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Induction of Extracellular Polyphenol Oxidase from Two White-rot Fungi

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木材 腐朽菌의 木質素 分解酵素 誘導에 관하여

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Abstract: Among the representative phenolic compounds in relation to lignin derivatives and protein synthesis inhibitors, the most effective inducer for the extracellular polyphenol oxidase (PO) of *Lentinus edodes* JA01 was gallic acid and ferulic acid for *Pleurotus ostreatus*. Optimum concentration of these inducers was 2.0 mM and 1.0 mM, respectively. Addition of gallic acid after two days culture had the best effect on production of PO enzyme of *L. edodes* JA01 and for *P. ostreatus*, and addition of ferulic acid after three days culture had the best effect. Also, in case of *L. edodes* JA01, polyphenol oxidase activity was parallel to growth curve, whereas the maximum enzyme activity of *P. ostreatus* was shown at exponential growth phase and declined thereafter.

Keywords: Polyphenol oxidase, Laccase, Inducer, *Pleurotus ostreatus*, *Lentinus edodes*, Gallic acid, Ferulic acid.

The production of extracellular polyphenol oxidase is a common feature of many higher fungi, particularly those associated with wood decay or the terminal stages of decomposition of leaf litter (Kirk, 1971; Higuchi, 1971).

The production, regulation and properties of fungal polyphenol oxidase have been examined in a number of species. In cultures of *Polyporus versicolor*, extracellular laccase could be induced by treatment of cultures with toluidine or xylidine (Fahraeus *et al.*, 1958). Similar inducing effect has been found with the extracellular laccase of *Neurospora crassa* by treatment with cycloheximide (Froehner & Eriksson, 1974). In *Botrytis cinerea*, gallic acid was an inducer of extracellular laccase (Marbach *et al.*, 1983).

In the present paper, we report the results of the most effective inducer for the extracellular polyphenol oxidase activity during growth of *L. edodes* and *P. ostreatus* on complete media.

Materials and Methods

Organisms

Lentinus edodes JA01 and *Pleurotus ostreatus* were obtained from Korean forest research laboratories and maintained on PDA (potato dextrose agar) slant at 4°C and subcultured every 4 weeks.

Media

For all cultures, a complete medium was used. The medium was composed of 2% malt extract, 0.5% peptone, 0.5% yeast extract, and 1% dextrose in distilled water, pH 5.5.

Culture and Enzyme Preparation

Two agar cubes (diameter 1.2 cm) cut from colonized PDA plates were inoculated on 100 ml sterilized medium in a 250 ml Elenmeyer flask and incubated with no shaking at 25°C for 10 days. For enzyme preparation, every day's sampling was carried out and centrifugated at 1,500 rpm for 10 min. The supernatant was used as the source of crude enzyme.

Enzyme Assay

Polyphenol oxidase (PO) activity was determined using L-dihydroxyphenyl alanine (DOPA) as a substrate and hallachrome production was measured.

In a final volume of 3.0 ml, the reaction mixture contained 5 mM of L-DOPA in 2.0 ml of 0.03 M phosphate buffer (pH 6.5) and 1.0 ml of enzyme solution. After incubation for 10 min at 30°C, optical density was measured at 475 nm. One enzyme unit was defined as 0.001 optical density changes.

Results and Discussion

In control (no inducer) cultures, no detectable PO enzyme was produced by two fungi at any growth stage. However, the inducer was added, PO enzyme activity was increased at a rapid rate. Table I was shown that *L. edodes* JA01 PO activity was induced by gallic acid, ferulic acid, vanillic acid and cycloheximide but not puromycine. Also, gallic acid was more effective inducer than any other compounds. This result agrees with that of Gigi *et al.* (1980). But, its concentration was ten times higher than ours. In case of *P. ostreatus*, all compounds were generally effective inducrs. Among them, ferulic acid was the most effective inducer (Table II).

As can be seen in Table I and II, *P. ostreatus* species is much more effective than *L. edodes* JA01 for all enzyme inducers and in both species puromycine did not induce polyphenol oxidase at all over a wide range of culture periods. Froehner *et al.* (1974) suggested that on this point the lack of induction by puromycine might be due to insufficient uptake of the drug by the species.

Table I. Induction of *L. edodes* polyphenol oxidase with phenolic compounds and protein synthesis inhibitors.

Inducer	Concentration (mM)	Total activity (unit)		
		24hr	48hr	72hr
None		0	0	0
Gallic acid	1.0	115	250	300
Ferulic acid	1.0	40	45.8	50
Vanillic acid	1.0	35.5	40	43
Cycloheximide	0.003	30	30	10.2
Puromycine	0.003	0	0	0

Table II. Induction of *P. ostreatus* polyphenol oxidase with phenolic compounds and protein synthesis inhibitors.

Inducer	Concentration (mM)	Total activity (unit)			
		24hr	72hr	120hr	168hr
None		0	0	0	0
Ferulic acid	1.0	48.5	135	320	520.5
Gallic acid	1.0	17.6	100	212	320.5
Vanillic acid	1.0	50	115	250	425
Cycloheximide	0.003	48.5	130.6	255	240.3
Puromycine	0.003	0	0	0	0

According to Haars *et al.* (1981), it was reported that addition of phenolic compounds to the culture medium caused a significant stimulation of extracellular laccase activity whereas no change in the production of the intracellular enzyme activity could be observed. Leonowicz *et al.* (1975) reported that ferulic acid was an inducer for endolaccase formation. In this experiment, as Table II shows, it was also a good inducer for exotype laccase production as well as 2, 5-xylidine.

According to Haars and Hütttermann (1983), the surface of the fungal hyphae may contain different receptor sites for phenolic compounds

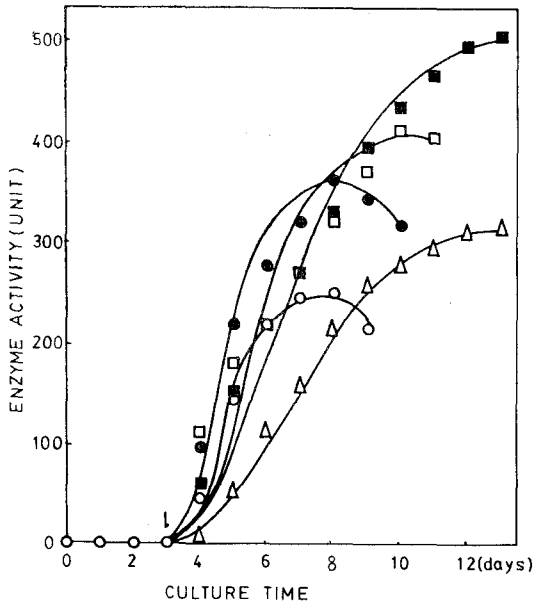

Fig. 1. Effect of gallic acid concentration on extracellular polyphenol oxidase activity from *Lentinus edodes* JA01. Arrow indicated that inducer was added into medium. Symbols: ○, 0.5 mM; ●, 1.0 mM; □, 1.5 mM; ■, 2.0 mM; △, 2.5 mM of gallic acid.

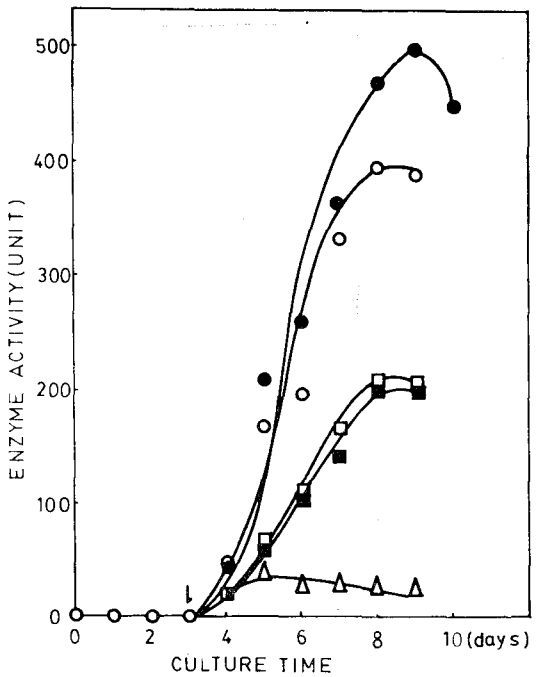

Fig. 3. Effect of inducer added time on extracellular PO activtty from *L. edodes* JA01. Symbols: ○, 0 day; ●, 2 days; □, 3 days; ■, 4 days of culture.

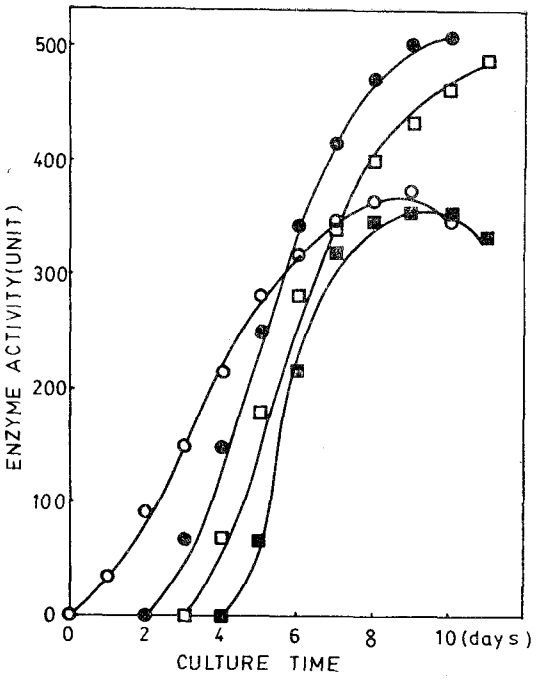

Fig. 2. Effect of ferulic acid concentration on extracellular polyphenol oxidase activity from *Pleurotus ostreatus*. Symbols are the same as figure 1 but, ferulic acid wa sapplied instead of gallic acid.

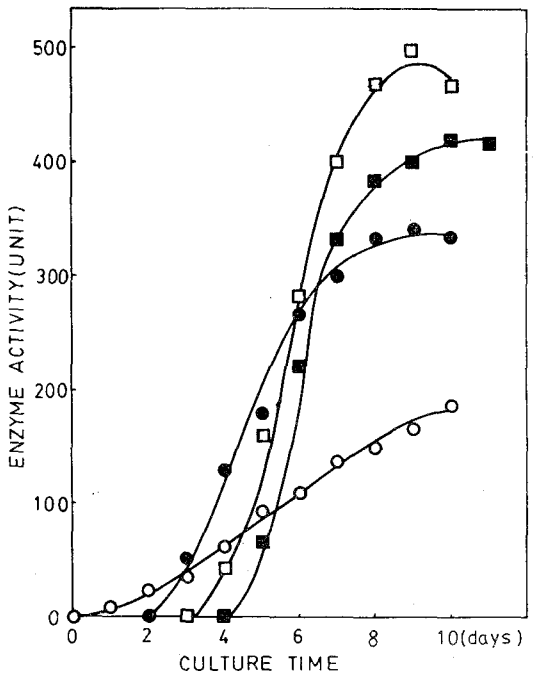

Fig. 4. Effect of inducer added time on extracellular PO activity from *P. ostreatus*. Symbols are the same as figure 3.

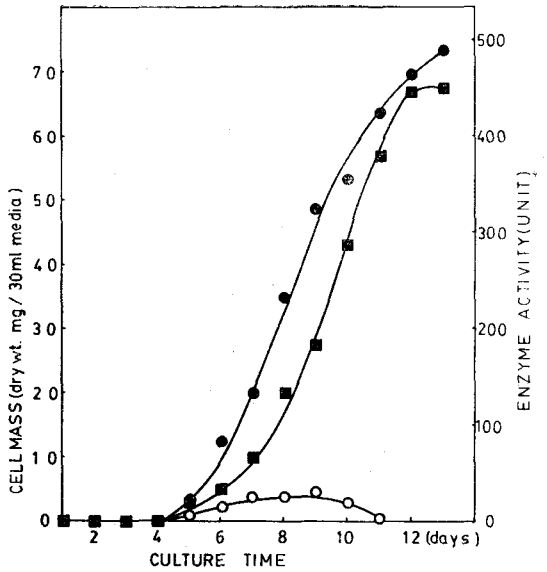

Fig. 5. Growth and polyphenol oxidase production of *L. edodes* JA01 at 2.0 mM gallic acid. Symbols: ■, growth curve; ●, 2.0 mM gallic acid; ○, control (no inducer).

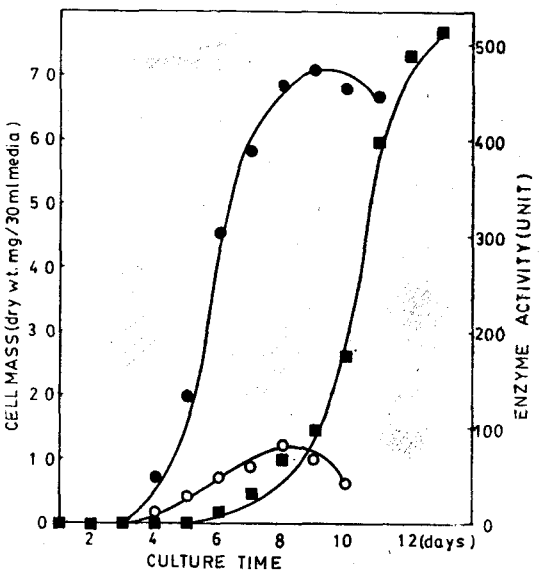

Fig. 6. Growth and polyphenol oxidase production of *P. ostreatus* at 1.0mM ferulic acid. Symbols: ■, growth curve; ●, 1.0mM ferulic acid; ○, control (no inducer).

from species to species. Induction of PO enzyme by antibiotics at low concentrations seemed due to the reflection of the conditions necessary for membrane uptake and not the effectiveness of the drug, because it was reported that the production of laccase in the presence of cycloheximide was related to Ca^{2+} concentration (Froehner *et al.*, 1974).

The concentration of inducers was another important factor for enzyme production, because phenols generally inhibits fungal growth. PO activity according to gallic acid concentration for *L. edodes* JA01 were shown in Fig. 1. From 0.5 mM to 2.0 mM, PO activity was increased. But, over 2.0 mM, PO activity was decreased rapidly, and *P. ostreatus* PO activity was dependent of ferulic acid concentration. one mM of ferulic acid was optimum for *P. ostreatus*. Above the optimal concentration of inducers, PO activity was rapidly decreased because of growth inhibitory effect. However, the growth of these basidiomycetes was not inhibited at optimal concentration of inducers.

According to inducer treatment time, PO activity was variable (Fig. 3 and 4). In *L. edodes*, inducer addition after 2 days' culture showed the highest PO activity and after 3 days' culture for *P. ostreatus* PO activity. Under optimum conditions, the production of enzyme after the addition of inducer and the corresponding growth curves are shown in Fig. 5 and 6. Generally, PO activity was linear with the growth curve. However, in case of *P. ostreatus*, enzyme activity peak appeared at the exponential phase. In both species, PO activity was not shown in the non-induced cultures except that *P. ostreatus* produced polyphenol oxidase in the range of 10~60 enzyme units. Therefore, it is concluded that the addition of inducer in the enzyme producing medium is necessary for the high production of polyphenol oxidase.

적 요

리그닌과 관련된 대표적인 페놀화합물인 ferulic acid, vanillic acid 및 gallic acid와 단백질합성저해항생제인

cycloheximide와 puromycine 중에서 *Lentinus edodes* JA01 경우는 gallic acid가 *Pleurotus ostreatus*는 ferulic acid가 각각 가장 효과적인 inducer였다. gallic acid는 2.0mM에서, ferulic acid는 1.0mM에서 induction이 가장 잘 되었다. Inducer의 처리시기는 접종후 *L. edodes*는 2일후에, 그리고 *P. ostreatus*는 3일후에 최고의 활성을 나타내었다. 또한 polyphenol oxidase activity는 균주의 생장곡선에 대체적으로 비례하여 증가하였으나, *P. ostreatus* 경우는 대수기에서 최고의 활성을 나타내고 그 이후 감소하였다.

References

Bollag, J.M. and Leonowicz, A. (1984): Comparative studies of extracellular fungal laccases. *Appl. Environ. Microbiol.* **48**:849-854.

Dubernet, M., Ribereau-Gayon, P., Lerner, H., Harel, E. and Mayer, A.M. (1977): Purification and properties of laccase from *Botrytis cinerea. Phytochemistry* **16**:191-193.

Fahraeus, G., Tullander, G. and Ljunggren, H. (1958): Production of high laccase yields in cultures of fungi. *Physiologia Plantarum.* **11**:631-643.

Froehner, S.C. and Eriksson, K.E. (1974a): Induction of *Neurospora crassa* laccase with protein synthesis inhibitors. *J. Bacteriol.* **120**:450-457.

Froehner, S.C. and Eriksson, K.E. (1974b): Purification and properties of *Neurospora crassa* laccase. *J. Bacteriol.* **120**:458-465.

Haars, A., Chet, I. and Hüttermann, A. (1981): Effect of phenolic compounds and tannin on growth and laccase activity of *Fomes annosus. Eur. J. For. Path.* **11**:67-76.

Haars, A. and Hüttermann, A. (1983): Laccase induction in the white-rot fungus *Heterobasidion annosum* (Fr.) Bref. (*Fomes annosus* Fr. Cooke). *Arch. Microbiol.* **134**:309-313.

Higuchi, T. (1971): Formation and biological degradation of lignins. *Advances in Enzymol.* **34**:207-283.

Kirk, T.K. (1971): Effects of microorganisms on lignin. *Ann. Rev. Phytopathol.* **9**:185-210.

Kurtz, M.B. and Champe, S.P. (1982): Purification and characterization of the conidial laccase of *Aspergillus nidulans. J. Bacteriol.* **151**:1338-1345.

Leonowicz, A. and Trojanowski, J. (1975): Induction of new laccase form in the fungus *Pleurotus ostreatus* by ferulic acid. *Microbios* **13**:167-174.

Marbach, I., Harel, E. and Mayer, A.M. (1983): Inducer and culture medium dependent properties of extracellular laccase from *Botrytis cinerea. Phytochemistry* **22**:1535-1538.

Motoda, S. (1979): Purification and some properties of polyphenol oxidase from *Alternaria tenuis. J. Ferment. Technol.* **57**:71-78.

Schoemaker, H.E., Harvey, P.J., Bowen, R.M. and Palmer, I.M. (1985): On the mechanism of enzymatic lignin breakdown. *FEBS Letters* **183**:7-16.

Wood, D.A. (1980): Production, purification and properties of extracellular laccase of *Agaricus bisporus. J. Gen. Microbiol.* **117**:327-338.

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