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Phenol oxidation by DyP-type peroxidases in comparison to fungal and plant peroxidases☆

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ABSTRACT

Over the last years, novel peroxidases secreted by lignocellulose-degrading agaricomycetes have been discovered. Among them, the so-called DyP-type peroxidases (DyPs) that are secreted under conditions close to nature (i.e. in wood cultures) are of particular interest, since they are able to oxidize diverse substrates including veratryl alcohol, non-phenolic lignin model dimers as well as recalcitrant phenols and dyes. In spite of their unique protein structure and their catalytic versatility, the estimation of the redox potential of this new peroxidase group is still pending. To solve this problem, we used a catalytic approach developed by Ayala et al., 2007 [21], which is based on the Marcus equation and the determination of the redox thermodynamics between heme-peroxidase compound II and the resting state enzyme. Five fungal DyPs (among them four wild-type enzymes and one recombinant protein) were tested regarding phenol oxidation in comparison to other well-studied plant and fungal peroxidases (soybean peroxidase, SBP, *Coprinopsis cinerea* peroxidase, CiP, lignin peroxidase of *Phanerochaete chrysosporium*, LiP). DyP-type peroxidases have a high affinity for phenols and can oxidize even recalcitrant representatives such as *p*-nitrophenol. Based on this "phenol oxidation method", their redox potential was estimated to range between 1.10 ± 0.02 and 1.20 ± 0.1 V, which is between the values calculated for high-redox potential LiP (1.26 ± 0.17 V) and low-redox potential, phenol-oxidizing plant (0.93 ± 0.04 V for SBP) and fungal (1.06 ± 0.07 V for CiP) peroxidases.

1. Introduction

Heme-containing peroxidases (donor: H_2O_2 oxidoreductases, EC 1.11.x) are versatile catalysts oxidizing diverse substrates. They are ubiquitous in fungi, plants, animals and eubacteria. On the basis of sequence homologies, they are classified within different superfamilies, among which those of animal and non-animal peroxidases (former plant peroxidases) are the largest groups (PeroxiBase; http://peroxibase.toulouse.inra.fr/index.php). Non-animal peroxidases comprise three classes: class I – intracellular, organelle-associated and bacterial peroxidases (e.g. cytochrome c peroxidase), class III – secreted plant peroxidases (e.g. horseradish peroxidase, HRP) and class II – secretory fungal peroxidases including ligninolytic peroxidases [1]. The latter are exclusively found in wood and leaf-litter decomposing Basidiomycotina (mostly agaricomycetes) and comprise manganese, lignin and versatile peroxidases (MnP, LiP and VP). They possess high redox potentials (1.1–1.5 V), which enable them to oxidize a huge number of recalcitrant substrates including methoxylated aromatics present in wood and other lignocelluloses [2,3].

Two new superfamilies of fungal peroxidases were discovered over the last decade, namely unspecific peroxygenases and dye-decolorizing peroxidases (DyPs). The enzymes of the latter superfamily are, according to genetic databases, widespread not only among agaricomycetes but also among bacteria[2] and

Abbreviations: DyPs, DyP-type peroxidases; *Aau*DyP, *Auricularia auricula-judae*; *Egl*DyP, *Exidia glandulosa*; *Mep*DyP, *Mycena epipterygia*; *Msc*DyP, *Mycetinis scorodonius* (formerly *Marasmius scorodonius*); CiP, *Coprinopsis cinereus* peroxidase; LiP, lignin peroxidase; MnP, manganese peroxidase; VP, versatile peroxidase; SBP, soybean peroxidase; HRP, horseradish peroxidase; TOP, tobacco peroxidase.

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[2] It has to be noted that bacterial and fungal DyPs share hardly sequence homology (<5%), except for a few key residues [5] so that an old age or a convergence evolution can be assumed [4].

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ascomyceteous fungi [4,5]. DyPs possess an "atypical" molecular architecture and divergent mechanistic behavior (that is not fully understood yet [4]), which does not allow their classification within the class II peroxidases [5–7]. Despite the structural differences, the physico-chemical properties of DyPs resemble those of classic heme proteins, e.g. regarding UV–vis spectral characteristics, molecular masses or isoelectric points [5,8,9].

Meanwhile twelve DyPs – eight fungal and four bacterial proteins – have been characterized [5,6]. We have recently found that two DyP isoforms of the jelly fungus *Auricularia auricula-judae* (*Aau*DyP1 and 2) can oxidize non-phenolic aromatics (e.g. veratryl alcohol and a β-*O*-4 lignin model dimer) [6], a catalytic property that is actually characteristic for high-redox potential peroxidases (LiP, VP) [10–12]. Since these reactions showed just moderate catalytic efficiency at pH 3 and very low pH optima (pH < 2), where it is easier to abstract single electrons from aromatic rings [13,14], we concluded that the redox potential of DyPs may range between those of ligninolytic peroxidases (e.g. ~1.5 V for LiP H8 calculated via oxidation of methoxybenzenes; [15]) and low-redox potential plant peroxidases (e.g. ~0.8 V for HRP; [13,16]).

The catalytic cycle of heme peroxidases is initiated by a rapid two electron transfer from the native ferric heme protein ($PorFe^{III}$) to H_2O_2, to give a higher oxidation state of the enzyme called compound I ($^{\bullet+}PorFe^{IV}=O$). It is a ferryl π–cation radical with a formal oxidation state of +5 for the iron (Fe^{V}) and oxygen coupled to this iron by a double bond (Eq. (1); [17]). In the peroxidative cycle, compound I is reduced by two consecutive one-electron reductions via compound II ($PorFe^{IV}=O$) to the native ferric enzyme (Eq. (2)). In dependence of the redox potentials of compound I and II, numerous substrates can serve as electron donors in these reactions, e.g. phenolic and non-phenolic aromatics, metal ions or complex dye-stuff molecules [5].

$$PorFe^{III} + H_2O_2 \rightarrow {}^{\bullet+}PorFe^{IV} = O + H_2O \quad (1)$$

$$^{\bullet+}PorFe^{IV} = O + 2e^- + 2H^+ \xrightarrow{k_{ET}} PorFe^{III} + H_2O \quad (2)$$

$$H_2O_2 + 2e^- + 2H^+ \rightarrow 2H_2O \quad (3)$$

There are different methods for the direct estimation of the redox potential of oxidative enzymes (redox couple compound I/native enzyme) like rapid kinetic techniques (e.g. stopped-flow kinetics, voltammetric methods; [18–20]) but also indirect methods based on the spectral determination of the equilibrium between high oxidation state species of peroxidases have been developed [16,21].

The Marcus theory of outer-sphere electron transfer expects a semi logarithmic dependence between the rate of an electron transfer reaction (e.g. k_{ET}) and a thermodynamic driving force [22]. This correlation can also be considered between the specific activity of a peroxidase-catalyzed reaction and the ionization energy of the substrate, which has successfully been used for the calculation of the redox potentials of some plant and fungal oxidoreductases (e.g. laccase, HRP and VP) [21,23–26]. It was postulated that the observed initial rate for the oxidation of a one-electron donor substrate (e.g. k_{cat}) reflects the electron transfer from the substrate to the enzyme (i.e. k_{ET}). Under saturated and optimized reaction conditions (e.g. by using small polar substrates such as phenol derivatives), this correlation can be used for the estimation of the standard reduction potential. Thus, kinetic data obtained from the initial oxidation rates of *p*-substituted phenols were normalized with the help of the maximum activities obtained for a number of peroxidases and extrapolated against the substrates' redox potential [21,27].

Our report describes for the first time the calculation of the redox potential of several DyP-type peroxidases by a simple and fast method based on the Marcus theory [22], which has been adapted from Ayala et al. [21]. To this end, five of the eight so far characterized fungal DyPs were studied with respect to the oxidation of different phenolic substrates.

2. Materials and methods

2.1. Organisms and enzyme production

All fungi used for the production of wild-type DyPs are deposited in the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig) or in the culture collections of the Department Food and Environmental Sciences (University of Helsinki, Finland) and the Department of Environmental Biotechnology (International Institute Zittau, Germany). The fungi were stored on 2% malt-extract agar (MEA) in culture slants at 4 °C in the dark and routinely pre-cultured on 2% MEA plates for 2 weeks.

Wild-type DyPs of *Exidia glandulosa* (*Egl*DyP), *Mycena epipterygia* (*Mep*DyP) and *Auricularia auricula-judae* (*Aau*DyP 1 and 2) were produced and purified as described recently [8,28]. Recombinant DyP of *Mycetinis scorodonius* (r*Msc*DyP) was provided by DSM (Delft, The Netherlands) [28]. For comparison, *Coprinopsis (Coprinus) cinerea* peroxidase (CiP) was produced according to Ikehata et al. [29] in a glucose–peptone–malt extract medium. Soybean peroxidase (SBP) was purchased from Sigma–Aldrich (Weinheim, Germany). Both enzymes were subjected to purification by FPLC and the final step was carried out on an anion exchanger (MonoQ, 10 mM sodium acetate buffer; pH range 4.0–5.5, gradient 0 to 0.3 or 1.0 M NaCl) as described in [8]. Crude LiP from *Phanerochaete chrysosporium* was purchased from JenaBios GmbH (Jena, Germany) and purified as described recently [28]. The purity of all used peroxidases was proved by SDS-PAGE and by determination of the Reinheitszahl ($RZ = A_{407}/A_{280}$) [8,28].

2.2. Enzymatic reactions

Activity of DyPs was routinely measured with ABTS as described previously [8]. The pH optima for the oxidation of phenolic substrates were determined using 2,6-dimethoxyphenol (2,6-DMP) instead of ABTS as the substrate; the pH of the sodium citrate buffer (50 mM) was varied in the range from 3.0 to 6.0. In case of SBP and CiP, the enzyme activity was determined accordingly but at their own optima of pH 5.5 and 6.8, respectively [21,30]. LiP activity was determined with veratryl alcohol as described elsewhere [31].

The following *p*-substituted phenols with increasing redox potentials were used for the oxidation studies: *p*-methoxyphenol, *p*-chlorophenol, *p*-bromophenol, phenol, *p*-hydroxyacetophenone, *p*-hydroxybenzoic acid, *p*-hydroxybenzonitrile as well as *p*-nitrophenol [21]. All phenols were purchased from Sigma–Aldrich with the highest purity available. Organic solvents and hydrogen peroxide (H_2O_2, 30%) were obtained from Merck (Darmstadt, Germany).

In-vitro reactions were carried out in 0.5 ml reaction tubes (Eppendorf, Hamburg, Germany) in a total volume of 250 μl and under slightly modified conditions as described by Ayala et al. [21]. Compared to the latter report, a lower concentration of hydrogen peroxide (0.1 mM) was applied in order to avoid protein damaging and heme-bleaching effects [32]. Reactions were started by the addition of H_2O_2 and kept under permanent stirring at a constant temperature of 23 °C. They were stopped by addition of trifluoroacetic acid (final concentration 2%) and the phenol derivate concentration was detected by HPLC. Preliminary experiments for each enzyme were performed to verify the kinetics of the enzymatic reactions. Every 10 s, samples were collected and analyzed over a total reaction time of 40 s. After evaluation of these data, a reaction time of 10 s was ascertained to be optimal (Fig. 1A).