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学 位 論 文 要 旨
Dissertation Abstract

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学位論文題目：
Title of Dissertation

Studies on laccases produced by the basidiomycete *Flammulina velutipes*
(担子菌*Flammulina velutipes*が生産するラッカーゼに関する研究)

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Mushrooms are large fruiting bodies including basidiomycetes. Basidiomycetous mushroom-forming fungi sense several environmental conditions such as light, temperature, and nutrient to form fruiting bodies for the spore dispersal, as well as to find the suitable location and timing of sexual reproduction. However, the molecular basis of fruiting body formation in basidiomycetous fungi has not been well understood yet. Thus, comprehension of the fruiting body initiation and development especially in basidiomycetes plays an important role in the aspect of research and commercial production of mushrooms.

Basidiomycetous fungi are also known as excellent laccase producers. Laccase is a blue multicopper oxidase catalyzing the oxidation of a broad range of aromatic substrates concomitantly with the reduction of molecular oxygen to water. Basidiomycetous fungi produce a number of laccase isozymes. Fungal laccase isozymes have various physiological functions including lignin degradation, fungal morphogenesis, pigment production, sporulation, stress defense, and plant pathogenesis. Laccase production can be influenced by some factors including the type of cultivation.

All in all, this study focused on the functions of laccase isozymes produced by the edible basidiomycetous mushroom *F. velutipes*.

Chapter I: Relationship between fruiting body development and extracellular laccase production in the edible mushroom *F. velutipes*

The biochemical mechanism underlying the development of fruiting bodies in *F. velutipes* was investigated using the YBLB colorimetric assay including bromothymol blue (BTB) to distinguish between the normal strain (FVN-1) exhibiting normal fruiting body development and the degenerate strain (FVD-1) with no fruiting body formation. The color of the YBLB medium (blue-green) inoculated with FVN-1 changed to yellow, while the color of the medium inoculated with FVD-1 changed to blue. The reason of this color difference was not known. The pH values of the media inoculated with these strains showed a slight alkaline shift during the incubation period. The absorbance of BTB at 615 nm decreased during the growth of FVN-1 in YBLB medium, and increased in FVD-1. Phenol-oxidizing enzymes including laccase, lignin peroxidase, and manganese peroxidase are involved in the decoloration of various aromatic dyes. Neither lignin peroxidase nor manganese peroxidase activity was found in the media inoculated with both strains. However, much higher laccase activity was observed in FVN-1 compared to FVD-1 during the growth in YBLB medium. To confirm that laccase changed the color of the YBLB medium from blue-green to yellow, the absorption spectra of YBLB medium incubated with the commercial laccase from

Trametes versicolor and the medium incubated with the culture filtrate of the FVN-1 strain were compared. Findings showed that this color difference originated from extracellular laccase produced by FVN-1. Additionally, the time course of laccase production in sawdust medium (mushroom cultivation condition) inoculated with FVN-1 and FVD-1 strains was monitored. Laccase activity in the sawdust medium gradually increased during the initial 14 days of cultivation of FVN-1 strain. Subsequently, a low temperature shift (from 23 °C to 15 °C) was applied, which is essential for fruiting body formation. A considerable increase in laccase activity was observed in FVN-1 culture, with the highest activity detected in the 16-old-day culture, followed by a gradual decrease in laccase activity. In contrast, the laccase activity in FVD-1 culture remained low even after the temperature shift. These findings suggest that extracellular laccase is involved in the fruiting body development process in the edible mushroom *F. velutipes*.

Chapter II: Heterologous expression of laccase isozyme (Lcc2) from *F. velutipes* in *Aspergillus oryzae*

In the time course of fruiting body formation, one of the laccase isozymes was expressed majorly in the sawdust medium. An explicit difference in expression of this major laccase isozyme, named as Lcc2, between the FVN-1 strain (expressed) and the FVD-1 strain (not expressed) was observed. In order to investigate Lcc2 more deeply, heterologous expression system was established using a filamentous fungus, *Aspergillus oryzae* with the aim of the production of only Lcc2 in large amount in a short time. Expression vector containing Taka-amylase A signal peptide sequence, Taka-amylase A gene (*amyB*) promoter, and *amyB* terminator was inserted into *A. oryzae* using polyethylene glycol method. The highest laccase production in the liquid culture inoculated with the transformant *A. oryzae* was determined in 9 days. Altogether, recombinant Lcc2 was successfully produced in *A. oryzae* in high level in a short time for the further studies.

Chapter III: Production and properties of a laccase isozyme (FvLcc3) from the edible mushroom *F. velutipes*, which is undetectable under the culture condition for fruiting body formation

Extracellular laccase isozyme (FvLcc3) from the edible mushroom *F. velutipes* was found to be undetectable under the culture condition for fruiting body formation. FvLcc3 was purified and determined to be an approximately 53-kDa monomeric protein. FvLcc3 showed the highest catalytic efficiency (k_{cat}/K_m) toward 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) followed by 2,6-dimethoxyphenol and guaiacol and did not oxidize 3,4-dihydroxy-L-phenylalanine and L-tyrosine.

Overall, laccase isozymes produced by the basidiomycete *F. velutipes* were studied in this dissertation. Possible important relationship between laccase isozyme (Lcc2) and fruiting body formation was determined. The high-efficient Lcc2 expression system was also constructed using *A. oryzae* which is known as cell factory. The results offer valuable information for further studies related to fruiting body formation in *F. velutipes* and other basidiomycete mushrooms. In addition, the effect of cultivation type (solid-state and liquid-static cultures) on the production of laccase isozyme (FvLcc3) was determined in *F. velutipes* for the first time, and its enzymatic properties were characterized.