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Manganese peroxidases, laccases and oxalic acid from the selective white-rot fungus *Physisporinus rivulosus* grown on spruce wood chips

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Abstract

The white-rot fungus *Physisporinus rivulosus* T241i produced manganese peroxidase (MnP), laccase, and oxalic acid when it was grown on spruce (*Picea abies*) wood chips. This white-rot basidiomycete degrades lignin selectively and is promising for use in biopulping of softwood. During solid-state cultivation on wood chips the fungus produced oxalic acid (28 μmol/g dry wood) and a high MnP activity (24 nkat/g dry wood), while measured laccase activity was negligible. The prepurified laccase and MnP isoforms were fractionated with anion exchange chromatography. Characterization of the isolated MnP fractions indicated the presence of several MnP isoforms with p*I* values between 3.4 and 3.9 and molecular masses between 47 and 52 kDa. The four laccase isoforms had p*I* values between 3.1 and 3.3 and molecular masses between 66 and 68 kDa. The MnP and laccase isoform profiles did not vary during the 4-week cultivation. The N-terminal amino acid sequences of four laccase and two MnP isoenzymes resembled those of the two other selective white-rot fungi applied in biopulping, i.e. *Ceriporiopsis subvermispora* CZ-3 and the strain IZU-154.

Keywords: Manganese peroxidase; Laccase; White-rot fungus; Oxalic acid; Solid-state cultivation; Norway spruce; Lignin degradation; Biopulping

1. Introduction

Lignin is an aromatic and heterogeneous constituent of plant cell walls that ensures strength and resistance towards microbial attack. White-rot basidiomycetes are the only organisms known to efficiently degrade and mineralize lignin into CO_2 and H_2O [1,2]. Several white-rot fungi, e.g. *Ceriporiopsis subvermispora*, cause selective lignin degradation, whereupon the wood enriches with cellulose [3]. This ability is beneficial in fungal biopulping for example to decrease energy consumption in mechanical pulping of wood [4]. The extracellular enzymes involved in lignin degradation are laccase (EC 1.10.3.2, benzenediol:oxygen oxidoreductase), lignin peroxidase (LiP, EC 1.11.1.14), manganese peroxidase (MnP, EC 1.11.1.13), versatile peroxidase (VP), and H_2O_2-forming enzymes such as glyoxal oxidase and aryl alcohol oxidase, all secreted by various species of white-rot fungi in different combinations [2,5]. Most common is the secretion of MnP together with laccase [2,5].

Today MnP is considered to be the key enzyme in the degradation of lignin and aromatic xenobiotics, and it is produced by most of the white-rot fungi studied so far [2,6]. MnP oxidizes Mn^{2+} to Mn^{3+} in an H_2O_2-dependent reaction [7]. To stabilize the formation of Mn^{3+}, an organic chelator is needed, which in general is a dicarboxylic acid secreted by the fungus [7,8]. In lipid-mediated peroxidation reactions, MnP oxidizes even the more recalcitrant non-phenolic substructures of lignin [9,10]. MnP of *C. subvermispora* can also oxidize oxalate and glyoxylate to form H_2O_2 and thus, in the presence of these organic acids, MnP is independent of added

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H_2O_2 [11]. Recently, it was found that fungal MnP is able to mineralize synthetic lignin (DHP) to CO_2 [10,12], and cause fragmentation of milled softwood [13] in vitro in the presence of a chelating organic acid and unsaturated lipids. Thus, the link between MnP and dicarboxylic acids is dual; carboxylic acids may stabilize the formation of Mn^{3+} ions by chelation and act as substrates for MnP to form the oxidant, H_2O_2.

While MnP is able to degrade lignin on its own, almost all white-rot fungi secrete also laccase. Laccase can normally attack only phenolic substructures of lignin. However, it is able to degrade the non-phenolic structures of lignin via the action of small-molecular weight mediators like hydroxybenzotriazole [14].

Although the enzymology of lignin degradation by white-rot fungi has been widely studied during the last few decades, fairly little is known about the expression of the different LiP, MnP, and laccase isoenzymes during fungal growth on lignocellulose substrates, such as wood or straw. Solid-state cultivation of *C. subvermispora* and *Phanerochaete chrysosporium* on wood results in a different composition of MnP and LiP isoforms than previously isolated from liquid cultures [15–17]. Since some of the isoforms are clearly expressed only under solid-state conditions, it is important to study the enzyme production on wood chips, in order to elucidate the role of ligninolytic enzymes during selective lignin degradation and biopulping.

In our recent study, lignin-degrading activity of 300 strains of white-rot fungi was evaluated to find suitable fungi for biopulping, i.e. fungal pretreatment of wood chips prior to Kraft or mechanical pulping [18]. One of these new isolates, *Physisporinus rivulosus* (Berk. & Curt.) (syn. *Poria albipellucida*, *Poria rivulosa*, *Ceriporiopsis rivulosa*) strain T241i, was found to cause selective degradation of lignin over cellulose on Norway spruce (*Picea abies*) and grow in a wide temperature range (15–40 °C) [18]. In laboratory scale biopulping experiments on spruce, *P. rivulosus* decreased energy consumption in refining up to 20% [19]. To elucidate the mechanism of selective lignin degradation by *P. rivulosus*, a detailed study of the properties of ligninolytic system of *P. rivulosus* was undertaken. Secretion of organic acids and ligninolytic enzymes during growth on spruce wood chips was followed under conditions similar to those under which the fungus causes selective lignin degradation [18]. The fungus was found to secrete several isoforms of MnP and laccase, which were isolated, purified and partially characterized.

2. Materials and methods

2.1. The fungus and inoculum

P. rivulosus strain T241i (DSM 14618) was found and isolated in the south of Finland. The isolate was maintained on 2% (w/v) malt extract (Biokar), 1.5% (w/v) agar (Biokar) with a piece of spruce wood as a supplement. The inoculum was grown on malt extract broth (2%, w/v) for 9 days at 28 °C and homogenized with a Waring blender, four times 10 s with 30 s intervals.

2.2. Wood chip cultures

2.2.1. Time-scale experiment in conical flasks

Production of ligninolytic enzymes and organic acids was followed in two separate cultivations at 28 °C in 250 ml conical flasks, which contained 25 g dw of Norway spruce (*P. abies*) wood chips (approx. 3 mm × 20 mm × 30 mm) as described by Mäkelä et al. [20]. Samples for analysis of enzyme activities, determination of organic acids, and pH were taken once, twice, or three times a week from three parallel culture flasks each time. Before extraction of enzymes or organic acids, the pH of two individual wood chips from each culture flask was determined using a surface pH electrode (Ingold, Pharmacia Amersham).

2.2.2. Production of MnP and laccase in polypropene bioreactors

Two cultivations to produce MnP (14 days) and laccase (7 days) for purification were carried out in five polypropene bioreactors (12 cm × 28 cm × 18 cm) each supplied with 300 g dw of Norway spruce wood chips. Moisture content of the wood was 60% before autoclavation (121 °C, 20 min). The wood chips were placed on a perforated plastic plate in the bioreactors and inoculated with 150 ml of homogenized mycelium prepared as described above. Cultures were continuously aerated with sterile air through the perforated plate during the incubation.

2.3. Extraction of enzymes and organic acids from wood chips

Fungal extracellular proteins were extracted from the wood chip cultures as described by Mäkelä et al. [20] with 25 mM sodium phosphate buffer (pH 7.0, 10 ml/g dw of wood) supplemented with 0.05% (w/v) Tween 80. The buffer was first impregnated into the wood chips using vacuum suction [19] and then the chips were pressed using high pressure N_2 compression [21]. Organic acids produced by the fungus were extracted from the wood with warm 1.5 M HCl (5 ml/g dw of wood) as described by Mäkelä et al. [20].

2.4. Enzyme assays

Laccase activity was determined using syringaldazine (4-hydroxy-3,5-dimethoxybenzaldehyde, EGA-Chemie) as substrate [22] in 100 mM citrate phosphate buffer (pH 5.0) at 25 °C. LiP was determined using veratryl alcohol as substrate at pH 3.0 in 0.1 M sodium tartrate buffer at 25 °C [23]. MnP was determined by following the formation of