Biochimie 142 (2017) 183–190

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Research paper

Properties of two laccases from the *Trametes hirsuta* 072 multigene family: Twins with different faces

Olga S. Savinova*, Konstantin V. Moiseenko, Ekaterina A. Vavilova, Tatiana V. Tyazhelova, Daria V. Vasina

Bach Institute of Biochemistry, Research Center of Biotechnology of the Russian Academy of Sciences, 33, Bld. 2, Leninsky Ave., Moscow, 119071, Russia

ARTICLE INFO

Article history:
Received 12 July 2017
Accepted 18 September 2017
Available online 21 September 2017

Keywords:
White-rot fungi
Laccase multigene family
Constitutive and inducible isozymes
Differential expression

ABSTRACT

Utilization of laccases in biotechnology and bioremediation has created a strong demand for the characterization of new enzymes and an increase in production of known laccases. Thus, additional research into these enzymes is critically needed.

In this study, we report a comparative study of the biochemical and transcriptional properties of two different laccase isozymes from *Trametes hirsuta* 072 – the constitutive and inducible forms. A recombinant LacC enzyme was expressed in *Penicillium canescens* to characterize its properties. LacC is single-purpose enzyme, unlike LacA, which can operate efficiently under a wide range of temperatures and pHs (55–70 °C and pH 3–5, respectively). LacC has a lower RedOx potential than LacA and does not oxidize substrates containing amine groups. Expression of the *lacC* gene was selective compared to that of the *lacA* gene and increased significantly in the presence of complex synthetic compounds such as dyes and xenobiotics.

This study shows that laccases from the multigene families of basidiomycetes differ significantly in their properties, thus providing a complementary effect during lignin degradation.

1. Introduction

Fungal laccases are part of a large family of extracellular (in some cases intracellular) proteins of microorganisms, fungi and insects and are of particular biotechnological interest compared to other multicopper oxidases. These enzymes have a wide spectrum of industrial uses ranging from delignification in biopulping and decolorization of wastewater to utilization in the food industry. Laccases have been intensively studied with the goal of creating an ideal biotechnologically oxidative enzyme. The desired attributes include being an ecologically sustainable biocatalyst with a broad substrate specificity, high stability, and efficiency. Their catalytic, physicochemical and structural properties have been studied for years [1–3]. There are a number of reviews devoted to laccase transcription, biosynthesis, and induction; development of effective recombinant laccase producers [4]; and investigations of the role of laccases in organisms [5,6]. Nevertheless, until now, there has been a lack of knowledge of the molecular mechanisms underlying transcriptional regulation of individual laccases, as well as their biogenesis and secretion.

Currently, white-rot fungi are the main source of laccases that are expressed both naturally and from recombinant organisms. This explains why laccases from basidiomycetous have been studied the most thoroughly. With the advent of the Next-Generation Sequencing era and active accumulation of genomic and transcriptomic data, it has become apparent that laccases are encoded in the genomes of basidiomycetes by multigene families with different regulatory patterns. Although the number of laccases encoded in genomes can reach 10–17 genes [5], substantial protein production is generally limited to a modest spectrum of isozymes. For some fungal species, several isozymes can be detected at once. For example, *Pleurotus ostreatus* produces two to four isozymes, but only six laccases out of 11 contained in the genome have been isolated and characterized to date [7]. Two laccases are universally

Abbreviations: ABTS, 2,2′-azino-bis-(3-ethylbenzthiazoline)-sulfonic acid; GP, glucose peptone; HBT, 1-hydroxybenzotriazole; NHE, normal hydrogen electrode; RQ, relative quantities; STRE, Stress Response Elements; TF, transcription factors.

* Corresponding author.
E-mail addresses: savinova_os@rambler.ru (O.S. Savinova), mr.moiseenko@gmail.com (K.V. Moiseenko), vavilovka@yandex.ru (E.A. Vavilova), t.tyazhelova@gmail.com (T.V. Tyazhelova), d.v.vasina@gmail.com (D.V. Vasina).

https://doi.org/10.1016/j.biochi.2017.09.013
0300-9084/

expressed by the saprotrophic fungus *Coprinopsis cinerea* [8]). In other cases, for example, the genus *Trametes*, fungi typically produce one major enzyme that has multiple isoforms with varying levels of protein glycosylation. Therefore, the question of diversity is important within laccase multigene families, and the existence of silent laccase isozymes has become relevant. An integrated study on the features of the expression of different laccase isozymes, as well as their biochemical properties could shed light on these questions.

Studies of the evolution and structural diversity of laccase genes [9] have shown that, according to the topology of the phylogenetic trees, the genes for laccase isozymes may have evolved independently, possibly through duplication–divergence events. Laccases are assumed to perform various physiological functions in fungi. They are involved in the degradation of lignin, detoxification, pigment production, fruiting body formation, and vegetative growth [1,5,10,11] or serve as virulence factors in fungal pathogens [12]. This hypothesis is supported by studies in which changes in the production of laccase isozymes in fungi have been shown over time, depending on the stage of fungal growth or substrate degradation [13,14].

It is known that the synthesis and secretion of laccases, including different isozymes in the same strain, are strictly influenced by the level of nutrients, developmental stage, and addition of a wide range of inducers to the culture media. These effects were demonstrated for laccase gene transcription and secretion in many white-rot fungi [6,15–17]. The presence of numerous regulatory elements in the promoter region of laccase genes is indirect proof of the close biosynthetic induction mechanisms for laccases from different sources [2]. Alternatively, the quality and quantity of the *cis*-regulatory elements vary depending on the particular gene [6], implying that the regulation of transcription depends on the composition of the promoter region as well as the culture conditions. In spite of the extensive data on laccases, it is difficult to predict the relevant conditions for the production of minor isozymes, to reveal global patterns of protein biosynthesis at the transcriptional and translational levels. Each microorganism has unique transcriptional and production activators. In several fungal species, the conditions for inducible laccase production were optimized. For example in *Trametes* sp. I-62, guaiacol, *p*-coumaric and ferulic acids selectively induced expression of three laccase genes [15]. In *Trametes* AH28-2, laccase isozyme B expression and production were selectively induced by 3,5-dihydroxytoluene [18], while production of laccase Lcc9 from *C. cinerea* was enhanced by co-cultivation with the zygomycete *Gongronella* sp. [8]. Since it has been suggested that the constitutive and inducible laccase isozymes differ in their properties [19], complex characterization of the expression and biochemical features of laccase isozymes from the same multigene family may lead to the investigation of new enzymes that show potential for industrial use and should allow researchers to develop an efficient strategy to enhance their production.

It has been shown previously that the basidiomycete *Trametes hirsuta* 072 is characterized by constitutive production of the single major laccase isozyme LacA [19]. This form predominates at both the expression and protein production levels under all conditions studied. However, a second laccase, isozyme LacC, was also found at a late stage of cultivation in the presence of a lignocellulosic substrate [14]. We assumed that inducing expression of the additional isozyme will depend on the accumulation of specific inducers at different degrees of lignin degradation. However, it was not possible to correlate this with any compound or class of compounds.

In this study, we aim to compare the constitutively produced LacA and inducible LacC isozymes from *T. hirsuta* 072 according to their expression patterns as well as their biochemical properties. The results are discussed in the context of a potential role of laccase isozymes in the life cycle of the fungus.

2. Materials and methods

2.1. Fungal strain and culture conditions

The mycelia of the basidiomycete *T. hirsuta* 072 that was used in this study were provided by the Collection of Komarov Botanical Institute, Russian Academy of Sciences (St. Petersburg, Russia). The fungi were pre-cultivated at 26 °C-28 °C in 750-ml Erlenmeyer flasks containing 200 ml of glucose peptone (GP) medium (3.0 g of peptone, 10.0 g of glucose, 0.6 g of KH_2PO_4, 0.4 g of K_2HPO_4, 0.5 g of $MgSO_4$, 50 mg of $MnSO_4$, 1 mg of $ZnSO_4$, and 0.5 mg of $FeSO_4$ per 1 l of water, pH 6.0) and ceramic beads [20]. Then, the mycelium was disrupted into small fragments by the ceramic beads and used for submerged cultivation of *T. hirsuta* 072 in GP medium on a rotary shaker at 180 rpm.

2.2. Laccase enzyme purification

The LacA protein was purified from the culture broth of the basidiomycete *T. hirsuta* 072 as previously described [20] using ion exchange chromatography on DEAE-Toyopearl 650M resin (Tosoh, Japan) eluted with a 5–200 mM gradient of potassium phosphate buffer, pH 6.5, and an additional purification step with a Superdex 75 column (HiLoad 26/600, GE Healthcare, UK) previously equilibrated with 5 mM potassium-phosphate buffer, pH 6.5.

Since the production of the native LacC was insufficient for protein purification, a recombinant protein was obtained and characterized. Previously, a protocol for heterologous expression of *T. hirsuta* 072 laccase in *Penicillium canescens* was developed [21,22]. The recombinant laccase *P. canescens* strain with the *lacC* gene from *T. hirsuta* 072 was constructed and cultivated as previously described (GB accession # KP027479.1).

The recombinant LacC isozyme was purified from the culture broth of *P. canescens* utilizing the same methods as those described for LacA.

2.3. Biochemical assays

2.3.1. Protein concentration measurement

Samples of the culture broth were centrifuged for 5 min at 9000×*g*, and the protein concentrations in the supernatants were determined by the method of Bradford according to the manufacturer's instructions (Pierce, USA). The same method was utilized for protein measurements in all samples. All of the measurements were performed in triplicate.

2.3.2. Electrophoretic testing of isozymes

Twelve percent sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed at a constant voltage (200 V) on a Mini Protean 3 system (Bio-Rad, USA) according to the method of Laemmli [23]. The molecular mass was determined under denaturing conditions and was performed by comparison with a PageRuler Prestained Protein Ladder (Fermentas, USA). Analytical isoelectric focusing on polyacrylamide gels (IEF-PAGE) in the ampholytes range of pH 2.0–4.0 (Serva Electrophoresis, Germany) was performed on a 111 Mini IEF Cell (Bio-Rad, USA). The pH gradient was measured with the Low Calibration Kit (GE Healthcare, UK). The proteins were stained using Coomassie Brilliant Blue R-250 (AppliChem, Germany). Native polyacrylamide gel electrophoresis (PAGE) was performed with the same conditions for SDS-PAGE without denaturation agents (including SDS) and reducing agents and without boiling of the samples. The native gels were