Effect of Environmental Conditions on Extracellular Protease Activity in Lignolytic Cultures of *Phanerochaete chrysosporium*

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Two different types of extracellular protease activity were identified in the culture fluid of *Phanerochaete chrysosporium* wild-type BKM-F grown in submerged batch culture on N-limited media. The first activity, which appears to be inherent to the active growth phase, displayed a maximum on day 2 and decreased to a very low level on day 4. The second activity, which appeared at day 8 following the peak of ligninase activity, seems to be characteristic of late secondary metabolism and is stimulated by carbon starvation. Cultures started with half the amount of glucose of other cultures showed a remarkably earlier development of secondary activity. In contrast, the fed-batch addition of glucose started when ligninase activity was at a maximum (day 6) completely repressed secondary protease activity and enhanced ligninase production. The addition of exogenous veratryl alcohol increased the level of secondary protease activity, whereas the oxygen supply pattern significantly affected both the time course and the level of overall proteolytic activity. The addition of phenylmethylsulfonyl fluoride to growing cultures (0, 1, or 6 days) diminished overall protease activity, while it significantly enhanced ligninase activity. In all cases, the time courses of protease and ligninase activities were negatively correlated, indicating that protease activity promotes the decline of ligninase activity in batch culture.

The production of extracellular enzymes by the white rot fungus *Phanerochaete chrysosporium*, wild type and mutants, has been extensively studied over the last decade (4, 18). A number of extracellular enzymes capable of cellulolytic, hemicellulolytic, or lignolytic activity have been reported (7, 8, 22); several of these enzymes may act synergistically (6). Of the isolated and purified lignolytic enzymes, H2O2-dependent lignin peroxidase (ligninase) and Mn-peroxidase are the most widely studied and characterized (17, 25). Ligninase was shown to play a major role in the oxidation of lignin-related model compounds (4, 17).

Interestingly, even though the production of extracellular proteases is a common feature among fungi (24) in general and wood-degrading basidiomycetes (20) in particular, very little work has been done on this aspect of *P. chrysosporium*. Eriksson and Pettersson (9, 10) reported the purification and partial characterization of two acidic proteases in shallow stationary cultures of *Sporotrichum pulverulentum* (= *P. chrysosporium*, ME-446) grown on cellulose. These two proteases, isolated from 10-day-old cultures, were shown to play a role in the activation of the endo-1,4-β-glucanases. However, no data on the effect of these proteases on the extracellular ligninase secreted by the fungus or their production and mode of action in shaken cultures with a soluble, noncellulolytic substrate were reported. Moreover, no data on the regulation of these proteases in either stationary or shaken lignolytic cultures of *P. chrysosporium* were reported.

The kinetic profile of ligninase activity in submerged batch cultures of *P. chrysosporium* with glucose as the substrate displays a relatively sharp maximum peak for most of the strains analyzed (4, 11, 12). Tien and Tu (27) showed that when ligninase activity in the extracellular fluid decreases, the mRNA-ligninase still tends to increase. Faison and Kirk (11) showed that although ligninase activity begins to decrease after maximum activity is reached, the lignolytic activity (14CO2) still increases in carbon-limited cultures of *P. chrysosporium*. Tonon and Odier (29) reported that veratryl alcohol enhances the ligninase activity of *P. chrysosporium*, protecting it against inactivation by endogenous H2O2. Whether enzyme inactivation or degradation of the protein structure of the enzyme is the major reason for the rapid loss of ligninase activity in batch culture remains uncertain.

The present study describes the kinetics of two stages of an extracellular, acidic, proteolytic activity found in submerged batch cultures of *P. chrysosporium*. Of these two activities, one appeared in the growth phase and the other was found in the late metabolic phase of lignolytic or nonlignolytic cultures. The relationship among the proteolytic and ligninase activities, as well as the conditions regulating them, was studied.

**MATERIALS AND METHODS**

**Organism.** *P. chrysosporium* Burds wild-type BKM-F-1767 (ATCC 24725) was maintained at 37°C on 2% malt agar slants (15). Prior to the experiments, the organism was subcultured on the same medium contained in petri dishes.

**Culture conditions and media.** All cultures were grown in an N-limited medium by the method of Tien and Kirk (26), but with 20 mM acetate (pH 4.5) instead of dimethylsulfoxide as buffer. The initial glucose concentration was 10 g/liter except in the study of the effect of the initial glucose concentration, for which the concentration was modified to 5 or 15 g/liter. All the other components and conditions remained the same. The inoculum was grown in stationary cultures initiated from fresh conidial suspensions by the method of Jäger et al. (15), but with a conidium concentration of 5 × 105 spores per ml. The inoculation ratio of the experimental cultures was 10% (vol/vol), and the incubation temperature was 37°C in all cases. All experiments were performed in an agitated submerged culture incubated in a

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G50 orbital shaker (New Brunswick Scientific Co., Inc.). The following culture sizes were employed: 50 ml/120-ml serum bottle and 90 ml/250-ml Erlenmeyer flask. Agitation speeds were 225 and 175 rpm, respectively. With the exception of the studies of the effects of oxygenation methods, all other experiments were carried out in Erlenmeyer flasks with periodic oxygenation. Flasks were sealed with rubber stoppers. Serum bottles were sealed with gas-impermeable butyl rubber septum-type stoppers (2048-11800; Belco Glass, Inc.) and covered with aluminum seals. For periodic oxygenation, the headspaces of the cultures were aseptically flushed (1 min at 20 lb/in2 gauge) with pure O2 at the time of inoculation and every 24 h just after samples were taken by means of two hypodermic needles (21G 1.5-in. [1 in. = 2.25 cm] gauge). Continuous oxygenation was achieved by continuously flushing the headspace (0.088 ml/ml per min; 21G 1.5-in. gauge) or the liquid phase (0.132 ml/ml per min; 21G 3.5-in. gauge) with pure O2.

All experiments were performed at least three times using three or four replicates for each condition each time. The results at the same conditions were averaged.

Enzymatic assays. (i) Lignin peroxidase activity. Lignin peroxidase activity was measured essentially as described by Tien and Kirk (26). Enzyme activity (in units per liter) was calculated with an extinction coefficient for veratrylaldehyde of 9.3/mmol per cm.

(ii) Mn-peroxidase activity. Mn-peroxidase activity was measured as described by Kuwahara et al. (21), with phenol red as the substrate. Activity was expressed as A4io-

(iii) Protease activity. Protease activity was measured with azocoll (Sigma Chemical Co.) as the substrate and 50 mM acetate buffer (2, 9, 23). Samples (1 ml) were incubated for 30 min with 1 ml of reaction suspension containing 20 mg of substrate per ml. As a blank, 1 ml of acetate buffer was incubated under the same conditions. Samples were shaken every 10 min, and the reaction was stopped by the addition of 0.4 ml of a 10% (wt/vol) trichloroacetic acid solution. The remaining unhydrolyzed substrate was removed by centrifugation (12,000 × g for 8 min). The colored supernatant was spectrophotometrically assayed at 520 nm against the blank. The optimal incubation pH and temperature were 4.5 and 37°C, respectively. Activity (in units per liter) was calculated assuming 1 U as the amount of enzyme which catalyzes the release of azo dye so that ΔA/Δt = 0.001/min (2), where ΔA is the change in absorbance and Δt is the change in time. Acceptable results were obtained with azoalbumin (28) or azocasein (5) as the substrate. However, because of its simplicity and better reproducibility, the azocoll technique was preferred.

Analytical techniques. Reducing sugar was determined by the dinitrosalicylic acid method with d-glucose as the standard, as described by Ghose (13). Protein was measured by the method of Bradford (3), with bovine serum albumin fraction V as the standard and with the reagent from Bio-Rad Laboratories. The presence of extracellular polysaccharides was qualitatively detected by precipitation at −20°C for 2 h after ethanol was mixed with extracellular fluid (3:1, vol/ vol).

Effect of additives. Unless otherwise stated, glucose was added daily immediately after sampling on the basis of the rate of consumption (1.3 to 1.5 g/liter per day), starting from day 6 of culture. Veratryl alcohol was added to a final concentration of 0.4 mM starting on day 6 and then every second day to 0.4 mM. Phenylmethylsulfonyl fluoride (PMSF) was added to a final concentration of 0.1 mM at inoculation time or after 1 or 6 days.

![FIG. 1. Effect of initial glucose concentration on the production of extracellular enzymes and proteolytic activity. (a) Protease; (b) linyinase; (c) Mn-peroxidase; (d) extracellular protein. Initial glucose concentrations were 5 (□), 10 (○), and 15 (△) g/liter.](http://aem.asm.org/)

**RESULTS**

Effect of initial glucose concentration. The kinetics of extracellular enzyme formation in N-limited cultures was characterized with three different initial glucose concentrations. The time courses (Fig. 1) of protease, linyinase, and Mn-peroxidases and of total extracellular protein were studied with 5, 10, and 15 g of initial glucose per liter. The results suggest that the production of secondary proteolytic activity (Fig. 1a) as well as the onset of the decay of the lignolytic system is regulated by the remaining glucose concentration (Fig. 1b and c and Fig. 2). Indeed, in the cultures started with 5 g of glucose per liter, a remarkably earlier development of secondary protease activity was found (Fig. 1a). Moreover, an increase of extracellular protein during the late metabolic stage was found to occur earlier for cultures with 5 g of initial glucose per liter than for those with higher glucose concentrations (Fig. 1d), suggesting autolysis of aged cells due to the effect of the proteolytic or other enzymatic activity. With 15 g of initial glucose per liter, there was a clear delay in the onset of secondary protease activity (Fig. 1a), while linyinase activity lasted longer (Fig. 1b). The glucose consumption rate and nitrogen uptake (data not shown) were practically identical during days 1 and 2 of the growth phase regardless of the initial glucose concentration. Furthermore, the glucose consumption rates were similar regardless of the initial substrate concentration (notice the nearly parallel slopes on Fig. 2). An inverse correlation between the extracellular protein and proteolytic activity was apparent (Fig.
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Glucose concentrations were 5 (□), 10 (○), and 15 (△) g/liter. Note the virtually parallel curves regardless of the glucose concentration.

1a and d) during the 2 to 3 days of culture, suggesting the involvement of this protease in either mycelium growth or pellet formation. While in general there was a good correlation between extracellular protein and both Mn-peroxidase and ligninase during the lignolytic phase, the protein profile was influenced by other factors, perhaps cell autolysis, during the late metabolic stage. Whether the change in extracellular protein content at this stage was due to the formation of protease itself or to its effect on other extracellular or cell proteins was not studied. In general, clear inverse correlations between the time course of proteolytic activity and total extracellular protein during the growth phase and between protease and ligninase activities during secondary metabolism were found in all cases.

Effect of glucose addition. To test the regulatory influence of the remaining glucose concentration on both protease and ligninase, the effect of fed-batch glucose addition, initiated when ligninase activity reached its maximum, was compared with that of the standard procedure. The results for no glucose addition and glucose addition are shown in Fig. 3a and b, respectively. Daily glucose addition, based on an average glucose consumption rate of 1.3 to 1.5 mg/ml per day, completely repressed the protease and simultaneously kept the ligninase near its highest activity from days 6 to 11. During the period of addition, the cultures had practically a constant glucose concentration (Fig. 3b), which indicates that the rate of glucose oxidation was equal to the rate of addition. The cultures displayed an increase in medium viscosity (data not shown) with the addition of glucose, probably because of the formation of high amounts of extracellular polysaccharides. Glucose addition displayed a similar stabilizing effect on Mn-peroxidase beyond day 6 (Fig. 4c). In each case, the extracellular protein content followed the ligninase profile during the lignolytic stage (Fig. 3), indicating that ligninase is the main extracellular protein in this stage. The decrease in both extracellular protein and ligninase activity and the increase in protease activity (Fig. 3a) during the late metabolic stage suggest that protease is involved in the breakdown of the ligninase-related protein.

The trends presented here were also noticed in similar experiments performed in an air-lift fermentor (C. G. Dosoretz, H. C. Chen, and H. E. Grethlein, Program Abstr. 1989 ASM Conf. Biotechnol., abstr. no. 36).

Effect of veratryl alcohol addition. To study the time course of proteolytic activity under defined conditions that stimulate ligninase production, 0.4 mM veratryl alcohol alone or in combination with glucose was added to growing cultures. This concentration, corresponding to that originally present in the medium, was added on day 6 at the maximum of ligninase activity and every second day thereafter.

The addition of veratryl alcohol (Fig. 4) temporarily increased ligninase production (Fig. 4a) when there was enough glucose without any effect on protease activity up to day 6 (Fig. 4b). There was a sudden decrease in ligninase activity (Fig. 4a) after a rapid increase in secondary protease activity (Fig. 4b). On the other hand, the combined addition of veratryl alcohol and glucose from day 6 stabilized ligninase activity at a value exceeding that resulting from the addition of veratryl alcohol alone (Fig. 4a). Furthermore, this combined addition and the addition of glucose alone repressed secondary proteolytic activity to similar extents, thus giving another clear indication of the direct involvement of glucose in the regulation of secondary proteolytic activity (Fig. 4b). As pointed out for the addition of glucose, the addition of combined glucose and veratryl alcohol had a similar stabilizing effect on the Mn-peroxidase activity (Fig. 4c) that remained at the time of the additions.

Effect of PMSF. To clarify whether protease directly affects ligninase decay or whether both enzymes are simul-
simultaneously regulated by the concentration of the remaining glucose, the addition of a specific protease inhibitor, PMSF, was studied. PMSF has been reported to be noninhibitory to ligninase activity in crude extracellular fluid (25). To test its effect on growing cultures, 0.1 mM concentrations were added at three different stages of the cultures: inoculation (prior to pellet formation), day 1 (following pellet formation), and day 6 (following the maximum of ligninase). The results show that the additions of PMSF at days 0 and 1, which inhibited the primary and secondary proteolytic activities (Fig. 5a), significantly enhanced ligninase production and prevented its decay (Fig. 5b) and considerably affected the time course of Mn-peroxidase activity (Fig. 5c). A lag of 1 day in the onset of the lignolytic system was observed after the addition of PMSF during the growth phase. This lag probably indicated some temporary growth inhibition effect. Whether PMSF remained intact or was degraded with time is not known. Stronger protease inhibition (Fig. 5a), lower glucose consumption (Fig. 5d), and higher ligninase production (Fig. 5b) were found when the inhibitor was added at the beginning of culture instead of after the lignolytic system was established. When PMSF was added on day 6, it inhibited secondary protease activity and stabilized Mn-peroxidase and ligninase activities beyond 6 days. The effect of primary proteolytic activity on either the growth phase or the lignolytic stage is still unclear, but when this activity was delayed or partially suppressed, it significantly affected the time course and extent of ligninase activity.

**Effect of oxygenation conditions.** Since O₂ is necessary to support the lignolytic system, different methods of oxygen supply, which establish different environments, were tested. The effects of these different environmental conditions on the development of the proteolytic and lignolytic activities are presented in Table 1. These results show that increased oxygenation simultaneously increased protease activity, decreased ligninase activity, and increased the glucose consumption rate. Increased O₂ supply also increased (about three times) secondary protease activity more significantly than primary protease activity, which was in line with the corresponding increase in glucose consumption.

**DISCUSSION**

A clear dependence of either secondary proteolytic activity or the extent of the lignolytic phase on the level of glucose remaining (below 10 mM) was observed after cultures were initiated with different glucose concentrations. Remarkably, the times of appearance as well as the levels of maximum primary proteolytic activity were practically identical regardless of the initial glucose concentration. Furthermore, since the glucose concentration was the sole component modified in the media, one can assume the same N limitation in all cases. This behavior suggests that the establishment of this primary activity is independent of the glucose concentration or, in other words, is growth related.

The effect of the fed-batch addition of glucose indicated that both ligninase and secondary protease activities were regulated by the glucose concentration. There are indica-
The addition of veratryl alcohol at day 6 and beyond gives an example of the development of protease activity under conditions stimulatory for ligninase production. Our results were consistent with previous reports (1, 12, 29) that veratryl alcohol stimulates or protects ligninase production. However, the combined addition of glucose and veratryl alcohol stabilized ligninase activity for days at a level corresponding to the maximum reached by the addition of veratryl alcohol alone. In this case, the repressive effect on protease activity was similar to that of the daily addition of glucose. This behavior indicates that the development of protease activity during the late stage of lignolytic cultures of *P. chrysosporium* is regulated by the amount of glucose present in the medium.

The effect of the addition of PMSF to growing cultures shows that inhibiting the early development of proteolytic activity enhances the time course of lignolytic activity. The direct effect of primary protease activity in the development of ligninase activity is not known. Our results show that inhibiting primary protease (as in the case of PMSF addition) or promoting it (as in the case of an increase in the O₂ supply) enhances or depletes ligninase activity, respectively. While primary proteolytic activity seems to be involved in the growth process, the role of secondary activity is in line with the general phenomena of autolysis displayed by fungi (9) under conditions of nutrient starvation. Indeed, the rise in protease activity in this work occurred after the complete depletion of glucose, denoting starvation conditions. The pronounced increase in extracellular protein found in cultures grown on 5 g of initial glucose per liter and incubated over the period of the lignolytic phase also supports this assumption.

The proteolytic activity identified in the present work is probably related to the acid proteases reported by Eriksson and Pettersson (9, 10) for the ME-446 strain grown on cellulose. Interestingly, they observed that the increase in protease activity was followed by rapid lysis of the fungal cell wall and an increase in endoglucanase activity. However, since glucose was used as the substrate, endoglucanase activity was completely repressed in our work; thus, the role assigned to these proteases may have to be reconsidered in the absence of cellulase activity. The fact that the addition of a specific protease inhibitor keeps both ligninase activity and extracellular protein at their maximums during our experiments suggests the involvement of the protease in the development and decay of ligninase in batch cultures. Furthermore, the good qualitative correlation between extracellular protein and ligninase obtained not only during the onset and development of the lignolytic system but also during its decay supports this assumption. Hellebust et al. (14) reported the activity of an exoprotease in *Staphylococcus aureus* that was responsible to some extent for the degradation of extracellular growth-related protein A. They reported findings, similar to ours, that the development of protease activity started after product formation and disappeared after prolonged incubation.

A similar picture for the involvement of protease in the lignolytic cultures of *P. chrysosporium* was found for mutant strain SC-26 (data not shown). Generally, all cases of protease inhibition or repression in the mutant strain led to a more significant enhancement and stabilization of ligninase than in the wild type.

The data presented above indicate the involvement of protease and the factors regulating it in the regulation of ligninase activity in submerged batch cultures of *P. chrysosporium* grown in nitrogen-limited media. The fact that one protease activity peaks during the growth phase under conditions in which the glucose concentration is relatively high while the second peaks in the late idiophasic stage after the glucose is completely dissipated suggests that these two peaks represent two different enzymes. Further work is ongoing in our laboratory to determine whether these peaks represent one or two enzymes.

**ACKNOWLEDGMENTS**

We are grateful for the support of this work by the Research Excellence Fund from the State of Michigan and the Michigan Biotechnology Institute.

**LITERATURE CITED**

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