholines which interfere with staining of the gel.

After the final wash, the gel was stained for 30 to 60 minutes with 40% ethanol, 10% acetic acid, 0.125% Coomassie Blue and destained with 40% ethanol, 10% acetic acid.

Veratryl Alcohol Oxidase Assay

Measurement of veratryl alcohol oxidase activity was based on the method described by Tein and Kirk (20). Extracellular fluid (0.135 mL) from fungal cultures was mixed with 0.8 mL of 120 mM sodium tartrate buffer pH 2.5, and 0.015 mL of 100 mM veratryl alcohol. Hydrogen peroxide (0.05 mL of 10 mM) was then added and the change in optical density was monitored at 310nm. The final concentration of sodium tartrate, veratryl alcohol, and hydrogen peroxide was 0.1 M, 1.5 mM, and 0.5 mM respectively. One unit of VAO activity corresponded to the oxidation of 1 micromole of veratryl alcohol per minute.

Protein Determination

Protein levels were determined by a modification of the Lowry assay (27). Diluted protein solutions were added to 0.200 mL of BCA Protein Reagent. Absorbance at 570nm was monitored using the EIA Plate Reader (model EL-307, Biotek Instruments Inc.) and compared to absorbances obtained from BSA standards in order to

quantitate protein concentrations.

Enzyme-Linked Immunoabsorbant Assays

An indirect non-competitive Elisa for the ligninases was developed based on the method of Paye et al (28). Antigen (0.050 mL) diluted in 50 mM sodium bicarbonate, pH 9.6, 2% sodium cholate, 0.02% azide was added to triplicate wells of microtiter plates. The plates were incubated 3 hrs at 37°C or over night at 4°C. The antigen solution was then replaced with 0.200 mL of 0.1% gelatin in 10 mM potassium phosphate, pH 7.4, 0.15 M NaCl, and 0.02% azide, and incubated for 30 minutes at 37°C. The plate was then rinsed with tap water using a microtiter plate washer and IgG was added in 0.1 M potassium phosphate, pH 7.4, containing 0.4 M KCl, 0.4 mM EDTA, 0.5% cholate, 0.1% Emulgen 0.5% cholate, 0.1% Emulgen 911, and 1% BSA at a concentration of 10 micrograms IgG/mL in a total volume of 0.100 mL/well and allowed to bind for 1.5 hrs at 37°C. After thoroughly washing the plates, 0.100 mL of the color reagent (2 mM o-phenylendiamine HCl, 2.5 mM hydrogen peroxide in 0.1 M sodium citrate pH 5.0) was added. Color development was stopped with 0.050 mL of 4N H_2SO_4 after approximately 5 minutes and the absorbance in each well at 490nm was read using a Biotech E1A Model 307 plate reader.

The percent cross-reactivity between purified

proteins and the antibody was determined by comparing the slopes obtained from the results of the ELISA curves which plotted Abs 490nm (which is proportional to the amount of antibody bound to antigen) versus the amount of antigen. A slope of zero would indicate no reaction between the antibody and antigen. One hundred percent cross-reactivity was assumed for Lig-4, since it had the largest slope. This was not unexpected since the antibody was made with partially purified protein which eluted during FPLC at the same percent HSB as Lig-4.

Amino Acid Composition of Ligninases

Two FPLC purified ligninases were dialized extensively against distilled deionized water, and 30 micrograms of each protein was lyopholized and given to the macromolecular facility at MSU for an amino acid composition. Only a 24hr hydrosylate of the proteins was analyzed.

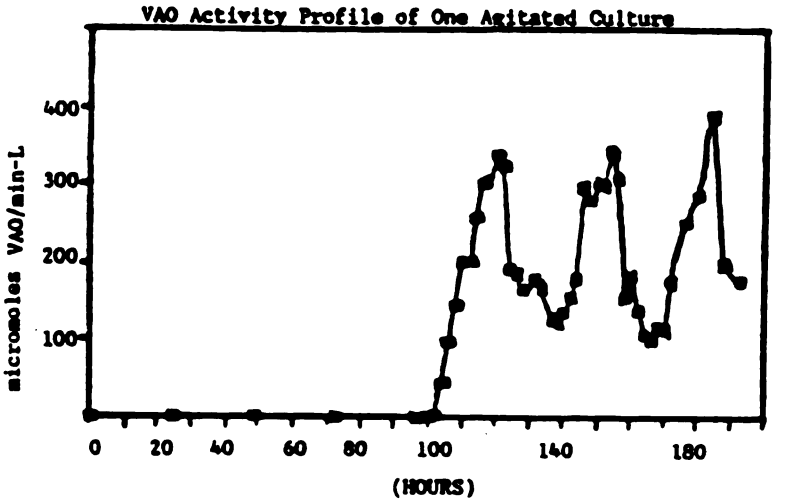
Results

Growth of Fungus in Agitated Cultures

Phanerochaete chrysosporium was grown in agitated cultures as described by Kirk et al (24) except the total volume was increased to one liter in an attempt to scale up ligninase production. Cultures were assayed for veratryl alcohol oxidase (VAO) activity every day. When VAO activity was detected, enzyme assays were performed every two to four hours in order to determine the cultures peak activity. Figure 4a shows VAO activity plotted versus hours of incubation. The onset of veratryl alcohol oxidase activity occured by day 4, and reached peak activity by day 5. Interestingly, the activity cycles from day to day were approximately 30 hrs from peak to peak. There are reports of activity profiles similar to those shown in Figure 4b and 4c (18). Figure 4b is a plot of the average daily activity for three cultures. The graph is nearly identical to that of Kirk et al (18) in Figure 4c. Figure 4 also shows that the agitated cultures exhibited higher levels of enzyme activity than that which has been reported in the literature. This data is summarized in Table I, which lists the values of ligninase activity and total activity

production per culture found in the literature. All researchers used P. chrysosporium strain BKM-F-1767 (ATCC 24725) under the growth conditions indicated. Stationary cultures refer to fungus cultured in either 200 mL or 125 mL Erlenmeyer flasks in a total volume of 10 mL of growth media. Agitated cultures are also grown in either Fernbach or Erlenmeyer flasks with total volumes ranging from 30 mL to 1000 mL of growth media. Nutrient levels were the same in all conditions. Ligninase activity levels were 40 to 250 times higher per culture in this study than levels of ligninase reported in the literature. Another aspect of increasing enzyme production is the time at which the fungus produces the enzyme. Figure 5 shows a diagram that outlines the culture growth of the fungus versus time. The fungus forms a mat after two days of growth from spores. The mat was then homogenized and inoculated with 1 liter of growth media. The fungus then grew rapidly, forming small pellets in the agitated cultures after two days, and producing ligninase as the nutrients began to run out by day four. Pellet formation has been shown to be very important in ligninase production (31), and under our growth conditions this process takes four days. One possible way to speed up ligninase production therefore, would be to use preformed fungal pellets, which according to our growth conditions should eliminate about four days

4a)



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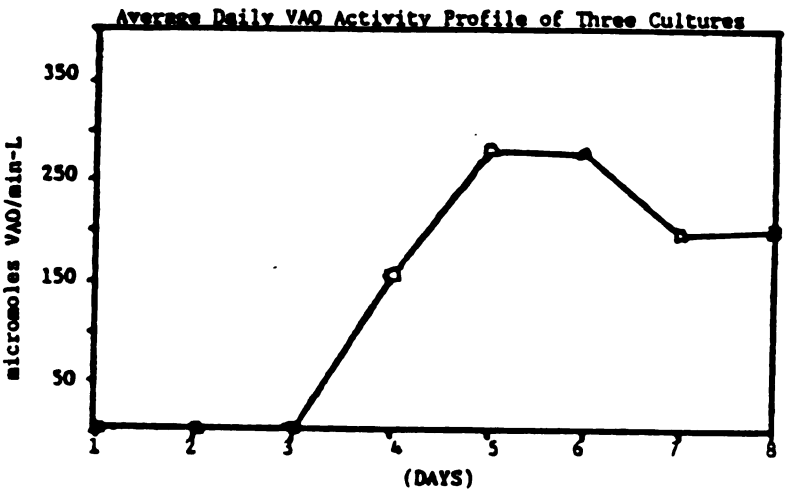


4c)

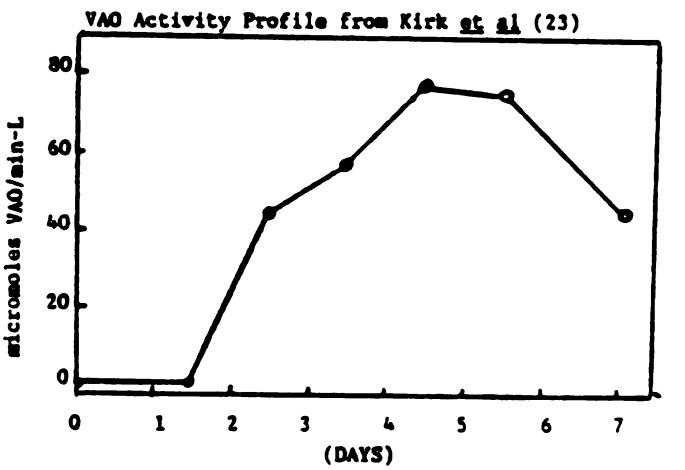


Figure 4 VAO Activity Profile of Agitated Cultures

Figure 4 VAO Activity Profile of Agitated Cultures

a) A one liter agitated culture of *P. chrysosporium* **was monitored every two hours for VAO activity. The plot shows ligninase activity expressed in micromoles of VAO per min-L plotted versus the hours after inoculation.**

b) The VAO activity data from three one liter agitated cultures having VAO activity profiles as in 4a) were averaged on a daily basis and plotted versus days after inoculation.

c) The VAO activity profile obtained from Kirk et al (23).

TABLE I. Summary of Ligninase Production by *P. chrysosporium*

CULTURE CONDITION	ACTIVITY (units/mL)	TOTAL VOL. (mL)	TOTAL ACTIVITY (units)	REF.
Stationary	0.180	10	1.8	16
Agitated	0.118	50	5.9	16
Agitated	0.144	30	4.3	17
Stationary	0.095	10	0.95	18
Agitated	0.250	1000	250	Ashbaugh

One unit of activity = 1 micromole of veratryl alcohol oxidized per minute

TABLE II. Ligninase Production by Preformed Fungal Pellets

Inoculation Cycle	Time to Peak VAO Activity (HRS)	Total Activity (units/L)
	UNTREATED CULTURES	
1	171	345
2	91	373
3	100	254
4	---	0
	OXYGEN-TREATED CULTURES	
1	192	62
2	96	260
3	104	316
4	205	292
5	---	0

Oxygen treated cultures were flushed with 100% Oxygen for one min each day. One unit of activity is equal to one micromole of veratryl alcohol oxidized per min.

of time from the total time required to produce the ligninases.

The data from this experiment, that is the time required for ligninase production in agitated cultures using preformed fungal pellets is reported in Table II. Oxygen treatment (100% oxygen flush for 1 min daily) was observed to slow fungal pellet growth. The data clearly shows that the time required to produce the ligninases was considerably decreased when preformed fungal pellets were used (inoculation cycles 2 and 3 in untreated, and oxygen-treated cultures).

The first inoculation cycle of both treated and untreated cultures contained steps I through III as shown in Figure 5, and required 7 and 8 days to reach peak ligninase activity. The remaining inoculation cycles which used preformed fungal pellets took only 4 days to complete, with the sole exception of the fourth inoculation cycle of the oxygen-treated cultures. By the third inoculation, the fungal pellets had visibly doubled in diameter. Leisola et al (31) showed that pellet size was very important in ligninase production, and the onset of ligninase activity. The onset of activity was delayed with large pellets which produced the the least amount of ligninase activity in their study. Their study indicated that optimum pellet size was between 1-2 mm, although smaller and larger pellet sizes did produce the

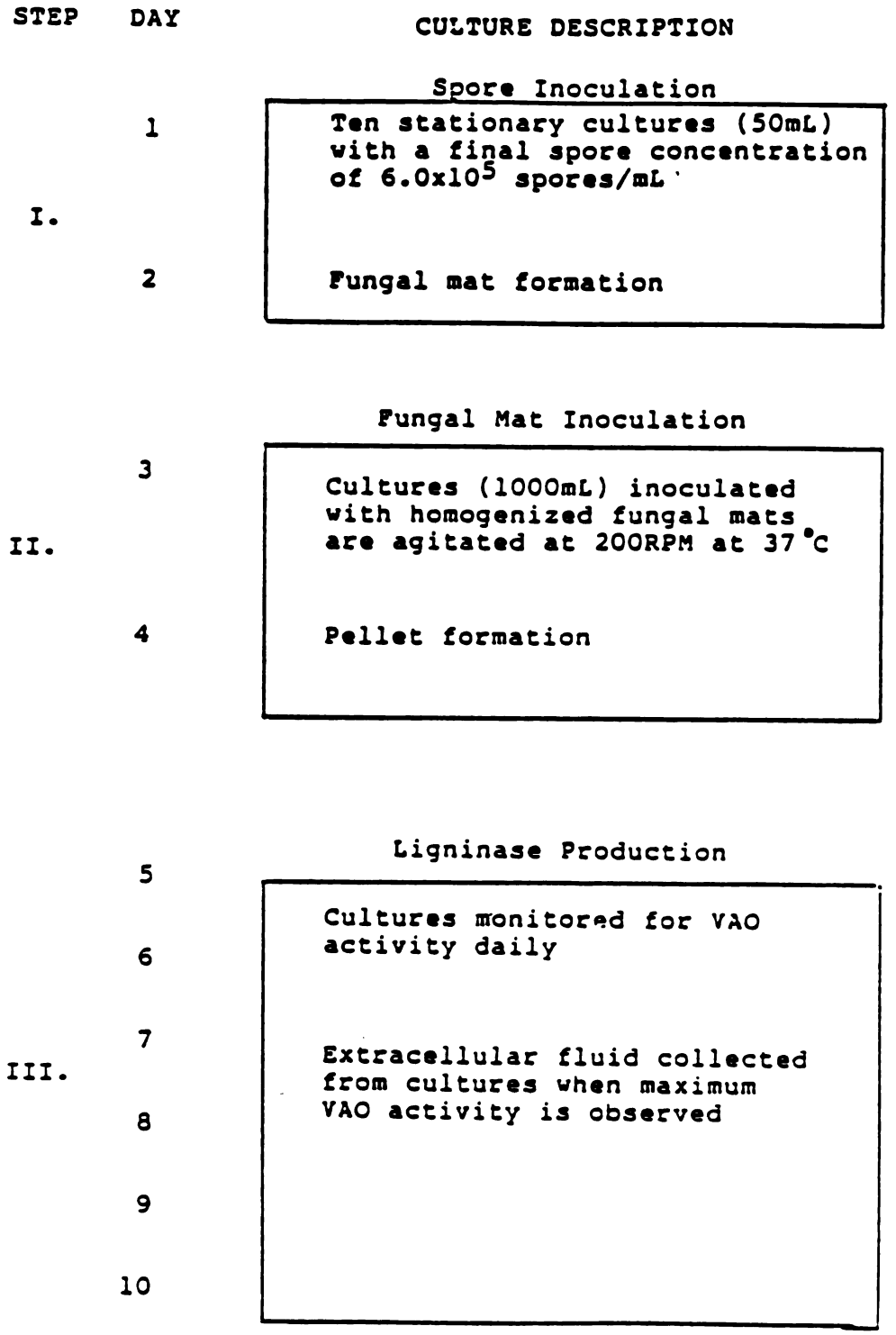


Figure 5. Time Course of Fungal Growth in Agitated Cultures

Figure 5 Time Course of Fungal Growth in Agitated Cultures

The growth of P. chrysosporium in agitated cultures can be divided into three steps. Step I includes inoculating growth media (see Materials and Methods) with a spore suspension. The spores grow to form a fungal mat in 2 days. Step II includes fungal mat homogenization and formation of fungal pellets on a rotary shaker, which takes another 2 days. Step III includes the monitoring of VAO activity in ECF, and the collection of ECF for ligninase purification, which takes from 1-6 days.

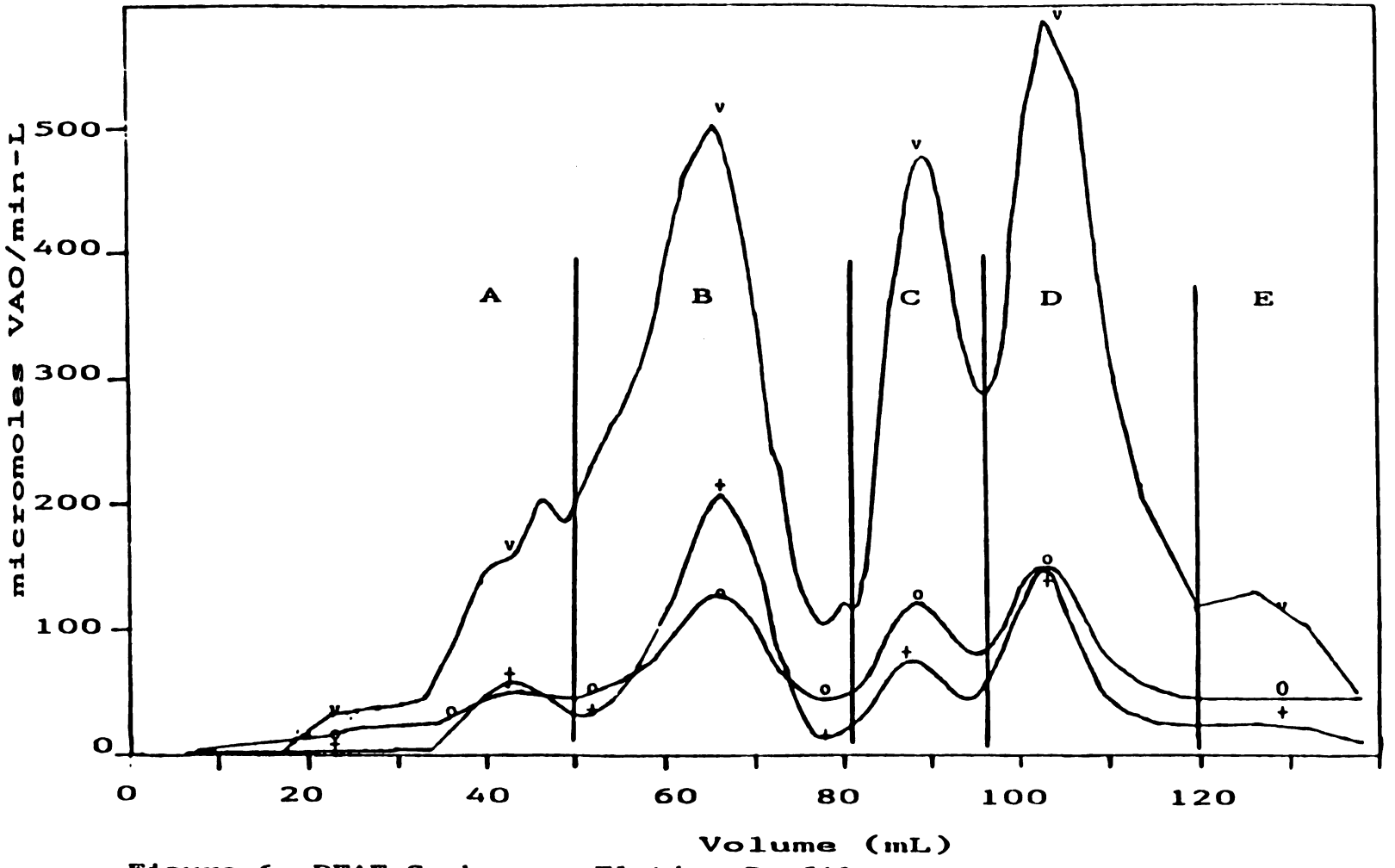


Figure 6 DEAE-Sepharose Elution Profile

Plot (+)- micromoles VAO/min-L Plot (o) - Abs 280 x 200 Plot (v) - Abs 410 x 200

Figure 6 DEAE-Sepharose Elution Profile

Concentrated ECF (80 mg of protein) was eluted in 140 mL of somM sodium succinate, pH 6.0 by an isocratic salt gradient (0.0 M to 0.4 M NaCl). The absorbance at 280nm and 410nm as well as VAO activity was monitored on each collected fraction (2 mL). Five major peaks (A-E) were pooled for further purification.

ligninases. The data in Table II also supports their conclusions. The fungal pellets initially formed were very small, and grew slowly especially in the oxygen treated cultures. Ligninase activity was seen as expected at day four as the fungus entered secondary metabolism (metabolism under starvation conditions). The fungal pellet's size and the time required for peak activity continued to increase during subsequent inoculations. When the pellets became large (5 mm in diameter) no ligninase activity could be measured.

Purification of the Ligninases

To purify the ligninases, most researchers have used some type of ion-exchange chromatography (16-18,29). In our purification scheme, approximately 10 liters of extracellular fluid is concentrated to 300-400 mL, then frozen and thawed to precipitate the slime which makes further purification difficult. At times, the freezing and thawing process had to be repeated. Enzymatic activity is not adversely affected by this procedure. The fluid is then concentrated further and passed over a number of ion-exchange columns.

The first column is a DEAE-sepharose column equilibrated with 20 mM sodium succinate, pH 6.0. Usually five major peaks based on VAO activity, Abs 280, and Abs 410 can be seen as in figure 6. The proteins are

eluted with a 0 to 0.4 M NaCl gradient. Peak B usually contained the majority of the protein and activity. The fractions corresponding to each peak were pooled and dialized against 10 mM sodium acetate, pH 6.0, for further purification.

FPLC Chromatography

Protein Liquid Chromatography (FPLC) using a Pharmacia Mono-Q column greatly facilitates purification of the ligninases. A typical elution takes 1 hour giving superior resolution to that obtained by DEAE-sepharose chromatography (Figure 7). Table 3 summarizes data from the elution profiles of three different preparations of crude ligninases. A total of 18 to 19 peaks can be detected, however only 9 or 10 predominate peaks can be seen in each chromatogram. Highly reproducible chromatographic elution patterns accompanied with variation in peak size were obtained between samples. This is a strong indication that the same proteins are present from preparation to preparation, but in varying amounts. This has also been found by other researchers (17,30).

The five peak fractions from the DEAE-sepharose chromatography were then purified by FPLC. Figure 8 shows that numerous peaks are associated with each fraction. Due to the complexity of the elution profiles,

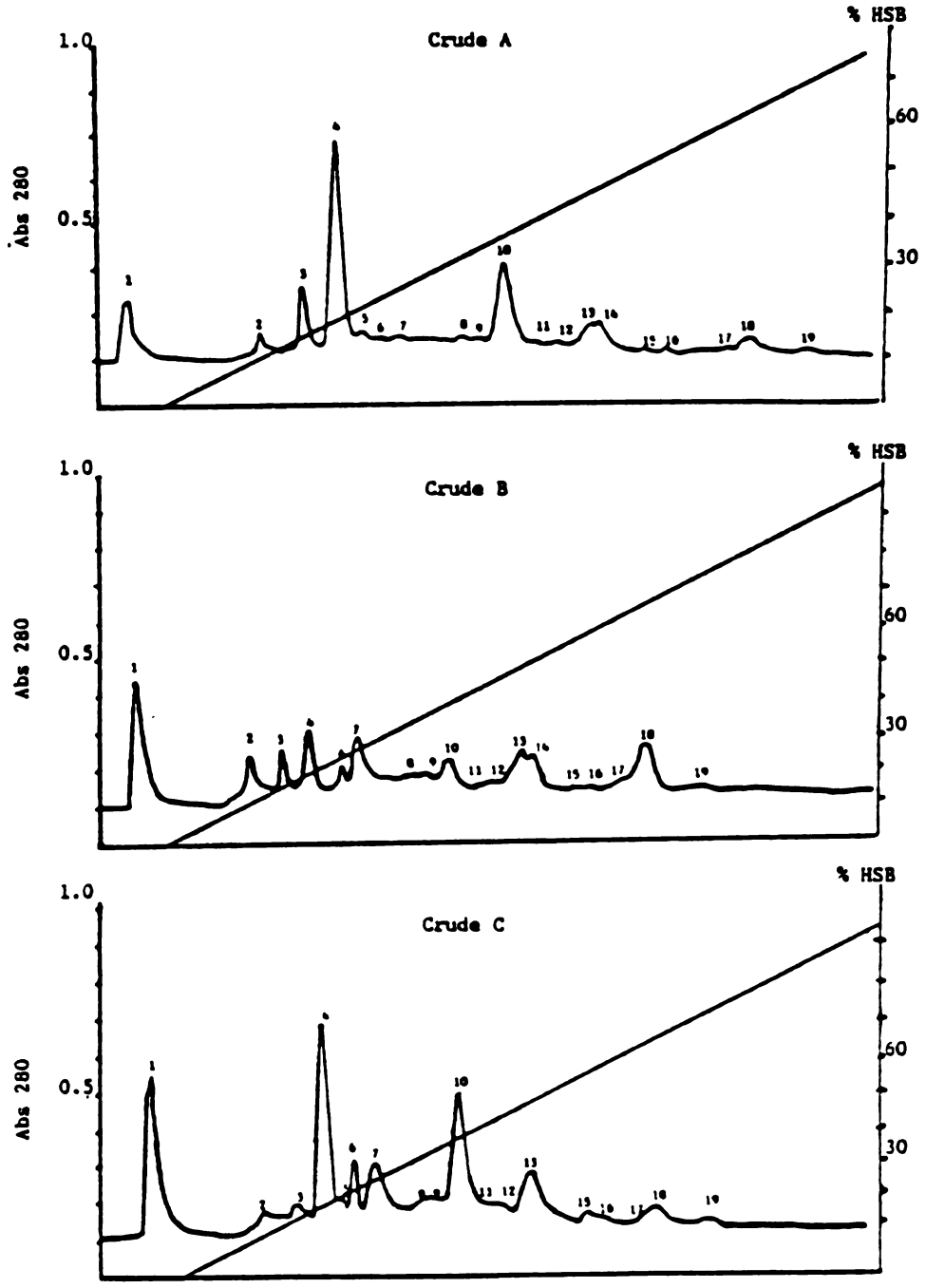


Figure 7 FPLC of Concentrated Extracellular Fluid from Agitated Cultures

Figure 7 FPLC of Concentrated Extracellular Fluid from Agitated Cultures

Three samples of concentrated ECF were run over a Mono-Q, Fast Protein Liquid Chromatography Column equilibrated with 10 mM sodium acetate, pH 6.0. The proteins were eluted with an isocratic gradient (10 mM to 1.0 M) of sodium acetate, pH 6.0. The absorbance at 280nm was monitored and plotted versus the elution volume.

TABLE III. FPLC Elution of Ligninase Isozymes

	CRUDE A	CRUDE B	CRUDE C
PEAK	% HSB	% HSB	% HSB
1	0	0	0
2	11	11	11
3	15	15	16
4	19	19	19
5		22	22
6	23	23	23
7	26	26	26
8	33	33	33
9	35	35	35
10	37	37	37
11	41	41	41
12	43	43	43
13	47	47	47
14	49	48	
15	54	53	54
16	56	55	56
17	61	61	60
18	64	64	64
19	71	70	70

Retention times are expressed as % HSB, the high salt buffer (1 M sodium acetate, pH 6.0)

further purification was performed mainly on the predominate peaks from each fraction. Fractions eluting at the same percentage of the high salt buffer were pooled, dialized in 10 mM sodium acetate and purified again by FPLC. A few proteins were repeatedly subjected to FPLC until single peaks were obtained, as shown in figure 9. Table IV summarizes the purification steps taken to isolate five of these ligninases. Their specific activities are much higher than those reported in the literature (18,19). Only the recent report by Leisola et al (30) cites values that are comparable. This is probably due to the large amount of starting material used in the purification, which is at least 7-fold greater than that reported in the literature. In this particular purification, 70% of the activity was lost after passing the extracellular fluid over the DEAE-sepharose column. In other purifications, 30% of the activity was lost. The total recovery after FPLC was 5%, in part due to the fact that only 5 proteins were purified.

SDS-Polyacrylamide Gel Electrophoresis

The crude and purified ligninases were subjected to SDS-polyacrylamide gel electrophoresis. Two bands can be seen with the crude material, giving apparent molecular weights of 47,000 and 42,000. Other researchers have

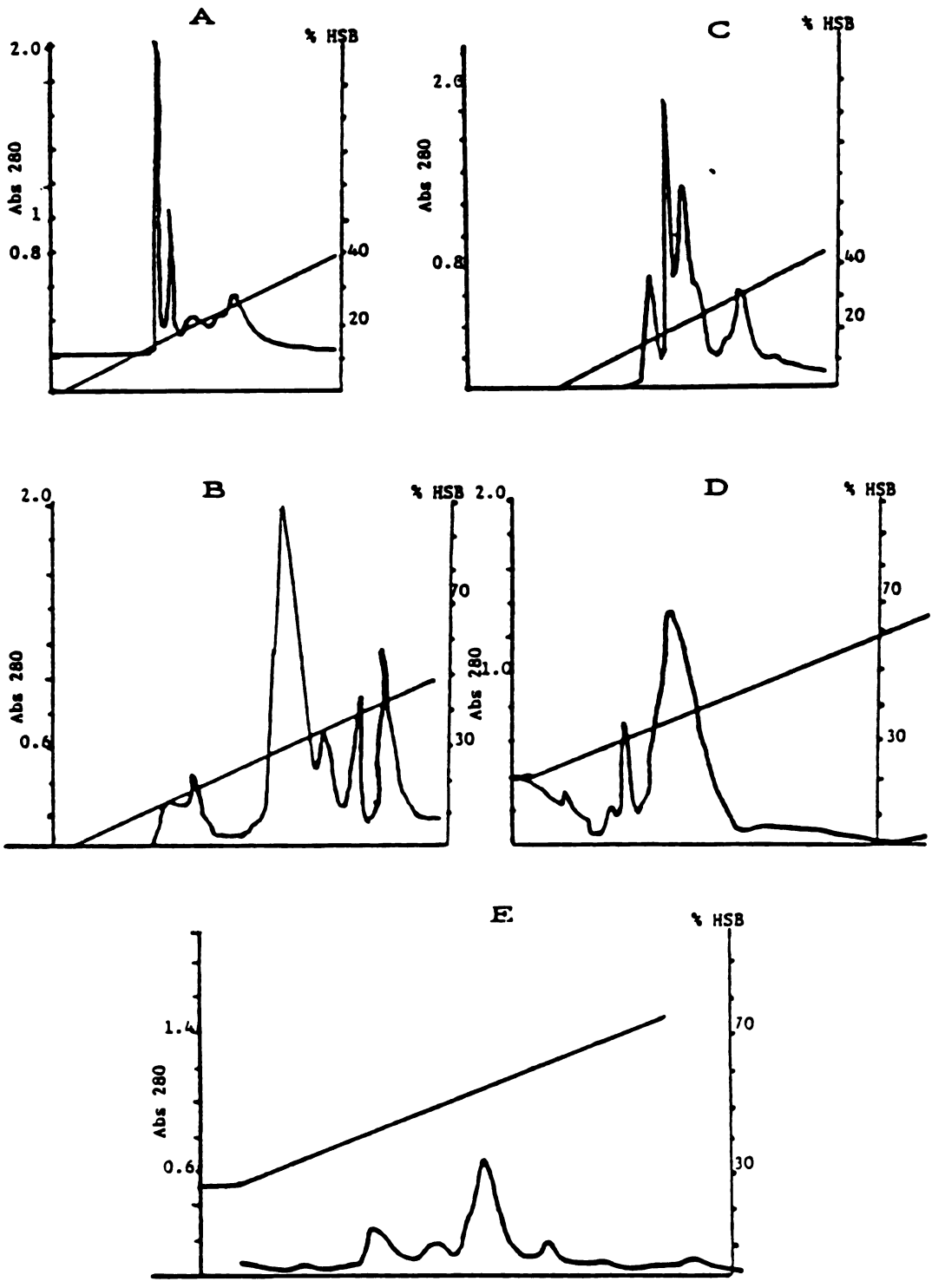


Figure 8 FPLC of DEAE-Sepharose Fractions

Figure 8 FPLC of DEAE-Sepharose Fractions

Protein fractions A through E from the DEAE-Sepharose elution profile (Figure 6) were eluted from a Mono-Q column equilibrated with 10 mM sodium acetate, pH 6.0, by an isocratic gradient (10 mM to 1.0 M) of sodium acetate, pH 6.0. The absorbance at 280nm as well as the percent high salt buffer are plotted versus the elution volume.

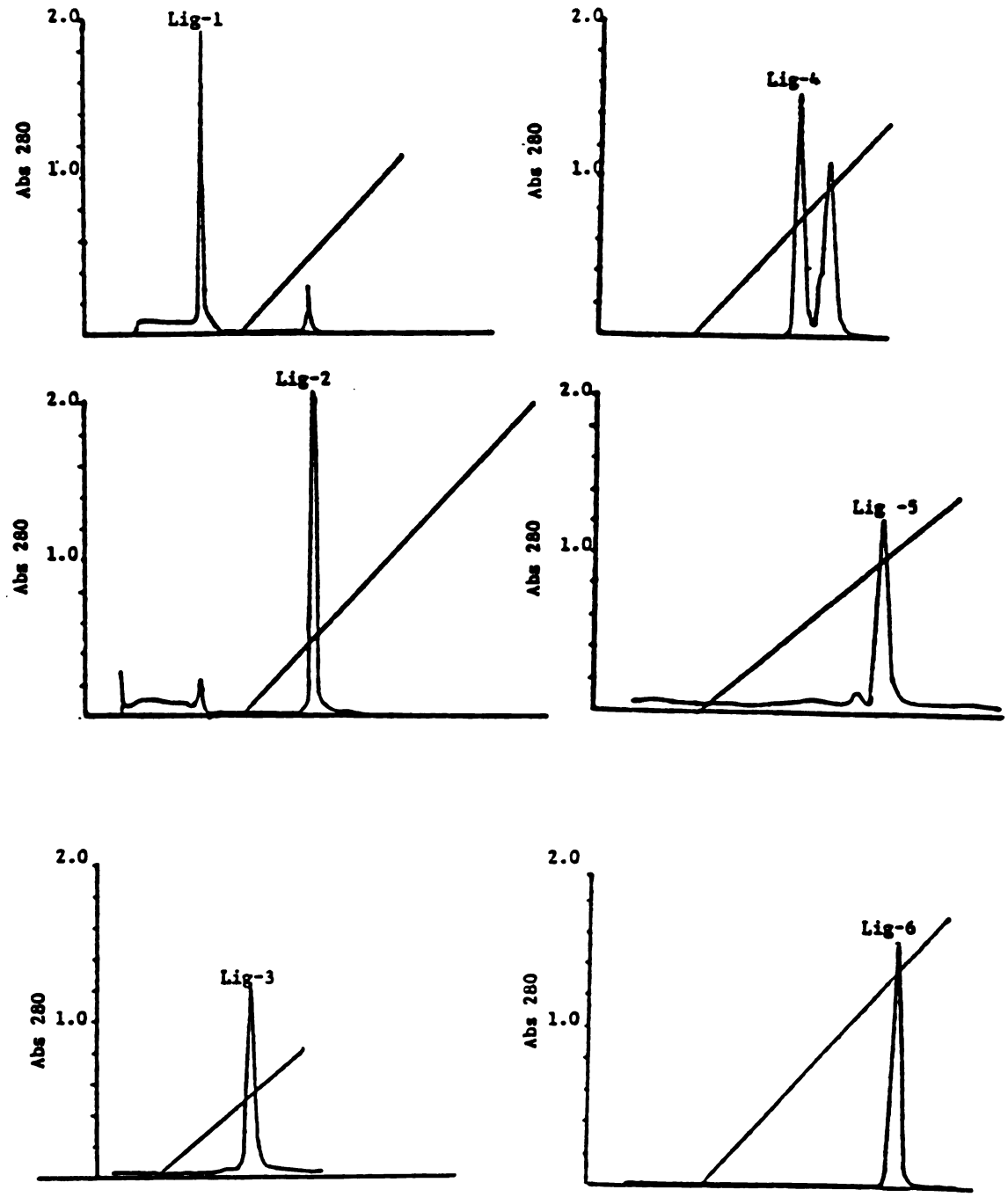


Figure 9 FPLC Profiles of Six Ligninases

Figure 9 FPLC Profiles of Six Ligninases

Major FPLC fractions eluting at the same percent high salt buffer were pooled, dialyzed against 10 mM sodium acetate, pH 6.0, and were repeatedly subjected to FPLC purification (proteins were eluted with an isocratic gradient from 10 mM to 1.0 M sodium acetate, pH 6.0). Six protein peaks were obtained as determined by the absorbance at 280nm. All peaks absorbed at 410nm indicating heme group, and all had VAO activity. The proteins were labeled Lig-1 through Lig-6 according to their order of elution (Lig-1 elutes at the lowest %HSB).

TABLE IV. Purification of the Ligninase Isozymes

Sample	Vol. (mL)	Activity (units/mL)	Protein (mg)	Total Activity (units)	Specific Activity (units/mg)	% Recov.
ECF	9200	0.191	----	1760	-----	100
Conc. ECF	8.0	171	82.4	1370	17	78
DEAE-Sepharose Fractions						
A	1.8	18	1.7	32	19	1.8
B	2.3	120	10	276	28	16
C	2.3	47	5.8	108	19	6.1
D	2.9	34	4.6	99	21	5.6
E	2.0	22	1.8	44	24	2.5
FPLC FRACTIONS						
From A	0.9	17	0.30	15	52	0.9
From B	0.7	51	0.58	36	61	2.0
From C	2.0	9	0.82	18	22	1.0
From D	1.6	11	0.85	17	21	1.0
From E	1.6	2	0.21	3	15	0.2

ECF- Extracellular Fluid from agitated fungal cultures

one unit of activity = one micromole of veratryl alcohol oxidized per min

also shown that there may be slight variations in molecular weights between the ligninase isozymes (16,19,29), but these variances are small. Therefore, SDS-page is not a suitable technique in determining the purity of the ligninases. Table V summarizes the molecular weights and specific activities of six ligninases. Four of the proteins have the same molecular weight and cannot be distiguished from one another on an SDS gel as seen in Figure 10. However, Table V shows that these four proteins are easily distinguished by their FPLC elution, and can readily be distinguished from the higher molecular weight isozymes.

Isoelectric Focusing Gels

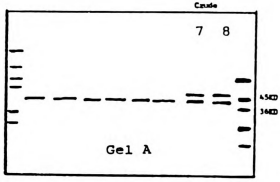
Isoelectric focusing is another analytical technique used to determine the purity of an enzyme preparation. The ligninases, while very similar in size, have different charges, as can be seen by ion-exchange chromatography. Figure 11 shows an isoelectric focusing gel run under denaturing conditions on the fractions obtained from the DEAE-sepharose elution. A total of thirteen different proteins could be seen, all having pI's between 4.05 and 5.10. Table VI summarizes the pI's of the proteins in each fraction.

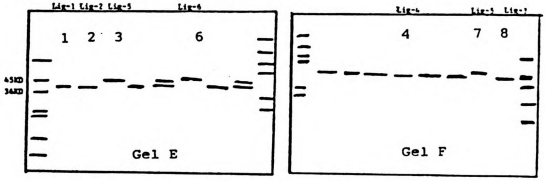
Isoelectric focusing gels (Figure 12) were also run on six different crude preparations to verify that the

TABLE V. Retention times, Specific Activities, and Molecular Weights of FPLC-Purified Ligninases

Protein	%HSB	Specific Activity	Mol. Weight by SDS-Gel
Lig-1	0	76	42,000
Lig-2	25	61	42,000
Lig-3	27	21	42,000
Lig-4	37	22	42,000
Lig-5	49	30	47,000
Lig-6	68	33	47,000

Retention times are expressed as % HSB, the high salt buffer (1M Sodium Acetate, pH 6.0).





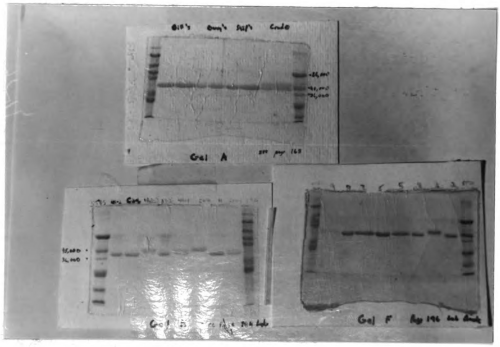


Figure 10 SDS-PAGE of FPLC-Purified Ligninases

Figure 10 SDS-PAGE of FPLC-Purified Ligninases

The molecular weights of the ligninases were determined by SDS-PAGE as described in Materials and Methods. Prestained molecular weight markers included: 180, 116, 84, 58, 48.5, 36.5, and 26.6 kilodaltons and unstained molecular weight markers included: 66, 45, 36, 29, 24, 20.1, and 14.2 kilodaltons. Three different gels, A, E, and F are shown. Gel A shows the two apparent molecular weights of 42 KD and 47 KD, present in the unpurified ECF of fungal cultures (lanes 7 and 8). Gel E shows the apparent molecular weights of Lig-1, Lig-2 (42 KD), and Lig-5, Lig-6 (47 KD) in lanes 1, 2, 3, and 6. Gel F shows the apparent molecular weights of Lig-3, Lig-4 (42 KD), and Lig-5 (47 KD) in lanes 4, 8, and 7 respectively.

same proteins were present in each preparation (Figure 7, Table 3). Lane 6 was the only lane that contained a sample of extracellular fluid that had no VAO activity. All lanes have proteins in common with one another, but the relative amounts of each protein differs between lanes. This verifies what was found by FPLC. Also interesting is the fact that the predominate proteins in lane 6 (pI = 4.91 and 4.86) are only weakly visible in other lanes, while the predominate proteins in other lanes are only weakly visible in lane 6. This is consistent with what has been found by other researchers who purify a peroxidase that contains no VAO activity along with the ligninases (19,30). The production of this enzyme has been shown to precede ligninase production (30). Table VII lists the pI's of the proteins in Figure 12. As in table VI, the majority of the proteins have pI's between 5.10 and 4.05.

Enzyme-linked Immunoabsorbant Assay

The ligninases are all heme-containing enzymes capable of oxidizing veratryl alcohol and they also exhibited cross-reactivity to an antibody prepared against one of the enzymes. Polyclonal antibodies were made to Lig-4 and an Elisa was performed to determine the percent cross-reactivity (see Materials and Methods) between the the isozymes (Table VIII). Nearly all the

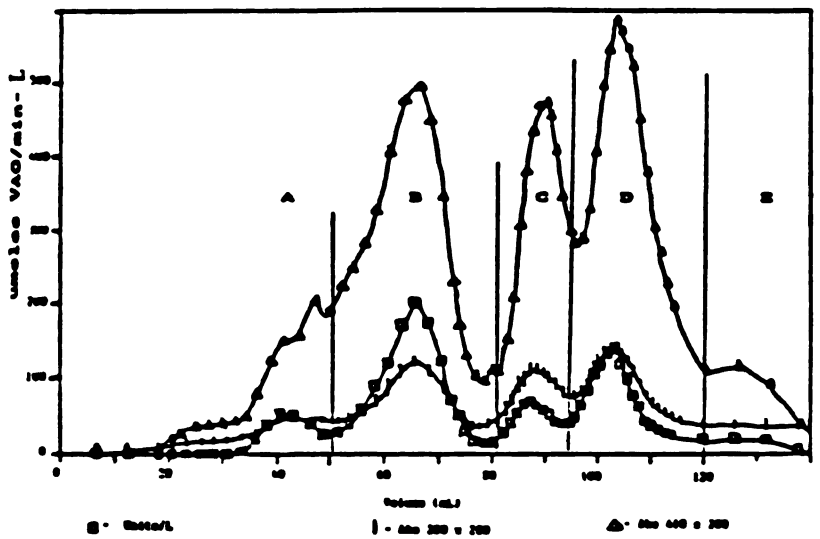


Figure 6 DEAE-Sepharose Elution Profile

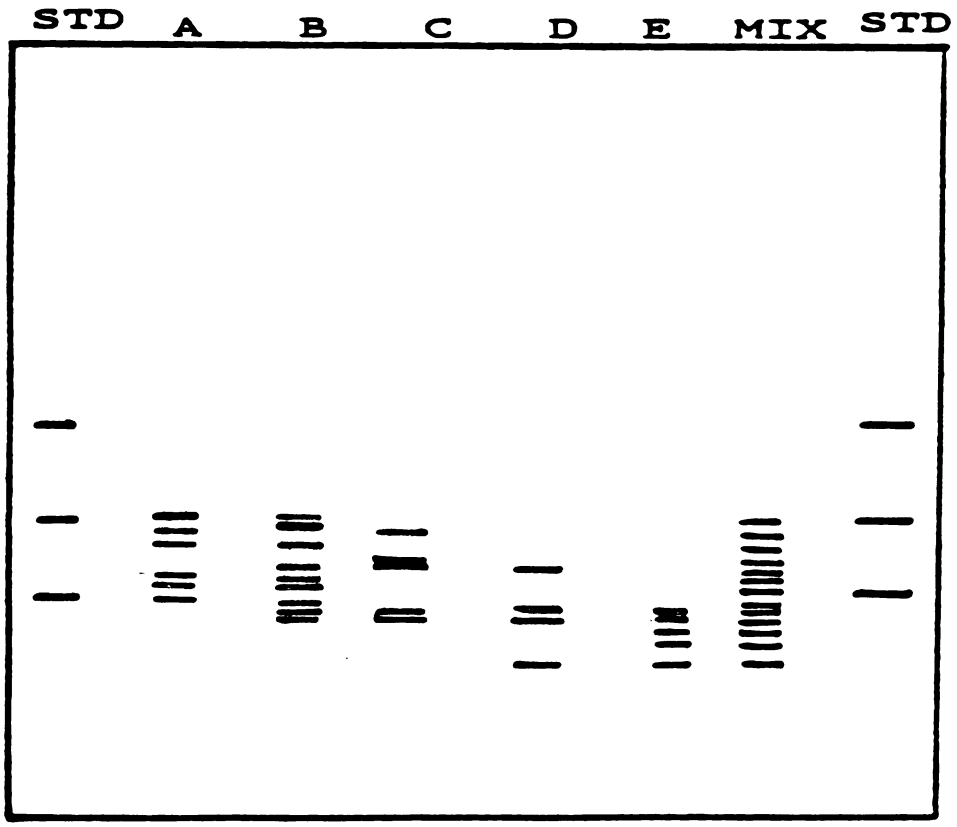


Figure 11 Isoelectric Focusing Gel of DEAE-Sepharose Fractions

Figure 11 Isoelectric Focusing Gel of DEAE-Sepharose Fractions

Protein fractions A through E (Figure 6) were subjected to isoelectric focusing under denaturing conditions as described in the Materials and Methods. Standards had pI's of 5.85, 5.13, and 4.55.

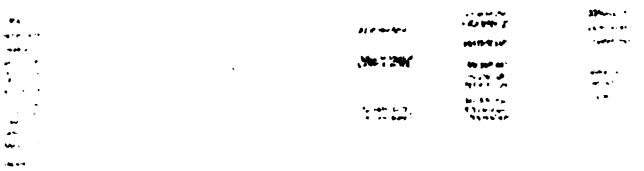


Table VI. Isoelectric Points of Proteins from DEAE-sepharose Chromatography

FRACTION					
A	B	C	D	E	Mix
5.10	5.10				5.10
5.02	5.02	5.02			5.02
4.92	4.92				4.92
	4.81	4.81			4.81
		4.73	4.73		4.73
4.68	4.68				4.68
4.60	4.60				4.60
4.50	4.50				4.50
	4.44	4.44	4.44	4.44	4.44
	4.37	4.37	4.37	4.37	4.37
				4.29	4.29
				4.18	4.18
			4.05	4.05	4.05

The pI's were determined through the use of a least squares program which fit the best line through pI standards 5.85, 5.13, and 4.55. The pI's were obtained under denaturing conditions (see Figure 11).

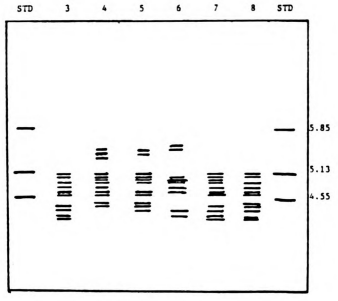


Figure 12 Isoelectric Focusing Gels of Six Samples of ECF from Agitated Cultures of P. chrysosporium

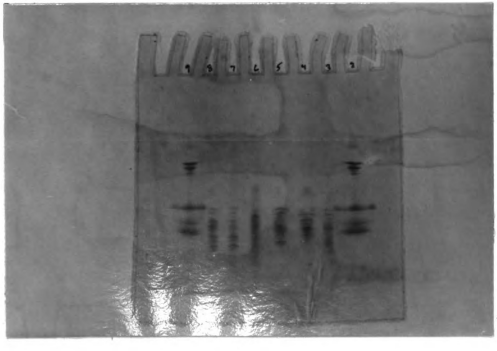


Figure 12 Isoelectric Focusing Gel of Six Samples of ECF from Agitated Cultures of *P. chrysosporium*

Six different preparations of ECF from agitated fungal cultures were subjected to ISF under denaturing conditions as described in Materials and Methods. Samples were run in lanes 3 through 8, while standards having pI's of 5.85, 5.13, and 4.55 were run in the outer lanes (lanes 2 and 9 in photograph).

Table VII. Isoelectric Focusing of Crude Ligninase Preparations (Figure 12)

Crude-1	Crude-2 DEAE-Sepharose Wash	Crude-3	Crude-4 No VAO Activity	Crude-5	Crude-6
			5.74		
	5.66	5.66	5.66		
	5.57	5.57			
	5.49				
5.10	5.10	5.10		5.10	5.10
4.99	4.99	4.99	4.99	4.99	4.99
	4.91	4.91	4.91	4.91	
4.86	4.86	4.86	4.86	4.86	4.86
4.75	4.75		4.75	4.75	4.75
4.66	4.66	4.66	4.66	4.66	4.66
4.58	4.58	4.58		4.58	4.58
	4.47	4.47			4.47
4.39	4.39	4.39		4.39	4.39
4.28		4.28	4.28	4.28	4.28
4.17			4.17	4.17	4.17
4.06				4.06	4.06

The isoelectric points were determined under denaturing conditions using a standard curve obtained from the least squares plot of three standards, pI= 4.55, 5.13, and 5.85 .

proteins tested cross-reacted 50% or more, indicating a great deal of homology between isozymes. Very low cross-reactivity was found in BSA and microsome controls as well as the crude preparation that had no VAO activity (4% cross-reactivity). Again this supports what has recently been found by Leisola et al (30); that ligninase antibodies do not react with the second type of peroxidase (MnII-dependent peroxidase) whose production by the fungus precedes ligninase production.

Amino Acid Analysis of Lig-1 and Lig-6

The amino acid composition for two ligninases, Lig-1 and Lig-6 were obtained through the macromolecular facility at MSU. Lig-1 and Lig-6 were chosen because of their differences in molecular weight, and their differences in FPLC elution. Table IX compares the composition of the two proteins as well as the recently reported composition obtained by sequencing one of the ligninase genes (23). What is quickly apparent is the high amounts of acidic residues, aspartic acid, and glutamic acid, and the high amounts of the small amino acids, alanine and glycine, that are present in each protein. Proline is also present in high levels. All residue levels are comparable to the deduced amino acid composition by Tein et al (23), suggesting a high degree of homology between the enzymes. It is not known which

enzyme (Lig-1 thru Lig-6) corresponds to Tein's sequenced protein.

Table VIII. Cross-Reactivity of the Purified Ligninases

Sample	Average % Cross-Reactivity
Lig-1	64
Lig-2	78
Lig-3	53
Lig-4	100
Lig-5	53
Lig-6	15
Bovine Serum Albumin	5
Rat liver microsomes	0
Crude Preparation -No VAO Activity	4
Crude Preparation- With VAO Activity	75

Rat liver microsomes were obtained from Dr. Richard Voorman. The average percent cross-reactivity from four experiments was determined as described in Materials and Methods.

Table IX. Amino Acid Composition of Lig-1 and Lig-6

Amino Acid	Lig-1 (M.Wt.=37,000)		Lig-6 (M.Wt.= 42,000)		Sequenced (23) Ligninase Gene (M.Wt.=37,000)	
	Residues	%	Residues	%	Residues	%
ASP	19*	5.1	34*	9.0	39*	11.3
GLU	28*	7.5	43*	11.4	35*	10.1
SER	28	7.5	34	9.0	27	7.8
GLY	47	12.6	47	12.5	31	9.0
HIS	10	2.7	7	1.9	8	2.3
ARG	12	3.2	12	3.2	14	4.1
THR	25	6.7	20	5.3	16	4.6
ALA	40	10.7	20	5.3	31	9.0
PRO	58	15.5	47	12.5	27	7.8
TYR	2	0.5	1	0.3	0	0
VAL	22	5.9	23	6.1	22	6.4
MET	7	1.9	8	2.1	5	1.4
ILE	21	5.6	21	5.6	20	5.8
LEU	19	5.1	24	6.4	23	6.7
PHE	27	7.2	29	7.7	27	7.8
LYS	8	2.1	6	1.6	10	2.9
CYS	--	---	--	---	7	2.0
TRP	--	---	--	---	3	0.9
ASN	--	---	--	---	13	---
GLN	--	---	--	---	19	---

* - Residue number is the sum of either ASP+ASN or GLU+GLN.

Discussion

Ligninases can be produced in high amounts in agitated (1L) cultures (approximately 10-20mg/culture) of P. chrysosporium. One very important aspect of ligninase production besides nutrient levels is the fungal pellet size. Pellet size can be affected by the volume of the culture, the shape of the flask, the partial pressure of oxygen, the agitation speed, and the nutrient conditions of the media (17,31). Very small pellets and large pellets do not produce desired amounts of ligninase (31), so care must be taken during the homogenization and subsequent agitation not to alter any one of the above factors by any large degree. It also may be of interest to point out that the appearance of the fungal pellets changes as the cultures approach, or become nutrient deficient. Fungal pellets are usually white and filamentous, like very small cotton balls, by day three of the inoculation cycle. Very suddenly, however, the pellets begin to take on a tan color, usually by the end of day three, which progresses to a coffee brown color by day four. The cultures usually had VAO activity a short time after they turn this coffee color.

Fungal pellets can be reused to produce more enzyme. It must be said, however, that pellet size varies considerably even within a given culture. To gain more control of this parameter, pellets should be suspended in a large graduated cylinder and allowed to settle. The larger pellets will settle first, and should

not be used again without homogenization. If properly developed, this technique could be used to help increase ligninase production.

The ligninases themselves have been shown only recently to be a group of very similar isozymes (16,18,19,30,31). All are glycosylated, heme-containing peroxidases which react to some degree with polyclonal antibodies made to a pure ligninase isozyme. The total number of isozymes is not known. FPLC analysis of crude samples showed 19 possible proteins (Table III). Isoelectric focusing gels also showed 17 proteins with different isoelectric points, some of which had no VAO activity. Recently, Leisola et al (30) reported 15 different ligninase isozymes and 6 different Mn(II)-dependent peroxidases. Tein et al (23) has showed that ligninase production is probably controlled at the mRNA level. How and what regulates the expression of the ligninase genes will be of great interest in the future.

However, much work still needs to be done to purify the ligninases. Fortunately, the ligninases are very durable enzymes. They do not denature at room temperature, or from repeated freezing and thawing. They are stable when stored in distilled water for weeks in the refrigerator or can be lyophilized and stored freeze-dried for months without losing their activity when brought into solution. The enzymes seem to be as tough as the substances they degrade.

The amino acid composition (Table IX) gives some clues to their durability. There is a high content of

alanine, glycine, and proline in the enzymes, as well as possibly 7 cysteines. The high proline content means that the ligninase's secondary structure must undergo many twists and bends. Beta-sheet secondary structure is very probable. The high proline content undoubtedly gives the protein a great deal of structural rigidity and if the cysteines (three grouped at the N-terminus and three grouped at the C-terminus) are crosslinked, one can envision a very sturdy enzyme.

Purification of the ligninases is only hindered by the inability to produce large amounts of crude enzyme. Unfortunately the lack of enzyme is compounded during ion-exchange chromatography. The ligninases are very acidic proteins and will bind tightly to the column. In fact, as can be seen by the purification table (Table IV), 71 % of the protein loaded on the DEAE-sepharose column remained on the column. Much of the remaining protein was lost in further FPLC purification. What gives more evidence that the enzymes are remaining bound to the column is the frequent washings that are required between elutions. Without the washings, the column would soon clog and cause not only longer FPLC runs, but also would alter the elution profile of the run. That is why isoelectric focusing should be employed to aid in purifying the ligninases. Isoelectric focusing not only provides more confidence to the researcher when it comes time to combine fractions of an FPLC elution, but is also allows the purity of FPLC proteins to be verified. Unfortunately, only the purity of one of the six

FPLC-purified ligninases was verified in this manner (Lig-6, pI= 4.05) because of insufficient quantities of the purified proteins. Isoelectric focusing gels are also adaptable to further analysis by western blots which will clearly establish the position of the ligninase isozymes on FPLC elution, and their respective denatured pI's. A quick and easy labeling system can then be established for each isozyme to benefit all researchers interested in working with the ligninases.

Future work with the pure isozymes should focus on their mechanism of xenobiotic degradation, their tertiary structure, and eventually the mechanism that regulates their production. Sequence analysis of the pure isozymes will allow the synthesis of oligonucleotide probes, which will eventually lead to the cloning of the different ligninase genes and overproduction of active enzymes.

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