KOR. JOUR. MICROBIOL. June. 1986 p. 141-146 Vol. 24, No. 2

Properties of Extracellular Polyphenol Oxidase Isolated from *Lentinus edodes* JA01

Shin, K.S., K.J. Kim,* S.W. Hong and Y.C. Hah

Department of Microbiology, College of Natural Sciences, Seoul National University, Seoul, 151

*Department of Biology, Kang-Reung National University, * Kang-Reung, 210, Korea*

Lentinus edodes JA01에서 분리한 細胞外 polyphenol oxidase의 부분적 성질에 관하여

申光洙 · 金圭衆* · 洪淳佑 · 河永七

서울대학교 자연과학대학 미생물학과

강릉대학 생물학과*

Abstract: To find the role of polyphenol oxidase in lignin biodegradation, chracteristics of extracellular polyphenol oxidase activity from *Lentinus edodes* JA01 was investigated. Polyphenol oxidase had its optimum activity at pH 4.5 and 45°C respectively. Also, the enzyme was very unstable in various pHs and comparatively heat stable up to 60°C. In lignosulfonate-NH_4 salts medium, the growth rate of *L. edodes* JA01 was relatively slow and polyphenol oxidase activity appeared 2 and 14 days after inoculation. No significant relationships were found between polyphenol oxidase activity and the amounts of lignosulfonate present in the culture medium.

Key Words: lignosulfonate-NH_4 salts, polyphenol oxidase, *Lentinus edodes*

Aromatic plant polymer lignin is the second most abundant organic compound on Earth, and that cellulose is the only other organic substance present in the biosphere in larger quantities than lignin (Crawford *et al.*, 1978). This aromatic macromolecule is biodegradable, but it's biodegradation is very slow.

The most effective decomposers of lignin in nature are found among the wood-rotting *Basidiomycetes*. However, many of these fungi cannot, without previous adaptation, use isolated lignin or lignosulfonate as their sole source of carbon and energy. Nonetheless, some utilization of lignosulfonates has been observed upon incubation of various mixed microbial populations in media where lignosulfonate served as the sole source of energy (Sundman *et al.*, 1970).

Gel permeation studies and thin layer chromatography have shown that utilization of lignosulfonate by microorganisms is accompanied by polymerization to dark compounds of higher molecular weight (Ferm, 1972). In an investigation of the stability of waste lignins in aquatic systems, Bouveng and Solyom (1973) noted a gradual increase in the color of sulfite waste lignin exposed to the action of a complex water flora and fauna during 40 weeks.

Wood-rotting fungi of the white-rot type, although actively degrading lignin in wood, have received little attention in work on lignosulfonate

degradation. Sundman *et al.* (1972) has shown that lignins and lignosulfonates can both be degraded by wood-rotting fungi. It therefore seemed profitable to extend the investigation on lignosulfonate utilization to various wood-rotting fungi.

Polyphenol oxidase was an important enzyme in lignin biodegradation, play a role in detoxifying low molecular weight phenols released during lignin degradation (Gierer *et al.*, 1973), initiate lignin degradation by performing some critical initial chemical transformation (Kirk *et al.*, 1975) and function in regulating the production of both lignin degradation and polysaccharide degradation enzyme (Ander *et al.*, 1976). But the exact role of polyphenol oxidase remains to be firmly established.

In this paper, we investigated some properties of polyphenol oxidase in white-rot fungus *Lentinus edodes* JA01 and the relationships between polyphenol oxidase activity and lignin transformation.

MATERIALS AND METHODS

Fungal strain

Lentinus edodes JA01 was obtained from Korean Forest Research Institute, stored on PDA (Potato Dextrose Agar) slant at 4°C and subcultured every 4 weeks.

Medium

Fungal cells were grown on minimal medium which containing the followings; 1.0g KH_2PO_4, 0.5g $CaCl_2 \cdot 2H_2O$, 0.5g $MgSO_4 \cdot 7H_2O$, 2.0g NH_4Cl and trace amounts of $FeSO_4 \cdot 7H_2O$, $MnSO_4 \cdot 4H_2O$, $ZnCl_2 \cdot 2H_2O$ and $CuSO_4 \cdot 5H_2O$ per 1L of distilled water. After autoclaving (20min at 120°C), 1.0mg of thiamine hydrochloride was added and initial pH was adjusted to 5.5. And for the enzyme induction, 0.1% lignosulfonate-NH_4 salts was added.

Cultivation and Crude enzyme preparation

Disintergrated mycelium (Omni mixer, Du Pont Instrument Co.) was used as inoculum. Mycelium suspension was inoculated to 250ml Erlenmayer flask with 100ml of above medium, and incubated with shaking (120rpm) at 25°C. Every days' sampling was centrifuged (15,000 rpm, 10min) and the supernatant fraction was used as enzyme solution.

Assay of enzyme activity

The reaction mixture contains 0.1ml of the enzyme solution and 10 umol guaiacol solution in a 50 umol acetate buffer (pH 5.0). The reaction mixture was incubated at 25°C for 2hrs.

The colour intensity was measured at 400nm with Cecil spectrophotometer CE272 (Tadakazu *et al.*, 1976). The initial rate of formation of colored products was taken as a relative measurement of polyphenol oxidase activity.

Gel filtration chromatography

Culture filtrate was fractionated on a column (2.5 × 70cm) packed with Sephadex G-75 (Pharmacia Fine Chemicals). The column was equilibrated with 0.25 M $CaCl_2$ and eluted with the same solution. Fractions of 3ml were collected at a flow rate of 9ml h^{-1}. Absorbance at 280nm was measured for each fraction with Giford spectrophotometer (Model No. 250) for the estimation of lignin quantities (Kern, 1983).

RESULTS AND DISCUSSION

When *L. edodes* JA01 was grown in a 0.1% lignosulfonate-NH_4 salts medium, the exponential phase observed between 6 to 18 days and any pH change was not detected during this period (Fig. 1).

The effect of pH on the enzyme activity was examined with 0.1 M citrate buffer, 0.1M acetate

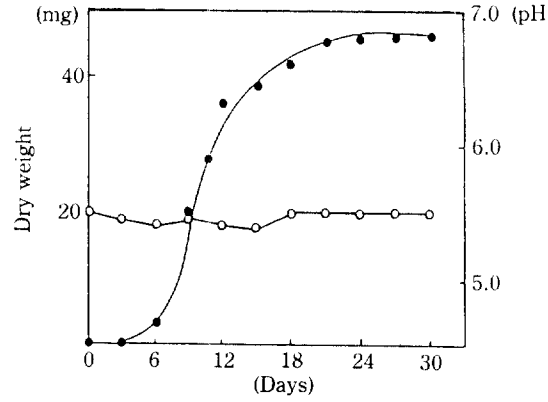

Fig. 1. *Growth and pH variation of L. edodes JA01 in lignosulfonate medium; pH(○) and dry weight (●)*

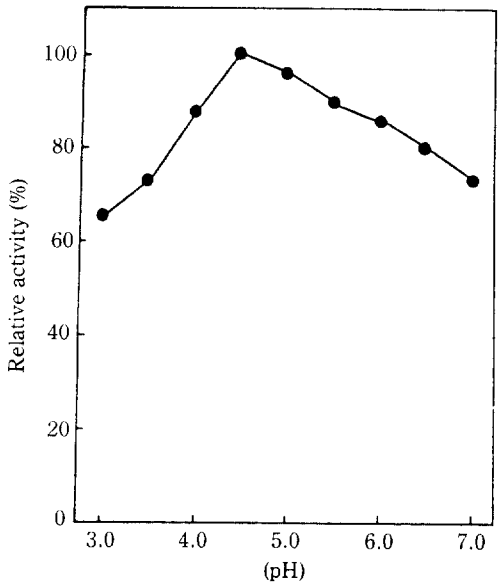

Fig. 2. *Influence of pH on the activity of the polyphenol oxidase produced by Lentinus edodes JA01.*

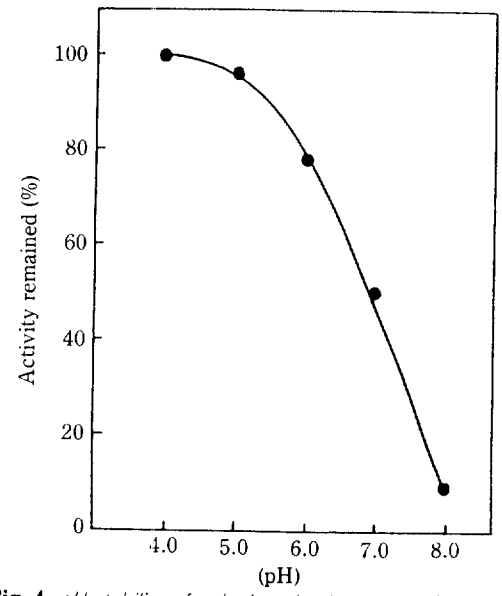

Fig. 4. *pH stability of polyphenol oxidase from L. edodes JA01.*

buffer and 0.1M phosphate buffer. The enzyme was active over the pH range of 4.0 to 6.5 and maximum activity was attained pH 4.5 (Fig. 2). It was slightly higher than the activity of the *Botrytis cinerea* laccase which had its maximum activity at pH 4.0 (Marbach *et al.*, 1983). The effect of temperature on the enzyme activity was shown in Fig. 3. The reaction was carried out for 60min at various temperature in 0.1M acetate buffer pH 4.5. The enzyme was active over the temperature range of 25°C to 50°C, with the maximum activity at 45°C. The optimum and maximum temperature of the enzyme was similar to that of the polyphenol oxidase from *Alternaria tenuis* (Motoda, 1979).

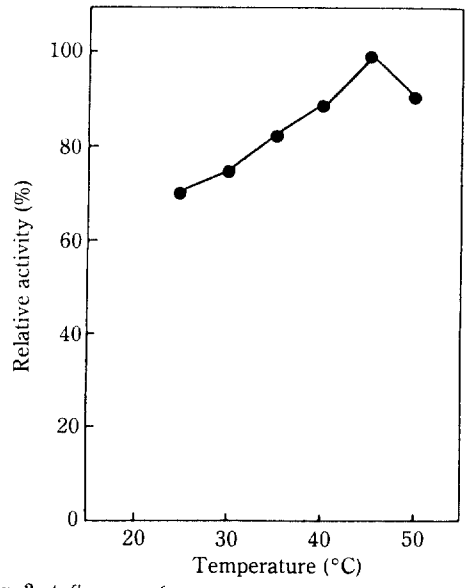

Fig. 3. *Influence of temperature on the activity of the polyphenol oxidase produced by L. edodes JA01.*

The effect of pH on the enzyme stability was examined by incubating the enzyme with 0.1M citrate buffer, 0.1M acetate buffer and 0.1M phosphate buffer at various pH values for 120 min at 25°C. The enzyme was fairly stable in the pH range of 4.0 to 5.0, and unstable above pH 5.5. Fifty percent of the initial activity was lost at pH 7.0 (Fig. 4). The effect of temperature on the enzyme stability was examined in 0.1M acetate buffer (pH 4.5). After incubation for 100min at various temperatures, the remaining activity was measured (Fig. 5). The enzyme was found to be stable on the temperature range of 30°C to 60°C, and fifty percent of the initial activity was lost at 70°C.

In lignosulfonate medium, polyphenol oxidase activity was appeared 2 day and 14 days after the inoculation (Fig. 6). According to Tadakazu and Eriksson (1976), the activity of *Pleurotus ostreatus* polyphenol oxidase was appeared 10 days and 30 days later.

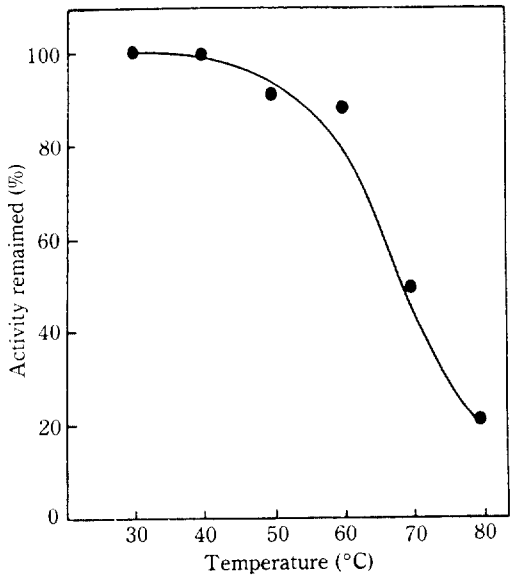

Fig. 5. *Thermal stability of polyphenol oxidase from L. edodes JA01.*

It was demonstrated earlier by Selin and Sundman (1972) that, when a commercial lignosulfonate was decayed by the white-rot fungus *Polyporus dichrous,* a significant decrease of the lignosulfonate content was accompanied by polymerization. In addition to polymerization, there was also some loss of lignosulfonate which causes a strong darkning of the cultivation medium due to the action of polyphenol oxidase.

The importance of polyphenol oxidase activity in lignin degradation has been clearly demonstrated by Ander and Eriksson (1976). They compared the lignin degradation caused by a wildtype, a polyphenol oxidase less mutant, and a polyphenol oxidase positive revertant of *Sporotrichum pulverulentum.* The polyphenol oxidase less mutant could not degrade lignin in wood, while the revertant could degrade lignin to the same extent as the wildtype. After addition of highly purified laccase to Kraft lignin agar plates, the phenol oxidase less mutant could also degrade lignin.

Haars and Hütterman (1980) treated lignosulfonate with a purified laccase from the white-rot fungus *Fomes annosus* and found that laccase brought about polymerization of the lignosulfonate. But in the prescence of thioglycolic acid which totally inhibited extracellular laccase activity, *Fomes annosus* cleaved high molecular weight lignosulfonate with a molecular weight ranged 2 $\times$ 10^6 to 1000.

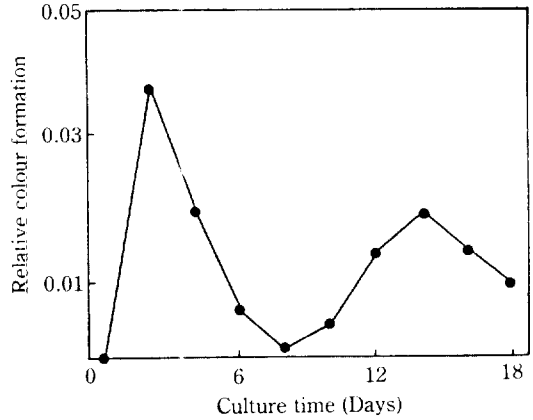

Fig. 6. *Changes of polyphenol oxidase activities on lignosulfonate-NH_4 salts medium.*

On the other hand, lignolytic activity of *L. edodes* has been demonstrated by Oki *et al.* (1981). They reported that the changes in polyphenol oxidase activity of this fungus was not responsible for regulating delignification.

In our experiments, we obtained the similar results as Oki *et al.* As shown in Fig. 7, lignosulfonate was slightly degraded by the 2 day culture filtrate and spectrum was shifted to the low molecular region. But, 8 and 14 days later, the spectrum was moved back to the initial region.

As a results, it is concluded that extracellular polyphenol oxidase from *L. edodes* JA01 was not effective on large scale lignin biodegradation, but can transform small amount of lignin.

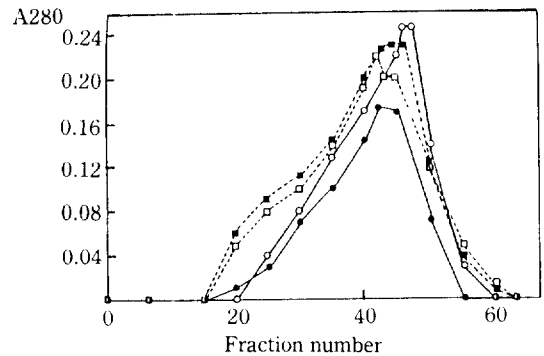

Fig. 7. *Gel permention chromatography of the crude extract of lignosulfonate medium; control (○), 2 days (●), 8 days (□) and 14 days (■) culture.*

적 요

Polyphenol oxidase와 리그닌 분해의 상관관계를 조사하기 위하여 표고균주 JA01로 부터 추출한 polyphenol oxidase의 특성을 살펴본 결과 pH 4.5와 45℃에서 각각 최대의 활성을 나타내었고, pH의 변화에 대하여서는 불안정하나, 온도변화에 대해서는 상당히 안정한 것으로 나타났다. 표고균주를 lygnosulfonate 배지에서 배양한 결과 생장은 비교적 미약하였고, polyphenol oxidase는 2일과 14일에 활성을 나타내었으며 그 당시 배지내 lignin 함량을 gel filtration chromatography로 분석하니 큰 변화가 없었음을 알 수 있었다. 따라서 표고균주의 polyphenol oxidase는 리그닌 분해에 크게 관여하지 않는 것으로 추정된다.

ACKNOWLEDGEMENTS

The authors wish to express their gratitude to Prof. Kang, Sa-Ouk for his sinciere help in the full experimental discussions.

REFERENCES

1. Amer, G.I. and S.W. Drew. 1980. Microbiology of lignin degradation. *Ann. Rep. Fermen. Pro.* **4:** 67-103.
2. Ander P. and K.E. Eriksson, 1976. The importance of phenol oxidase activity in lignin degradation by the white-rot fungus *Sportotrichum pulverulentum. Arch. Microbiol.* **109:** 1-8.
3. Bligny, R. and R. Douce. 1983. Excretion of laccase by sycamore (*Acer pseudoplatoanus L.*) cells. *Biochem. J.* **209:** 489-496.
4. Bollage, J.M. and A. Leonowicz. 1984. Comparative studies of extracellular fungal laccase. *Appl. Environ. Microbiol.* **48(4):** 849-854.
5. Bouveng, H.O. and P. Solyom. 1973. Long-term stability of waste lignins in aquatic systems. *Sven. Papper.* **76:** 26-29.
6. Crawford, R.L. and D.L. Crawford. 1978. Radioisotopic methods for the study of lignin biodegradation. *Dev. Ind. Microbiol.* **19:** 35-49.
7. Durand, H., P. Soucalle and G. Tiraby. 1984. Comparative study of cellulases and hemicellulases from four fungi; mesophiles *Trichoderma reesei* and *Penicillium* sp. and thermophiles *Thielavia terrestris* and *Sporotrichum cellulophilum. Enzyme Microb. Technol.* **6:** 175-180.
8. Ferm, R. 1972. Molecular-weight changes in lignin and lignosulfonate caused by phenoloxidizing enzymes. *Sven. Papper.* **75:** 863-865.
9. Gierer, J. and A.E. Opara. 1973. Studies on the enzymatic degradation of lignin. the action of peroxidase and laccase on monomeric and dimeric model compounds. *Acta Chem. Scand.* **27:** 2909-2922.
10. Haar, A. and A. Hütterman. 1980. Function of laccase in the white-rot fungus *Fomes annosus. Arch. Microbiol.* **125:** 233-237.
11. Kern, H.W. 1983. Transformation of lignosulfonates by *Trichoderma harzianum. Holzforschung* **37:** 109-115.
12. Kirk, T.K. 1975. Effects of a brown-rot fungus, *Lenzites trabea,* on lignin in spruce wood. *Holzforschung* **29(3):** 99-107.
13. Kirk, T.K. and H.M. Chang. 1975. Decomposition of lignin by white-rot fungi. 11. Characterization of heavily degraded lignins from decayed spruce. *Holzforschung* **29:** 56-64.
14. Kurtz, M.B. and S.P. Champe. 1982. Purification and characterization of the conidial laccase of *Aspergillus nidulans. J. Bacteriol.* **151(3):** 1338-1345.
15. Marbach, I., E. Harel and A.M. Mayer. 1983. Inducer and culture medium dependent properties of extracellular laccase from *Botrytis cinerea. Phytochemistry* **22(7):** 1535-1538.
16. Motoda, S. 1979. Purification and some properties of polyphenol oxidase from *Alternaria tenuis. J. Ferment. Technol.* **57(2):** 71-78.
17. Oki, T., H. Watanabe and H. Ishikawa. 1981. The biodegradation of lignin by Shiitake *Lentinus edodes* (Berk.) Sing. *Mokuzai Gakkaishi* **27(9):** 696-702.
18. Räihä, M. and V. Sundman. 1975. Characterization of lignosulfonate induced phenol

oxidase activity in the atypical white-rot fungus *Polyporus dichrous*. *Arch. Microbiol.* **105(1):** 63-70.

19. Sundman, V. and J.F. Selin. 1970. Microbial utilization of lignosulfonates of various molecular sizes. *Paper and Timber* **52:** 473-479.
20. Sundman, V. and L. Näse. 1972. The synergistic ability of some wood-degrading fungi to transform lignins and lignosulfonates on various media. *Arch. Microbiol.* **86:** 339-348.
21. Tadakazu, H. and K.E. Eriksson. 1976. Microbiological degradation of lignin. Part 1. Influence of cellulose on the degradation of lignins by the white-rot fungus *Pleurotus ostreatus*. *Sven. Papper.* **nr5:** 157-161.
22. Wood, D.A. 1980. Production, purification and properties of extracellular laccase of *Agaricus bisporus*. *J. Gen. Microbiol.* **117:** 327-338.

(Received Mar. 28, 1986)