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Induction and Stabilization of Lignin Peroxidase from *Phanerochaete chrysosporium*

SANG, BYEONG IN, YONG HWAN KIM AND YOUNG JE YOO*

Department of Chemical Engineering, Seoul National University, Seoul 151-742, Korea

Veratryl alcohol which has been reported as an inducer for lignin peroxidase showed different effects on the enzyme biosynthesis in *Phanerochaete chrysosporium* depending on the addition time. Enzyme expression was optimally induced by adding veratryl alcohol when the carbon source began to be depleted. Hydrogen peroxide, to some extent, stimulated production of lignin peroxidase, but beyond a certain concentration, inactivated lignin peroxidase. Tween 80 induced the formation of small pellets, which were resistant to the deactivation by shear stress. Lignin peroxidase production was increased twice compared with that of the control by adopting all the optimal factors in the culture system.

The synthesis and degradation of lignin play a major role in carbon recycling on earth. Knowledge of the biodegradation of lignin has grown with the discovery of lignin degrading enzymes (14). This finding has generated interest in researching lignin degrading enzymes. The biodegradation of lignin has been most extensively studied with the white rot fungi, and in particular with the filamentous fungus *Phanerochaete chrysosporium*. *Phanerochaete chrysosporium* has a relatively high optimum growth temperature and actively degrades lignin, which has made it the most well known lignin degrader.

This fungus is capable of degrading lignin to carbon dioxide. During vegetative growth, the ligninolytic system of this organism is not expressed. The lignin-degrading system requires hydrogen peroxide for activity. Veratryl alcohol, a secondary metabolite of *P. chrysosporium*, and certain trace elements have been shown to improve lignin peroxidase activity (14, 9). Some surfactants like sorbitan polyoxyethylene monooleate (Tween 80) and oleic acid positively affect the activity of lignin peroxidase (1); however, the mechanism for the effects of the surfactants on enzyme activity is not yet clear.

Many environmental factors including nutrition, pH and other special chemical reagents are known to affect the growth and lignin peroxidase production of this microbe. Many research works have been performed on aspects of environmental microbiology, but relatively few works have considered environmental factors in production of lignin peroxidase. So this research is focused on the production of lignin peroxidase by determining the optimal values of the many environmental factors relating to the induction and stabilization of the enzyme.

*Corresponding Author

Key words: lignin peroxidase, veratryl alcohol, Tween 80, *Phanerochaete chrysosporium*, induction, deactivation

MATERIALS AND METHODS

Microorganism and Medium

The microorganism used in this research for lignin peroxidase production was *Phanerochaete chrysosporium* ME-446 (ATCC 34541). Stock cultures were prepared by growing the organism on yeast malt extract agar slopes at 25°C (see Table 1). Cultures were stored at 4°C. Spores were harvested from six-day-old yeast malt extract agar cultures, incubated at 25°C.

The cultures for lignin peroxidase production were grown in liquid medium derived from that of Tien and Kirk (15) in a jar fermenter, except that 0.5% (w/v) glucose, 1.2 mM ammonium tartrate, 0.1 M sodium succinate buffer (pH 4.75) and 0.04% (w/v) oleic acid (from Junsei) emulsified with 0.05% (w/v) Tween 80 (from Sigma) (1) were added. When *P. chrysosporium* was cultivated in the fermenter, 0.05% (w/v) Tween 80 was again added in the medium at the beginning of the carbon-limited phase (6) in order to stabilize the secreted lignin peroxidase. The medium for agitated flask cultures had the same composition as that for fermentation cultures except that 0.3% (w/v) glucose was added. The flask cultures were made in 500 ml Erlenmeyer flasks containing 200 ml of medium. After inoculation with approximately $(4\pm2)\times10^6$ spores/ml, cultures were flushed with 100% oxygen for 5 min

Table 1. Medium composition.

Component	Concentration (g/l)
Yeast extract	3.0
Malt extract	3.0
Peptone	5.0
Glucose	10.0
Agar	20.0
KH_2PO_4	2.0
$MgSO_4 \cdot 7H_2O$	1.0

and rubber-stopped. Incubation was carried out at 37 °C using a rotary shaker operating at 150 rev/min.

The inoculum for fermentation was prepared by growing the fungus from spore suspension ($(4\pm2)\times 10^6$ spores/ml) in shaking 500 ml Erlenmeyer flasks containing 200 ml of the above medium at 37°C, at 150 rev/min, for 4 days. At the beginning of the carbon-limited phase, veratryl alcohol (2 mM final concentration, from Aldrich) and Remazol Brilliant Blue R dye (3 mg/l, from Sigma) (5) were added aseptically to the cultures. The decolorization of the dye could be an indication of the presence of a ligninolytic system. The initial pH was 4.75 and the pH was allowed to change freely during the experiment. To select a nitrogen source, ammonium chloride and ammonium nitrate (from Junsei) and ammonium tartrate (from Sigma) were used. To optimize initial pH, the following buffers were used: 0.2 M sodium citrate buffer (pH 3, 5); 0.2 M sodium succinate buffer (pH 4, 4.75); 0.2 M sodium phosphate buffer (pH 6, 7); 0.2 M sodium borate buffer (pH 8).

The cultivation of *Phanerochaete chrysosporium* for the production of lignin peroxidase was made in a 5 l stirred tank reactor without baffles, which had a single six-flat-blade turbine impeller and the following measurements: tank diameter, 0.17 m; impeller diameter, 0.08 m; impeller width, 0.0167 m; reactor content, 2 l; aeration rate, 1.1 vvm. The agitation rate was maintained, unless otherwise stated, at 150 rpm and the temperature, at 37°C.

Assays

1. Cell Concentration

Samples of 20 ml were taken aseptically from the reactor and centrifuged at 9000 g. for 5 min. The cells were washed with 0.85% (w/v) NaCl solution and centrifuged again. Then the cells were washed with distilled water and dried at 90°C for 24 h. The supernatant was used for glucose, nitrogen and enzyme assays.

In the case of the immobilized cell, cell concentration was determined by the following method (7): after measuring dry carrier-cell weight, the immobilized cell was removed using 10% (v/v) sodium hypochlorites solution; then, the dried carrier weight was determined; cell weight was calculated from the difference between carrier-cell weight and carrier weight.

2. Nutrient Concentration

Glucose in the culture medium was determined by DNS (dinitrosalicylic acid) method. 1 ml of the supernatant of the centrifuged medium was reacted with 1 ml of DNS color reagent at 100°C for 5 min. 10 ml of distilled water was added after the reaction and optical density was measured at 546 nm.

Ammonium concentration in the culture medium was determined by Berthelot reaction (13).

Hydrogen peroxide level was quantified via the peroxidase-dependent oxidation of *o*-dianisidine (17).

3. Lignin Peroxidase Activity

The lignin peroxidase activity was measured from the centrifuged culture broth as the H_2O_2-dependent oxidation rate of veratryl alcohol to veratraldehyde (extinction coefficient 9,300 $M^{-1}cm^{-1}$), detected by its absorbance at 310 nm at room temperature (16). The reaction mixture contained 0.2 M sodium tartrate buffer (pH 2.9), 2 mM veratryl alcohol and 0.27 mM H_2O_2, in a final volume of 3 ml. The activity was reported as units per liter. One unit was defined as the amount of enzyme required to oxidize one μmole of veratryl alcohol per min.

4. Spore Concentration

Spore concentration was determined by Haemocytometer under a microscope and by optical density at 650 nm. The spore suspension was prepared by suspending spores in sterilized 0.02% (w/v) Tween 80 solution. After samples of several different spore level were made, the spore concentration was measured by Haemocytometer. Then, the samples' optical densities were measured at 650 nm and compared with a calibration curve.

RESULTS AND DISCUSSION

Optimal Induction Time

Veratryl alcohol and Remazol Brilliant Blue R dye (3 mg/l) (5) were added aseptically to the cultures. These compounds were known to stimulate lignin peroxidase synthesis (2). Therefore the decolorization of dye is an indication of the presence of ligninolytic system. Kirk *et al.* (9) have shown that the optimum concentration of veratryl alcohol for increase of activity was 0.4 mM, whereas 2 mM was better according to our results. However, in the former study, veratryl alcohol was added at the time of inoculation and could have interfered with fungal metabolism, whereas in the present study this reagent was added at the several different phases of cell growth. Leisola

Table 2. Effects of induction time on the enzyme biosyntheis.

Time	Maximum lignin peroxidase activity (U/l)
inoculation time	17.0
1st day	16.5
2nd day	14.0
3rd day	19.0
4th day	23.0
carbon limitation time (5th day)	33.0

et al. (12) reported maximal enhancement of lignin peroxidase activity for veratryl alcohol concentrations between 1 and 2 mM. Benzyl alcohol and 3,4, 5-trimethoxybenzyl alcohol, which were not good substrates for lignin peroxidase, were inefficient in preventing a decrease of lignin peroxidase activity. However, the former was reported to increase lignin peroxidase activity in the presence of veratryl alcohol by Kirk *et al.* (8). To determine the optimal induction time, 2 mM veratryl alcohol was added to the culture medium at different times. Table 2 shows that the time when carbon began to be limited was the optimal time to add veratryl alcohol for stimulating lignin peroxidase from *P. chrysosporium.* This result agrees well with general knowledge of the optimal induction time for enhancing secondary metabolites biosynthesis.

Effects of Hydrogen Peroxide

It was reported that *P. chrysosporium* during the production of lignin peroxidase secreted hydrogen peroxide extracellularly (3). The increase of lignin peroxidase activity in dialyzed concentrated supernatant under various concentrations of hydrogen peroxide was reported, which is in good agreement with previous reports (4) that hydrogen peroxide at a physiological level caused inactivation of the lignin peroxidase. These results showed that veratryl alcohol acted as an enzyme protector against inactivation of lignin peroxidase by hydrogen peroxide. Hydroxyl radical HO· and superoxide anion radical O_2· were known to cause protein degradation (19); both radicals can be formed from hydrogen peroxide. Also, horseradish peroxidase can be converted to an inactive form in the presence of excess peroxide, and a cholesterol oxidase from *Streptomyces* sp. is rapidly inactivated in the presence of produced or added hydrogen peroxide (10). The detailed mechanism of enzyme inactivation by hydrogen peroxide remains unknown.

The carbon source used in the cultures strongly influenced the rate of hydrogen peroxide production as well as the loss of lignin peroxidase activity in non-

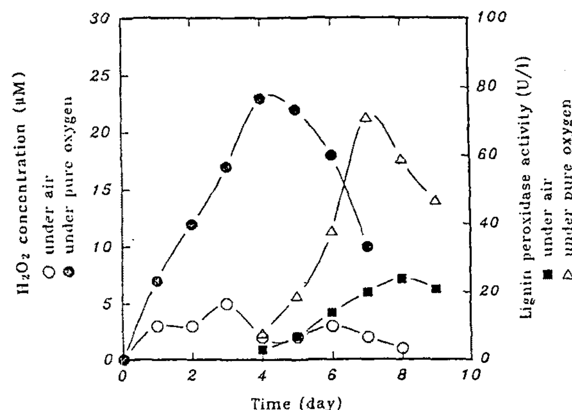

Fig. 1. Time courses of hydrogen peroxide production and lignin peroxidase production under air or oxygen.
Cultures were flushed with 100% oxygen for 5 min a day.

protein-synthesizing cultures (17). Particularly, when pure oxygen was used during fermentation, secreted hydrogen peroxide and lignin peroxidase activity were enhanced as shown in Fig. 1. These results did not coincide with reported results. So, in the present work the following postulation can be made: because the hydrogen peroxide secreted from *P. chrysosporium* caused damage to the cell wall (lipid and protein degradation), *P. chrysosporium* secreted lignin peroxidase in order to reduce the toxic effect of hydrogen peroxide.

To test whether the suggested postulation was true or not, 24 μM and 50 μM hydrogen peroxide were added to the medium. In the case of the air-sparged culture, if the postulation was true, the production of lignin peroxidase would increase when 24 μM and 50 μM hydrogen peroxide were added. As shown in Fig. 2, in the case of air-sparged culture, lignin peroxidase activity increased when hydrogen peroxide concentration was 24 μM and decreased when 50 μM. And in the case of cultivation using pure oxygen, lignin peroxidase decreased with the increase of hydrogen peroxide concentration. From these results, it may be found that hydrogen peroxide somewhat stimulated the biosynthesis of lignin peroxidase, but it inactivated lignin peroxidase beyond a certain concentration. Tonon and Odier (17) reported that hydrogen peroxide seemed to be the main factor, apart from protein turnover, determining the decrease in lignin peroxidase activity through the inactivation of the enzyme during cultures. And they showed that veratryl alcohol could protect lignin peroxidase and the protective effect depended on the mutual balance among the concentrations of hydrogen peroxide, veratryl alcohol and the enzyme in the medium. Since the present studies did not investigate the exact

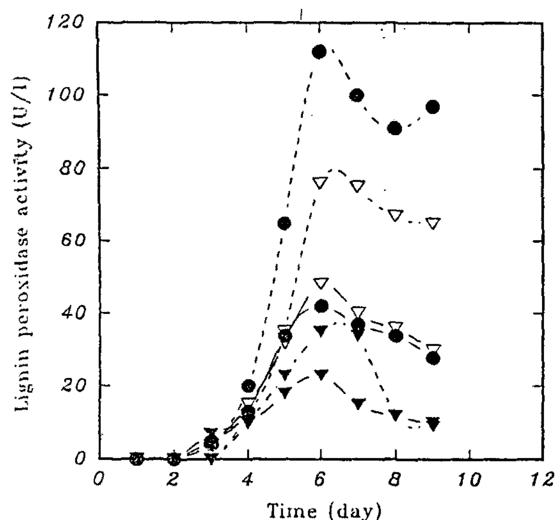

Fig. 2. Effects of hydrogen peroxide addition under air (——) or oxygen (······) sparged condition.
Cultures were flushed with 100% oxygen for 5 min a day. ●: control, ▽: 24 μM, ▼: 50 μM.

Table 3. Effect of oxygen on lignin peroxidase production.

Oxygen supply mode	Maximum lignin peroxidase activity (U/l)
non-aeration	40
oxygen flushing for 5 min a day	100
oxygen flushing twice for 10 min a day	130

effects of hydrogen peroxide, more specific experiments need to be done for a valid conclusion.

From Fig. 2., it seems that oxygen stimulated lignin peroxidase biosynthesis compared to the result with air, in both agitated and static cultures. Table 3. clearly shows the effects of oxygen on lignin peroxidase production in flask cultures.

Role of Tween 80

Early research showed that agitation in submerged flask cultures led to the formation of mycelial pellets and suppression of both lignin degradation and lignin peroxidase activity. Later, some success was achieved with agitated flask cultures. Jaeger *et al.* (6) showed that lignin peroxidase production in agitated pelleted cultures was made possible by including detergents Tween 20, Tween 80 or 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) in the culture medium. Jaeger *et al.* (6) and Asther *et al.* (1) did not explain the mechanism by which these detergents allowed the accumulation of lignin peroxidase. The detergents Tween 20, Tween 40, Tween 60, Tween 80, and CHAPS were shown to protect both purified lignin peroxidase and extracellular lignin peroxidase in culture fluids (free of cell) against mechanical inactivation by Venkatadri and Irvine (18). But the mechanism of protection by detergents was not fully explained. The present study verified that Tween 80 was a protector against inactivation of lignin peroxidase. Several researchers reported that Tween 80 led to small pellets in the fungal cultures. In the present study, Tween 80 was also found to take part in making small pellet as shown in Fig. 3. The smaller the pellet size, the more stable the pellet will be by contrast to the fluid shear force.

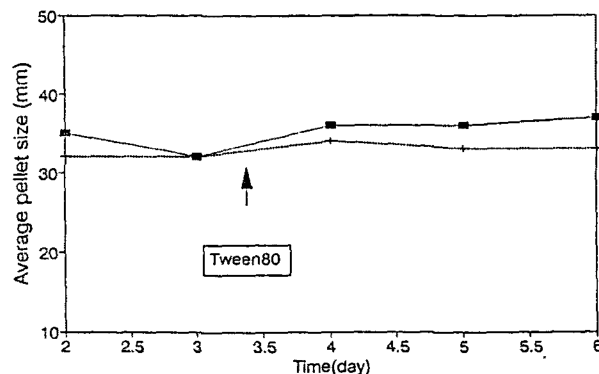

Fig. 3. Effect of Tween 80 on pellet size.
■ : control, +: when Tween 80 was added.

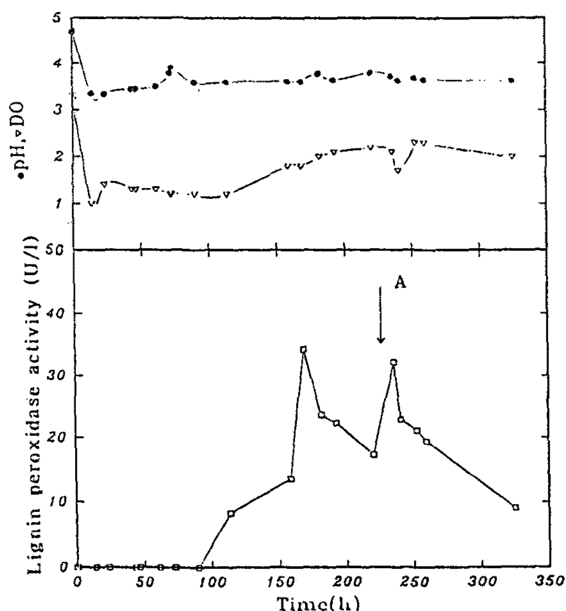

Fig. 4. Time courses of lignin peroxidase activity, pH, and DO in 5 l fermenter.
Arrow A indicates that the time PEG 6000 was added.

Additionally, the increased lignin peroxidase activity demonstrated by Asther *et al.* (1) could simply be due to the increase in the observable activity in the presence of Tween 80.

Jansheker and Fiechter (5) showed that the fa-

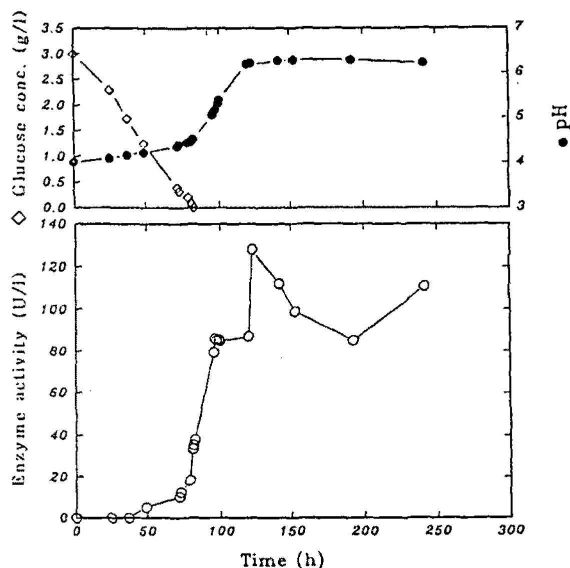

Fig. 5. Time courses of lignin peroxidase activity, glucose concentration and pH under optimal conditions in 5 l fermenter.

cilitated lignin peroxidase production in a 42-liter stirred-tank reactor was due to the use of polypropylene glycol, which probably served to protect lignin peroxidase in a manner similar to that described above for detergents. As shown in Fig. 4., the present study showed the lignin peroxidase production in a 5-liter fermenter with 2-liter medium involved the use of Tween 80 and that polyethylene glycol (M.W. 6,000). PEG 6,000 probably served to protect lignin peroxidase in a manner similar to that described above for Tween 80.

Cultivation under Optimal Conditions

Fermentation to produce lignin peroxidase was carried out in a 5-liter stirred-tank fermenter containing 2-liter medium at 150 rev/min under the optimal conditions previously determined(oxygen was flushed twice for 10 min a day and 2 mM veratryl alcohol was added into medium when carbon was depleted). As shown in Fig. 5, lignin peroxidase activity increased two times over that of the control. Furthermore, this activity was maintained even after 250 h. This result showed that the optimal conditions determined in the flask batch experiments could be applied to large scale fermentation systems.

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