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学位論文全文に代わる要約
Extended Summary in Lieu of Dissertation

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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)

学位論文要約：
Dissertation Summary

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*

F. velutipes, which is also known as Enokitake, is a commercially important cultivated edible basidiomycetous mushroom, especially in Japan. Degenerate strains of *F. velutipes*, which exhibit the loss of fruiting body formation, have been reported, resulting in substantial financial losses in the mushroom industry. Additionally, the biochemical mechanism taking part in fruiting body formation has not been known.

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 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 showed the relationship between fruiting body development and extracellular laccase production using normal and degenerate strains of *F. velutipes*.

Chapter II: Heterologous expression of the major laccase isozyme from *F. velutipes* in *Aspergillus oryzae*

The expressions of laccase isozymes are regulated according to various factors including developmental stages. Previous results (Chapter I) revealed the importance of extracellular laccase in the fruiting body development of *F. velutipes* by comparing the FVN-1 and FVD-1 strains. Remarkable effect of low temperature shift on the laccase activity was also seen in the FVN-1 strain cultivated in the sawdust medium (Chapter I). In line with this information, native polyacrylamide gel electrophoresis followed by laccase activity staining using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as the substrate was performed to analyze the laccase isozyme patterns of FVN-1 and FVD-1 strains under different growth stages.

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 between the FVN-1 and FVD-1 strains was observed. In order to investigate this major laccase isozyme more deeply, heterologous expression system was established using a filamentous fungus, *Aspergillus oryzae* with the aim of the production of only this laccase isozyme 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. Altogether, recombinant laccase isozyme 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 from the edible mushroom *F. velutipes*, which is undetectable under the culture condition for fruiting body formation

The expression of various genes, including laccase genes, which can affect enzyme production, can be changed by differences in culture conditions between solid-state and submerged cultures. One of the extracellular laccase isozymes from the edible mushroom *F. velutipes* FVN-1 was found to be undetectable under the culture condition for fruiting body formation. This laccase isozyme was purified and determined to be a monomeric protein. The effect of pH on laccase activity was examined at pH values ranging from 2.0 to 10.0. Temperature stability, ranging from 4 to 60 °C, was also determined. To determine the optimum pH for laccase activity, ABTS, 2,6-dimethoxyphenol (2,6-DMP), and guaiacol were used. The highest catalytic efficiency (V_{max}/K_m) was observed for ABTS, followed by that for 2,6-DMP and guaiacol. Chloride ion is common inhibitor of fungal laccases. To determine the effect of chloride ion on laccase activity, different concentrations of NaCl were applied. Furthermore, the effects of different concentrations of metal ions, including Fe^{2+}, Zn^{2+}, Cu^{2+}, and Mg^{2+}, on laccase activity were determined. Some putative laccase inhibitors such as ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT), sodium azide (NaN_3), and L-cysteine were also used to determine their effects on laccase activity. NaN_3 exerted considerable inhibitory effect on the activity of laccase isozyme. Furthermore, DTT and L-cysteine demonstrated considerable inhibitory effects compared to that exerted by the metal chelator EDTA.

Overall, laccase isozymes produced by the edible basidiomycetous mushroom *F. velutipes* were studied in this dissertation. Important relationship between the major laccase isozyme and fruiting body formation was determined. The high-efficient major laccase isozyme expression system was also constructed using *A. oryzae* which is known as cell factory. These offer valuable information for further studies aimed at deducing the molecular basis of fruiting body formation in *F. velutipes* and other basidiomycetous mushrooms. In addition, it was shown here for the first time that one of the extracellular laccase isozymes is undetectable under fruiting body forming condition in *F. velutipes* and characterized it in terms of its enzymatic properties. This may contribute to literature given the knowledge gap regarding the laccase secretion patterns of *F. velutipes* depending on its cultivation type and enzymatic characteristics of laccase isozymes.

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