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Proteolytic modifications of laccase from *Cerrena unicolor*

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ABSTRACT

Laccase from the wood-degrading *Cerrena unicolor* FCL139 has been proved many times to be useful as a decolorizing enzyme, antibacterial agent, and important part of biosensors. In recent papers, there have been attempts to modify its structure and thereby alter its features and activity. In this work, proteolytic modification of laccase isoforms was performed. To achieve the goal, commercially available proteases: proteinase K, trypsin, pepsin, papain, and *Rhizopus* pepsin were applied. The results obtained proved that only proteinase K was able to digest laccase resulting in lowering its activities to only 7%. The other four proteases enhanced the activities of this multicopper oxidoreductase up to 140%. Proteolytic modification of laccase structure resulted also in enhancement of its prooxidative potential. Moreover, analysis of kinetic constants proved that V_{max} of modified laccase increased over 2 times, the highest k_{cat} (321.9 ± 14.83 L/s) was observed when analyzing laccase incubated with pepsin from *Rhizopus*. The electrochemical investigations of anodic peak (650 mV vs Ag/AgCl), and cathodic peak (400 mV) showed that pepsine-treated laccase had 2 times higher peaks than trypsin digested and four times higher than control experiment. Additionally, the remaining forms seem to inhibit proteolytic activities leaving even only 20% of its initial activity.

1. Introduction

Fungal laccase seems to be the best-examined enzyme engaged in ligninolysis; moreover, it plays an important role in mushroom morphogenesis and pigmentation processes [1–3]. A growing number of papers describe not only the enzyme itself and its physicochemical and kinetic characteristics [4], but also the structure of genes coding for laccase and its promoter region. Due to their physiological function that is common for all fungi and at the same time despite their broad substrate specificity, all fungal laccases have similar features and they are glycoproteins composed of ca. 520–550 amino acids with a 20 aa signal peptide. Moreover, the active site in all laccases is composed of four conserved regions of one cysteine and ten histidine residues that form a cage-like chelating ligand environment for copper ions [5,6]. In laccase genes, further similarities in the number (average 8–13 for *Basidiomycetes* and 1–6 for *Ascomycetes*) and size of introns (50–90 bp) can be found [7]. With the growing number of laccase gene sequences deposited in GenBank, there is a notable increase in sequenced and characterized promoter regions of this enzyme, which gives better insight into laccase expression regulation. In the past decades, many papers have been produced describing the medium composition and culture optimization of many fungal species; this resulted in enhancement of enzyme production, which is so important in biotechnology. Analysis of these optimization data together with characteristics of the laccase promoter region provides better understanding on how the production of this enzyme is regulated at the transcriptional level and, together with transcriptomes and real-time analysis, may be used to deduce how wood decomposition is realized in nature [5,7]. In the promoter region, carbon and nitrogen regulatory elements (Mig, Sp1, CreA, NIT2) can be found, which supports the hypothesis that most fungal laccases are produced when the carbon and/or nitrogen source is depleted [8–13]. Moreover, laccase synthesis seems to be regulated by a number of environmental factors such as temperature (Heat Shock Element in the promoter region), heavy metals (Metal Responsive Elements), xenobiotics (Xenobiotic Responsive Elements), and antioxidants (Antioxidant Responsive Elements, Ap1) [7,14–16]. At the transcriptional level, expression of laccase seems to be more complicated as the laccase genes are clustered [17,18], but there is scant evidence relating such transcriptional regulation [19]. Despite the many papers describing laccase production under

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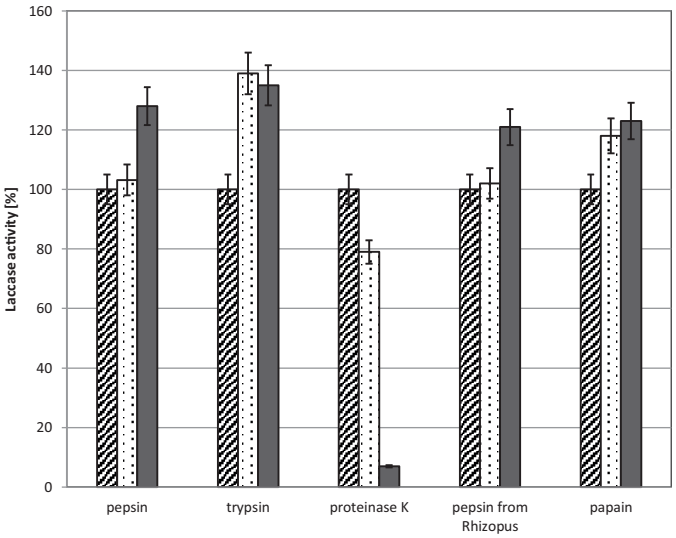

Fig. 1. Laccase activities depending on the time of incubation with proteases. Samples were collected at 0 (dashed bar) point and after 60 (dotted bar) and 120 min (grey bar) of incubation (0.247 U/mg of proteins was defined as 100% of LAC activity).

different conditions and the growing number of promoter regions characterized, it is clear that the regulation of expression of the gene encoding this enzyme is a highly complex process with various factors and their interactions [7]. However, transcriptional regulation of laccase synthesis seems to be better described in comparison to posttranslational modifications of this enzyme. It is well known that fungal laccases are N-glycosylated and modified by removal of N-terminal residues [20]; however, only few papers have identified the sugar moiety of purified proteins [21–23]. Glycosylation of laccase is responsible for secretion, activity, copper retention, and thermal stability [24,25]. It is presumed that glycosylation also protects laccase from proteolysis; however, the thesis seems to be rather a result of heterologous expression in the fungal host than of an analysis of laccase interaction with homologous proteases [26]. Even if it is obvious that fungal extracellular proteases may affect their laccases, little is known about their interactions in nature. Only few papers have proved that this relationship may be crucial for laccase activity [27,28] and therefore useful in biotechnology. Recent investigations conducted by means of mutagenesis and heterologous expression have proved the importance of the so-called C-plug in maturation of laccase in *Ascomycetes* and its kinetic properties as a recombinant protein [29,30]. However, little is known about how proteases may affect laccase structure and properties in nature mainly due to the complexity of the endogenous system of fungal proteases. Therefore, in this study commercially available proteases were used to analyse their influence on *Cerrena unicolor* laccase activity, kinetics, and antioxidant and electrochemical properties.

2. Materials and methods

2.1. Microorganism, growth conditions, and production of laccase

C. unicolor C-139 was obtained from the culture collection of the Regensberg University. The fungus was maintained in 2% (w/v) malt agar slants. As an inoculum, pieces of agar were grown in the Lindenberg and Holm medium in non-agitated conical flasks for 7 days at 28 °C. The mycelial mats were subsequently collected and homogenized in a waring blender. The fragmented mycelial culture (2.5% v/v) was used as a standard inoculum for further studies.

The fermentor-scale cultivation was performed at 28 °C in a 2.5-L Bioflo III (New Brunswick Scientific, New Brunswick, NJ) fermentor containing 2 L of the Lindenberg and Holm medium sterilized at 121 °C for 30 min and optimized as in Janusz et al. [31]. The fermentor was inoculated with crumbled fungal mats (10% of total volume), aerated at 1 L air per minutes, and stirred at 100 rpm. Antifoam B emulsion (Sigma, St. Louis, MO) was occasionally added to the fermentor cultures to break the foam. 10 mL of the sample culture was sampled every 24 h.

2.2. Laccase isolation and purification

The post-culture liquid was centrifuged at 10,000 × *g* on a 6K15 (Sigma, Osterode am Harz, Germany) centrifuge for 15 min. The supernatant was concentrated 10 times on the ultrafiltration system Pellicon 2 Mini holder (Millipore, Bedford, MA) with an Ultracel mini cartridge (10 kD cut-off) and used as a source of the crude enzyme. Chromatography was performed using a chromatographic EconoSystem (Bio-Rad, Richmond, VA). The enzyme solution was loaded on a DEAE-Sepharose column (2.5 × 15 cm) pre-equilibrated with 20 mM Tris–HCl buffer (pH 6.5) and 0.1 M NaCl, and proteins were eluted with a 0–0.5 M linear gradient of NaCl at a flow rate of 1 mL/min. Semi-purified laccase was then lyophilized on a FreeZone 18 system (Labconco, Kansas, USA).

2.3. Laccase–protease experiments

The solution of the lyophilized laccase (activity 0.25 U/mg of protein) in ultra-pure water was incubated in a ratio 1:1 with the solution of pepsin (4.2 U/mg), trypsin (10.3 U/mg), papain (2.5 U/mg), proteinase K (10.1 U/mg), and *Rhizopus* pepsin (3.8 U/mg) (Sigma) at 37 °C. The LAC activity was determined periodically (at 0 point and after 60 and 120 min) using syringaldazine (4-hydroxy, 3,5-dimetoxybenzaldehyde) as the reaction substrate and the other part of the reaction mixtures were subjected to native PAGE, electrochemical experiments and antioxidant activity.

2.4. Analytical procedures

2.4.1. Protein assay

The protein concentration was determined with the use of the Coomassie Brilliant Blue (G-250) dye-binding method [32] using a Bio-Rad dye stock solution with bovine serum albumin as a standard.

2.4.2. Laccase activity and kinetics parameters determination

Laccase activity was measured on a Shimadzu UV-160A spectrophotometer with syringaldazine (Aldrich, USA) as a substrate [33]. The LAC activity was measured following oxidation of 0.025 mM of syringaldazine (4-hydroxy, 3,5-dimetoxybenzaldehyde) in 0.1 mM citrate-phosphate buffer at pH 5.3 [35]. The oxidation of the substrate was recorded at 525 nm at 20 °C. The activity of LAC was expressed as U per mg of protein (U/mg).

Kinetics parameters (K_m, V_{max} and k_{cat}) for digested laccase were determined by direct regression of the Michaelis–Menten hyperbola obtained experimentally. The assays carried out using a laccase were analyzed with syryngaldazine as a substrate in concentration that varied from 0.25 to 0.75 mM. The kinetics parameter values were obtained by nonlinear curve fitting according to the Michaelis–Menten equation: $V = V_{max}S/(K_m + S)$, where V is the laccase activity, and S is the syryngaldazine concentration. The OriginPro 8 software (OriginLab Corporation, Northhampton, MA, USA) was used for data analysis.

2.4.3. Assay of protease activities

The protease activities were estimated at optimal pH according to the Anson [34] procedure using hemoglobin as a reaction