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SHORT COMMUNICATION

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Changes in phenol oxidases and superoxide dismutase during fruit-body formation of *Pleurotus* on sawdust culture

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Abstract Peroxidase and laccase activities increased rapidly up to the formation of primordia and then declined throughout the entire stage of fruiting. In the case of *Pleurotus ostreatus*, the level of Mn-dependent peroxidase was very low in primordia and fruiting stages but gradually increased with the growth of the fruit-body, whereas no activity was detected in *Pleurotus sajor-caju* during all growth stages. Superoxide dismutase activity was observed mainly at the fruiting stages. These results show that changes in concentration of lignin-related enzymes are associated with the fruiting process.

Key words Fruit-body · Lignin-degrading enzymes · Phenol oxidase · *Pleurotus* · Superoxide dismutase

Fruit-body development of mushrooms generally consists of two stages. One stage is primordia formation, which requires relatively lower temperature, light exposure, and relatively higher water content of culture media. In addition, primordia development also depends on mycelium quantity. In the second stage, fruiting from the primordia is induced by low temperature, watering, concentration of carbon and nitrogen sources, medium pH, aeration, and the association of some enzymes (Terashita et al. 1981; Ishikawa et al. 1983; Matsumoto 1988; Ohga 1992; Ohga et al. 2000).

The fruiting process in higher fungi is connected with several biochemical changes. Among others, these relate to levels of laccase, peroxidase, and phenol oxidase, which take part in the metabolism of phenolic compounds and in the degradation of lignin (Eriksson et al. 1990; Hammel et al. 1993; Leonowicz et al. 1999). Phenolic substances are active in the process of building mushroom cell walls (Kitamoto et al. 2000). Phenols are very sensitive to the presence of active forms of oxygen and can be subject to various oxidative processes. Superoxide radicals are also able to initiate these processes, and it is known that autooxidation of diphenols decreases in the presence of superoxide dismutase (SOD) (Misra and Fridovich 1975). During enzymatic oxidation of diphenols, H_2O_2 is also generated, becoming a good electron acceptor for peroxidase, and it activates enzyme change (Jiang and Miles 1993).

The purpose of this article is to report on the changes in phenol oxidases [peroxidase, laccase, and Mn-dependent peroxidase (MnP)] and SOD, which take part in lignin metabolism, and on the level of phenols and free radical during the fruiting process.

Two strains of *Pleurotus* species from the Fungi Collection of Cheongju (FCC) and the Fungi Collection of Lublin (FCL) were used; these were *P. ostreatus* (Jacq.: Fr.) Kummer (FCC-822) and *P. sajor-caju* (Fr.) Singer (FCL-215). The fungi were cultivated in polypropylene bags containing 1.5 kg of solid beech sawdust–wheat bran medium (80:20 w/w). The bags were incubated for 30 days at 25°C and moved to a conditioned room at 8°–15°C and 90% humidity for about 5–10 days for primordia formation. When the first primordia appeared, the culture bag was opened and moved to an illuminated room (about 500 lx) at 10°–15°C for 10 days for fruit-body growth. Samples were taken from cultures at various stages of development: mycelium before fruiting (Mbf) on the 30th day from inoculation, primordia (P) on the 35th day, small fruit-body (F1) on the 42th day, medium fruit-body (F2) on he 45th day, large mature fruit-body (F3) on the 50th day, and mycelium after fruiting (Maf) on the 60th day. Five liters of 50 mM

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phosphate buffer (pH 7.0) was added to the fresh material (200 g dry wt) and agitated for 30 min to extract extracellular enzymes. The suspension fraction was centrifuged at 24000 *g* for 20 min. The supernatant was immediately desalted with deionized water on a G-25 Sephadex column and used as the crude enzyme source. All procedures were carried out at 5°C.

The activity of peroxidase was assayed with *o*-dianisidine as a substrate (Clairborne and Fridovich 1978). Laccase activity was measured with syringaldazine. SOD activity was determined colorimetrically on the basis of decrease in pyrogallol autooxidation at pH 8.4 (Marklund and Marklund 1974; Nebot et al. 1993). The MnP level was measured by monitoring phenol red oxidation of $MnSO_4$ and H_2O_2. The relative level of superoxide free radicals in the whole supernatant based on absorption at 560 nm was measured colorimetrically with nitrotetrazolium blue (NTB) in 0.1 M NaOH. All experiments were performed in triplicate.

During the fruiting process of *P. ostreatus* and *P. sajor-caju*, fluctuations in enzyme activities were observed in various stages of fruit-body development. As shown in Figs. 1 and 2, peroxidase and laccase activities increased rapidly up to the formation of primordia, declined throughout the fruiting stage, and increased a little in the mycelia after fruiting. The natural substrates for the extracellular laccase are likely to be lignin-derived phenolic compounds (Leonowicz and Grzywnowicz 1981). Nevertheless, the high activity levels of these two enzymes indicated that changes in the level of peroxidase and laccase are closely linked to the development of the fruit-body as in *Agaricus bisporus* (Lange) Singer (Wood and Goodenough 1977). It has been shown that laccase accumulates during its colonization by the mycelium of *A. bisporus* and that laccase activity falls rapidly at the time of fruiting (Turner 1974). Particularly, similar changes in laccase activity have been reported in sawdust-based media of *Lentinula edodes* (Berk.) Pegler (Matsumoto 1988; Ohga 1992; Ohga et al. 2000) and *P. ostreatus* (Ishikawa et al. 1983). Although it was reported that laccase activities in the fruit-body of *L. edodes* (Leatham and Stahmann 1981) and in bed logs after taking off the fruit-body (Tokimoto and Fukuda 1997) were increased, we did not find a great increase of laccase activity in mycelial parts during fruiting. Although the role of peroxidase and laccase during fruit-body formation is not clear, it is possible that these enzymes are required mainly at the contact point of wood-rotting basidiomycetes with an environment containing lignin. Ross (1982) observed a general increase of intracellular laccase just before the fruiting of *Coprinus congregatus* (Fr.) S.F. Gray, whereas in the case of *L. edodes* and *Schizophyllum commune* Fr., increased extracellular and intracellular activity of laccase was observed during the fruiting process. The foregoing difference could be explained by the fact that peroxidase and laccase not only oxidize phenolic compounds but also create a new oxidative cross-linking substance in mushroom cell walls. The absence of peroxidase and laccase activities in the cap of the fruit-body could result from its inactivation during fruiting (Wood 1980).

It has been observed, in the case of *P. ostreatus* (Fig. 3), that the level of MnP is very low in primordia and fruiting stages but gradually increases with the growth of the fruit-body. In contrast, no MnP activity was shown in *P. sajor-caju* during all the growth stages. The activity of SOD showed almost the same tendency as MnP activity (Fig. 4). The activity of SOD was shown to be higher in *P. sajor-caju* than in *P. ostreatus*. The role of SOD seems to consist of the regulation of activity of all oxygen-dependent enzymes

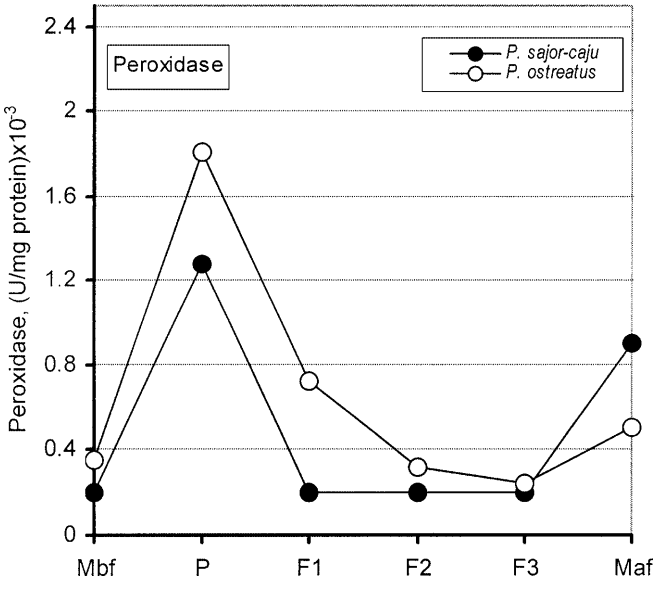

Fig. 1. Activity of peroxidase of *Pleurotus ostreatus* (*open circles*) and *P. sajor-caju* (*solid circles*) in sawdust culture. *Mbf*, mycelia before fruiting; *P*, primordia; *F*, fruit-bodies (*F1*, *F2*, *F3* are stages from small to large forms); *Maf*, mycelia after fruiting

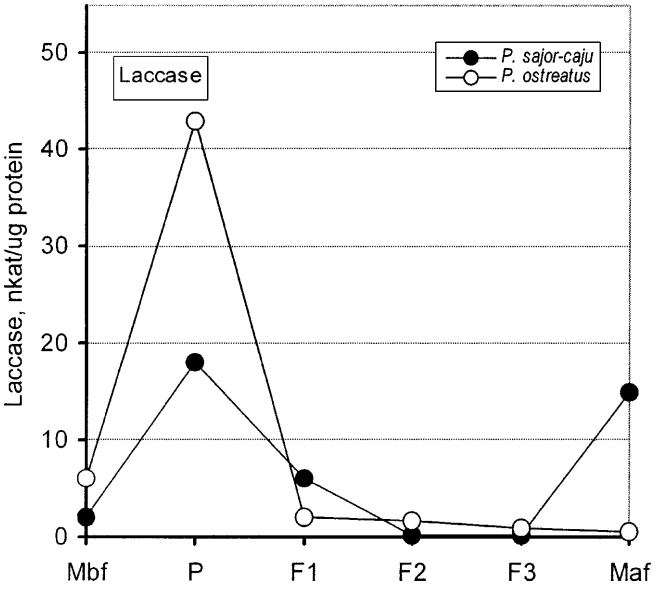

Fig. 2. Activity of laccase of *P. ostreatus* and *P. sajor-caju* in sawdust culture

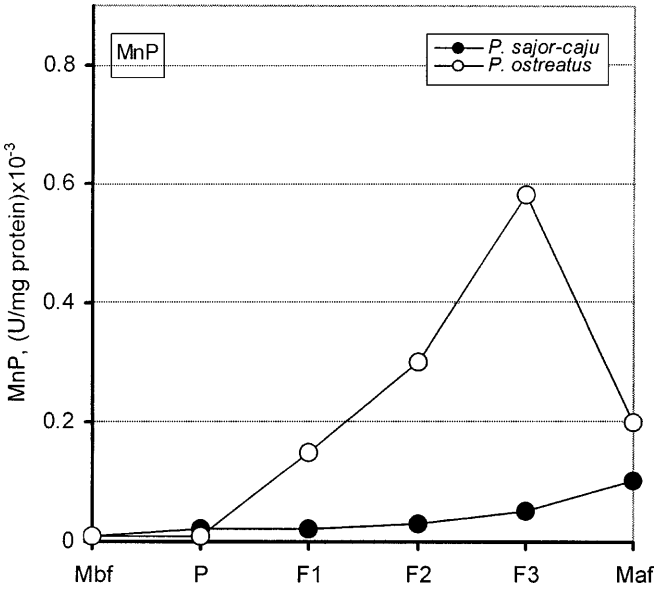

Fig. 3. Activity of Mn peroxidase (*MnP*) of *P. ostreatus* and *P. sajor-caju* in sawdust culture

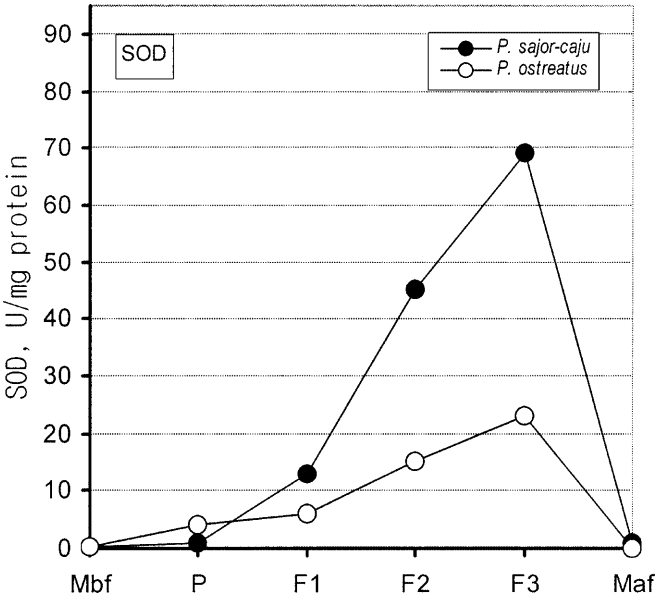

Fig. 4. Activity of superoxide dismutase (*SOD*) of *P. ostreatus* and *P. sajor-caju* in sawdust culture

Table 1. Changes in superoxide radicals and methoxyphenols during the development of *Pleurotus* strains

Fruit-body development[a]	Superoxide radicals A_{560nm}		Methoxyphenols A_{560nm}	
	P. ostreatus	*P. sajor-caju*	*P. ostreatus*	*P. sajor-caju*
Mbf	0.70	0.48	17.5	0.5
P	0.22	0.25	18.2	0.5
F1	0.17	0.28	25.0	0.9
F2	0.15	0.34	36.0	1.2
F3	0.13	0.40	41.0	2.0
Maf	0.05	0.10	20.0	2.0

[a] Mushroom development stages: Mbf, mycelia before fruiting; P, primordia; F1, small fruit-bodies; F2, medium fruit-bodies; F3, large fruit-bodies; Maf, mycelia after fruiting

taking part in fruiting, achieved by decreasing the level of free radicals, which are very common in all phenol-related processes. The SOD is a ubiquitous metalloprotein that catalyzes the dismutation of superoxide into hydrogen peroxide and molecular oxygen. Superoxide is the first reduction product of molecular oxygen. In addition, superoxide is an important source of hydroperoxides and free radicals (Fridovich 1986). The level of SOD activity seems to be opposite to the level of peroxidase and laccase activities. SOD activity is mainly observed at the fruiting stages, the activities of peroxidase and laccase at that period being much lower.

The level of superoxide radicals (Table 1) was very high in the stage of mycelial aggregate before primordia formation, gradually decreased in the case of *P. sajor-caju*, increased during fruit-body formation, and suddenly decreased in the mycelium after fruit-body harvesting. A decreasing tendency of superoxide radicals throughout the fruiting stages was shown only in *P. ostreatus*. Intensive production of the methoxyphenolic compound as well as free superoxide radicals was observed during fruiting stages of *P. ostreatus*, whereas no methoxyphenols formed in *P. sajor-caju* (Table 1). The levels of superoxide radicals and phenolic compounds seem to be not directly related to the activities of peroxidase, laccase, and SOD. Moreover, a high level of methoxyphenols accompanies high activity of MnP in the fruiting stage in *P. ostreatus*.

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