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Substrate specificity, de novo synthesis and partial purification of polyphenol oxidase derived from the wood-decay fungus, *Coriolus versicolor*

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SUMMARY

Coriolus versicolor, a white-rot Basidiomycete, secretes cellulolytic and ligninolytic enzymes as well as polyphenol oxidase (PPO). Whereas the former degrade wood polymers, the latter can convert diphenols to diquinones and oligomerize syringic acid, a lignin derivative. Certain phenolic compounds can serve as disease-resistance factors controlling the proliferation of wood-decay fungi within host tissues. Because *C. versicolor* can be 'batch-cultured', overproduction and enhanced secretion of enzymes of biological and commercial interests are feasible. Reported here are the results of attempts to define the timed appearances of intracellular and extracellular PPO, to assess substrate specificity as well as distinguish synthesis versus activation of intracellular PPO and to partially purify extracellular PPO. These efforts were to provide data enabling cell-free synthesis of PPO, cloning of the gene(s) for the oxidase and the establishment of its subcellular route of secretion. Whereas two protein peaks (6 and 12 days in a 16 day time-course) were observed for dialyzed mycelial homogenates, the homogenates' PPO specific activity rose between 4 and 12 days and then declined. Total extracellular protein content climbed from 6 to 15 days for dialyzed growth medium and the medium's PPO specific activity rose at 4 days post-inoculation and except at 9 days increased linearly to 15 days. When aliquots of dialyzed 12 and 15 day media were added to PPO assay mixtures containing catechol and either syringic or gallic acids, statistically significant differences in PPO specific activity between phenolic substrates were noted. Supplementation of cultures with 1.91 μg cycloheximide ml growth medium^{-1} (control, growth medium only) together with 0.5 μCi [^{14}C]-leucine revealed that cycloheximide inhibited PPO activity and suppressed [^{14}C]-leucine incorporation into TCA-insoluble cytoplasmic protein. As for PPO partial puri-

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fication, growth medium dialysis followed by 0–30% $(NH_4)_2SO_4$ fractionation and subsequent 12 000 × *g* dialyzate centrifugation yielded a 3.27-fold enhancement in PPO specific activity within the 12 000 × *g* supernatant. Chromatography of the latter upon DEAE-Sephadex indicated that PPO exchanged with the DEAE counterion as it could be eluted with high ionic strength salt. These results suggest that: the occurrences of intracellular and extracellular PPO are time-dependent, intracellular PPO is de novo synthesized, the preferred substrate for extracellular PPO appears to be catechol and extracellular PPO can be partially purified by a combination of dialysis and ammonium sulfate fractionation as well as possibly DEAE chromatography and/or Sephadex G-150 gel filtration.

INTRODUCTION

Wood decay is caused primarily by certain basidiomycetes [22] which can degrade cellulose, hemicellulose and lignin, the main polymers of wood by secreting enzymes [11,20]. In addition, some white-rot fungi can elaborate polyphenol oxidase (PPO), an enzyme capable of converting diphenols to diquinones [8] as well as oligomerizing syringic acid, a lignin derivative [18]. In this connection, phenolic compounds appear to be disease-resistance factors produced by certain trees as a host-response to infection [10,25]. These compounds can influence the growth of some fungi [28] thereby possibly 'playing' a role in the wood decay process [21].

Because *Coriolus versicolor*, a white-rot fungus, secretes cellulolytic and ligninolytic enzymes and PPO [26,27] into its culture medium when grown in a defined liquid medium, it may constitute a model system for achieving overproduction and enhanced secretion of enzymes of biological and possibly commercial interests. Here are reported the timed-appearances of intracellular and extracellular PPO, substrate specificity and de novo synthesis of intracellular oxidase as well as the partial purification of extracellular PPO. The rationale for these efforts was to provide data enabling PPO overproduction through 'batch-culture' of mycelia [5] as well as substrate-induction and/or recombinant DNA technologies [31]. Provision of the time-course for intracellular PPO appearance affords an opportunity to determine the subcellular route of PPO secretion via ultrastructural cytochemistry [29].

MATERIALS AND METHODS

Growth conditions

Mycelia of a *Coriolus versicolor* (L. ex fr.) Quel isolate which was provided by the USDA-Northeastern Forest Experiment Station (Durham, NH) and maintained at 4°C upon agar slants were aseptically transferred to a defined medium [13] containing 2% agar. Following mycelial growth in the dark at 25 ± 2°C upon an agar plate's surface to a disc 30 mm in diameter, discs were aseptically excised and then homogenized for 30 s into 25 ml autoclaved distilled H_2O within a sterile Wareing blender cup. One ml aliquots of the mycelial homogenates were transferred with a sterile manostat to autoclaved 250 ml Erlenmeyer flasks containing 25 ml of the above sterile medium lacking agar. The cultures were maintained at 25 ± 2°C and 150 rpm within a model 3529 Lab-line gyrotory shaker (Melrose Park, IL) for various times depending upon the described experiments.

For dry wt. analyses (a growth measure), mycelia were separated from their growth media by gentle vacuum filtration in order to collect mycelia onto oven-dried, pre-weighed glass fiber discs (Gelman Sciences Inc., Ann Arbor, MI). The latter together with the collected mycelia were oven-dried at 77°C until constant weights were achieved. Then, the discs together with the mycelia were weighed and the weights of the discs subtracted.

Time-dependent appearances of intracellular and extracellular protein and PPO

To establish the timed appearances of intracellular and extracellular 280 nm-absorbing substances

(total protein) and PPO mycelial (EC 1:10:3:1) activity, mycelia were cultured as above for 15 days with harvesting occurring at 3 day intervals post-inoculation. The mycelia were separated from the growth media by gentle vacuum filtration onto Whatman #1 filter paper contained within a Buchner funnel suspended in a suction flask.

To obtain intracellular protein, the harvested mycelia were homogenized 5 min into 2 ml of pH 5.0, 100 mM, acetate buffer within an 'ice-cold' mortar and pestle. The resultant homogenates were 'pulled' through a single layer of Miracloth (Calbiochem, LaJolla, CA) and the filtrates rapidly frozen in liquid nitrogen prior to lyophilization. For quantifying 280 nm-absorbing substances and PPO, the collected media were rapidly frozen in liquid nitrogen, lyophilized and then stored over drierite at −20°C until use.

Assay of total protein and PPO activity

Total protein — The residues from lyophilization of either homogenized mycelia or media were each reconstituted in pH 5.0, 100 mM, acetate and subsequently divided into two equal portions. Whereas one of these was dialyzed (Spectrofluor tubing, MW 'cutoff' = 14 000) with stirring for 18 h at 4°C against 2–4 l acetate buffer, the other fraction was maintained for 18 h at 4°C. Then, non-dialyzed and dialyzed fractions were assayed for both 280 nm-absorbing substances and PPO activity as below.

To quantify total protein, 60–140 μl aliquots of both dialyzed and non-dialyzed homogenates and 60-80 μl aliquots of both dialyzed and non-dialyzed media were diluted to 2 ml and assayed at 280 nm using quartz cuvettes and a stock of 140 μg bovine serum albumin (Sigma, St. Louis, MO) 100 μl acetate buffer^{-1} for standard curve construction. The quantified 280 nm-absorbing substances within aliquots were corrected to μg total protein total fraction volume^{-1} and then mg 280 nm-absorbing substances.

Intracellular and extracellular PPO for both dialyzed and non-dialyzed homogenates (intracellular) and media (extracellular) were assayed by pippetting 10 μl of the reconstituted fractions (usually 2 ml acetate buffer) into a one ml reaction mixture consisting of 100 mM catechol (Fisher Scientific, Norcross, GA) dissolved in 'pre-warmed' pH 5.0, 100 mM, acetate buffer and then maintaining the mixture for 10 min at 30°C with shaking [4]. At 2, 4, 6, 8 and 10 min (occasionally for longer time periods) PPO activity was measured by determining absorbances at 440 nm. To generate a 0 time 'reading', 10 μl aliquots of the fractions were added to 'ice-cold' reaction mixtures and their absorbances at 440 nm recorded immediately. The 0 time was subtracted from the 2, 4, 6, 8 and 10 min readings and the resultant values divided by time to yield ΔA min^{-1} to obtain units of PPO activity. This value was multiplied by 1000, corrected to total volume of the reconstituted fractions and divided by the appropriate mg protein. Thus, PPO activity was expressed as Units where

$$1\ \text{Unit} = \frac{\Delta\text{A min}^{-1} \times 1000}{\text{mg protein}} \qquad [33]$$

rather than μmol product formed min^{-1} mg protein^{-1} because of the lack of certitude regarding the product's identity.

Substrate specificity of PPO

The growth media from 9, 12 and 15 day old 26 ml cultures were collected as above, frozen in either liquid nitrogen or dry ice/acetone and then lyophilized. The lyophilized media were reconstituted in 2 ml of pH 5.0, 100 mM, acetate buffer and subsequently dialyzed as above. Ten μl aliquots of the media were added to the aforementioned reaction mixture containing either 100 mM gallic acid (Sigma, St. Louis, MO), 100 mM syringic acid, or 100 mM catechol. Polyphenol oxidase activity was quantified as above.

De novo synthesis vs activation of intracellular PPO

Augmenting cultures with cycloheximide. 25 ml cultures prepared as above were supplied with either 1 ml growth medium or a stock of 1 mg cycloheximide (Calbiochem, LaJolla, CA) ml growth medium^{-1} [1] to yield 1.91 μg ml^{-1} at the day of mycelial inoculation. The cultures were maintained for 12 days at 25 ± 2°C within the previously men-

tioned shaker when the mycelia and growth media were separated by gentle vacuum filtration prior to homogenizing the mycelia and deriving a Miracloth filtrate of the homogenate as previously described. The filtrates were lyophilized, reconstituted in acetate buffer, dialyzed against acetate buffer, and then both total protein and PPO activity quantified as above.

In a separate experiment, 25 ml cultures were supplied with either 1 ml growth medium of 1 mg cycloheximide ml medium^{-1} together with 0.5 μCi uniformly labeled [^{14}C]-leucine (specific activity 33 mCi/mmol, New England Nuclear Corp.) flask^{-1}. At days 0, 2, 4, 6, 8, 10 and 12 days post-inoculation, 200 μl aliquots of media were aseptically withdrawn and added to 10 ml Aquasol (New England Nuclear Dupont) liquid scintillation cocktail contained within plastic scintillation vials.

At day 12 post-inoculation, the mycelia were collected by centrifugation at 500 × *g* for 5 min. Whereas the 500 × *g* supernatants were frozen in liquid nitrogen and stored at −20°C, the pellets were suspended in 2 ml homogenization buffer (25 mM Tris, pH 7.2, 3 mM EDTA, 250 mM sucrose) and homogenized for 5 min within a mortar and pestle on ice. The resultant homogenates were centrifuged at 500 × *g* for 15 min at 4°C to 'pellet' cell walls which were suspended in 1 ml distilled H_2O and then solubilized in 1 ml 0.6 N NCS tissue solubilizer (Amersham, Arlington Heights, IL) prior to adding the wall fraction to the above cocktail. Two, 100 μl aliquots of the 500 × *g* supernatant (cytoplasm) were added to the above cocktail for liquid scintillation counting. In addition, 200 μl aliquots of the supernatant were delivered to Whatman 3 MM filter paper discs. These were immersed in 3 ml cold 10% trichloroacetic acid (TCA) for 36–42 h. At this time, the 10% TCA was removed and replaced with 3 ml 5% TCA which was followed by 3 ml absolute EtOH [19]. One ml aliquots of the pooled 5 and 10% TCA washes as well as the EtOH rinsings were added to 10 ml liquid scintillation cocktail. The air-dried discs were added to 10 ml cocktail (Econoflour-2, New England Nuclear Dupont). Radioactivity was measured with a Packard 300C Tri-carb liquid scintillation counter equipped with automatic quench calibration. Each sample was counted twice for 10 mins each time. In addition, background and the [^{14}C]-efficiency of the counter were determined. These were employed to convert cpms to dpms.

Deuterium oxide labeling: 1 ml aliquots of homogenates of 30 mm mycelial discs upon agar (see above) were added to 80% deuterium oxide (control growth medium) and then cultured from 12 days at 25 ± 2°C [32]. At day 12, the mycelia were separated from the growth medium as above. The collected mycelia were homogenized, the homogenate 'pulled' through a single layer of Miracloth by vacuum filtration, and the filtrate dialyzed as described. Both the total protein and PPO activity were quantified.

To assess whether PPO could be density labeled with deuterium, aliquots equivalent to 10 mg protein were layered onto $CsCl_2$. In addition, 10 mg authentic PPO was dissolved in 1000 μl acetate buffer and layered onto a $CsCl_2$ gradient. Following centrifugation at 10 000 rpm for either 20 or 91 h in a Beckman Type 65 fixed angle rotor with a Beckman L8-70M ultracentrifuge, the tubes were punctured with a Haake-Buchler gradient collector (Saddle Brook, NJ) and 0.2-0.5 ml fractions collected for both total protein and PPO assays.

Partial purification of extracellular PPO

Following collection and lyophilization of growth media from 13 day-old cultures as above, the growth media from four, 26 ml replicate cultures, were reconstituted in 2000 μl pH 5.0, 100 mM, acetate buffer and then 60–180 μl aliquots were assayed for the presence of 280 nm-absorbing substances. Ten μl aliquots of the reconstituted media were utilized for the assay of PPO as described. The resultant mg 280 nm-absorbing substances and PPO (ΔA min^{-1} × 1000) quantifications were employed to calculate the PPO specific activity within the crude medium.

The remainder of the dialyzate was subjected with stirring to a 0–30% ammonium sulfate saturation 'cut' within a stirred ice bath. Next, the sulfate-treated growth medium was centrifuged at 12 000 × *g* for 30 min at 4°C to yield a pellet and a super-

natant. Whereas the former was suspended in 2 ml acetate buffer, transferred to Spectrofluor tubing and dialyzed 18 h against 2–4 l acetate buffer, the supernatant was added to tubing and dialyzed directly. Subsequent to dialysis, the specific activity of the dialyzate's PPO was quantified through assay of both 280 nm-absorbing substances and PPO. This permitted calculation of fold-purified through two purification steps.

To further develop a purification protocol leading toward PPO homogeneity upon a Laemmli SDS-PAGE gel [15], 10 mg lots of authentic PPO (mushroom, Sigma Chemical Co., St. Louis, MO) in 100 μl aliquots of 25 mM phosphate buffer, pH 6.5 were layered onto 0.7 × 15.0 cm Bio-Rad Econo columns containing either phosphate buffer equilibrated DEAE-Sephadex A50 (Pharmacia, Uppsala, Sweden) or CM-Sephadex C50 (Pharmacia) ion exchange resins. These resins were eluted with 5–10 ml buffer lacking 1 M NaCl into a beaker and then with the same buffer containing high ionic strength salt into a separate beaker. To establish the distributions of 280 nm-absorbing substances and PPO within the non-salt and salt resin elutions, the 280 nm-absorbing substances were quantified using a BSA standard curve and PPO by enzyme assay. Because authentic PPO could be eluted with high ionic strength salt from a DEAE resin (see Results, Table 5), the dialyzed and salt-fractionated medium from a 26 ml culture was lyophilized and reconstituted in 100 μl pH 6.5, 25 mM, phosphate buffer and layered onto a 0.7 × 15.0 cm Bio-Rad Econo column containing phosphate buffer-equilibrated DEAE-Sephadex A-50. This resin was eluted with 10 ml buffer lacking 1 M NaCl into a beaker and then with the same buffer containing high ionic strength salt into another beaker. To determine the distributions of 280 nm-absorbing substances and PPO within the non-salt elutions, 280 nm-absorbing substances and PPO were quantified as above. Since PPO could be eluted with high ionic strength salt, a logical subsequent purification step would be Sephadex gel filtration as de-salting could be accomplished. Therefore, 100 μl 'crude medium' containing 1.47 mg 280 nm-absorbing substances was layered onto a 0.7 × 15.0 cm column of Sephadex G-150 (Pharmacia). The column, whose void volume had been marked with blue dextran, was eluted with acetate buffer and 1 ml fractions collected with a fraction collector. Each fraction was assayed for both PPO activity and total protein as previously detailed. In addition, 1.2 mg of authentic PPO within 100 μl acetate buffer was also layered onto a column of Sephadex G-150 which was eluted as above and PPO assayed at 280 nm since it was the only protein gel filtrated.

Statistical analyses

Each experiment was routinely replicated and, therefore, data are means and standard deviations throughout with the number of replicates being experiment-dependent as detailed in the Results. When appropriate, data were analyzed by either a student *t*-test or a two-way analysis of variance [23].

RESULTS

Time-dependent alterations in mycelial dry wt

The growth curve for *C. versicolor* in liquid appeared to be sigmoidal during 15 days of culture at 25 ± 2°C (Fig. 1).

Time-dependent appearances of intracellular and extracellular total protein and PPO

Because the results from comparing 280 nm-absorbing substance levels and PPO specific activities

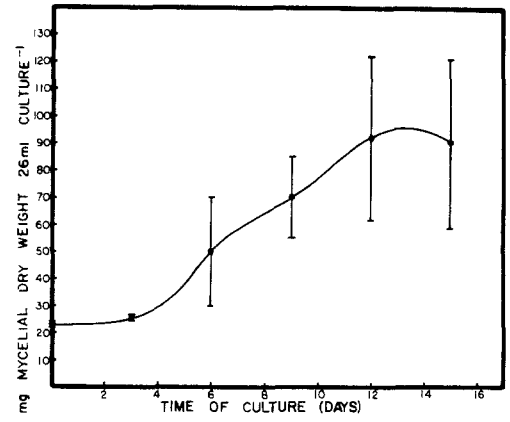

Fig. 1. Time-course for the growth of *Coriolus versicolor* in liquid culture. Data are $\bar{x}$s and σs where $N = 3$.

from non-dialyzed and dialyzed growth medium revealed that dialysis reduced the amount of 280 nm-absorbing substances from 10 to 6 mg 26 ml culture^{-1} at 15 days of a 16 day culture period and enhanced the specific activity of PPO throughout the 16 day time-course, the time-dependent appearances of 280 nm-absorbing substances and PPO within dialyzed mycelial homogenates and growth media were established. These were performed to generate PPO specific activities in the absence of 280 nm-absorbing substances $< 14\,000$ MW.

The time-dependent appearances of total protein (280 nm-absorbing substances) and PPO for dialyzed homogenates are depicted in Fig. 2A. Two peaks of total protein were observed, i.e., 6 and 12 days in a 16 day time-course. In contrast, the specific activity of PPO accelerated from day 4 to day 12 post-inoculation followed by a slight diminution.

With regard to the time-dependent occurrences of 280 nm-absorbing substances and PPO within dialyzed growth medium, with the exception of day 3 of a 16 day time-course, 280 nm-absorbing levels remained nearly constant between 1 and 6 days of culture and then climbed to day 15 during the time-course (Fig. 2B). As for the timing of PPO appearance within the growth medium, the specific activity of extracellular PPO increased from day 4 to day 15 of the time-course except for a slight decline at day 9 (Fig. 2B).

Substrate specificity of PPO

Table 1 summarizes the specific activities of extracellular PPO employing three phenolic compounds, catechol, gallic acid and syringic acid as possible substrates. For day 9 medium, the PPO specific activity utilizing catechol as a substrate was 1.35- and 1.22-fold greater than those for gallic and syringic acids, respectively. At day 12, PPO's specific activity with catechol was 1.62-fold greater than that for gallic acid and almost the same as that for syringic acid. In contrast, when 15 day medium was employed, PPO's specific activity using catechol as a substrate was 6.32- and 1.57-fold greater than those with gallic acid and syringic acids, respectively. When these data were analyzed by a two-way analysis of variance, there was no significant difference in substrate effects for the 9 day medium samples ($F_{2,40} = 3.23$) and 15 ($F_{2,17} = 3.59$) day samples.

De novo synthesis vs. activation of intracellular PPO

Cycloheximide addition: To assess whether intracellular PPO was either de novo synthesized or activated, 25 ml cultures were exposed at the time of culture inoculation with mycelia to 1.91 μg cycloheximide ml growth medium^{-1} for 12 days with subsequent harvesting, mycelial homogenization and quantification of both total protein and PPO activity. A twelve day exposure to cycloheximide

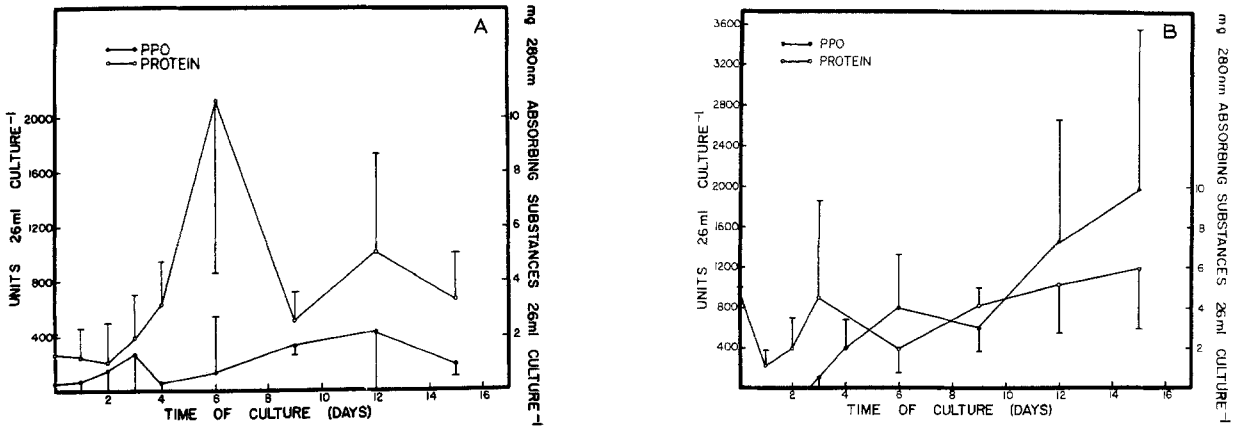

Fig. 2. Time-related appearance of intracellular 280 nm-absorbing substances and PPO for dialyzed mycelial homogenates (A) and growth medium (B). Data are $\bar{x}$s and σs where $N = 9$–15 for the homogenates and $N = 21$–29 (280 nm-absorbing substances) and 12–47 (PPO) for the medium.

Table 1

Comparison of specific activities as units of polyphenol oxidase when assayed with various phenolic substrates[a]

Phenolic substrate	Medium (day of collection)	Polyphenol oxidase activity (Units)[b]
Catechol	9	930.08 ± 823.12
	12	323.69 ± 180.25
	15	1974.06 ± 702.51[c]
Gallic acid	9	686.54 ± 176.40
	12	198.76 ± 63.64
	15	311.87 ± 112.75
Syringic acid	9	759.58 ± 668.40
	12	393.30 ± 279.20
	15	1255.28 ± 942.37

[a] Data are means and standard deviations where N = 9–15.
[b] Significant differences for day 12 ($F_{2,40}$ = 3.23) and day 15 ($F_{2,17}$ = 3.59) were observed amongst the substrates.
[c] Six data points from one sample.

resulted in a 24% inhibition of intracellular protein content and a 73% diminution in PPO spc. act. The latter inhibition was statistically significant ($t_{0.05}$ (28 df) = 1.701).

To further test whether PPO was synthesized rather than activated, cell cultures were labeled with [^{14}C]-leucine in the presence and absence of 1.91 μg cycloheximide ml growth medium^{-1} for 12 days. To ascertain whether [^{14}C]-leucine was taken up, aliquots of the medium were harvested and radioactivity quantified by liquid scintillation counting (see Materials and Methods). At day 12, mycelia were separated from the growth medium, homogenized and centrifuged. Radioactivity within the pellet (cell wall) was measured by liquid scintillation counting after solubilizing the wall. In contrast, protein within the supernatant (cytoplasm) was precipitated with trichloroacetic acid (TCA) onto filter paper discs and radioactivity within the TCA-soluble (disc 'washings') and insoluble (discs) fractions quantified by liquid scintillation counting.

Fig. 3 presents the time-dependent uptake of [^{14}C]-leucine by mycelia grown in medium with and without cycloheximide. Whereas substantial uptake did not occur over time for those cultures grown in medium containing cycloheximide, those mycelia

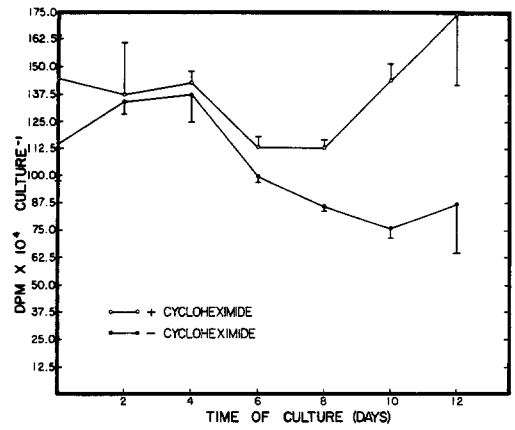

Fig. 3. Time-related uptake of [^{14}C]-leucine for cultures grown with and without cycloheximide. Data are $\bar{x}$s and σs where N = 4 replicate flasks.

cultured in medium lacking cycloheximide began to remove [^{14}C]-leucine from the medium by day 4 of the time-course and continued to until day 10 when the difference in uptake between cycloheximide treatment and the control was statistically significant. Approximately 45% of the exogenously-supplied [^{14}C]-leucine was 'taken-up' in cycloheximide's absence.

Table 2 depicts the amounts of free (TCA-soluble) and incorporated (TCA-insoluble) [^{14}C]-leucine (assuming that the amino acid was not metabolized) 'taken-up' by the mycelia. It is apparent that the majority of the [^{14}C] recovered from the cytoplasm was incorporated into TCA-insoluble protein without cycloheximide. However, inclusion of cycloheximide within the growth medium resulted in a 87.5% reduction in [^{14}C]-leucine incorporation into acid insoluble protein. This reduction was statistically significant at P = 0.05. In addition, cycloheximide administration lowered the amount of [^{14}C]-leucine within the TCA-soluble cytoplasmic and cell wall fractions by 67.9 and 97.5%, respectively.

The effects of cycloheximide administration on intracellular protein levels and PPO activity relative to the incorporation data are summarized in Table 3. The addition of cycloheximide to growth medium completely inhibited PPO activity and correlates well with the cycloheximide-induced suppression of [^{14}C]-leucine incorporation into acid-insoluble protein.

Table 2

Effects of cycloheximide on the uptake and incorporation of [^{14}C]-leucine by *Coriolus versicolor* mycelia grown 12 days in liquid culture

Cell fraction	Treatment	DPM culture^{-1}
Cytoplasm		
Homogenate minus wall	without cycloheximide	31 692 ± 12 507[a]
	with cycloheximide	6 817 ± 1 796
Homogenate subjected to trichloroacetic acid (TCA)		
TCA soluble	without cycloheximide	3 143 ± 1 536
	with cycloheximide	1 007 ± 186
TCA insoluble	without cycloheximide	23 656 ± 16 829
	with cycloheximide	2 957 ± 5 915[b]
Cell wall	without cycloheximide	136 987 ± 82 827
	with cycloheximide	3 555 ± 4 692

[a] Data are means and standard deviations were $N = 4$ replicate cultures.
[b] Statistically different from without cycloheximide at $P = 0.05$.

Deuterium oxide labeling: In addition to employing cycloheximide to differentiate between either PPO de novo synthesis or activation, attempts were made to combine density labeling of intracellular PPO with $CsCl_2$-gradient centrifugation and PPO assays to provide the differentiation. Table 4 presents the results which resulted when intracellular protein and PPO from both deuterated and non-deuterated cultures were quantified. It is seen that mycelial growth in 80% D_2O severely inhibited PPO specific activity while not reducing total protein. In this connection, *C. versicolor* hyphal PPO activity could not be recovered from $CsCl_2$ gradients.

Table 3

Effects of cycloheximide on intracellular total protein and polyphenol oxidase for 12 day cultures

Treatment	mg 280 nm absorbing substances	Polyphenol oxidase activity (Units)
Without cycloheximide	7.9 ± 4.4[a]	29.6 ± 17.5
With cycloheximide	0.0 ± 0.0	0.0 ± 0.0

[a] Data are means and standard deviations where $N = 8$.

Partial purification of extracellular PPO

To achieve partial purification, 'crude' growth medium was subjected to dialysis and then a 0–30% ammonium sulfate fractionation 'cut'. The alterations in extracellular PPO specific activity which occurred as a result of applying these procedures are summarized in Table 5. Whereas dialysis of 'crude' growth medium yielded a 2.17-fold enhancement in PPO specific activity, a 0–30% ammonium sulfate saturation 'cut' resulted in 94% of the PPO activity being recovered within the 12 000 × *g* supernatant of the sulfate-fractionated, dialyzed media. When the PPO specific activity within this supernatant was compared to that for PPO within the crude medium, a 3.27-fold elevation was observed.

Table 4

Total protein contents and polyphenol oxidase activities of mycelial homogenates obtained from *Coriolus versicolor* cultured with and without deuterium oxide[a]

Culture condition	mg 280 nm absorbing substances	Polyphenol oxidase activity (Units)
Without D_2O	16.70 ± 0.42	73.28 ± 35.97
With D_2O	21.75 ± 5.58	6.67 ± 2.72[b]

[a] Data are means and standard deviations where $N = 4$ and 10 replicates for protein and polyphenol oxidase, respectively.
[b] Significantly different from without D_2O at $P = 0.002$.

Table 5

Polyphenol oxidase activity following sequential purification of extracellular polyphenol oxidase[a]

Medium fractionation step	Polyphenol oxidase activity (Units)
Crude medium	1332.7 ± 367.5
Dialyzed medium	2893.6 ± 1310.3
Dialyzed plus $(NH_4)_2SO_4$-fractionated medium	
Pellet	285.2 ± 308.5
Supernatant	4359.0 ± 2762.5

[a] Data are means and standard deviations where N = 27–64 assays for two replicate experiments each containing 3 replicate flasks.

Ion exchange chromatography: To assess whether PPO within the growth medium could be further purified by ion exchange chromatography, experiments centering about chromatography of commercial PPO on both DEAE-Sephadex A_{50} (anionic resin) and CM-Sephadex C_{50} (cationic resin) were performed as mentioned. Layering of authentic PPO onto phosphate buffer-equilibrated resins followed by elution revealed that PPO did not exchange with the counterion of CM-Sephadex C_{50} but did with DEAE-Sephadex A_{50} (data not shown). The bulk of the PPO (90.9 ± 5.5) could only be eluted from the latter exchange resin with phosphate buffer containing high ionic strength salt. Therefore, 13 day growth media from 26 ml cultures which had been dialyzed and subsequently subjected to ammonium sulfate fractionation was layered onto a column of Sephadex DEAE-A50 prior to eluting the resin with phosphate buffer lacking 1 M NaCl and then containing the salt. The results of three replicate experiments are presented in Table 6. Like authentic PPO, the bulk of the medium PPO activity recovered from the DEAE-A50 resin was eluted with buffer containing 1 M NaCl except for experiment 2. In contrast to PPO, the recoveries of 280 nm-absorbing substances for the three experiments differed with experiments two and three exhibiting either most or all of the recovered protein within the salt-eluted fraction. For experiment 1, the recovery was reversed, i.e., most of the protein was eluted without high ionic strength salt.

Sephadex Gel Filtration: A Sephadex G-150 elution profile of PPO within growth medium is depicted in Fig. 4A. Two 280 nm-absorbing peaks were witnessed with one of these eluting in the void volume and overlapping in part with the retarded protein peak. The position of this PPO peak relative to the void volume was similar to that of authentic, commercially-available PPO when it was chromatographed upon Sephadex G-150 (Fig. 4B).

DISCUSSION

Time-dependent alterations in mycelial dry wt

The growth for *C. versicolor* cultured within a defined liquid medium (Fig. 1) appeared to follow a sigmoidal curve although the elevated standard deviations allow for other interpretations. This repeats that reported by Taylor et al. [27] and suggests that this curve does indeed reflect the time-dependent growth pattern of *C. versicolor* for a defined liquid medium [13]. The value of the curve is that it offers a chance to investigate the possible regulation of *C. versicolor* in vitro growth by tree-synthesized phenolic compounds, purported disease-resistance factor [25]. This was initiated by Taylor et al. [26] who demonstrated that *C. versicolor*'s growth in liquid culture responded in a time-dependent bimodal fashion to catechol, which may provide 'useable' information regarding the mechanism(s) by which certain fungi degrade wood [21].

Time-dependent appearances of intracellular and extracellular protein and PPO

The demonstrated time-dependent occurrences (Fig. 2A) of two intracellular 280nm peaks subsequent to mycelial homogenates' dialysis (removes substances < 14 000 MW) may reflect the 'turnover' of two different classes of enzymes as *C. versicolor* has been reported to elaborate both cellulolytic and ligninolytic enzymes into its culture medium. To test this, the proteins which compose peaks one and two could be both isolated and characterized by standard protein purification procedures as well as assay of cellulases [3] and ligninases [2]. The signif-

Table 6

Recovery of protein and polyphenol oxidase from DEAE-Sephadex following chromatography of dialyzed and ammonium sulfate fractionated growth medium

Eluate condition	Source of polyphenol oxidase	% of total 280 nm-absorbing substances recovered			% of total polyphenol oxidase recovered		
		Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3
pH 6.5, 25 mM K_2HPO_4	Mushroom thallus		20.1 ± 6.9[b]				
KH_2PO_4 minus 1 M NaCl	*C. versicolor* growth medium	82.8 ± 1.0[a]	28.4 ± 8.5[c]	0.0 ± 0.0	6.2 ± 6.0[a]	62.9 ± 28.8[c]	8.6 ± 7.1[d,c]
pH 6.5, 25 mM K_2HPO_4	Mushroom thallus		79.9 ± 6.8[b]				
KH_2PO_4 plus 1 M NaCl	*C. versicolor* growth medium	17.2 ± 1.0[a]	71.6 ± 8.5[c]	100.0 ± 0.0	93.8 ± 6.0[a,c]	37.0 ± 28.9[c]	91.4 ± 7.1[d,c]

[a] Data are means and standard deviations from 3 replicate flasks.

[b] Sigma Chemical Co. commercial enzyme was subjected to DEAE-Sephadex chromatography only and enzyme detected at 280 nm; data are means and standard deviations from 4 replicate experiments.

[c,d] Data are means and standard deviations from 3 (280 nm-absorbing substances) and 4 (polyphenol oxidase) replicate flasks.

[c] Significantly different from the PPO eluted with buffer lacking salt at $P = 0.001$.

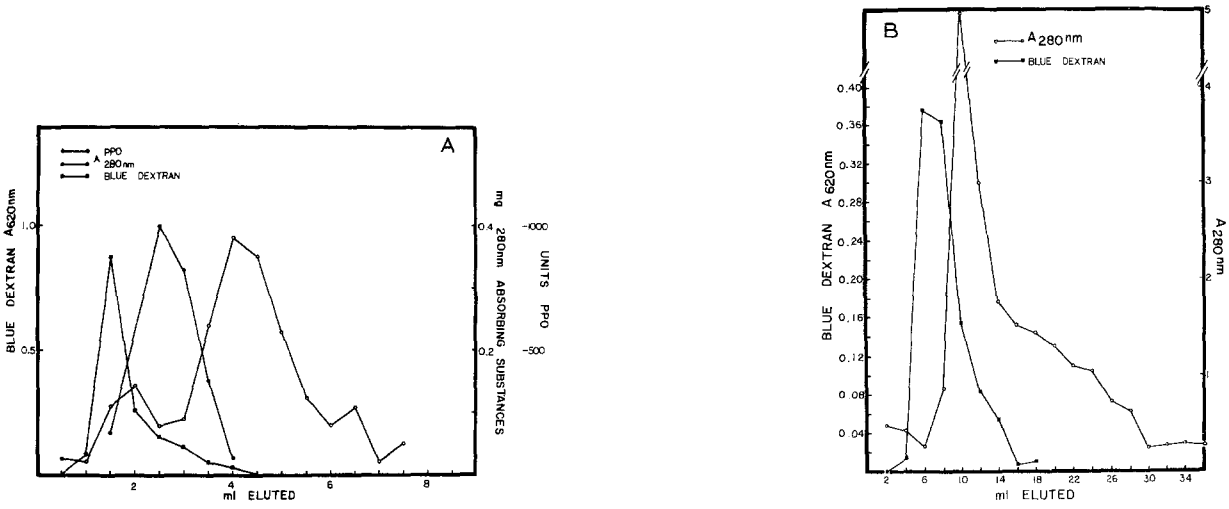

Fig. 4. Sephadex G-150 elution profiles of 280 nm-absorbing substances and PPO within crude growth medium (A) and authentic PPO(B). Data are $\bar{x}$s and σs where $N = 3$ (A) with complete recovery of the layered protein being acheived. The A280nm data for B represent absorbances corrected to fraction total volume.

icance of the latter to white rot fungal-promoted lignin biotransformation has recently been reviewed [9].

On the other hand, the two different 280 nm-absorbing peaks may represent proteins which are destined to remain intracellular and those which are to be elaborated into the growth medium. This could be tested by devising procedures which may inhibit secretion. For example, if *C. versicolor* extracellular enzymes are glycoproteins, then inhibitors of glycoprotein glycosylation may be of value.

In this connection, the time-dependent appearance of intracellular PPO (Fig. 2A) more closely coincided with the occurrence of the second protein peak. This correlation supports the possibility that one of the protein peaks represents those proteins which are to be secreted, e.g., PPO is elaborated into the *C. versicolor* external milieu [27].

The time-related occurrences of PPO activity within the growth medium (Fig. 2B) substantiate the previous findings of Taylor et al. [26] that *C. versicolor* elaborates PPO into the external milieu. However, some differences in the time-course for extracellular PPO were noted from that reported by Taylor et al. [27]. For example, extracellular PPO activity was detected prior (3 day post-inoculation) to that (8 day post-inoculation) by Taylor et al. There are various explanations for these differences, e.g., longer PPO assay times were employed in the present investigation than those performed by Taylor et al. This resulted in the detection of low PPO activity early in the time-course. In addition, dialysis of the growth medium yielded a reduction in the 280 nm-absorbing substances thereby enhancing the PPO specific activity throughout the time-course for extracellular appearance. This reduction was most likely due to low molecular weight 280 nm-absorbing substances as the dialysis tubing which was employed possessed a molecular weight 'cut-off' of 14 000. Thus, the time-dependent appearances of both extracellular protein and PPO displayed in Fig. 2B most likely reflect the temporal fashion by which *C. versicolor* elaborates non-PPO protein and PPO into its growth medium.

The time-courses for intracellular (Fig. 2A) and extracellular PPO (Fig. 2B) suggest that there may be a precursor-product relationship between intracellular and extracellular PPO, i.e., the latter may be derived through secretion of the former as intracellular PPO 'turns over' at day 12 of culture while extracellular continues to accumulate until day 14. This could be tested by calculating the rates at which intracellular PPO turns over and extracellular accumulates. In addition, intracellular PPO could be purified to determine whether it is similar in amino acid composition and MW to extracellular

PPO. In this connection, it is well-known that certain higher plants, e.g., *Daucus carota* [14] and *Mucuna pruriens* [30] contain a prophenoloxidase.

Substrate specificity of PPO

The data presented within Table I suggest that catechol can serve as a substrate for *C. versicolor* PPO since extracellular PPO within 15 day medium (extracellular used because of its ease of collection) exhibited a high specific activity when this phenolic compound was employed as a substrate. This is consistent with the widespread usage of this dihydroxphenolic compound as a substrate for fungal PPO. However, *Rhizotonia practicola*'s (a white-rot fungus) phenoloxidase has been reported to oligomerize syringic acid, a lignin derivative [18]. Leonowicz and Grzywnowicz [17] have employed syringaldazine to assay the laccase component of the polyphenol oxidase complex. Laccase (EC1.10.3.2) catalyzes the conversion of either *p*-hydroquinones or *p*-phenylenediamines to either *p*-quinones or *p*-quinonediimines [8].

To definitively establish the substrate specificity of both intracellular and extracellular PPO, the utilization of purified intracellular and extracellular *C. versicolor* PPO together with Line-Weaver Burke plots to obtain a K_m is required. This parameter can then be employed to draw more certain conclusions regarding the affinity of *C. versicolor* PPO for its appropriate substrate. In this connection, Wichers et al. [30] have successfully involved Michaelis-Menten kinetics to assess the affinity of *Mucuna pruriens* phenol oxidase for catechol.

De novo synthesis vs. activation of intracellular PPO

As for the question of whether intracellular PPO was either de novo synthesized or activated, the combined results of Tables 2 and 3 which employed the protein synthesis inhibitor, cyclohemimide, as well as [^{14}C]-leucine incorporation into mycelial cytoplasmic protein, strongly supports the alternative that intracellular PPO was de novo synthesized. However, this conclusion could not be verified via another but quite different technique, namely, density labeling of intracellular PPO with deuterium oxide (D_20) and a consequent demonstration of PPO band shift (indicator of synthesis) upon $CsCl_2$-gradient centrifugation of mycelial homogenates. This technique was successfully applied in an investigation of whether β-amylase of barley aleurone layers was either de novo synthesized or activated [6]. A possible explanation as to why density labeling with D_2O was not successful can be found within Table 4. It is apparent that in vitro growth of *C. versicolor* in 80% D_2O coupled with assay of intracellular PPO resulted in a loss of PPO activity when compared to that for PPO derived from cultures grown in D_20's absence. This raises the possibility that D_20 inactivated the enzyme. The mechanism by which D_20 would do so is worthy of experimentation.

The conclusion that *C. versicolor* PPO was de novo synthesized rather than activated differs from that for certain higher plant PPOs where intracellular enzyme occurs in a latent form and can be activated by various treatments such as pH, fatty acids, detergents and proteases especially trypsin (cf. literature in [12]).

Recently, these authors demonstrated by electrophoresis that trypsin could convert inactive broad bean PPO to an active form possessing a slightly reduced molecular weight. Other higher plants than broad bean have been reported to contain a prophenoloxidase, e.g., Soderhall et al. [24] reported that *Daucus carota* L. cells grown in culture contain an inactive PPO which was activated by divalent cations such as Ca^{2+} and Mn^{2+}.

This discussion raises the possibility that *C. versicolor* mycelia could contain two types of PPO, one of which is synthesized and another that is activated; the latter of which went undetected in the present investigation. Recently developed immunoblotting procedures originated by Lanker et al. [16] offer the possibility of detecting inactive PPO. However, these procedures were developed for *Vicia faba* PPO and may require modification for adoption to the *C. versicolor* system.

Partial purification of extracellular PPO

Although there are some previous reports within the literature related to the purification of *C. versicolor* PPO [5,14], these appear to have been con-

cerned with the laccase component of the PPO complex rather than PPO itself. Fahraeus and Reinhammer [5] have employed catechol as a substrate to quantify laccase activity which was partially purified by dialysis and ammonium sulfate to saturation. Fric [8] has clarified much of the terminological confusion which exists within the polyphenol oxidase literature by distinguishing PPO activity (EC 1:10:3:1) from laccase activity (EC 1:10:3:2) of the polyphenol oxidase complex via substrate specificates. The present investigation dealt with PPO activity against catechol rather than laccase activity. It would be of interest to assess whether our preparations can exhibit laccase activity against p-hydroquinones. The data within Table 1 indicate that extracellular PPO can be purified 3.07-fold via sequential dialysis and 0–30% ammonium sulfate fractionation. As previously mentioned, the fact that the specific activity of extracellular PPO increased after dialysis indicated that the growth medium contained 280 nm-absorbing substances $<14\,000$ MW. Furthermore, the enhancement of PPO specific activity within the $12\,000 \times g$ supernatant of the 0–30% fractionation suggests that this 'cut' eliminated some non-PPO protein. This could be verified by subjecting aliquots (same amount of protein) of crude medium, dialyzed medium and dialyzed ammonium sulfate-fractionated medium to Laemmli SDS-PAGE electrophoresis [15] following Coomassie blue staining to determine whether protein bands were lost through the sequential protocol employed in Table 5.

With regard to ammonium sulfate fractionation, the PPO within the $12\,000 \times g$ supernatant resulting from ammonium sulfate fractionation may be further purified by partitioning between 40 and 90% ammonium sulfate as this 'cut' was successfully applied in the purification of *Mucuna pruriens* PPO [30].

Ion exchange chromatography: Because mushroom (a fungus) PPO eluted with high ionic strength salt from a DEAE-Sephadex A50 column (Table 6), dialyzed and ammonium-sulfate fractionated *C. versicolor* growth medium was subjected to DEAE chromatography. The PPO within fractionated medium could be eluted with high ionic strength salt in 2 of 3 experiments.

The elution of PPO with high ionic strength salt from a DEAE resin is consistent with that observed by Wichers et al. [30] who used this anionic resin following ammonium sulfate fractionation and celite chromatography to partially purify the phenol oxidase from suspension cultures of *Mucuna pruriens*. In addition, Soderhall et al. [24] used DEAE cellulose chromatography following ammonium sulfate precipitation in an attempt to purify a prophenoloxidase from *Daucus carota* L. cell cultures. This observation coupled with the present findings suggest that there may be some chemical similarities between fungal and higher plant phenol oxidases. This could be tested by establishing the co-factor requirements as well as the molecular weight of *C. versicolor* PPO once purified to homogeneity. In spite of the widespread involvement of DEAE chromatography in purifying PPO from various plants, an elevation in PPO spc. act. was not consistently noted in the present investigation prompting a requirement of repetition of PPO chromatography upon DEAE.

Related to purification, filtration of crude medium upon Sephadex G-150 separated PPO from some non-PPO protein raising the possibility that an enhancement in PPO specific activity may be obtained via gel filtration. In this connection, preliminary Sephadex G-150 gel filtration of dialyzed, ammonium sulfate treated and DEAE salt-eluted medium PPO yielded a similar PPO elution profile to that for crude medium but a reduction in the protein content of peak two.

Finally, we are continuing to develop a purification protocol which will ultimately lead to a single band upon a Laemmli SDS-PAGE gel [15] and a significant fold-purified. This purification is centering about the combined protocol of Sephadex G-150 gel filtration and hydrophobic interaction chromatography on phenyl sepharose C-L4B and may also involve celite and DEAE-sepharose chromatographies [30] as well as hydroxylapatite chromatography [16]. Once purified to homogeneity, PPO's molecular weight will be established by calibrated Sephadex gel filtration and Laemmli SDS-PAGE. This together with purification of *C. versi-*

color PPO will be the subjects of a forthcoming paper as purified PPO is required in an effort to 'raise' antibody to the enzyme [7]. The antibody will serve as a probe for the immunocytochemical localization of hyphal PPO and the products of in vitro translation of *C. versicolor* poly-adenylated RNA.

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