FINAL TECHNICAL REPORT

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Project Title: Producing a True Lignin Depolymerase for Biobleaching Softwood Kraft Pulp
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SUMMARY

This project constituted an intensive effort devoted to producing, from the white-rot fungus *Trametes cingulata*, a lignin degrading enzyme (lignin depolymerase) that is directly able to biobleach or delignify softwood kraft pulp brownstock. To this end, the solutions in which *T. cingulata* was grown contained dissolved kraft lignin which fulfilled two functions: it behaved as a lignin depolymerase substrate and it also appeared to act as an inducer of enzyme expression. However, the lignin depolymerase isoenzymes (and other extracellular *T. cingulata* enzymes) interacted very strongly with both the kraft lignin components and the fungal hyphae, so that isolating these proteins from the culture solutions proved to be unexpectedly difficult. Even after extensive experimentation with a variety of protein purification techniques, only one approach appeared to be capable of purifying lignin depolymerases to homogeneity. Unfortunately the procedure was extremely laborious: it involved the isoelectric focusing of concentrated buffer-exchanged culture solutions followed by electro-elution of the desired protein bands from the appropriate polyacrylamide gel segments.

Despite the difficulty encountered in isolating purified proteins, at the beginning of the second annual reporting period two lignin depolymerase isozymes were successfully obtained for the first time from *T. cingulata* culture solutions. Differing in pI by ~0.9 units, these two isozymes possessed the same apparent molecular weight of 35 kDa (much lower than that of laccase) determined by SDS-PAGE. Moreover, they were both capable of degrading, in a homogeneous solution without a cofactor or mediator, high molecular weight kraft lignin components. Most importantly, unlike the lignin peroxidases and laccases implicated in other lignin biodegradation studies, these two isoenzymes did not exhibit any lignin polymerization activity. The efficacy of the lignin depolymerase isozymes was supported by preliminary studies indicating that they also reduce the kappa number of softwood kraft pulp brownstock, although more extensive confirmation with larger quantities of the lignin depolymerases would be desirable.

It was soon recognized that the lignin depolymerase isozymes were produced by *T. cingulata* in extremely small quantities and that the expression of these proteins was very sensitive to the culture conditions being employed. The need to accumulate sufficient lignin depolymerase for characterization and sequencing purposes, a prerequisite for identifying the corresponding genes, thus dictated that attention be preferentially directed to only one of the two isoenzymes. Initially the one with the higher pI was chosen because of the relatively simple protein pattern in that region of the gel after isoelectric focusing (IEF). However, shortly after the two lignin depolymerase isozymes had been purified to homogeneity for the first time, the ability of *T. cingulata* to degrade kraft lignin components in cultures spontaneously began to diminish quite drastically; the conditions adopted for propagating the cultures had proven not to support indefinite expression of these two important proteins by the microorganism. Such a setback could hardly have been more serious; a monumental effort was mounted to re-establish *de novo* a viable *T. cingulata* source that sustains vigorous kraft lignin biodegradation. This goal was

ultimately achieved by repeatedly selecting, for the inoculation of fresh solutions, mycelium derived only from those earlier cultures where lignin biodegradation had been occurring the most rapidly. Moreover, the inoculum sources were routinely incubated beyond the primary growth phase in aqueous 1.5% malt extract because otherwise a premature drop in pH of the culture solution after inoculation would invariably accompany rapid fungal development and cause kraft lignin precipitation onto the hyphae before significant biodegradation could take place.

After appropriating the new *T. cingulata* inoculum source and selecting for faster lignin biodegradation, the cell-free culture solution was found to hold a protein that behaved during IEF and SDS-PAGE almost indistinguishably from the original higher-pI lignin depolymerase isozyme. Yet this extracellular protein did not exhibit any lignin degrading activity; indeed the same protein was also detected in a particular chromatofocusing fraction obtained from culture solutions that did not contain kraft lignin, a putative inducer of lignin depolymerase. Actually, the first time that lignin degrading activity was eventually recovered successfully from *T. cingulata* cultures was from the surfaces of the hyphae (grown in solutions containing kraft lignin) by washing them with aqueous Tris phosphate (pH 7.5). However, it now became evident that only a small proportion of the proteins electro-eluted from the electrofocused gel segments spanning the pH range of the newly recovered higher-pI lignin depolymerase isozyme bore lignin degrading activity. Even after electro-elution and refocusing (by IEF), the proteins in the region of gel encompassing the higher-pI isoenzyme tended to exhibit multiple bands. Moreover, as work progressed during the third annual reporting period this isozyme was no longer routinely detected through silver staining of culture solution proteins that had been subjected to IEF. Obviously such inconsistencies had begun to complicate the characterization of the purified higher-pI lignin depolymerase isozyme quite considerably.

Owing to the difficulties confronting the purification of the newly recovered higher-pI lignin depolymerase isozyme, the lower-pI isoenzyme then became the primary focus of attention. However, a similar situation was encountered here also: the proteins electro-eluted from the electrofocused gel segments encompassing the lower-pI isozyme typically manifested three conspicuous bands (and some minor ones). Nevertheless, assays of the electro-eluted components with the customary high molecular weight (polymerized) kraft lignin substrate revealed that lignin depolymerase activity was confined to a faint band located +0.04 pI units from the middle one of the three major bands. Despite these difficulties, the gradual accumulation of each lignin depolymerase isozyme seemed to provide the only reliable means of obtaining sufficient quantities for amino acid sequencing and characterization studies.

Despite the provision for isolating lignin depolymerases, sizable losses of active enzyme during a laborious purification protocol involving IEF in polyacrylamide gels followed by electro-elution was a continuing concern. Alternative approaches were therefore always being sought. The purification of lignin depolymerase isozymes by means of gel-free IEF in a Rainin RF3 instrument has been collaboratively investigated at the National Renewable Energy Laboratory, and the first set of viable conditions to have been developed furnished some encouraging results. Once the running conditions of the instrument have been optimized, this gel-free IEF system should significantly simplify the isolation procedure and in the process preserve a greater proportion of active lignin depolymerases.

Preliminary amino acid analyses of the higher-pI lignin depolymerase have suggested an *N*-terminal sequence of Glu-Ala-Tyr-Pro-Gln-X-Asp-X, where the results from the Edman

degradation ceased to be reliable after the 5th residue. Sequencing appeared to be taking place at a level that was over 10-fold lower than expected from the overall quantities of the amino acids present. Such findings could indicate that the *N*-terminus is compromised by the presence of a pyroglutamate residue since the first amino acid identified in the sequence was glutamic acid. Internal amino acid sequencing through cyanogen bromide cleavage of the polypeptide chain was not successful. These difficulties can only be overcome by isolating more of the lignin depolymerase isozymes. Owing to the enormous amount of labor needed for purifying these enzymes, it has been recognized that MALDI-TOF mass spectrometry is likely to be preferable for amino acid sequencing purposes. The resulting sequence information is required for identifying the lignin depolymerase genes, either by screening the *T. cingulata* cDNA library (which has been successfully constructed), or by employing the reverse-transcriptase polymerase chain reaction (RT-PCR) with the total RNA isolated when lignin biodegradation is occurring *in vivo* (a protocol which has also been explored—albeit with inadequate primers).

The insights gained from this project have been extremely valuable despite the enormous experimental difficulties with which the research and development effort has had to contend. The purification of the two lignin depolymerase isozymes from *T. cingulata* represents a genuine breakthrough in the field of lignin biodegradation, but the work has fallen far short of producing recombinant lignin depolymerases for biobleaching softwood kraft pulp. It is tremendously important that the investment made in this project not be lost. Accordingly the work is continuing in collaboration with the Enzyme Technology Team at the National Renewable Energy Laboratory, and the results secured around the time when the present report was being completed have been very promising.

BACKGROUND

This research project rests upon discoveries that differ fundamentally from the results of all other biobleaching studies. Here the means are being developed for producing the first enzyme that *directly* delignifies softwood kraft pulp. Serious concerns about the effects of chlorinated organic compounds have been prompting the pulp and paper industry to use less chlorine and chlorine compounds in bleaching kraft pulp. From an environmental perspective, the most attractive bleaching agents for kraft pulp would be natural lignin-degrading enzymes. White-rot fungi are the only organisms that produce lignin depolymerizing enzymes, and thus they represent the most realistic source of potential biobleaching agents that can *directly* delignify kraft pulp. From the white-rot fungus *Trametes cingulata*, a new enzyme has been isolated at the University of Minnesota that *completely* depolymerizes high molecular weight softwood kraft lignin components. This lignin depolymerase has been found to reduce the kappa number of softwood kraft pulp brownstock by at least 50%. Despite its unprecedented effectiveness in degrading macromolecular lignin components, the lignin depolymerase activity naturally expressed by *Trametes cingulata* is low. Consequently this research project is dedicated to demonstrating how the first genetically engineered lignin depolymerase production technology will be created for biobleaching softwood kraft pulp. By optimizing biobleaching conditions, the work is committed to achieving acceptable levels of brightness for softwood kraft pulp through a bleaching sequence of lignin depolymerase, alkaline extraction and peroxide stages alone.

STATUS

1. Development of White-Rot Fungal Cultures

Throughout the course of this project, between 80 and 150 x 50-mL portions of homogenous solution were routinely inoculated with *Trametes cingulata* mycelium in 14 – 45 day-old aqueous 1.5% malt extract solutions. Each portion of growth medium containing 0.244 gL⁻¹ MgSO₄, 0.018 gL⁻¹ FeSO₄·7H₂O, 0.22 gL⁻¹ ammonium tartrate, 0.697 gL⁻¹ KH₂PO₄, 1.875 gL⁻¹ Na₂HPO₄·7H₂O, 10.0 gL⁻¹ glucose, 0.55 gL⁻¹ Jack pine kraft lignin, 0.10 gL⁻¹ CaCl₂·2H₂O, 1 mL L⁻¹ trace element solution without MnSO₄·7H₂O (*Eur. J. Appl. Microbiol. Biotechnol.* **1982**, *14*, 174-181), and 1 mL L⁻¹ vitamin solution (*Arch. Microbiol.* **1978**, *117*, 277-285) was filter-sterilized through a 0.2 µm 37GF Serum Acrodisc (Gelman no. 4525) and placed into an autoclave-sterilized (60 min. at 121°C) 500-mL conical flask. The kraft lignin (*vide infra*: section 8) was added to ensure that, if its presence is essential for inducing lignin depolymerase expression, it would be present to interact with a putative positive regulator to increase transcription from the lignin depolymerase gene. A neoprene stopper fitted with a glass tube containing a glass wool plug was inserted into the mouth of each 500-mL culture flask to allow air-exchange for *T. cingulata* growth. The cultures were incubated at room temperature whereupon the brown color of each solution darkened during the first two weeks as the kraft lignin underwent polymerization during primary vegetative growth of the white-rot fungus. After a brief delay, degradation of the polymerized kraft lignin began, causing the culture solution to become much lighter in color. During this period, when deemed appropriate, the fungal mycelia would be harvested and incubated with wash solution (*vide infra*: section 3) while the culture solutions were collected for lignin depolymerase purification (*vide infra*: section 3).

As a profitable basis for selecting and handling source cultures for inoculation purposes was being developed (*vide infra*: section 2), substantial enhancements in lignin biodegradation rate were being realized, but at a price: the pH of the culture solution concomitantly began to fall more quickly during primary vegetative growth of the white-rot fungus. This resulted in a greater tendency for the kraft lignin substrate to be adsorbed or precipitated from solution onto the hyphae. Such a phenomenon is undesirable as far as the accessibility of the kraft lignin components to the lignin depolymerase is concerned. To counteract the effect, the buffer capacity of the culture solution was modified by changing the ratios of the mono- and dibasic-phosphates that were initially present (the pH of the growth medium being raised in the process to 7.1 – 7.3). Although the adsorption and/or precipitation of kraft lignin was greatly reduced as a result, the majority of these modifications did not yield noticeable improvements in lignin biodegradation. It was later found that, when the capacity of the buffer was increased 1.5-fold (at pH 7.0) allowing more of an opportunity for the pre-emptive formation of hydrophilic functional groups through the action of fungal oxidases on the kraft lignin components before the pH had reached too low a value, a considerable improvement occurred in the proportion of *T. cingulata* cultures witnessing substantial biodegradation without adsorption and/or precipitation of the kraft lignin components onto the hyphae. However, complete compliance with substrate retention in solution proved difficult to achieve under such higher buffer capacity conditions despite repeated cycles of selection, where the mycelium from the best culture(s) was automatically used to produce the stock for inoculating the next batch of aqueous media. Moreover, depolymerization of (pre-polymerized) high molecular weight kraft lignin *in vitro* could not be secured from these culture solutions. It was therefore concluded that the increased buffer capacity did not significantly bring about a higher level of lignin biodegradation despite

its facilitating the retention of kraft lignin components in the culture solutions. It was ultimately the continuing selection, for producing inoculum source, of the best cultures grown under the original culture conditions (*vide infra*: section 2) which led to sustainable and complete lignin depolymerization both *in vivo* and *in vitro*. Toward the end of the project period, at least a 2-fold improvement in lignin biodegradation rate had been achieved routinely over what had been the very best encountered in the past. At the same time, kraft lignin precipitation onto the hyphae, which previously had often accompanied rapid fungal development, had ceased to occur. This is of particular importance because the retention of the kraft lignin in solution is a prerequisite for complete lignin depolymerization to take place within a reasonable time, and for subsequent protein isolation from the culture solutions to be reasonably effective.

2. Selection of Source Cultures

Much of the work during this project was confounded by a sudden and spontaneous drastic reduction in the lignin depolymerase activity expressed in the *T. cingulata* cultures. This occurred about 16 months after the initiation of the research project, soon after the two lignin depolymerase isozymes had been isolated for the first time (*vide infra*: section 5). It remains to be determined whether *de facto* selection occurs after a number of generations of hyphal growth that gradually engenders a change in the level of lignin depolymerase expression, or whether a relatively minor change in culture conditions can cause a decrease in the enzymatic activity responsible for lignin depolymerization. Identification of the underlying cause would probably have necessitated an extensive series of studies, and thus it was decided that a different source of *T. cingulata* for inoculation should be sought while continuing to work as productively as possible with the original culture.

Other sources of *Trametes cingulata*, designated as *Tc2*, *Tc3* and *Tc4*, were obtained from different cultures which were available to us. The *Tc2* and *Tc3* sources had been refrigerated on 1.5% malt extract agar in separate vials while the agar plug from which *Tc4* was obtained had been immersed in sterile water within a refrigerated vial. These three sources were allowed to grow in aqueous 1.5% malt extract solution for 2 weeks before being used to inoculate culture solutions (*vide supra*: section 1). Unfortunately, as they grew vegetatively in the culture solutions, the dissolved kraft lignin tended to undergo precipitation or adsorption onto the fungal hyphae. This contrasted sharply with what had been observed in the original *Tc1* culture solutions where all the kraft lignin components remained in solution. Previous work had shown that initial light (phosphate) buffering at pH 7.0 was necessary to prevent the adsorption of kraft lignin from solution onto the fungal hyphae. When this was not done, the kraft lignin that associated with the fungal cell walls required 1M NaOH at elevated temperatures for redissolution to occur. All of the culture media used before had therefore been buffered to allow enough polar groups to be introduced onto the kraft lignin components, through oxidation by the microorganism, so that precipitation could be avoided when the pH began to fall during primary fungal growth. For the use of the *Tc2*, *Tc3* and *Tc4* sources to be profitable for inoculating culture solutions, association of the kraft lignin components with the fungal mycelium had to be prevented. Thus an increase in buffer capacity of the culture medium might have allowed the kraft lignin to remain in solution if sufficient oxidation of the components could have taken place before the pH fell as *T. cingulata* developed. The phosphate buffer concentration was therefore increased to 1.3 – 1.5-fold greater levels than initially employed, but this modification did not

significantly reduce the precipitation or adsorption of kraft lignin components onto the fungal hyphae when the *Tc2*, *Tc3* and *Tc4* sources were used for inoculation.

Culture solutions inoculated with *Tc2*, after acetate buffer (0.02 M, pH 4.5) exchange and concentration 80 – 100-fold, were analyzed by isoelectric focusing (IEF, *vide infra*: section 3) in the pH range between 4.0 – 6.5. In contrast to *Tc1* (the original *T. cingulata* source), only three prominent protein bands (instead of 7) and four faint bands (instead of 6) materialized after silver staining. The pI's of these major bands were 5.6, 5.25 and 4.85, respectively, while those of the four minor bands all fell between 4.40 and 4.70. The greater simplicity of the protein pattern produced by *Tc2* allowed for the possibility of much easier purification. Therefore, to determine if any of the extracellular proteins expressed by *Tc2* bore lignin depolymerase activity, chromatofocusing and electro-elution (*vide infra*: section 3) were employed in attempts to separate the components in the three most prominent bands from one another. The remaining species in the four minor bands were destined to be isolated together as a group. The visible characteristics exhibited by *T. cingulata* sources *Tc3* and *Tc4* were actually very similar to those of *Tc2* and they were all believed to be identical.

Two other inoculum sources of the original *Tc1* were also explored. Namely, *Tc1a*, which had been preserved for an extended period of time on 1.5% malt extract agar at room temperature, and *Tc1b*, which had been kept in (what had originally been) aqueous 1.5% malt extract solution under ambient conditions. These two sources behaved in the same distinctive way as *Tc1* in the culture solution containing dissolved kraft lignin. Nevertheless, *Tc1a* was shown to exhibit greater lignin depolymerase activity than either *Tc1* or *Tc1b* judging by the extent of decolorization of culture solution during a particular period of time, which had been shown earlier to correlate with lignin biodegradation *in vivo*. This enabled the enzyme to be isolated for the first time as an individual protein. However, the lignin biodegradation rate was still limited and a more viable source culture was continuously being sought during the course of the project.

Ultimately, a culture (designated as *Tc1a2*), that had been incubated for an extended period of time at room temperature on 1.5% malt extract agar plates, was found to display some desirable attributes, *viz.* a greater lightening in the color of the culture solutions (which indicates more lignin biodegradation) with less lignin precipitation; it therefore began to be used exclusively as the source for inoculating new groups of cultures. IEF of *Tc1a2* culture solutions, after acetate buffer exchange and concentration, displayed a slightly different protein pattern from that of *Tc1a*, but the lignin depolymerase isozymes appeared to be present, as would be expected. However, its performance was sometimes disappointing and it appeared necessary constantly to select mycelium from the most rapidly lignin biodegrading cultures for transfer into aqueous malt extract solutions and onto malt extract agar (the latter being destined for transfer to the former after a suitable incubation period) to produce inoculum for the next set of culture flasks. Nevertheless, when the lignin biodegradation rate was as a result undergoing improvement, lignin precipitation/adsorption onto fungal hyphae from culture solutions continued to represent an obstacle to complete lignin depolymerization *in vivo* and protein purification. All possible means to improve the source cultures further were therefore sought on a continuing basis, and a protocol was ultimately established that led to further increases in the levels of lignin depolymerase activity while at the same time minimizing lignin precipitation/adsorption. Thus, for inoculum source production, aqueous 1.5 % malt extract solutions were regularly inoculated with (*Tc1a2*) mycelium from the most active lignin biodegrading cultures and then incubated beyond the rapid hyphal growth phase before use. As a result, the growth of the newly

inoculated *T. cingulata* in culture solution was more moderate and the concomitant drop in pH was more gradual, tending to minimize kraft lignin precipitation onto the hyphae (*vide supra*: section 1). This is a result that had been sought for a considerable period. Moreover, the pH of the culture solution at the time of harvest (2½ – 4 weeks after inoculation) had fallen to approximately 4.3, which was significantly lower than before and correlated better with empirical expectations for higher levels of lignin depolymerase activity being expressed by *T. cingulata*.

For the batches of cultures most recently inoculated, roughly half had begun to exhibit extensive lignin biodegradation before 3 weeks had elapsed after inoculation; and 80% of the cultures overall had progressed into the lignin biodegradative phase by the end of the 4th week. This represents at least a 2-fold improvement in lignin depolymerization by *T. cingulata* over what had been previously typically encountered. At the same time, kraft lignin precipitation onto the hyphae, which earlier had often accompanied rapid fungal development, did not occur any more. This is of particular importance because the retention of the kraft lignin in solution is a prerequisite for complete lignin depolymerization to take place within a reasonable time, and for subsequent protein isolation from the culture solutions to be reasonably effective.

3. Enzyme Isolation

Typically 3 – 5 weeks after inoculation with *T. cingulata* mycelium, culture solutions were harvested automatically as the media were turning from dark brown to light yellow, which signified that the polymerized kraft lignin (the polymerization of which occurred during the first two weeks) was being degraded by the lignin depolymerase activity expressed by the organism. This was repeated at different times for sets of flasks that had been incubated for varying numbers of days after inoculation. Tables I and II indicate the number of flasks and dates of inoculation for each set of 50 mL cultures. They also report the dates on which culture solution was collected, and the number of flasks involved on each occasion.

The contents of each flask were filtered through Mira-cloth (Calbiochem) disposed in a Büchner funnel using a vacuum. On appropriate occasions the fungal mycelium from the most decolorized cultures, in which the kraft lignin components were most degraded, was saved for RNA isolation (*vide infra*: section 11). The combined culture solutions from each harvest were treated with two protease inhibitors, Pepstatin A and PMSF (phenylmethanesulfonyl fluoride) for 16 – 24 hours. The excess (solid) Pepstatin A and PMSF were then filtered off with glass-fiber circles, and the majority of the filtrate was reduced in volume to 200 mL whereupon it was equilibrated with 2.0 L acetate buffer (0.02 M, pH 4.5) in an ultrafiltration cell fitted with a 10,000 nominal molecular weight cutoff (Amicon PM10) membrane and finally concentrated ~80–100-fold. The resulting solution, which was stored at 4°C, was ready for subsequent isoelectric focusing for separating the proteins. For chromatographic studies, on the other hand, portions of the acetate-buffer-equilibrated culture solutions were concentrated 20-fold by ultrafiltration. Otherwise portions of unconcentrated culture solutions prepared for lignin depolymerase activity assays were filter-sterilized with a 0.2 µm 37GF Serum Acrodisc (Gelman no. 4525) and transferred into sterile 500 mL flasks each closed by a stopper fitted with a glass tube containing glass wool that allowed air-exchange.

There have, however, been indications that the lignin depolymerase may not be readily released from the hyphal walls into the culture solution. Therefore, in an attempt to secure as much lignin

depolymerase as possible, after removal of the culture solutions, the mycelia were extracted under more alkaline conditions with aqueous 0.05 *M* Tris phosphate buffer at pH 7.5 containing 0.10 *M* NaCl in the presence of PMSF at the time of harvesting; following 12 – 16 h incubation, the mycelia were removed by filtration through Mira-cloth (Calbiochem) and the resulting wash solution was handled in the same way as the culture solution. However, because such wash solutions did not consistently exhibit substantial levels of (polymerized) kraft lignin degradation, the mycelia from some harvests, were submerged in 0.02 *M* acetate buffer in the presence of PMSF. The resulting wash solution was otherwise processed in the same way as that obtained with aqueous 0.05 *M* Tris phosphate at pH 7.5.

(Text continues after Tables I and II on page 12)

Table I. Inoculation and Harvesting of *T. cingulata* Culture Solutions.

Inoculation date	Number of flasks	Culture collection Date, number	Culture collection Date, number	Culture collection Date, number	Culture collection Date, number
5/01/98	128	6/08/98, 12	6/17/98, 12	6/25/98, 10	7/15/98, 13
7/27/98	55	8/24/98, 14	8/31/98, 10	9/09/98, 8	9/16/98, 15
8/13/98	52	9/30/98, 17			
9/02/98	26	10/15/98, 5	10/21/98, 4	10/27/98, 4	
9/08/98	48	10/15/98, 5	10/21/98, 9	10/27/98, 18	10/28/98, 6
9/15/98	36	10/28/98, 11	11/04/98, 18		
9/17/98	24	11/09/98, 6	11/11/98, 18		
9/21/98	50	11/09/98, 12	11/13/98, 18	11/16/98, 10	
9/22/98	53	11/16/98, 8			
9/23/98	47	discarded			
10/30/98	44	11/30/98, 12	12/02/98, 12	12/07/98, 10	
11/13/98	48	12/14/98, 10	12/21/98, 12	12/28/98, 16	
11/19/98	49	1/05/99, 4	1/06/99, 10	1/13/99, 10	
12/31/98	10	2/15/99, 10			
1/05/99	35	2/16/99, 12	2/22/99, 22		
1/15/99	19	2/26/99, 10	3/31/99, 9		
1/27/99	19	3/11/99, 12	3/31/99, 6		
1/27/99	7 (-KL)	3/03/99, 7			
2/08/99	33	3/24/99, 14	4/05/99, 19		
2/12/99	18	4/21/99, 17			
3/01/99	22	4/15/99, 18			
3/05/99	24	4/08/99, 6			
3/11/99 ^a	13	4/07/99, 13			
3/11/99 ^b	14	4/21/99, 14			
3/25/99 ^a	10	4/27/99, 10			
3/25/99 ^b	9	5/04/99, 9			
4/07/99 ^a	15	5/11/99, 15			

Table I continued

4/07/99 ^b	17	5/17/99, 17			
4/13/00 ^c	18	6/01/99, 18			
4/22/99 ^a	11	6/01/99, 11			
4/22/99 ^c	29	discarded			
4/27/99 ^a	15	6/01/99, 14			
4/27/99 ^c	13	6/15/99, 13			
5/11/99 ^a	13	6/17/99, 13			
5/11/00 ^c	14	discarded			
5/20/99 ^a	30	6/23/99, 30			
5/20/99 ^c	14	discarded			
5/27/99 ^a	21	6/28/99, 21			
5/27/99 ^c	9	discarded			
5/27/99 ^d	11	6/16/99, 11			
6/03/99 ^a	20	6/21/99, 20			
6/03/99 ^c	7	discarded			
6/03/99 ^d	11	7/08/99, 11			
6/08/99 ^a	27	7/21/99, 27			
6/16/99 ^d	64	7/06/99, 19	7/08/99, 11	7/21/99, 7	7/22/99, 16
		8/05/99, 13			
6/18/99 ^b	27	7/18/99, 27			
6/24/99 ^d	20	7/27/99, 15	8/05/99, 4		
6/30/99 ^d	35	8/10/99, 35			
7/06/99 ^d	78	7/21/99, 17			
7/08/99 ^d	29	7/26/99, 25	8/05/99, 2		
7/21/99 ^d	75	8/09/99, 15			
7/27/99 ^d	19 (-KL)	8/17/99, 17			
8/09/99 ^d	77	8/26/99, 14	9/01/99, 19	9/08/99, 17	
8/12/99 ^d	38	9/08/99, 2	9/13/99, 25		
8/19/99 ^d	19	9/08/99, 5	10/15/99, 13		
8/26/99 ^d	55	9/14/99, 14	9/23/99, 5		
9/08/99 ^d	39	9/28/99, 6	10/15/99, 26		
9/14/99 ^d	78	10/08/99, 8	10/21/99, 36		
9/22/99 ^d	41	10/15/99, 10	10/28/99, 14	10/28/99, 12	
9/23/99 ^d	40	10/28/99, 23	10/28/99, 6		
9/24/99 ^d	40 (-KL)	10/22/99, 40			
9/28/99 ^d	36	10/26/99, 11	11/03/99, 16	11/04/99, 8	
10/08/99 ^d	59	11/10/99, 14	11/11/99, 35		
10/15/99 ^d	57	11/10/99, 4	11/18/99, 23	11/29/99, 2	12/01/99, 17
10/20/99 ^b	29	11/18/99, 29			
10/26/99 ^d	58	11/17/99, 12	11/23/99, 12	12/01/99, 17	12/03/99, 16
11/03/99 ^d	77	12/03/99, 7	12/08/99, 14	12/14/99, 24*	
11/10/99 ^d	77	12/03/99, 6	12/14/99, 20	12/15/99, 13	
11/17/99 ^d	58	12/07/99, 20	12/10/99, 8	12/20/99, 27	

Table I continued

11/23/99 ^d	77	12/22/99, 13	12/27/99, 40	12/28/99, 24	
12/03/99 ^d	58	12/29/99, 13	1/05/00, 24	1/06/00, 13	
12/10/99 ^d	39	1/11/00, 29			
12/15/99 ^d	78	1/13/00, 10	1/19/00, 52	1/19/00, 4	
12/22/99 ^b	36	1/18/00, 36			
12/29/99 ^d	59	1/21/00, 14	1/25/00, 4	2/04/00, 25 [*]	
1/04/00 ^b	39	2/07/00, 39 [*]			
1/06/00 ^d	59	1/25/00, 6	1/28/00, 17	1/31/00, 11	2/07/00, 25
1/13/00 ^d	59	1/31/00, 9	2/04/00, 17	2/07/00, 8	2/18/00, 2
		2/21/00, 23 [*]			
1/21/00 ^d	84	2/11/00, 24	2/15/00, 16	2/20/00, 14	2/29/00, 14
1/28/00 ^d	87	2/18/00, 14	2/25/00, 7	2/27/00, 37	2/29/00, 28
2/04/00 ^d	78	3/13/00, 35 [*]			
2/11/00 ^d	79	3/03/00, 11	3/08/00, 18	3/21/00, 33 [*]	
2/18/00 ^d	79	3/22/00, 65 [*]	4/16/00, 3	4/24/00, 4	
2/25/00 ^c	5	discarded			
2/25/00 ^d	54	3/16/00, 10	3/28/00, 31 [*]	4/16/00, 4 [*]	4/24/00, 2
3/03/00 ^d	79	3/24/00, 7	4/02/00, 18	4/16/00, 35 [*]	4/24/00, 6
3/08/00 ^d	59	4/27/00, 4	4/28/00, 6	5/02/00, 37	
3/16/00 ^d	59	4/27/00, 4	5/10/00, 50 [*]		
3/24/00 ^d	39 (-KL)	4/03/00, 3	4/24/00, 23		
3/30/00 ^d	39	5/15/00, 39 [*]			
4/03/00 ^d	39 (-KL)	5/08/00, 39			
4/19/00 ^d	78	5/05/00, 26	5/15/00, 32		
5/04/00 ^d	78	5/31/00, 44			
5/11/00 ^d	58	6/06/00, 10	6/09/00, 34		
5/17/00	78	6/12/00, 52			
5/25/00	75	6/19/00, 48			
6/01/00	77	6/26/00, 70			
6/08/00	76	7/03/00, 49			
6/14/00	19 ²	7/18/00, 10			
6/15/00	77	7/10/00, 74			
6/22/00	97	7/17/00, 76	7/20/00, 20		
6/24/00	10 ³	7/13/00, 10			
	10 ⁴	8/02/00, 1			
6/29/00	78	7/24/00, 75			
7/06/00	74	7/31/00, 71			
7/13/00	40	8/17/00, 40			
	40 ³	8/18/00, 38			
7/20/00	79	8/21/00, 38	8/28/00, 6		
7/28/00	60	8/28/00, 60			
	41 ⁴	8/28/00, 24			
8/03/00	40 ⁵	9/05/00, 34			

Table I continued

8/24/00	80 ⁴	9/21/00, 4	9/25/00, 68		
8/31/00	80 ⁴	10/02/00, 60			
9/04/00	100 ⁴	10/16/00, 28			
9/07/00	100 ⁴	10/02/00, 23	10/11/00, 59		
9/21/00	100 ⁴	10/27/00, 64			
9/28/00	100 ⁴	11/06/00, 85	7/03/01, 20		
11/13/00	52	12/12/00, 35	12/14/00, 2		
	43 ⁴	12/11/00, 40			
11/24/00	59	12/27/00, 47			
12/09/00	60	1/06/01, 52			
12/10/00	60 ⁴	1/20/01, 20			
12/22/00	40 ⁴	2/04/01, 20			
12/24/00	60	2/04/01, 41			
2/28/01	60	4/02/01, 29	3/26/01, 6	4/10/01, 13	
3/02/01	40	4/04/01, 20	4/10/01, 18		
4/09/01	60	5/07/01, 34	5/21/01, 20		
4/13/01	60	5/09/01, 17	5/21/01, 32		
4/26/01	60	5/03/01, 36	5/23/01, 25	7/03/01, 20	
4/27/01	60	6/04/01, 23	5/31/01, 22		
5/10/01	80	6/09/01, 29	6/14/01, 32		
5/24/01	60	6/23/01, 26	6/30/01, 22		
5/29/01	60	6/23/01, 10	6/30/01, 35		
6/01/01	60	7/03/01, 7	7/10/01, 40		
6/28/01	120	7/16/01, 46	7/21/01, 26	7/28/01, 24	8/02/01, 8

* These cultures, where the kraft lignin was polymerized very effectively but subsequent depolymerization was marginal, were used for preparation of polymerized kraft lignin as the most appropriate substrate for lignin depolymerase assays.

¹-KL: Cultures without kraft lignin

² 20% change in phosphate (pH 7.17)

³ 30% change in phosphate (pH 7.25)

⁴ 50% change in phosphate (pH 7.42)

⁵ 10% increase in nitrogen content

^a Tc1a transferred from 1.5% malt extract agar on which it had been stored at room temperature.

^b Tc1b transferred from a dormant 1.5% malt extract solution in which it had been stored at room temperature.

^c *Trametes cingulata* newly supplied by ATCC (American Type Culture Collection)

^d Tc1a2 transferred from 1.5% malt extract agar on which it had been stored at room temperature.

Table II. Harvesting of Culture Solutions Inoculated with *Tc2*, *Tc3* and *Tc4* *T. cingulata* Sources.

Inoculation date	Number of flasks	Culture collection date, number	Culture collection date, number	Culture collection date, number	Source*
12/02/98	33	1/11/99, 12	1/13/99, 6	1/15/99, 4	<i>Tc2</i>
12/04/98	39	1/22/99, 12			<i>Tc2</i>
12/09/98	24	1/28/99, 20			<i>Tc2</i>
12/16/98	34	2/10/99, 29			<i>Tc2</i>
12/18/98	35	2/10/99, 27			<i>Tc2</i>
12/23/98	34	2/26/99, 23			<i>Tc2</i>
12/31/98	38	2/26/99, 19			<i>Tc2</i>
1/08/99 ¹	10	discarded			<i>Tc2</i>
1/12/99 ²	10	discarded			<i>Tc2</i>
1/27/99	10 (-KL)	3/03/99, 10			<i>Tc3</i>
2/02/99	18	discarded			<i>Tc2</i>
2/02/99	15	discarded			<i>Tc4</i>
2/22/99	24	discarded			<i>Tc4</i>

-KL Cultures without kraft lignin.

¹ The buffering capacity of culture solutions was increased 1.3-fold.

² The buffering capacity of culture solutions was increased 1.5-fold.

**Tc2* and *Tc3* *Trametes cingulata* sources that had been refrigerated on 1.5% malt extract agar in separate vials.

**Tc4* *T. cingulata* source that had been refrigerated on a 1.5% malt extract agar plug immersed in sterile water within a vial.

Isoelectric Focusing of T. cingulata Culture Solution

The *T. cingulata* solution cultures developed for expressing lignin depolymerase activity are different from those used by other investigators in that they include dissolved kraft lignin (*vide supra*: section 1). The presence of the kraft lignin is expected to induce lignin depolymerase expression, which is not likely to be constitutive, as has often been supposed in the past by other investigators. The task of purifying the extracellular enzymes in these cultures, as a result, turned out to be unexpectedly difficult: the proteins of interest interact very strongly with both the kraft lignin components (which are inevitably still present in smaller quantities in harvested culture solutions) and the white-rot fungal mycelium. The problem was finally solved by the straightforward, although extremely laborious, procedure of isoelectric focusing (IEF) in polyacrylamide gels followed by electro-elution of proteins from the appropriate gel segments. Isoelectric focusing, an electrophoretic technique, separates amphoteric molecules such as proteins from one another. A pH gradient is generated in an electric field across a polyacrylamide gel wherein proteins migrate toward the anode or the cathode until they reach the respective positions where their net charges are zero. These pH values are the corresponding isoelectric points (pIs) of the proteins being subjected to IEF. The concentrating effect of this technique is due to the continual tendency of the protein molecule to return to its pI in the face of opposing effects resulting from diffusion.

Initial studies used a pH range of 4.0 to 6.5, where ~1 mL (80 – 100-fold) concentrated culture solutions were applied onto the cathode side of the Ampholine gel. Much of the kraft lignin, being negatively charged, gave rise to blurred features in the pattern resulting from IEF, but silver staining (*Electrophoresis* **1985**, 6, 427-448; *Anal. Biochem.* **1982**, 119, 325-329) revealed a number of darker putative protein bands characteristic of the culture solution that were not observed in the lanes containing kraft lignin alone. Two (unstained) gel regions embodying these stronger bands (pI ~4.9 and pI ~5.9, respectively) were excised and refocused on a new IEF polyacrylamide gel in a pH 4.0 – 6.5 gradient. The results from the second IEF gel indicated that these bands were indeed probably proteins but there was still considerable kraft lignin contamination. The most prominent bands evident after silver staining were located in the region between pH 4.8 and 6.5, but it should be borne in mind that the presence of the kraft lignin could have significant effects of the electrophoretic pattern of even some of the standard marker proteins.

Kraft lignin components, due to their inherent negative charges, always migrate from the cathode to the anode side of the Ampholine gel during IEF, which causes a smear along the entire length of the lane in the gel. This has interfered with the protein patterns being examined. To avert such a problem associated with the kraft lignin, which is still present in smaller quantity than in the original culture solution, during IEF the culture solution was applied onto the anode side of the gel. If the pH is sufficiently low, this would allow the proteins, which are expected to be positively charged under such conditions, to enter the gel and migrate toward the cathode to their respective isoelectric points, while the lignin moves still further toward the anode. Thus the interference from kraft lignin components on the electrophoretic protein patterns could be minimized. Moreover, in employing IEF for the purpose of separating proteins by their respective isoelectric points, running conditions were developed to produce highly focused protein bands which separate from one another reasonably well. The most favorable IEF running conditions discovered were as follows. About 1 mL (80–100-fold) concentrated culture solution was first loaded onto a polyacrylamide gel in a pH 4.0 – 6.5 (or pH 3.5 – 9.5) Ampholine gradient, which was subjected to 1 watt (constant) at 200 volts (constant 22 volts cm⁻¹) for two hours. The voltage was then set at 350 while the power remained steady during the overnight period (14 – 16 h). The gel was next run with increased power at 600 volts for one hour, which was then changed to 1,000 volts for the next hour; for the subsequent ½ hour the voltage was set at 1,200 and for the final ½ hour the gel was run at 1,500 volts. These extended running conditions allow dissociation of kraft lignin components from the proteins to occur almost completely and produce IEF gels that contain highly focused protein bands and thus greatly aid in the isolation of the desired enzyme.

There was a concern over operationally irreversible binding between carrier ampholytes and the proteins during IEF which would engender difficulties in amino acid sequencing of the protein of interest. Consequently, attention was also directed to IEF in Immobiline gels (pH 4.0 – 6.5) where the buffering groups responsible for creating the pH gradients are covalently attached to the polyacrylamide gel matrix. The pH in such gels are created by a continuous change in the ratio of acidic to basic Immobilines which is effected by mixing two polymerizing solutions from a gradient maker (*J. Biochem. Biophys. Methods* **1982**, 6, 317-339). In this context, ~1mL (80 – 100-fold concentrated acetate buffer exchanged) *T. cingulata* culture solution alongside pI standards were applied onto the anode side of the gel; electrophoresis was carried out by first allowing sample entrance into the gel at 500 volts and 5 watts overnight and increasing the

voltage to 3500 volts for ~8 hours. As in the case of IEF using Ampholines, the lignin migrated toward the anode and away from the interior of the gel, whereas the proteins moved to their respective isoelectric points. Using this reproducible technique 13 bands were visualized after silver staining, of which 6 bands were more prominent with the pI's ranging from 5.0 to 6.2 while 7 bands were much fainter. This technique appears to be an effective tool to separate kraft lignin components from the proteins and the proteins from each other. Nevertheless, the methodology routinely adopted for isolating proteins from *T. cingulata* culture and wash solutions was electrofocusing in Ampholine (pH 4.0 – 6.5) gels which were run in the appropriate manner to optimize focusing and separation of the proteins. This was followed by electro-elution of the appropriate protein bands as described in the forthcoming section.

Electro-Elution of Protein Bands

After IEF to separate the proteins in (~1 mL 80 – 100-fold concentrated) solutions from lignin-induced *T. cingulata* cultures, as described above, the sample lanes located at the two outer edges of the gel were silver stained to allow visualization of the protein bands. The intervening lanes were first divided into 14 segments, each embodying a narrow pH range containing a possible protein band(s). Gel slices containing the protein bands of interest were obtained by cutting out the appropriate segments from the gel; these were subjected to electro-elution in 0.03 M Tris phosphate at pH 7.0 or 7.2 in a Centrilutor micro-electroelutor (Amicon). Thus about 7 gel slices were arranged vertically, for efficient elution, within each of nine 0.5 mL microcentrifuge tubes, into which narrow-bore holes had been inserted at the top lid and the bottom to allow current flow and protein migration. Each microcentrifuge tube containing gel slices was positioned in a Centricon-10 microconcentrator (Amicon), above the 10,000 nominal molecular weight cutoff (YM10) ultrafiltration membrane held within the unit, and the entire assembly was placed into the micro-electroelutor between the upper and the lower solution chambers, which were then filled with 0.03 M Tris phosphate buffer.

Figure 1 illustrates the electro-elution apparatus employed and its components. Several aspects of the electro-elution protocol that were implemented proved essential to the successful isolation of the enzymes and the preservation of their activity. Before electro-elution, each Centricon-10 microconcentrator was made passive to minimize protein adsorption by treatment with 5% polyethylene glycol (PEG) overnight followed by thorough rinsing with distilled water, washing by centrifugation (3,200 x *g* for 35 min.) and removal of any residual solution from the tubes. The washing procedure was repeated once and the tubes were ready for electro-elution. To prevent possible protein denaturation engendered by any extensive pH changes in the upper and lower chambers of the apparatus, the solutions in the chambers were changed several times (every 35 min.) during the course of the electro-elution period (a total of 140 min.). Any bubbles forming beneath the YM10 membranes and inside the microcentrifuge tubes containing the gel slices were also removed to prevent disruption of the current flow and migration of proteins from the gel slices into the Centricon-10 devices. In addition, as a precautionary measure, the current (~30 mA) was reversed at the end of the procedure for 3 min., which is a short enough time to allow proteins to become free of the YM10 membrane without risking their exit from the tubes into the upper chamber where they would be lost. All these measures have been shown to increase the yields of proteins purified from *T. cingulata* cultures.

The electro-eluted solutions were concentrated and subjected to buffer-exchange (0.02 M acetate, pH 4.5) using the same centrifugal filter (Centricon-10) devices. In this way, small quantities of

protein samples in solution were recovered from particular polyacrylamide gel segments. These equilibrated solutions were concentrated to small volumes (the final total volume being 2 – 3 mL). A portion (70 μ L) of the resulting solution was loaded onto IEF gels (pH 4.0 – 6.5) in sample wells on the anode side to analyze the protein components in the eluents. The results after silver staining showed that such an electro-elution protocol leads to a substantial level of separation of the proteins from the kraft lignin components, and most gel segments had released more than one protein band on the basis of the IEF results.

In order to identify which electro-eluates contained lignin depolymerase activity, kraft lignin and the respective protein samples obtained from the Micro-Electroelutor were initially incubated after buffer exchange together in solution having the same composition as the culture medium, but lacking glucose, under ambient conditions. Thereupon IEF (pH 3.5 – 9.5) and size exclusion chromatography (SEC) were employed to analyze the resulting changes in the kraft lignin components. IEF analysis yielded a distinctive lignin pattern that appeared in the presence of the protein sample corresponding to the gel segment with apparent pI $\sim$ 5.2 as more “low-pI” lignin components were formed in this case in comparison with other samples or the kraft lignin

Figure 1. Schematic diagram of the Centrilitur Micro-Electroelutor (Amicon) used to electro-elute lignin depolymerase from Ampholine gel segments.

control. SEC of the kraft lignin components containing the same protein sample showed a higher proportion of lower molecular weight components compared to the control, confirming that ligninolytic activity was indeed present in this gel segment.

While SEC and IEF were primarily employed to analyze how the kraft lignin was modified after incubation with the protein solutions obtained after electro-elution and acetate buffer exchange, an alternative approach based on ultrafiltration was developed to monitor changes in the concentrations of low molecular weight kraft lignin components as a result of enzymatic degradation. Typically a 0.1 mL portion of the assay solution containing a particular electro-eluent and 0.55 gL⁻¹ kraft lignin was diluted to 2.0 mL with 0.10 M aqueous NaOH (to dissociate the huge kraft lignin complexes that are formed spontaneously at lower pH's) and transferred into a centrifugal concentrator incorporating a 10,000 nominal molecular weight cutoff (Amicon YM10) membrane which was then subjected to 3,200 x g for 30 min. The kraft lignin components passing through the membrane into a collection tube were monitored through their absorbance at 280 nm (A₂₈₀), which would increase when degradation had occurred and decrease when polymerization had taken place. This approach only succeeded in identifying lignin polymerase activity as a 26% reduction in A₂₈₀ after 10-day incubation in the presence of the protein(s), which was readily amenable to confirmation by SEC.

As described previously (*vide supra*: section 2), *Tc2* exhibited quite different characteristics from *Tc1* (the original *T. cingulata* source) during growth and development, and the culture solution from *Tc2* had fewer dissolved proteins than did that from *Tc1*. The *Tc2* culture solution did become lighter in color during the course of fungal growth, which was believed to be a combined result of lignin depolymerase activity and precipitation/adsorption of kraft lignin onto the hyphae (*vide supra*: section 2). Owing to the increased likelihood of identifying lignin depolymerase if it were present among the smaller complement of proteins, the more prominent bands in the *Tc2* culture solution observed after IEF were electro-eluted and proteins from four gel segments encompassing different pH ranges (containing proteins with pI ~4.6, ~4.85, 5.25 and 5.6) were isolated. IEF of the electro-eluted components indicated that such an approach successfully yielded proteins from each gel segment. A portion of each electro-eluent with its dissolved proteins was incubated with the original kraft lignin as a plausible substrate, which was then analyzed by SEC over appropriate time intervals (2 – 6 days). Among proteins in these four pI regions, those possessing a pI of ~4.85 bore some ligninolytic activity but the result needed to be confirmed as the extent of depolymerization was quite small.

Gel-Free Isoelectric Focusing

The predominant method that had been employed for purifying the lignin depolymerase isoenzymes was isoelectric focusing (IEF) in polyacrylamide gels followed by electro-elution (*vide supra*), which was a laborious process and sizable losses of active enzyme during such a purification protocol was always a concern. Alternative approaches to purification had always been sought. A new means of enzyme isolation has more recently been provided by a gel-free Rainin RF3 unit at the National Renewable Energy Laboratory (NREL). The RF3 is designed for preparative protein purification by isoelectric focusing. The flow path consists of a closed-loop circuit of 30 parallel channels between the (high pI) cathode and (low pI region) anode connected to an in-line focusing cell. A 30 channel peristaltic pump recirculates the sample solution rapidly through the loops and as the solution passes through the focusing cell it is exposed to an electric field perpendicular to the flow. During each pass through the focusing

cell, the proteins move from channel to channel as they migrate incrementally toward their isoelectric points. After each pass, the solution is cooled by a heat exchanger. When focusing is complete, the flow path is diverted and the solutions in the 30 lines of tubing are collected. Such a gel-free IEF apparatus offers several advantages over IEF in polyacrylamide gels. First of all, much larger sample quantities (100 mL) can be loaded into the instrument for a single run, whereas only ~1 mL can be used with a typical polyacrylamide gel. Secondly, the instrument separates proteins into 30 fractions, all of which can be subsequently concentrated and subjected to buffer-exchange, if necessary at the same time, using centrifugal filter devices. Therefore, a considerable effort has more recently been devoted to optimizing the running conditions for separating the proteins in *T. cingulata* culture solutions by means of this recycling free-flow IEF system when the RF3 apparatus became available for protein purification purposes.

The pH gradient in the apparatus was strongly influenced by the solutions employed in the anode and cathode compartments. Phosphoric acid was first used in the anode compartment of the apparatus, which produced a low pH close to 3.0 in channel 30 (near the anode) within just a few hours of running time. Ordinarily the kraft lignin components in the (10-fold) concentrated *T. cingulata* culture solution being electrofocused would have precipitated under these conditions, but at NREL it had been discovered that 8 M urea effectively prevents this from occurring. The retention of the kraft lignin in solution is a prerequisite for the successful electrofocusing and subsequent isolation of the extracellular enzymes from *T. cingulata* cultures; this is particularly relevant for those lignin-degrading proteins that tend to interact with the kraft lignin components at the molecular level. The effect of 8 M urea in preventing kraft lignin precipitation was remarkable, but at the same time such conditions would tend to bring about protein denaturation, which is highly undesirable as far as any subsequent lignin depolymerase assays are concerned.

Consequently, in an attempt to obviate the need for 8 M urea, acetate buffer with a pH of 4.0 was introduced into the anode compartment of the Rainin RF3 unit, but this engendered an even lower pH in the channels close to the anode, channel 30 reaching a pH below 2.5 after only 2 hours! Yet, when a pH 4.0 solution containing 0.2 M gly-gly and 0.2 M MES was introduced into the anode compartment, with aqueous 0.1 M NaOH being employed at the cathode, kraft lignin precipitation did not occur even after a 44 hour electrofocusing period at 200 volts. (However, some of the channels developed a faint blue tint, the reason for which remains to be determined.) Nevertheless, the pH of the solution in the anode compartment still ultimately dropped sharply to 1.3 during the course of electrofocusing, even when IEF had been performed for shorter periods of time (23 h) after the same culture solution had been freshly loaded into the apparatus. The identification of (buffer) solutions, the pHs of which would remain reasonably stable in the anode compartment of the Rainin RF3 unit during extended electrofocusing time periods, is clearly an important goal. It is anticipated that, once proper anode solutions have been developed, substantially improved yields of active lignin depolymerase isozymes could be secured within a short period of time.

Three sets of fractions obtained from the solution channels of the Rainin RF3 gel-free IEF unit were analyzed. The first of these resulted from an experiment where 8 M urea was employed to prevent kraft lignin precipitation. The second was derived from a corresponding experiment without urea, where kraft lignin precipitation occurred in some of the channels close to the anode when the pH fell below 4.0. The third set was generated from a Rainin RF3 run in which kraft lignin precipitation was avoided by the introduction of 0.2 M gly-gly/0.2 M MES solution into the anode compartment, with aqueous 0.1 M NaOH being employed at the cathode.

To identify the proteins present in the fractions, isoelectric focusing (IEF) with polyacrylamide gels was used. Most attention in this regard was directed toward the fractions with pH values between 4.0 and 5.0 (which encompass the range of the lower-pI lignin depolymerase isozyme) that were produced when no kraft lignin precipitation occurred. In the set generated in the presence of 8 *M* urea, three bands were observed through silver staining in fractions 16 – 23 (pH 5.97 – 4.94) with pIs 4.6, 4.8 and 5.1; the band with the highest pI (5.1) was absent from fractions 24 – 30, which were closer to the anode. Similar patterns were obtained when broad range (pH 3.5 – 9.5) IEF was carried out. These protein bands included one with a pI remarkably close to that of the lower-pI lignin depolymerase isozyme. Two putative protein bands, on the other hand, were detected by SDS-PAGE in fractions spanning a pH-range between 4.5 and 5.0. These two bands were quite diffuse and exhibited molecular weights in the 21 – 31 kDa range, significantly lower than what had been previously observed for lignin depolymerase. Thus these results need to be checked by both IEF and SDS-PAGE after the fractions in question have been subjected to buffer-exchange and further concentrated.

In marked contrast, no bands were detected by IEF in polyacrylamide gels among the fractions from the experiment where kraft lignin precipitation had been prevented without using 8 *M* urea; in this regard the findings were the same whether IEF had been performed over a narrow (pH 4.0 – 6.5) or broad (pH 3.5 – 9.5) range. Such a result was surprising in view of what was obtained with the fractions produced in the presence of urea. It is conceivable that the urea was capable of facilitating the release of proteins from protein-lignin complexes, but whether protein denaturation is an obligatory part of the process is not known at the time of writing. If it is not, a chemical component, which has the same effect upon protein-lignin complexes as urea without denaturing the protein in the process, could play an important role in lignin depolymerase purification. Since none of the fractions from this RF3 run, where kraft lignin precipitation had been prevented without using 8 *M* urea, had exhibited any bands after being subjected to IEF in polyacrylamide gels, it was considered desirable to concentrate them further before analyzing them through SDS-PAGE. Accordingly six fractions (21 – 26 with pHs between 5.41 and 4.10) were concentrated 5-fold by precipitation with acetone followed by a brief wash of the solid with aqueous 80% acetone. After air-drying, the resulting pellets were redissolved in 0.02 *M* acetate at pH 4.5 and subjected to SDS-PAGE (using a 4% stacking and 10% resolving gel). The widths of the lanes in which these fractions had been loaded broadened substantially during SDS-PAGE, and no protein bands could be detected. Presumably solute components other than just proteins had been precipitated out of solution by the acetone, and these in turn would have been present in high concentrations in the solutions subsequently loaded onto the respective SDS-PAGE lanes; thereupon lateral diffusion may have been responsible for the observed effects. To investigate this possibility further, the same set of fractions from the RF3 unit were subjected to 0.02 *M* acetate buffer (pH 4.50) exchange and subsequent concentration using pacified centrifugal filter devices before analyzing them through SDS-PAGE and IEF. However, there were still no protein bands that could be detected through silver staining.

Chromatofocusing

Chromatofocusing is a column technique that separates proteins according to their isoelectric points (pI). A pH gradient is formed by the buffering action of the positively charged groups on an ion exchange resin toward a lower pH eluent buffer passing through the column which is initially at higher pH. Under such conditions, proteins with different pIs will migrate to different

extents through the column. Protein molecules above their pIs in the gradient are negatively charged and bind to the gel, while those below their pIs are repelled from the column. As the lower pH eluent buffer passes through the column, the protein is carried with it until the pH rises above the pI and it rebinds. This principle governing the interactions with the ion-exchange gel is maintained until the proteins emerge from the column at their respective pIs. One major advantage of chromatofocusing arises from the fact that the process focuses the proteins to their pIs and concentrates them as they are eluted from the column.

Typically, a concentrated portion of culture solution (9 – 15 mL 20-fold) was first equilibrated with the starting buffer (0.025 M imidazole-HCl, pH 7.4) and then applied to the chromatofocusing column packed with Polybuffer exchangers (PBE 94, Pharmacia), which had been equilibrated with the same starting buffer also. The proteins were then eluted with ~300 mL (8-fold diluted) Polybuffer 74 (Pharmacia) at pH 4.0 whereupon the total eluent was collected, concentrated (50mL) and equilibrated with 10 – 15 volumes of acetate buffer (0.02 M, pH 4.5) before final concentration to between 150- and 200-fold was performed. When the pH of the eluent emerging from the column matched that of the solution (pH 4.0) entering the column, it signaled the end of the chromatofocusing process. The elution profile (A_{280}) indicated the presence of possible protein peaks but lignin components also absorb strongly at 280 nm. Thus to confirm the presence of proteins in the eluent, isoelectric focusing (IEF) was employed. Typically a 50 μ L portion was subjected to IEF (by loading onto the anode side of the polyacrylamide gel) in a pH 4.0 – 6.5 Ampholine gradient. The results of silver staining after IEF suggested that the majority of the kraft lignin was remaining on the column while the protein bands appeared to mirror the pattern of those in the original culture solution. It appears that the Polybuffer employed facilitates the dissociation of the lignin components from the proteins so that the latter elute at their respective pIs.

Nevertheless, in view of the strong interactions prevailing between lignin depolymerase and the kraft lignin substrate, after chromatofocusing the culture solution as described above, the column was eluted with aqueous 1 M NaCl in an attempt to elute any proteins from the exchange resin-bound kraft lignin. The eluent salt solution was equilibrated with acetate buffer (0.02 M at pH 4.5), concentrated 100-fold and analyzed by IEF. The resulting silver-stained gel revealed similar protein patterns to the chromatofocusing eluent solution, but with a number of exceptions: certain bands were absent in the salt eluent but present in the chromatofocusing eluent and *vice versa*. UV-visible spectral analysis suggested that the chromatofocusing eluent was nearly devoid of kraft lignin contamination while the IEF patterns of the salt eluent from the chromatofocusing column indicated that the lignin was present. This underscored the fact that strong interactions existed between the protein(s) and kraft lignin substrate.

Chromatofocusing had been expected readily to effectuate lignin depolymerase purification, but the method has been unsuccessful in this respect. However, chromatofocusing did facilitate separation of at least nine culture solution proteins (14 – 75 kDa in apparent molecular weight, visible as silver-stained bands after SDS-PAGE) from the bulk of the kraft lignin components that were present. Moreover, this initial removal of the lignin from the culture solution would be essential for the next step of separating and fractionating the proteins from one another. It was later determined that a second chromatofocusing step was necessary. To secure enough of the protein that had been purified from kraft lignin for a second step, three to five separate initial chromatofocusing experiments were performed. The concentrated eluents (in starting buffer) from this first chromatofocusing step were pooled and applied to the second chromatofocusing

column. Fractions (17 – 25) were collected (6 – 10 mL each) and the pH of each was determined to establish the stage that had been reached in chromatofocusing as the respective samples eluted out of the column. The elution profile was monitored at A_{280} with an ISCO V⁴ detector. The resulting fractions were concentrated and subjected to buffer-exchange with 0.02 M acetate (pH 4.5) by ultrafiltration through a 10,000 nominal molecular weight cutoff (Amicon PM10) membrane. Rechromatofocusing of these proteins yielded fractions containing subsets of the same proteins, but in the vast majority of cases many electrophoretic bands (rather than just one) were found to be present in each fraction. Assays of the fractions encompassing the ranges of the two lignin depolymerase isozymes with high molecular weight polymerized kraft lignin components (*vide infra*: section 9) yielded no indication of lignin depolymerization through size-exclusion chromatography.

In addition to the rechromatofocusing described above, the proteins in the eluted fractions obtained from chromatofocusing were also further purified by electro-elution from Ampholine gel segments after IEF. The obvious advantage of this approach is that the apparent pI values of the proteins should no longer be affected by the presence of kraft lignin components, which had been largely removed during chromatofocusing as described above. Thus three such proteins with pI's of 5.0, ~5.2 and ~5.7, which all appeared to have been induced by the presence of kraft lignin in the culture solutions, were obtained by election-elution from the respective Ampholine gel segments. Further isoelectric focusing of each of the separate electro-eluted samples revealed one prominent band and some minor bands that had been co-isolated due to their proximity to the preponderant protein. However, lignin depolymerase assays of the three isolated proteins using the polymerized kraft lignin as the substrate did not yield detectable activity.

Size-Exclusion Chromatographic Separation of Proteins

While chromatofocusing has been used with reasonable success to remove kraft lignin components from culture solutions, there were other alternatives for separating the proteins from one another. Accordingly a technique that separates proteins on a different physicochemical basis from chromatofocusing may be advantageous for producing fractions with altered distributions of proteins. Such changes in the identities of the proteins in each fraction may facilitate the purification of lignin depolymerase by avoiding the problems of co-isolation with proteases and/or polymerases. This is important because the proteases may degrade lignin depolymerase even in the presence of protease inhibitors and the polymerases will act on any kraft lignin components that have not already been polymerized, causing the SEC profile to indicate, incorrectly, that no lignin depolymerase activity is present.

Thus, in work devoted to separating the proteins in the culture solution by molecular size, kraft lignin was first removed by chromatofocusing from five different concentrated culture solutions that had been subjected to buffer exchange with 10 volumes of 0.02 M acetate (pH 4.5), and the eluent was analyzed size exclusion chromatographically (SEC). For this purpose a 1.5 cm x 60 cm column was packed with Sephadex G-150 (Pharmacia) which has a formal separation range for globular proteins of 5 to 150 kDa. Phosphate buffer (0.012 M, pH 7.0) was used as the eluent. Combined chromatofocusing eluent solution (2.0 mL) was loaded onto the SEC column and 11 fractions (6 – 9 mL) were subsequently collected, concentrated and subjected to buffer exchange with 0.02 M acetate. The proteins in these fractions were visualized by silver staining of both Ampholine IEF (pH 4.0 - 6.5) and SDS-PAGE (12% resolving) gels. Of the 11 fractions

collected, only 2 through 7 contained proteins, displaying between 1 and 6 strong bands and a varying number of faint bands. Results from SDS-PAGE confirmed that the proteins had been separated according to their molecular weights. There appeared to be a discrepancy between the results from the SDS-PAGE and IEF gels in regard to the number of protein bands in each fraction. This may be due to a difference in staining sensitivity between the two gels or to an apparent coincidence in molecular weights for many of the proteins that would not be evident under IEF conditions.

Fractions 2 – 6 were analyzed for lignin depolymerase activity by incubating each of them (after concentration and buffer exchange) with kraft lignin in 0.02 *M* acetate. Subsequent SEC indicated that fraction 2 exhibited slight lignin polymerase activity while fractions 3 and 4 produced more significant polymerization of the kraft lignin substrate. The proteins in fraction 5 did not engender any noticeable changes in the molecular weight distribution of the kraft lignin components. On the other hand, fraction 6, which contained four strong and a number of weak protein bands, brought about a perceptible decrease in the leading SEC peak height, suggesting the presence of lignin depolymerase activity. However, this result was not sustained when high molecular weight polymerized kraft lignin components were later used as the substrate and this avenue of investigation was discontinued.

Cation-Exchange Chromatography of Culture Solution

An ion exchange gel consists of a stationary matrix to which charged groups have been covalently bound. The charged groups are associated with mobile counterions which can be reversibly exchanged with other ions of the same charge. Ion exchange has been widely used for separating closely similar biological macromolecules. The principle of the separation mechanism lies in the differences between the charges carried by the macromolecules. Most experiments are carried out in two stages. The first stage involves sample application and adsorption whereby unbound substances are washed out from the matrix with a start buffer. In the second stage, bound substances are eluted from the column in an order based on their different affinities for the ion exchange matrix, which is determined by the magnitudes of their charges. Thus weakly bound molecules would be eluted first and more strongly bound molecules later, especially after the counterion concentration has been increased. Due to the inherent net charges determined by their unique amino acid sequences, proteins, under particular solution conditions, can be isolated and separated from one another using this approach. For the purposes of purifying lignin depolymerase from culture solutions, cation exchange chromatography was employed. The pH of the sample and mobile phase in the column containing SP (sulphopropyl) Sephadex C25 was adjusted to pH 4.0 where most proteins become positively charged but kraft lignin components would still bear weakly negative charges. Under these conditions it would be expected that lignin components could simply pass through the column while the positively charged proteins would become adsorbed onto the cation exchange resin. To the head of a column (15 x 300 mm) containing SP Sephadex C25 equilibrated with acetate buffer (0.02 *M*) at pH 4.0, 20-fold concentrated culture solution (5 – 10 mL) was applied. The lignin in the culture solution was eluted from the column quite readily in 0.02 *M* acetate at pH 4.0, as was confirmed visually (brown-colored eluent) and in the A₂₈₀ elution profile. When kraft lignin elution was complete, the eluent was changed to 0.01 *M* sodium acetate containing 0.01 *M* NaCl at pH 7.5; under such conditions, the proteins of interest became negatively charged and thus began to be eluted from the column. The eluent fractions corresponding to the

peaks observed in the elution profile were collected. It should be noted that the pH of the eluent here had been carefully optimized. Three fractions were thus obtained, concentrated and exchanged with 0.02 M acetate buffer at pH 4.5; the resulting solutions were analyzed by applying them onto the anode side of an Ampholine IEF gel (pH 4.0 to 6.5). Silver staining revealed that the third fraction contained most of the proteins and exhibited a protein pattern very similar to that of the culture solution itself. However there was still some lignin contamination, as revealed by UV spectral analysis of the solution and silver staining of the IEF gel. These results suggested, on the one hand, that another viable technique had been devised for removing the majority of the kraft lignin components from the culture solution as a prelude to the complete purification of the lignin depolymerase(s). On the other, the interaction between the kraft lignin components and proteins was indeed very strong, as has repeatedly been observed under a variety of experimental conditions. A second chromatographic technique would probably need to be used in combination with the foregoing approach for complete separation of the proteins in culture solution from one another and from residual kraft lignin components.

Thus, fractions containing proteins eluted from the SP Sephadex C25 column were loaded onto the more weakly charged cation-exchange CM (carboxymethyl) Sephadex C50 column in an attempt to bring about further protein separation by allowing a slower rate in the change of pH during elution. Conceivably such an effect could engender better separation of the culture solution proteins from one another. After loading, the column was first equilibrated and eluted with 0.02 M acetate buffer (pH 4.0) to remove any remaining lignin components from the sample. Then the mobile phase was changed to 0.2 M sodium acetate containing 0.2 M NaCl at pH 7.5 or 8.0, whereupon the eluent from the column was monitored to detect putative protein fractions emerging from the column. However, there were no separate peaks visible in the profile, presumably because the elution conditions were difficult to optimize on account of two problems: the pH of the eluent was changing very slowly, leading to more diffuse protein bands during elution, and the lignin components which were still in the protein fractions from the SP Sephadex C25 column might have affected column resolution due to competition for binding with the weak cation-exchange sites. For this technique to be useful in isolating lignin depolymerase(s), an alternative method will be necessary after the initial chromatographic fractionation.

4. SDS PAGE Analysis of Culture Solution

Analytical electrophoresis of proteins according to size is typically carried out in polyacrylamide gels under conditions where proteins are dissociated into their polypeptide subunits and denatured. Most commonly, the strong anionic detergent SDS (sodium dodecyl sulfate) is used along with a reducing agent (dithiothreitol or β -mercaptoethanol) and heat to denature the proteins before they are loaded onto the gel. The denatured proteins bind to SDS and become overwhelmingly negatively charged regardless of their sequences. Thus SDS polyacrylamide gel electrophoresis (SDS-PAGE) separates proteins according to their molecular weights regardless of their inherent charges. To analyze the extracellular proteins produced by *T. cingulata* grown in media containing kraft lignin, culture solutions exhibiting lignin depolymerase activity were subjected to SDS-PAGE (employing a 4% stacking and 10% resolving gel) after buffer-exchange with 0.02 M acetate buffer at pH 4.5 (*vide supra*: section 3). As in the case when the culture solutions were loaded onto the cathode side of the gel during isoelectric focusing (IEF) (*vide supra*: section 3), the kraft lignin components which were still unavoidably present in small

quantities caused pronounced brown smears along the entire lengths of the gel lanes. This interferes with the protein patterns being examined. Moreover, kraft lignin as a control was also applied to the gel; it was strongly stained when employing either Coomassie blue or silver staining procedures. However, after some careful experimentation with various SDS-PAGE conditions and sample preparations, it was found that three or four darker putative protein bands were reproducibly evident over the more faintly stained uniform lignin background when using 7 to 10% resolving gels. All the bands were in the size range between 30 and 65 kDa. A putative lignin-protein complex was later obtained by acetone precipitation from a 5-fold concentrated culture solution. The complex was redissolved in sample buffer and then subjected to SDS-PAGE. The result revealed three prominent bands upon silver staining of the electrophoretic gel.

SDS-PAGE (employing a 10% resolving gel) was also employed to analyze the proteins in the eluents obtained from the chromatofocusing of culture solutions and the fractions in eluents resulting from rechromatofocusing (*vide supra*: section 3). There were nine bands (14 – 75 kDa) visualized by silver staining in the chromatofocusing eluents, and fractions resulting from rechromatofocusing contained subsets of these same protein bands. Thus this confirmed the results from IEF of the chromatofocusing eluents (*vide supra*: section 3) indicating that effective protein separation could not be obtained by chromatofocusing under the conditions employed. SDS-PAGE was also used routinely to determine the molecular weights of the purified proteins. It was found that the lignin depolymerase isoenzymes with apparent pI's of ~4.9 and ~5.8, that had been successfully isolated by electro-elution from electrofocused gels (*vide infra*: section 5), both exhibited identical molecular weights of 35 kDa when subjected to SDS-PAGE. The apparent molecular weight of these two lignin depolymerase isozymes was far below that of laccases, affirming that these two isoenzymes had not been previously implicated in lignin depolymerization *in vivo*.

5. Isolation of Lignin Depolymerase Isoenzymes by Isoelectric Focusing and Electro-Elution

Typically 3 – 5 weeks after inoculation, when the polymerized kraft lignin (formed as a result of the laccase activity in culture solution) was being degraded by the lignin depolymerase in the *T. cingulata* cultures (as indicated by the lightening of the color in the medium from dark brown to light yellow), the cultures were harvested for enzyme isolation (*vide supra*: section 3). Two proteins (with apparent pI's of ~5.1 and ~5.8), which appeared to have been induced by the presence of kraft lignin in the *T. cingulata* cultures (*vide infra*: section 6), and two others (with apparent pI's of ~4.9 and ~5.4), chosen on account of their overall prominence in the cell-free culture solutions, were selected for isolation by isoelectric focusing (IEF) and subsequent electro-elution from the appropriate polyacrylamide gel segments (*vide supra*: section 3). Silver-staining after IEF (in Immobiline gradients) of the respective electro-eluates indicated that single proteins could be isolated from each gel segment, albeit in low yields. After concentration and buffer exchange (0.02 M acetate at pH 4.5), portions of three of the four electro-eluted samples containing these individual proteins from *T. cingulata* culture solutions were assayed for lignin depolymerase activity by introducing a standard high molecular weight polymerized kraft lignin fraction in 0.02 M acetate buffer (*vide infra*: section 10). Size-exclusion chromatographic (SEC) profiles indicated that both of the proteins with apparent pI's of 4.9 and 5.4 bore weak lignin depolymerase activity. However, significantly stronger lignin depolymerase activity was established for the protein with an apparent pI of 5.8 on the basis of a decrease in the leading high molecular weight kraft lignin peak in the SEC profiles. The likelihood of more than one

lignin depolymerase isoenzyme being produced by *T. cingulata* is supported by the tendency of white-rot fungi to excrete several isoenzymes of different extracellular proteins such as the lignin peroxidases. Moreover, altered glycosylation patterns of a single protein could produce what might appear to be a number of distinct proteins with very similar catalytic properties.

It was later confirmed that the proteins with apparent pI's of ~4.9 and ~5.8 were indeed lignin depolymerase isozymes. These two isozymes, differing by 0.9 units in pI, exhibited identical molecular weights of 35 kDa (on the basis of SDS-PAGE using 4% stacking and 12% resolving gels, *vide supra*: section 4), far below the range characteristic of laccases. The incubation of these two lignin depolymerase isozymes separately in 0.02 M acetate at pH 4.5 engendered substantial degradation of high molecular weight polymerized kraft lignin components (*vide infra*: section 10) in homogeneous solution in the complete absence of a cofactor or mediator (Figures 2 and 3). The degradation observed in the absence of the isoenzymes was insignificant. Such results, which were established size-exclusion chromatographically by elution through Sephadex G100 in aqueous 0.10 M NaOH, affirmed that these isoenzymes were probably responsible for bringing about lignin depolymerization *in vivo* even though they had not been previously implicated in such processes. Moreover, preliminary studies on the ability of the purified lignin depolymerase isozymes to reduce the kappa number of softwood kraft pulp brownstock (*vide infra*: section 10) indicated they could indeed bring about delignification in this heterogeneous system also. This finding was substantiated by kappa number determinations on pulp samples extracted with aqueous 0.4% NaOH before and after incubation with the respective isoenzyme in 0.02 M acetate buffer under sterile conditions at pH 4.5, but these observations require confirmation with higher levels of active enzyme. Such experiments were postponed, however, in favor of producing as much of the purified lignin depolymerase isoenzymes as possible for characterization and sequencing purposes.

Now that it had been demonstrated that at least two lignin depolymerase isoenzymes are present in *T. cingulata* culture solutions and a protocol for protein isolation by IEF and electro-elution had been devised, all efforts were focused on producing enough of the enzyme to obtain sufficient N-terminal and internal amino acid sequence information, a prerequisite for identifying the genes encoding the proteins. For the purposes of purifying the lignin depolymerases from *T. cingulata* culture solutions using electro-elution, which had proven to be effective, the segments containing each of the two lignin depolymerase isozymes were sectioned from the polyacrylamide gel after IEF (*vide supra*: section 3). The remaining three sections from the gel were also set aside so that no proteins would be lost. Typically, an Ampholine (pH 4.0-6.5) gradient IEF gel, after electrofocusing was complete, was cut into five separate segments encompassing the entire range of proteins originally present in the culture solutions. The culture solution sample that was loaded onto the anode side of the gel had been concentrated 80- to 120-fold as described (*vide supra*: section 3). Segment 1 encompassed the pH range from 4.40 to 4.75, segment 2 from 4.75 to 5.09, segment 3 from 5.09 to 5.20, segment 4 from 5.20 to 5.50 and segment 5 from 5.50 to 5.80. The segments that were believed to contain lignin depolymerase, namely segments 2 and 5, were immediately subjected to electro-elution while the remaining segments were stored at 4°C.

It was soon discovered that the yields of these two isozymes were extremely low in absolute terms and only relatively small proportions of these purified proteins might actually bear lignin depolymerase activity. On the other hand, the lignin depolymerase expressed in *T. cingulata* cultures, that had undergone selection for more rapid biodegradation, is a remarkably powerful

enzyme: the fully purified protein, in quantities that are barely detectable through silver staining of electrofocused polyacrylamide gels, degrades polymeric kraft lignin components quite effectively. However, the expression of these two isoenzymes was very sensitive to the experimental conditions employed. For example, it was found that either weak lignin depolymerase or no lignin depolymerase nor lignin polymerase (laccase) activity was typically observed among the lower-pI proteins from the rapidly lignin biodegrading culture solutions. Consequently the culture conditions and inoculum sources were constantly being optimized (*vide supra*: sections 1 and 2). The need to accumulate sufficient quantities of proteins for preliminary

Figure 2. Degradation of high molecular weight polymerized kraft lignin fraction catalyzed by lignin depolymerase isozyme with lower pI in 0.02 M acetate at pH 4.5; molecular weight distributions depicted in terms of Sephadex G100/aqueous 0.10 M NaOH elution profiles after 0.025, 5 and 21 days of incubation.

characterization studies and determination of amino acid sequences had dictated that attention be directed primarily to one of the lignin depolymerase isozymes as the subject of the purification work. At first, the higher-pI isoenzyme was chosen for the purpose because visually there appeared to be fewer proteins in the region encompassing this component in silver-stained polyacrylamide gels after isoelectric focusing, so that identification should have been simplified.

Accordingly, an intensive effort was focused on securing this higher-pI lignin depolymerase isozyme. After a suitable amount of this protein had been isolated by means of isoelectric focusing (IEF) and subsequent electro-elution (*vide supra*: section 3), it was observed through IEF that the kraft lignin which had been originally present in the culture solution might not have

Figure 3. Degradation of high molecular weight polymerized kraft lignin fraction catalyzed by lignin depolymerase isozyme with higher pI in 0.02 M acetate at pH 4.5; molecular weight distributions depicted in terms of Sephadex G100/aqueous 0.10 M NaOH elution profiles after 0.025, 5 and 10 days of incubation.

been removed completely during purification. In view of potential interference from the remaining kraft lignin with the amino acid analysis of the protein, a second electro-elution step was considered necessary to remove the last traces of these components from the protein sample. After some experimentation it was found that IEF using broad range Immobiline (pH 4.0 – 10.0) gels prior to the second electro-elution step generated protein samples free of kraft lignin components. Briefly, the concentrated culture solutions (after exchange with 0.02 M acetate, pH 4.5) were first subjected to IEF in a pH 4.0 – 6.5 Ampholine gradient (the samples being loaded near the anode side), whereupon the appropriate protein bands (in this case the higher-pI lignin depolymerase isoenzyme) were electro-eluted from the gel in 0.03 M Tris phosphate buffer at pH 7.2. The resulting eluents containing the higher-pI lignin depolymerase isozyme were concentrated to 20 – 25-fold and equilibrated with 0.02 M acetate at pH 4.5. Then the lignin depolymerase samples were electrofocused in a pH 4.0 – 10.0 Immobiline gradient (after loading near the cathode) and electro-eluted as before. Ultraviolet spectrophotometric analysis did not thereupon detect any kraft lignin components remaining in the solution. A portion of the resulting purified protein sample was incubated with high molecular weight (pre)polymerized kraft lignin for the enzyme assay and SEC analyses (*vide infra*: section 10) indicated that lignin depolymerization could indeed be effectuated by this isoenzyme (Figure 3, see page 26).

The situation associated with the extremely low and sometimes diminishing yields of lignin depolymerase isozymes produced by *T. cingulata* represented a problem for the adequate accumulation of enough protein for amino acid analyses. Thus ways of improving the fungal cultures were sought by a more thorough selection of promising source cultures for inoculation purposes (*vide supra*: section 2). In the process of rejuvenating the white-rot fungal solution cultures in order to produce higher lignin depolymerase levels, a *T. cingulata* inoculum source was appropriated for use that had been more recently transferred from malt extract agar, but here a profoundly deceptive situation was unfortunately encountered. This arose from the fact that the electrophoretic protein pattern characteristic of the new culture solutions varied according to the time when they were isolated and differed appreciably from that previously obtained (Figure 4(A) and (B)); however, it included bands in the regions of both the higher- and lower-pI target lignin depolymerase isozymes. After selecting, as before, for much more rapid kraft lignin biodegradation, an extracellular protein materialized that, while not bearing lignin degrading activity, exhibited almost identical behavior during IEF and SDS-PAGE to the original higher-pI lignin depolymerase isozyme. Substantial resources were committed to purifying and assaying this protein before it was finally recognized that its inability to depolymerize high molecular kraft lignin components could not be overcome.

Nevertheless, it ultimately proved possible to obtain lignin depolymerase isozymes from *T. cingulata* by extracting the mycelium of rapidly lignin-degrading cultures under more alkaline conditions (pH 7.5, with 0.05 M Tris phosphate containing 0.10 M NaCl, *vide supra*: section 3) than those prevailing during kraft lignin biodegradation (pH 4.0 – 4.5). The protein pattern in the wash solution was more complicated than that in the culture solution (Figure 4(B) and (C)). In general, there were two major protein bands in the region of the higher-pI isoenzyme after IEF of the culture solution, whereas 6 bands (four prominent and two other minor ones) were typically manifested from wash solution. After electro-elution and refocusing (by IEF), this higher-pI isozyme region tended to exhibit multiband problems (Figure 4(D)). On the other hand, for the region encompassing the lower-pI isozyme, the bands from both culture solution and wash solution were often so closely spaced that it was difficult to distinguish between them

reliably (Figure 4(B) and (C)). Nevertheless, degradation of high molecular weight polymerized kraft lignin components was brought about by the electro-elutes from the gel segments in both the higher- and lower-pI lignin depolymerase isozyme regions manifested during IEF (Figures 2, 3, 4(D) and (E)).

Owing to the complicated protein patterns (Figure 4(D)) in the region of the higher-pI lignin depolymerase isozyme after IEF, *i.e.*, the persistence of multiple bands after refocusing, which made the purification of the correct protein for amino acid sequencing very difficult, the other (lower-pI) isoenzyme later became the primary focus of attention. After extracting the mycelium of the rapidly lignin-degrading cultures at pH 7.5 (with 0.05 *M* Tris phosphate containing 0.10 *M* NaCl, *vide supra*: section 3), however, a similar situation was encountered: the proteins electro-eluted from the electrofocused gel segments in the region of the lower-pI isozyme typically manifested three conspicuous bands (and some minor ones) in unblended Ampholine pH 4.0 – 6.5 gradients (Figure 4(E)). The same pattern of protein bands was also obtained from the kraft lignin degrading culture solutions themselves by electrofocusing the electro-elutes from the gel segments encompassing the region immediately around the lower-pI lignin depolymerase isozyme.

Figure 4. Isoelectric focusing (IEF) in Ampholine pH gradients of proteins from *T. cingulata* cultures.

Such results were very sensitive to the experimental conditions employed. For example, as far as the proteins were concerned that had been electro-eluted for shorter periods of time (~90 min. rather than 140 min.) from gel segments in the lower-pI isozyme range after electrofocusing pH 4.5 aqueous extracts from lignin biodegrading mycelia, IEF in unblended Ampholine pH gradients (4.0 – 6.5) generally produced only two prominent bands (without minor ones). Nevertheless assays of the corresponding electro-eluates obtained after 140-min. electro-elution from the lower-pI isozyme region showed that lignin depolymerase activity was located in a narrow pI-range around the middle protein band of the three prominent ones obtained under these conditions. Careful gel sectioning facilitated the isolation of the protein in the conspicuous middle band and that in a much fainter band situated +0.04 pI units away. Solutions containing the two proteins together, on the one hand, and that in the minor (slightly higher pI) band alone, on the other, both exhibited comparable levels of lignin depolymerase activity.

The protein in the conspicuous middle band alone was also isolated using a similar procedure beginning with a pH 7.5 aqueous extract from homogenized *T. cingulata* mycelium (*vide infra*: section 7), and it did not contain lignin depolymerase activity when subjected to the standard assay with a 0.08 gL⁻¹ solution of high molecular weight kraft lignin fraction that had been previously polymerized by the microorganism (*vide infra*, section 9). Taken together with the previous observations, this seemed to confirm that the lower-pI lignin depolymerase might have been confined to that faint band +0.04 pI units away from the prominent middle protein. These findings appeared to be in agreement with the supposition that the lignin depolymerase only constituted a small portion of the extracellular enzymes produced by *T. cingulata*.

The possibility of a connection based on redox state between the proteins in the faint active lignin depolymerase band and the prominent inactive band just 0.04 pI units below was subsequently investigated by incubating the latter under oxidative conditions in the presence of FAD (3×10^{-5} M) at 4°C for up to 48 h. The FAD was then removed by equilibrating the solutions with 10 – 15 volumes of acetate buffer (0.02 M, pH 4.5) using centrifugal filter devices (Centricon-10, Amicon) which were fitted with 10,000 nominal molecular weight cutoff (YM10) membranes. The equilibrated solution was further concentrated in the same filter devices and subjected to IEF in unblended Ampholine pH 4.0 – 6.5 gradients. However, no detectable traces of the faint lignin depolymerase band materialized after silver staining and thus no correlation between the prominent and faint protein bands which were just 0.04 pI units apart could be established.

It is noteworthy that the higher-pI isoenzyme could not reliably be isolated for a number of the later quarterly reporting periods from the culture solutions produced under the conditions developed for expressing higher levels of lignin depolymerase activity (*vide supra*: sections 1 and 2). Interestingly, this higher-pI lignin depolymerase isozyme once again prominently reappeared only in the most recent culture solutions witnessing rapid lignin biodegradation that had resulted from continued selection of the best cultures for inoculum source production during an extended period of time (*vide supra*: section 2). The absence of this isozyme in the culture solutions had coincided with more limited lignin depolymerase activity towards the (standard) polymerized kraft lignin assay substrate. Therefore, the presence of this isozyme seems to be correlated with greater lignin depolymerase activity in culture solutions. Such findings exemplify the sensitivity of the extracellular *T. cingulata* protein patterns to the experimental culture conditions employed. One of the higher priorities in continuing work is the isolation of

the newly reappearing higher-pI lignin depolymerase isozyme on an analytical scale from the cultures exhibiting rapid lignin biodegradation.

Dealing with each impediment to purifying enough active lignin depolymerase became as important as any of the tasks envisaged at the inception of the project. An ongoing difficulty in this respect was that the effect of any modification to the conditions under which *T. cingulata* (the microorganism producing the enzyme) was being grown could only be determined upon completion of the lignin depolymerase assays 5 – 6 weeks after inoculating the cultures. There was no alternative but the gradual accumulation of sufficient protein for amino-acid sequencing through MALDI-TOF mass spectrometry (preferred over the classical Edman degradation because it is more sensitive without being obstructed by a compromised *N*-terminus, a suspected problem as far as the lignin depolymerase is concerned).

6. Culture Solutions without Kraft Lignin

In the process of isolating the lignin depolymerase, it had been established that the lignin substrate interacted very strongly with the protein, which made the purification extremely difficult and laborious to carry out (*vide supra*: section 3). Although the expression of lignin depolymerase by *T. cingulata* is believed to be induced by degradation products derived from polymeric lignin components in the culture solutions, it would be anticipated that extremely minute quantities of the enzyme(s) must still be released from the hyphal cell walls in the complete absence of the substrate. In view of the prospective ease of purifying even the smallest detectable quantities of a lignin depolymerase isoenzyme under such circumstances, cultures without kraft lignin were prepared. The culture conditions were identical to the standard ones (*vide supra*: section 1) except that kraft lignin was absent from the growth medium.

After incubating for 22 – 28 days, the culture solutions were isolated, treated with PMSF and Pepstatin A, then subjected to exchange with a 10-fold volume of acetate buffer (0.02 *M*, pH 4.5) and concentrated 10-fold. Enzyme activity was assayed by incubating the resulting (10-fold concentrated) culture solution with a broad molecular weight range polymerized kraft lignin substrate. This polymerized kraft lignin sample was prepared from cultures where initial polymerization of the substrate had not been followed by particularly effective degradation. Filtered culture solution of such a kind was exchanged, through ultrafiltration using a 10,000 nominal molecular weight cutoff (Amicon PM10) membrane, with a 10-fold larger volume of acetate buffer (0.02 *M*, pH 4.5), and then the proteins present were denatured by rendering the solution alkaline with 0.5 *M* NaOH added until the pH reached 13. After ~16 h incubation at room temperature the pH was slowly reduced to ~5.5 with 0.5 *M* HCl. The resulting solution was then again exchanged with more than 10 volumes of acetate buffer (0.02 *M*, pH 4.5) using ultrafiltration through an Amicon PM10 membrane under conditions where the kraft lignin was 2-fold more dilute than that in the original culture solution. Size-exclusion chromatographic analyses of the assay solutions indicated that lignin polymerase (laccase) activity was clearly present in the samples collected 22 days after inoculation. On the other hand, the lignin polymerase activity was not as pronounced in culture solutions harvested 28 days after inoculation. It was possible that lignin polymerase could have masked competing lignin depolymerase activity, but chromatofocusing failed to yield any lignin depolymerase or lignin polymerase activity in any of the column fractions. It was possible that one or both of the enzymes, as the case may be, were retained on the column.

When the extracellular proteins accumulating in the *T. cingulata* solution cultures without kraft lignin were subjected to chromatofocusing (*vide supra*: section 3), one of the resulting fractions contained a protein that was almost identical in pI and molecular weight to the higher-pI lignin depolymerase, but lignin depolymerizing activity with a polydisperse polymerized kraft lignin substrate was not substantiated. Moreover, on one occasion, cell-free culture solution, that had been concentrated 130-fold four weeks after inoculation, appeared to bring about degradation of a high molecular weight polymerized kraft lignin fraction, but no further experimental confirmation of this single result was obtained. At the same time, the lignin-degrading effect was not substantiated with a broader range polymerized kraft lignin preparation containing an appreciable number of medium molecular weight components. Accordingly this avenue of investigation was discontinued.

7. Preparation of Mycelial Homogenate Extract

As another plausible enzyme source, an extract of lightly homogenized *T. cingulata* mycelium was investigated. The wash solution, which had been produced by submerging the hyphae at the time of harvest in 0.05 M Tris phosphate buffer containing 0.1 M NaCl (pH 7.5) for 12 – 16 h, seemed to contain more proteins than the culture solution alone, and it was therefore desirable to determine whether even more proteins could be obtained through a more invasive extraction protocol. The proteins liberated in this case would most likely originate from the slime matrix surrounding the hyphal material. As before, choosing the cultures that were being extensively decolorized (indicating that the polymerized kraft lignin was being degraded by lignin depolymerases in the *T. cingulata* culture solutions), the contents of each flask were filtered through Mira-cloth (Calbiochem) disposed in a Büchner funnel using a vacuum. The culture solutions were collected as before and the standard wash solution prepared as already described (*vide supra*: section 3). The fungal mycelium was thereupon removed from the Mira-cloth and placed in wash buffer (0.05 M Tris phosphate and 0.1 M NaCl, pH 7.5) to be blended in a laboratory blender for 20 s three separate times. The resulting mixture was then centrifuged (8,240 x g, 30 min.), and the supernatant was equilibrated with 10 – 15 volumes of 0.02 M acetate buffer at pH 4.5 by ultrafiltration through a 10,000 nominal molecular weight cutoff (Amicon PM10) membrane. Portions of the resulting solution were then concentrated ~100-fold by ultrafiltration for electrophoretic studies. Silver staining of electrofocused gels loaded with this solution from the mycelial homogenate revealed that a strong protein band situated in the same region as the lower-pI lignin depolymerase isoenzyme was present. Electro-elution from the appropriate electrofocused Ampholine (pH 4.0 – 6.5) gel segments was employed to purify this protein, but it did not exhibit any detectable lignin depolymerase activity. It was probably the same as the protein occupying the prominent middle band in the lower-pI lignin depolymerase isozyme region of the normal pH 7.5 mycelial extract (*vide supra*: section 5).

8. Kraft Lignin Isolation

The most important component in the *T. cingulata* culture solutions used during this project was the dissolved kraft lignin because it is believed to induce the expression of lignin depolymerase(s) by this fungus. Accordingly, kraft lignin was routinely isolated from an industrial Jack pine kraft black liquor produced by the Boise Cascade Corporation in International Falls, MN. A 220 mL volume of Jack pine black liquor was diluted to 1,600 mL with HPLC grade water. The resulting solution was filtered through a VWR grade 417 filter

paper to remove any fibrous materials. The black liquor was then acidified with aqueous 1.0 M HCl, which was added at a rate of 0.05 mL per 10 – 15 seconds. The progress of acidification was monitored with a pH-meter at the beginning, around pH 11 and near pH 3, with the volume of acid consumed being recorded. Vigorous stirring of the solution was continued throughout the process to break up the formation of the localized precipitate created by each drop of acid. Acidification was interrupted at pH 11 to remove any colloiddally suspended sulfur by centrifuging the solution in glass centrifuge bottles at 1,400 x g for 1 hour. The acidification was then continued until the pH of the solution reached 3.0. The kraft lignin precipitate was isolated by dividing the solution containing suspended solids among 4 polycarbonate centrifuge bottles, and centrifuging for 30 min. at 3,100 x g. The kraft lignin precipitate was consecutively washed three times by pouring 250 mL pH 3.0 aqueous HCl into each of the 4 polycarbonate centrifuge bottles and shaking until the suspension was homogeneous, whereafter the bottles were centrifuged again at 3,100 x g for 30 min. After each wash and centrifugation, any floating sulfur was carefully removed.

After the third wash, the supernatant was usually cloudy and so it was decanted from all four bottles into a 1,000 mL beaker. While stirring, the cloudy supernatant was basified with aqueous 0.10 M NaOH to pH 8.5 to redissolve the suspended solids; addition of aqueous base was discontinued only after the pH had remained stable at this value for about 30 min. The resulting clear solution was then rapidly re-acidified to pH 3.0 with aqueous 1.0 M HCl. The recovered precipitate could now be readily separated from the supernatant by centrifugation, whereupon it was combined with the main part of the precipitate obtained from the third wash. The combined kraft lignin precipitate was resuspended in 400 mL HPLC water, shaken to homogeneity, and basified with aqueous 0.10 M NaOH to pH 8.5; again the addition of aqueous base was discontinued only after the pH had remained stable at this value for ~30 min. The resulting clear solution was kept at 4°C overnight and then subjected to 1 h centrifugation at 36,000 x g to remove colloidal sulfur; thereafter the kraft lignin solution was distributed among 8 – 9 fast-freeze flasks, frozen in a dry-ice/acetone bath and loaded onto a freeze-dryer.

9. Preparation of High Molecular Weight (Polymerized) Kraft Lignin

In the context of the present study, the most obvious choice for a lignin depolymerase substrate was the polymerized components produced by *T. cingulata* (during primary vegetative growth) from the kraft lignin originally present in culture solution. A high molecular weight polymerized kraft lignin fraction, exhibiting a single excluded peak during SEC with aqueous 0.10 M NaOH/Sephadex G-100 (Sigma), has clear advantages as an assay substrate since kraft lignin components throughout the higher and intermediate molecular weight ranges would not have to compete with one another for the enzyme under such circumstances. To this end, after buffer exchange (2.0 L 0.02 M acetate, pH 4.5) of the (200 mL) cell-free culture solution and concentration to ~100 mL in final volume, 0.5 M NaOH (all manipulations from this step onwards being performed under N₂) was added until a final concentration of 0.10 M NaOH (pH ~13.0) was reached. The resulting polymerized kraft lignin solution was transferred to a 2,500-mL ultrafiltration cell fitted with a 100,000 nominal molecular weight cutoff (Amicon YM100) membrane, whereupon it was exchanged with over 10 volumes of 0.10 M NaOH. The volume was then reduced to ~200 mL and the pH was reduced to 9.5 – 9.0 by exchange, under the same ultrafiltration conditions, with distilled H₂O. In lowering the pH further to ~7.5, 10 volumes of (200-mL) triply distilled H₂O were employed in the same way. The volume of the solution was

finally reduced to ~100 mL and centrifuged at 47,900 x g for 20 min. to remove any undissolved material; thereupon it was frozen and lyophilized.

Initially the isolated high molecular weight polymerized kraft lignin was unstable in acetate buffer (0.02 M, pH 4.5): the lignin components tended to precipitate during the course of assays which resulted in a reduction in the excluded leading peak height of the control (without introduction of either lignin depolymerase isozyme) in the size exclusion chromatographic profiles. This caused false positive results from the assays and ways of overcoming the problem were investigated. Eventually a marked improvement in the reliability of the lignin depolymerase assay was realized through a relatively small modification in the high molecular weight polymerized kraft lignin fraction employed for the purpose. As already mentioned, this substrate is formed directly by the polymerization of the original kraft lignin in the *T. cingulata* cultures during primary vegetative white-rot fungal growth. It was established that significantly greater solubility and stability are engendered by the introduction of more hydrophilic groups in the polymerized kraft lignin produced during longer incubation periods in ((higher) pH > 5) cultures where the onset of degradation is naturally delayed. Under these circumstances, the components enjoy a greater opportunity to become oxidized and typically exhibit enhanced buffer capacity around pH 8.5. Thus the polymerized kraft lignin employed as an assay substrate was isolated only from culture solutions with pHs that had not fallen below 5.0. Moreover, imposition of a three-day prior incubation period on the polymerized kraft lignin fraction in (0.02 M acetate buffer) solution under standard assay conditions has been found to reduce considerably the incidence of falsely positive size-exclusion chromatographic results.

10. Lignin Depolymerase Activity Assays

Degradation of High Molecular Weight (Polymerized) Kraft Lignin

As the kraft lignin components that had been polymerized during vegetative growth of *T. cingulata* were degraded by lignin depolymerases, enzymatic activity could be monitored by successive size-exclusion chromatographic analyses of the dissolved high molecular weight kraft lignin components in solution. The molecular weight distributions of these lignin components were determined by means of their (A_{280}) profiles in carbonate-free aqueous 0.10 M NaOH being eluted from Sephadex G100 in a 2.5 x 100 cm column, to which an ISCO V⁴ detector was connected at the outlet. Standard lignin depolymerase assays were carried out in 0.02 M acetate buffer solutions (pH 4.5) containing 0.08 gL⁻¹ high molecular weight kraft lignin that had been previously polymerized by *T. cingulata* in culture solutions. The prepolymerized lignin solution was prepared and incubated under ambient conditions for 3 days before being filter-sterilized for assay purposes to ensure the stability of the lignin components in the buffer (*vide supra*: section 9). These assays were carried out with the cell-free culture solutions, wash solutions, or purified enzyme solutions as appropriate.

Extracellular *T. cingulata* culture solution, which had been harvested during the degradation of the polymerized kraft lignin substrate (*vide supra*: section 3), manifested a decrease in the average size of the high molecular weight kraft lignin components in successive elution profiles, thereby confirming the presence of lignin depolymerase activity. These findings corresponded to the decolorization of the culture solution during the secondary metabolic phase exhibited by the microorganism after primary vegetative growth had ceased.

Kraft Pulp Delignification

The ultimate goal of this project was to produce a lignin depolymerase which is directly able to delignify softwood kraft pulp. Thus the lignin depolymerase catalyzed delignification of softwood kraft pulp was determined in the following manner. Unbleached industrial softwood kraft pulp, primarily from Jack pine (*Pinus banksiana*), was washed with distilled water before and after sterilization by autoclaving at 121°C for 30 min.; the brownstock was then extracted with aqueous 0.10 M NaOH for 5 min. at room temperature and thoroughly washed with distilled water. Samples of this softwood kraft pulp (0.35 g) were subsequently apportioned into individual 250-mL conical flasks and autoclaved for a second time at 121°C. Each sterilized kraft brownstock portion was then washed 5 times with approximately 20 mL sterile aqueous 0.020 M acetate buffer at pH 4.50, after which acetate buffer containing the respective lignin depolymerase isoenzyme obtained by isoelectric focusing and electro-elution (*vide supra*: section 3) was added to each flask in such an amount that the final solution volume would be 32 mL. A neoprene stopper fitted with a glass tube, into which a glass wool plug had been loosely inserted to allow air-exchange, was then placed into the mouth of each flask.

After suitable incubation periods under ambient conditions, the kappa numbers of the pulp samples were determined using a modification of TAPPI Test Method T236 cm-85 (scaled down 10-fold) following thorough washing with distilled water before and after extraction (5 min.) with aqueous 0.10 M NaOH at room temperature. Thus in each case 10.0 mL 0.10 N KMnO₄ and 10.0 mL 4.0 N H₂SO₄ were added to a 100 mL slurry containing a 0.23 g portion of one of the pulp samples. Following a 10 min. reaction period at 25°C, 2.0 mL 1.0 N KI was added and the iodine formed was titrated with 0.010 N Na₂S₂O₃. The reductions in kappa number observed after 8 days were small (0.9% and 2.0% in the presence of the higher- and lower-pI isozymes, respectively) but significant compared to the controls (which varied by less than 0.10%).

These results were very promising but they needed to be confirmed with higher concentrations of the purified lignin depolymerases. The purification of the native isoenzymes was so challenging and time-consuming (*vide supra*: section 3), however, that it was deemed more appropriate to wait until adequate quantities of the recombinant enzyme(s) become available.

11. RNA Isolation

When the cultures had turned from dark brown to light yellow-brown in color, which signified extensive degradation of the polymerized kraft lignin in solution, the *T. cingulata* mycelia were harvested by filtration through Mira-cloth (Calbiochem). Owing to the high rate of lignin depolymerase turnover, it is reasonable to suppose that, if the enzyme is present, then mRNA corresponding to the enzyme will also be present. The mycelium was then scraped off the Mira-cloth into a pre-chilled mortar containing liquid N₂, which effectively froze the cells to prevent RNA degradation, and the solidified hyphae were ground into a powder to liberate their cellular contents. Total RNA from *T. cingulata* grown in the presence of kraft lignin (*vide supra*: section 1) was isolated using a method based on what was originally developed by Chomczynski and Sacchi (*Anal. Biochem.* **1987**, 162, 156-159). TRIZOL[®] reagent (Life Technologies), containing acid guanidinium thiocyanate-phenol which effectively dissolves DNA, RNA and protein, was used for the single-step isolation of RNA. To the ground hyphal sample from each 50 mL culture, 5 mL of cold TRIZOL[®] reagent (4°C) was added while the hyphae were still frozen in liquid N₂. This was repeated for each flask in the culture set being processed on any given

occasion. The resulting mixture was transferred to polyethylene centrifuge tubes and the fungal hyphae were homogenized, whereafter they were incubated for at least 5 min. at room temperature for complete dissociation of nucleoprotein complexes to occur. The undissolved material was removed by centrifugation (4,640 x *g* for 20 min.). Chloroform (0.2 mL per 1 mL of TRIZOL[®] reagent) was added to each tube containing the homogenized mycelia, and the tubes were shaken for 15 s and incubated for 15 min. at room temperature. After centrifugation (4,640 x *g* for 30 min. at 2 – 8°C) each mixture separated into three phases, where the upper aqueous phase containing the RNA was transferred to fresh polypropylene tubes. To it isopropanol (0.5 mL per 1 mL of TRIZOL[®] reagent for initial homogenization) was added and the resulting mixture was incubated at room temperature for 10 min., which led to RNA precipitation. Subsequent centrifugation (4,640 x *g* for 10 min. at 2 – 8°C) of the mixture yielded RNA pellets. The supernatant was removed and the RNA pellet was washed with 75% ethanol (at least 1 mL per 1 mL of TRIZOL[®] reagent for initial homogenization), whereafter the mixture was vortexed briefly and centrifuged at 4,640 x *g* for 5 min. at 2 – 8°C. The RNA pellet was air-dried and dissolved in RNase-free water; thereafter it was incubated for 10 min. at 55 – 60°C before being stored at -60 – 70°C. Each set of cultures treated in this way generated total RNA that could be used for mRNA isolation and subsequent cDNA library production (*vide infra*: sections 13 and 14) and RT-PCR (*vide infra*: section 16). Each total RNA preparation from a particular set of cultures was subjected to electrophoresis on a 1% denaturing agarose gel containing 2.2 *M* formaldehyde to evaluate the quality of the RNA. The gel was then stained with ethidium bromide which is intercalated between the bases of the RNA molecules and the result may be visualized under ultra-violet light. The fungal rRNAs (ribosomal RNA) corresponding to the 28S (~3.2 kb) and 18S (~1.8 kb) components generated strong and highly focused bands and did not appear to be degraded, suggesting that RNase activity was not present (which would have otherwise resulted in smears on the gel). These results were confirmed by the UV spectrophotometric characteristics of the RNA sample. In addition, the concentration of the RNA sample was estimated by measuring the absorbance of the components at 260 nm. In general, just under 1 mg total RNA was isolated for every ten 50 mL *T. cingulata* cultures. This procedure was highly reproducible.

12. Isolation of (polyadenylated) mRNA

The starting material for constructing a cDNA library is mRNA, which only constitutes a small portion of the total RNA (<10%). Thus it is desirable to isolate mRNA from a pool of total RNA so that there is more starting material for subsequent manipulation. The approach for achieving this takes advantage of the fact that most mature eukaryotic mRNA species have a polyadenosine region present at the 3' end. This 3' polyadenosine region is conventionally referred to as a poly(A) tail and mRNA possessing it is called poly(A) mRNA. The poly(A) mRNA of *T. cingulata* was thus isolated from total RNA (*vide supra*: section 11) using the PolyATtract mRNA Isolation System (Promega). The system uses a biotinylated oligodeoxythymidine (oligo(dT)) primer to hybridize to the poly(A) tail. The mRNA which hybridizes to the biotinylated oligo(dT) primers was captured and washed (at high stringency where a greater degree of complementarity is required for base pairing) using streptavidin (biotinylated substances have a high affinity for streptavidin) coupled to paramagnetic particles held in place using a magnetic separation stand. The washing removed the other RNAs (rRNA and tRNA) which do not have poly(A) tail thus did not bind to paramagnetic particles. The mRNA was then eluted from the biotin- oligo(dT)/paramagnetic particle complex by rinsing with RNase-free

deionized water and subsequently recovered by ethanol precipitation. Hereby mRNA (measured by A₂₆₀) was isolated from the total RNA of *T. cingulata* in its secondary metabolic phase when lignin depolymerase activity was being expressed in the culture solutions (*vide supra*: section 11). Electrophoretic analysis of ~3 µg of the isolated *T. cingulata* poly(A) RNA on a 1% denaturing gel containing 2.2 M formaldehyde indicated that the majority of the mRNA fell into the size range between 300 and 3000 nucleotides. This suggested that the mRNA preparation was sufficient for cDNA (complementary DNA) generation. Enough mRNA was secured for multiple attempts at generating a cDNA library, the next step for isolating the gene that encodes lignin depolymerase.

13. cDNA Synthesis

The first major objective of this project had been to obtain enough lignin depolymerase isozymes for amino acid sequencing. Once sufficient sequence information became available it could be used to generate probes for screening a *T. cingulata* cDNA library so that the genes encoding the lignin depolymerase(s) could be identified and characterized. The first step in constructing a cDNA library is to synthesize cDNA (complementary DNA) from mRNA using a reverse transcriptase. cDNA was thus generated from the isolated *T. cingulata* mRNA (*vide supra*: section 12) using the ZAP Express cDNA synthesis kit (Stratagene). First strand cDNA synthesis begins with the annealing of 5 µg *T. cingulata* mRNA to a hybrid oligo (dT) linker-primer (a 50-base oligonucleotide prepared with a "GAGA" restriction site protective region and an 18-base poly(dT) segment) containing a *Xho* I restriction site which allows completed cDNA to be cloned into the ZAP Express vector in a sense orientation with respect to the *lacZ* promoter in the construct. The primed mRNA is transcribed using Moloney murine leukemia virus reverse transcriptase (MMLV-RT) and dATP, dGTP, dTTP and the analog to dCTP, 5-methyl dCTP. Incorporation of a methyl group on each cytosine base protects the cDNA from the restriction enzymes used in subsequent cloning steps. The poly(dT) region binds to the 3' poly(A) tail of the mRNA template and MMLV-RT starts the synthesis of the first-strand cDNA. After completion of first strand synthesis, addition of RNase H during second strand synthesis nicks the RNA bound to the first-strand cDNA to produce a multitude of fragments, which serve as primers for DNA polymerase I (nick translation) and thus the second-strand cDNA is generated. Addition of the dNTP mixture and *Pfu* DNA polymerase fills the uneven termini of the double-stranded cDNA, thus producing blunt cloning ends on the cDNA.

To obtain directional cloning of the cDNA for subsequent protein synthesis, *EcoR* I adapters (9- and 13-base complementary oligonucleotides with an *EcoR* I cohesive end) are ligated to the blunt ends of the cDNA. The previously incorporated *Xho* I site (added as part of the linker-primer) located on the polyadenylated end of the cDNA is digested with *Xho* I, which releases the *EcoR* I adapter and residual linker-primer from the 3' end, producing a population of directionally oriented cDNAs with 5' *EcoR* I and 3' *Xho* I cloning ends. Without prior knowledge of the exact size of lignin depolymerase, the complete family of cDNA components from *T. cingulata* was used for library construction. A Sephacryl S-400 spin column (Promega) was used to remove contaminating residual enzymes, buffers, dNTPs and excess linker-primer. The cDNA was applied to the pre-washed column and centrifuged in a swinging bucket rotor at 800 x g for 5 min. so that the recovered directionally oriented cDNA sample could be collected.

14. Generation of *Trametes cingulata* cDNA Library

The directionally oriented cDNA (*vide supra*: section 13), originating from the mRNA of kraft lignin induced *T. cingulata*, was ligated to the λ ZAP Express vector (Stratagene) and, as a control in a separate experiment, a test insert was also ligated to the λ vector. The ZAP Express vector contains 12 unique cloning sites (polylinker) that can accommodate up to 12 kb DNA inserts. Inserts cloned into this λ vector can be excised out of the phage in the form of the kanamycin-resistant pBK-CMV phagemid vector, and in this form can be utilized in either a prokaryotic (driven by *lac* promoter) or eukaryotic (driven by cytomegalovirus (CMV) immediate early promoter) expression system for the production of protein. Color selection of recombinants is facilitated by the *lacZ* promoter, which is repressed in the presence of the *lacI* protein but is inducible in the presence of the gratuitous inducer, isopropyl-1-thio- β -D-galactopyranoside (IPTG). The polylinker is placed downstream from this promoter in the amino terminus of the α -complementing portion of the β -galactosidase gene. Without the insert, the functional β -galactosidase protein expressed is capable of hydrolyzing the substrate 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal) into galactose and 5-bromo-4-chloroindigo, the latter being blue. It thus allows blue/white screening of clones with insert: plaques containing vector without insert stain blue in the presence of X-gal and IPTG, while plaques containing vector with insert are white because of the functional inactivation of the 5' segment of the *lacZ* gene by the insertion of foreign DNA into the polylinker region.

After ligation, the recombinant λ phage was packaged using Gigapack III Gold packaging extract (Stratagene) along with a λ wild-type control (λ cI857 Sam7) for determining packaging efficiency. The host *E. coli* strain used with this λ library system was XL1-Blue MRF' which contained the F' episome. The F' episome is required for three purposes. First the λ M15 *lacZ* gene on the F' episome is required for the β -galactosidase-based α -complementation selection mentioned above. Second, this episome possesses the genes forming pili on the surfaces of the bacteria. Pili formation is essential for the infection of a filamentous helper phage which is required for the *in vivo* excision step that converts a recombinant ZAP Express clone to a phagemid containing the cloned insert. Third, the F' episome carries the *lac* repressor (*lacI^q*) gene which prevents transcription from the *lacZ* promoter in the absence of the inducer IPTG. This repressor is important for controlling the expression of fusion proteins which may be toxic to the host *E. coli* cells. The λ wild-type control is packaged in the host strain VCS257. The packaged λ phages (cDNA recombinant phage, test insert recombinant phage and wild-type phage) are then infected into the appropriate host *E. coli* strains. IPTG and X-gal were present in the top agar (color selection for vector with and without insert) to check for the test insert and cDNA recombinant packaged phages. The predicted packaging efficiency for 0.2 μ g of control DNA is 1×10^9 plaque forming units (pfu)/ μ g of vector (Stratagene ZAP Express cDNA/Gigapack Cloning Kit manual, p. 11).

Our results indicated that we were able to obtain 84% of the predicted efficiency with 8.4×10^8 pfu/ μ g of vector. The control (ligated test insert) recombinant packaged phage (3.0 μ L/500 μ L) produced plates with innumerable plaques (very little lawn of confluent *E. coli* growth was visible) and the cDNA (3.0 μ L/500 μ L) recombinant packaged phage was similar. The control plate had no blue colored plaques (indicating an insert-lacking vector), while the plate derived from infection by the cDNA ligated λ vector exhibited an estimated 5% of the total plaques in blue. In other words, the vast majority of the vectors did indeed harbor cDNA clones. These

results verified that the primary *T. cingulata* cDNA library generated in the ZAP Express λ vector possessed a sufficient frequency of cDNA cloned genes to be used for isolating the one that encodes lignin depolymerase.

It is usually highly desirable to amplify libraries constructed in λ vectors for a large, stable quantity of a high-titer stock of the library. This primary library was thus amplified by infecting the host *E. coli* strain (XL1-blue MRF', 266 μ L at OD₆₀₀~0.5) with recombinant λ phage (2.2×10^4 pfu) at 37°C for 15 min. The infected bacteria were mixed with (~48°C) 3.0 mL of NZY top agar (containing agarose rather than agar) and then spread evenly onto freshly poured NZY (a medium for *E. coli* containing, *inter alia*, MgSO₄·7H₂O, yeast extract, and casein hydrolysate) plates (100 mm). After incubating at 37°C for ~8 h, the plates were overlaid with 4.0 mL of SM buffer (a buffer used for storage and dilution of bacteriophage λ stocks) and stored at 4°C to allow the phage to diffuse into the solution. The SM buffer containing recombinant phages was pooled from eleven separate plates. Cell debris was removed from the buffer by adding 5% chloroform (v/v) and subsequently centrifuging for 10 min. at 500 x g. It was found that the amplification of this primary library containing *T. cingulata* cDNA had produced a titer of 1.4×10^6 pfu/mL. With this titer only 36 μ L was needed for each infection to generate 50,000 plaques/150 mm plate (twenty plates were needed to screen the desired 1×10^6 pfu), which is the recommended number for each plate when screening a cDNA library.

15. Genomic DNA Isolation from *T. cingulata*

It was possible that screening the cDNA library might have failed to identify the gene encoding the lignin depolymerase because evidently *T. cingulata* was only producing very a small amount of the enzyme. Screening of a cDNA library may not always detect sequences from genes expressed at very low levels. If this were to happen, a genomic DNA library would be employed instead. Consequently, genomic DNA was isolated (*Proc. Natl. Acad. Sci. USA* **1984**, *81*, 1470-1474) for the construction of a white-rot fungal genomic library. Mycelium (200 mL culture), grown to the secondary metabolic phase in 1.5% malt extract, was harvested by filtration through Mira-cloth, rinsed with ice cold water, flash frozen in liquid N₂, and powdered by crushing with a pestle in a mortar. The cells were then rapidly dissolved in 5 mL of cell suspension buffer (50 mM EDTA, pH 8.5, 0.2% SDS, 0.1% Diethylpyrocarbonate (DEPC) and shaken for 1 min. at room temperature. The mixture was then heated at 68°C for 15 min., cooled to room temperature and centrifuged for 15 min. at 12,000 x g. The supernatant (4 mL) was placed into a fresh tube on ice, 8.0 M potassium acetate (0.25 mL, pH 4.2) was added and the mixture was incubated for 1 h. The mixture was then centrifuged (25,000 x g for 15 min.) and 4 mL of the supernatant was added to 4 mL isopropanol to precipitate the nucleic acids. Centrifugation (25,000 x g for 15 min.) collected the precipitate, which was dissolved in 1.2 mL TER (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 10 mg/mL RNase A) buffer. The solution was then extracted sequentially with phenol-chloroform (1:1, v/v) and chloroform. The resulting upper aqueous phase after centrifugation was transferred to a fresh tube and the DNA was precipitated with isopropanol, whereafter the pellet was dissolved in water (40 μ L). Electrophoretic (1.0% agarose gel) and spectral (A₂₆₀) analyses indicated that this DNA isolation technique could be used successfully to acquire sufficient quantities of *T. cingulata* genomic DNA for the production of a genomic DNA library.

16. Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR)

Persuasive evidence indicated that only small quantities of lignin depolymerase are expressed in *T. cingulata* cultures under the best of circumstances; this had complicated the protein isolation work (*vide supra*: sections 3 and 5) and cDNA library screening was expected to be challenging. As a result, RT-PCR protocols were initiated toward the end of this research project for cloning the relevant gene(s). Such an approach can be used to clone particular cDNAs from minute amounts of mRNA without constructing a complete cDNA library—the screening of which may not always detect sequences from genes expressed at very low levels. From the 6 amino acids at the *N*-terminus of the higher-pI isozyme (*vide infra*: section 17), degenerate primers containing mixed-base residues or deoxyinosine at positions of codon degeneracy were synthesized by Genosys. These sense primers and oligo(dT) were employed with the Superscript One-Step RT-PCR System (Life Technologies) and total RNA (*vide supra*: section 11) as the starting material. New primers were, of course, destined to be synthesized in response to more amino acid sequence information.

Before specific amplification with satisfactory yields can be obtained, RT-PCR parameters often have to be optimized with respect to the starting RNA quantity, primer concentration, and annealing temperature. As a starting point, two parameters, the concentrations of RNA template (170 ng vs. 510 ng) and primers (0.2 μ M vs. 1.2 μ M), were varied. The negative controls included a reaction without RNA template (water only), a reaction without Taq polymerase, and RNase-treated RNA samples. The degenerate primers derived from the *N*-terminal amino acids of laccase were used as a tentative positive control (because *T. cingulata* expressed laccase-like polymerase activity). Since the primer designed from the amino acid sequence contained “universal” bases such as inosine, the melting temperatures (T_m) of hybrids between the oligonucleotide primer and target DNA sequences could not be known with certainty. Thus it was not possible to calculate from first principles an annealing temperature that would maximize the yield of the desired amplification product while minimizing nonspecific binding. Accordingly, touchdown PCR was employed whereby the onset of nonspecific amplification of DNA sequences should be delayed in relation to specific priming, which would bring about preferential amplification of the target sequence. RT-PCR reactions were carried out in 50- μ l reaction mixtures containing ~100 ng total RNA, 0.2 μ M sense degenerate primer, 0.2 μ M oligo(dT), and 1 μ l RT/Platinum Taq mix (Life Technologies). The cDNA synthesis was carried out in a Hybaid PCR Express unit at 50°C for 30 min., after which the reverse transcriptase was inactivated at 94°C for 2 min. and the polymerase chain reaction (PCR) immediately followed.

Because of the high degeneracy of the primers mentioned above, touchdown PCR was employed. The first cycle consisted of 15 sec at 94°C, 30 sec at 60°C and 1 min at 72°C. The annealing temperature was subsequently decreased by 1°C for every other cycle until it reached 48°C, at which point an additional 22 cycles were performed. The PCR products were subjected to analysis by electrophoresis on a 1.5% agarose gel in TAE buffer (Sambrook and Russell 2001), followed by staining with ethidium bromide. Unfortunately the results obtained only revealed nonspecific amplification. Therefore new primers needed to be synthesized in which the incorporation of inosine has been minimized, and of course sufficient amino acid sequence information will allow a completely new set of both the sense and antisense primers to ensure much better specific amplification of the gene encoding the lignin depolymerase isozymes.

17. Amino Acid Sequence Determination

In preparation for amino acid sequencing, the electro-eluted lignin depolymerase isoenzyme samples (*vide supra*: section 5) were electrofocused in a pH 4 – 10 (Pharmacia) Immobiline gradient (after loading near the cathode) and electro-eluted as before. Ultraviolet spectrophotometric analyses of the resulting electro-eluted protein samples indicated that all kraft lignin components had been successfully removed as the spectra in the 220 – 500 nm wavelength range displayed typical protein patterns. The protein samples were then sent to Commonwealth Biotechnologies, Inc., for amino acid analysis and sequencing.

The amino acid analyses indicated that the samples contained ~70 pmol protein, and *N*-terminal amino acid sequencing was carried out on ~60 pmol in each case. Sequencing appeared to be taking place at the 5 pmol level, however, with cycle yield differences during the Edman degradation of only 2 pmol. These findings suggested an *N*-terminal sequence of Glu-Ala-Tyr-Pro-Gln-X-Asp-X for the higher-pI lignin depolymerase isozyme, where reliable sequencing results vanished after the 5th residue. Such results from attempted *N*-terminal amino acid sequencing have been encouraging, although the data have been neither extensive nor reliable enough. The *N*-terminus could be compromised somehow, as for example by the presence of a pyroglutamate residue, and in this regard it is interesting that the first amino acid identified in the sequence was glutamic acid. Internal amino acid sequencing through cyanogen bromide cleavage failed altogether, on the other hand, possibly because the amount of protein may have been too small or because methionine may have been completely absent from the polypeptide chain. Therefore, MALDI-TOF mass spectrometry is being employed for all subsequent amino acid sequencing (on account of its greater sensitivity and better tolerance for certain kinds of contaminants).

CONCLUDING NOTE

The work proposed for this project at the outset represented a substantial but, in principle, straightforward research and development exercise. It involved isolating (for the first time) a genuine lignin degrading enzyme (lignin depolymerase) and sequencing it, cloning the corresponding gene and producing the recombinant enzyme in a suitable expression system. Despite the most heroic efforts, however, the entire project was delayed at the stage where the lignin depolymerase gene was about to be identified. Nevertheless the experience gained and the insights acquired will be invaluable in bringing this unique project to a successful conclusion. Accordingly the work is continuing in collaboration with the Enzyme Technology Team at the National Renewable Energy Laboratory. Complete publication of the interim results is being postponed until the underlying reasons for some of the profound experimental difficulties have been fully established, but two conference proceedings have appeared that provide a brief account about particular aspects of the work described in this report.

Chen, Y.-r.; Jacobson, B.; Sarkanen, S.; Wang, Y. "A True Lignin Depolymerase that Delignifies Softwood Kraft Pulp," *Proc. 10th Internat. Symp. Wood Pulp. Chem.* **1999**, III, 54-59.

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