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Exploring the biocatalytic potential of a DyP-type peroxidase by profiling the substrate acceptance of *Thermobifida fusca* DyP peroxidase

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ABSTRACT

Dye-decolorizing peroxidases (DyPs) represent a new class of oxidative enzymes for which the natural substrates are largely unknown. To explore the biocatalytic potential of a DyP, we have studied the substrate acceptance profile of a robust DyP peroxidase, a DyP from *Thermobifida fusca* (*Tfu*DyP). While previous work established that this bacterial peroxidase is able to act on a few reactive dyes and aromatic sulfides, this work significantly expands its substrate scope towards lignin related compounds, flavors, and various dyes.

1. Introduction

Substrate promiscuity has often been attributed to different oxidoreductases and in particular to peroxidases. These enzymes perform hydrogen peroxide driven one electron oxidations of a wide range of phenolic and nonphenolic substrates.[1] Substrate promiscuity of enzymes is an interesting biocatalytic property as it broadens up the applicability of an enzyme as a biocatalyst. Plant and animal peroxidases are notorious for their high activity and wide range of substrates. These eukaryotic biocatalysts are known already for several decades, have been thoroughly studied, and are currently used in numerous processes.[2,3] Despite being powerful catalysts, application of these enzymes is often hampered by their low temperature stability and sensitivity to salt and organic solvents. Furthermore, it has been proven to be difficult and often impossible to produce these peroxidases in recombinant form. For example, it has been shown that it is extremely difficult to produce horseradish peroxidase in a heterologous host.[4] As a result, horseradish peroxidase is still mainly produced by isolating it from plant roots which results in a mixture of various peroxidase isoforms.

As alternatives for the plant and animal peroxidases, the newly discovered DyP-type peroxidases (DyPs) may offer advantages. One advantage is the possibility to produce such peroxidases using bacterial expression hosts as most DyPs are of bacterial origin.[5] Except for facilitating the production of peroxidases and eliminating the existence of isoforms, the ability to produce DyPs in a recombinant form also allows engineering of these biocatalysts. The first DyPs were identified less than two decades ago.[6] DyPs are unrelated in sequence and structure to peroxidases belonging to the plant or animal peroxidase superfamilies.[7] While numerous putative DyP-encoding genes can be identified in sequenced bacterial genomes, only a small number of DyPs have been characterized. Originally, their activity was established based on the decolorization of dyes, and hence their name (DyP stands for *dye* decolorizing *p*eroxidase). DyPs are typically identified by their activity on anthraquinone dyes. While DyPs are efficient in oxidizing these synthetic dyes, the physiological substrates for DyPs are unclear and therefore their role in nature is enigmatic. Interestingly, recent studies suggest that bacterial DyPs may play an important role in the degradation of lignin which suggests that DyPs represent the bacterial counterparts of the fungal lignin peroxidases. Except for establishing their activity on synthetic dyes and possible role in lignin degradation, little data is available concerning their biocatalytic potential. Therefore, we set out a study aimed at profiling

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the potential of a newly identified DyP which can be easily produced as recombinant enzyme and is thermostable: DyP from *Thermobifida fusca* (*Tfu*DyP).

T. fusca is a moderate thermophilic soil actinomycete with a growth temperature of approximately 50 °C. It is a major degrader of plant cell walls in heated organic materials.[4] It produces many extracellular enzymes, including cellulases. A number of these secreted enzymes have been studied because of their thermostability, broad pH range and high activity.[8] *Tfu*DyP is a robust and secreted peroxidase described previously as a member of the DyP family.[9] Activity of *Tfu*DyP towards several reactive dyes was described in addition to enantioselective sulfoxidation.[9] In this paper we present an exhaustive substrate profiling study, which provides a better view on the biocatalytic repertoire of this newly discovered robust peroxidase.

2. Results and discussion

2.1. Establishing optimal conditions

To investigate the experimental boundaries at which *Tfu*DyP can be applied, the apparent melting temperature of *Tfu*DyP was measured at different pH values. In the pH range of 5–8 the enzyme shows a $T_{m,\ app}$ of ~56 °C (Fig. 1). This is in line with temperatures at which *T. fusca* thrives and it shows that *Tfu*DyP is a rather thermostable peroxidase. However, its thermostability decreases dramatically at a pH below 5 ($T_{m,\ app}$=35 °C at pH 3). This contrasts the pH optimum for optimal *Tfu*DyP activity which is in the range of pH 3–4 (vide infra). Such a low pH optimum for activity has also been observed for other DyPs.[10] These data indicate that there is a delicate balance in pH optima for activity and stability. Related to this, one should realize that *Tfu*DyP and many other DyPs are secreted and may have to operate at a pH different from neutral pH that is normal for intracellular enzymes. The broad pH optimum for stability is in line with the pH optima observed for other secreted enzymes of *T. fusca* that typically display a pH optimum of 4–10.[8] Another noteworthy observation is the fact that the pH optimum for activity seems to depend on the type of substrate (vide infra).

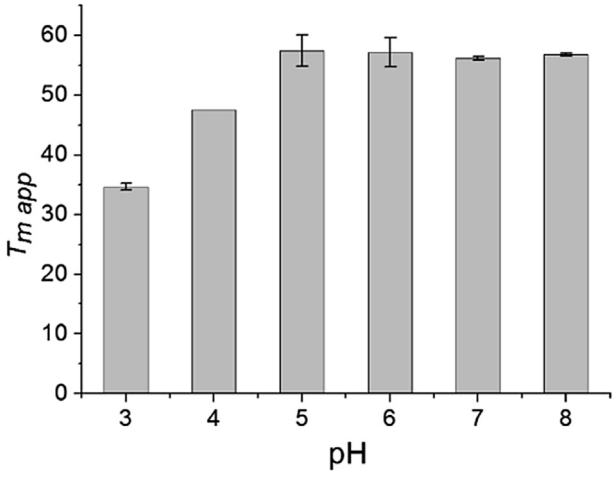


Fig. 1. Effect of pH on apparent melting temperature of *Tfu*DyP.

For peroxidases different from DyPs, it has been established that hydrogen peroxide can be replaced by organic peroxides such a *tert*-butyl peroxide.[11] To our knowledge, DyPs had not been tested before with these peroxide alternatives. However, when testing *Tfu*DyP activity with 0.10, 1.0 or 10 mM *tert*-butyl peroxide as electron acceptor and Reactive Blue 19 as substrate, no activity was observed. Moreover, when monitoring the UV/Vis absorbance spectrum of *Tfu*DyP upon the addition of 0.10 mM *tert*-butyl peroxide, no spectral changes were observed in the Soret band. This indicates that *Tfu*DyP is very selective for hydrogen peroxide. For the substrate profiling experiments performed in this study, 0.10 mM of hydrogen peroxide was used as cosubstrate.

2.2. Degradation of synthetic and natural dyes

DyP-type peroxidases are named after their ability to convert dyes. In previous studies, DyP activity has been mainly probed using a restricted number of synthetic anthraquinone and azo dyes for each reported DyP.[6] Activity towards triarylmethane dyes and natural pigment β-carotene has also been reported.[12,13] This study aimed at an extensive exploration of the substrate scope of a DyP-type peroxidase, *Tfu*DyP. The activity of *Tfu*DyP towards hemin, three natural carotenoids and 30 members of seven different classes of dyes was determined. For every dye, the initial activity (k_{obs}) and the amount of dye degraded in one hour were determined at pH 3, pH 4, and pH 5. The amount of dye degraded in one hour was defined as the observed decrease in absorbance at λ_{max}. One should note that the degree of dye degradation is an underestimation in case the product has a comparable absorption spectrum. The absorbance maxima of carminic acid and the copper phthalocyanine tetrasulfonic acid dye were pH dependent. For these compounds the isosbestic point of the spectra at pH 3, 4 and 5 was used to analyze the activities. A few dyes were found to be poorly soluble in buffer and were prepared in DMSO and used in the reaction mixture with a final concentration of 2.5% DMSO (resorufin) or 10% DMSO (Disperse Blue 1, curcumin, and β-carotene).

Only a small number of tested compounds did not show any activity with *Tfu*DyP: hemin, β-carotene, the azo dyes Direct Yellow 27 and Acid Yellow 23, and the heterocyclic dyes methylene blue, neutral red, and resorufin. The highest activities and conversions were observed for the anthraquinone dyes (see Tables 1 and 2, and Table S1). Most of the representatives of the other dye classes displayed 2–3 orders of magnitude lower initial activities. There were two exceptions: the xanthene dye Eosin Y and the copper phthalocyanine tetrasulfonic acid dye were good substrates with k_{obs} values of around 1 s^{-1} (Table 2). Anthraquinone dye Acid Blue 129 was the best substrate with a k_{obs} of 22 s^{-1} and a 82% decrease in absorbance at λ_{max} in one hour. While the initial rates of decolorization among the different dyes varied significantly, significant decolorization of most of the dyes was observed after 1 h. This can be caused by various factors, for example, the affinity of *Tfu*DyP for dyes can be different and/or the formed dye degradation products may inhibit decolorization by the peroxidase. For most dyes only one oxidation/decolorization step was observed as no other color developed during the decolorization. Only for the phthalocyanine dye two clear oxidation steps were visible. First, the color changed from light blue to dark blue, after which the solution decolored fully. The products of the decolorization reactions were not characterized. In fact, it is worth noting that, although DyPs can effectively decolor various dyes, they do not fully degrade dyes into regular metabolites. Still, such enzymes may develop as valuable biocatalysts for dye degradation, for example, for textile wastewater treatment, as the degradation products may be less toxic and/or easily degraded by follow-up microbial catabolic routes.[14,15]

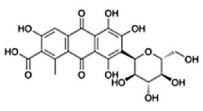
In general, higher initial activities were observed at pH 3 (Table S1). However, the enzyme is poorly stable at this pH and rapidly inactivates, resulting in lower dye degradation in the first hour. The measured initial rates of decolorization also revealed that the pH optimum for activity is clearly substrate dependent. Such phenomenon was also observed for other DyP-type peroxidases in previous studies.[6,16] In most cases, when taking both k_{obs} and the degree of degradation in one hour into account, *Tfu*DyP was most effective at pH 4 (Table S1). Some substrates were however an

Table 1
Structural formulas of the tested dyes

Dyes

Anthraquinone dyes


1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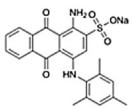
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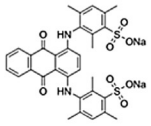
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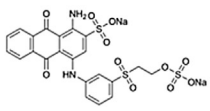
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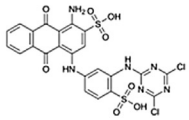
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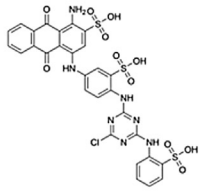
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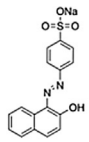
Azo dyes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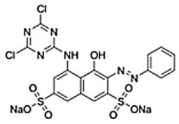
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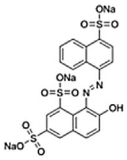
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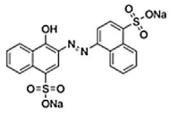
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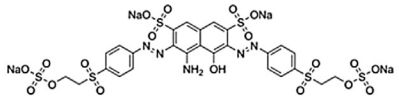
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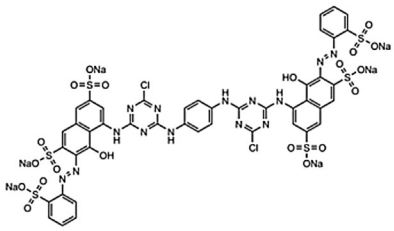
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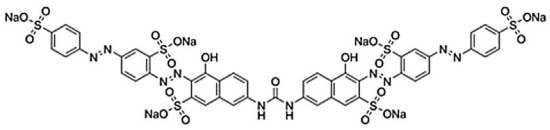
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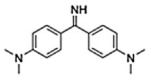
Arylmethane dyes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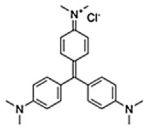
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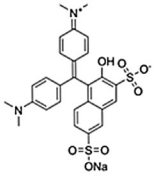
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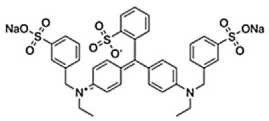
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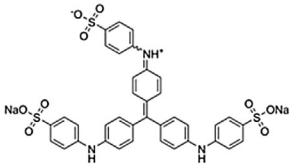
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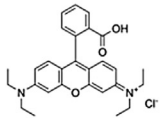
Xanthene dyes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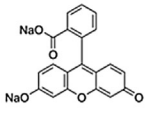
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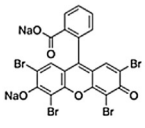
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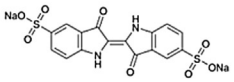
Indigoid dyes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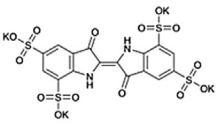
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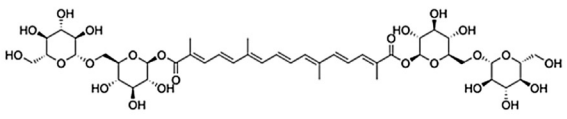
Carotenoids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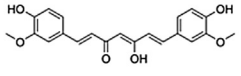
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Phthalocyanine dye

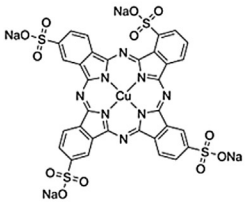
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Table 2
Activity of *Tfu*DyP on representatives of different dye classes

Nr.	Dye	λ_{max} at pH 4 (nm)	Dye concentration (μM)	k_{obs} at pH 4 (s^{-1})[a]	Dye degraded in 1 h (%)[b]
Anthraquinone dyes					
1	Disperse Blue 1	588	50[j,k]	10	48 (13)[c]
2	Carminic acid	503[h]	50	$2.4\cdots10^{-2}$	41
3	Acid Blue 129	629	50[j]	22	82
4	Acid Blue 80	629	50[j]	0.11	34
5	Reactive Blue 19	595	50[j]	1.7	22[d]
6	Reactive Blue 4	598	50[j]	1.4	12
7	Cibacron Blue 3G-A	615	50[j]	1.5	4.2
Azo dyes					
8	Acid Orange 7	484	25	$1.4\cdots10^{-2}$	16[e]
9	Reactive Red 2	512	25	$3.5\cdots10^{-3}$	2.1[d]
10	Acid Red 18	507	25	$2.7\cdots10^{-2}$	15
11	Acid Red 14	516	25	$4.7\cdots10^{-2}$	19[e]
12	Reactive Black 5	597	25	$1.4\cdots10^{-2}$	7.7
13	Reactive Red 120	510	10	$8.4\cdots10^{-3}$	3.2
14	Direct Red 80	543	10	$4.1\cdots10^{-3}$	4.1
Di/Tri-arylmethane dyes					
15	Basic Yellow 2	432	25	$5.0\cdots10^{-3}$	16
16	Crystal Violet	590	25	$5.2\cdots10^{-3}$	15
17	Acid Green 50	635	10	$3.2\cdots10^{-2}$	48
18	Acid Blue 9	630	25	—	35
19	Acid Blue 93	592	50	$6.1\cdots10^{-2}$	5.4[e]
Xanthene dyes					
20	Rhodamine B	554	25	$1.6\cdots10^{-2}$	12
21	Fluorescein	474[i]	25	$9.4\cdots10^{-3}$	12[f]
22	Eosin Y	517	25	0.93	92
Indigoid dyes					
23	Indigo carmine	611[i]	50	$2.2\cdots10^{-2}$	31 (1.9)[g]
24	Indigotetrasulfonate	590	50	$2.3\cdots10^{-2}$	8.2[e]
Carotenoids					
25	Crocin	441[i]	50	$8.8\cdots10^{-3}$	72 (1.2)[g]
26	Curcumin	431	50[k]	$7.2\cdots10^{-2}$	37 (4.1)[c]
Phthalocyanine dye					
27	Copper phthalocyanine-3,4′,4″,4‴-tetrasulfonic acid	612[h]	25	0.85	64[e]

[a] If necessary k_{obs} was corrected for the background activity.
[b] Percentage of dye degraded in one hour is based on the observed decrease in absorbance at λ_{max}. The actual amount of degraded dye is higher in case the product absorbs in the same range. High background activities of dye degradation with H_2O_2 but without enzyme are given in parenthesis.
[c] Higher k_{obs} and more degradation after 1 h at pH 5.
[d] Higher k_{obs} and more degradation after 1 h at pH 3.
[e] Lower k_{obs} but more degradation after 1 h at pH 5.
[f] Measured at pH 5 as only activity at pH 5 could be observed.
[g] Measured at pH 5, dye is not stable at pH 4.
[h] In this case the isosbestic point at pH 3, 4 and 5 was taken as wavelength to monitor activity.
[i] At pH 5.
[j] 30 nM enzyme was used instead of 300 nM.
[k] Containing 10% DMSO.

exception. *Tfu*DyP showed a higher activity for the anthraquinone dye Reactive Blue 19 and azo dye Reactive Red 2 at pH 3 while it performed better with the anthraquinone dye Disperse Blue 1 and curcumin at pH 5.

When inspecting the reactivity of *Tfu*DyP towards the tested dyes, it is not obvious why some substrates were degraded fast while others were not. Except for a general preference for anthraquinone dyes, neither the size nor the charge of the substrates seemed to have a large influence on the activity. To shed light on this, the redox potentials of the dyes were determined using cyclic voltammetry. Interestingly, the lowest redox potentials were observed for the anthraquinone dyes, $E_{½}$=0.3–0.65 V (Table S1). This may explain why *Tfu*DyP is most active towards this class of dyes. Unfortunately no redox potentials could be obtained for Eosin Y or the copper phthalocyanine tetrasulfonic acid dye, the other two compounds to which *Tfu*DyP displayed a high activity. The oxidation of the azo dyes was found to be irreversible when measuring the redox potentials and high oxidation potentials were obtained, Ep=0.7–1.1 V. The observed peak potentials of the azo dyes Direct Yellow 27 and Acid Yellow 23 were both above 0.95 V, which might explain why *Tfu*DyP could not degrade these dyes. The arylmethane dyes and Rhodamine B, showed a high observed oxidation peak potential as well, with values between 0.6–1.1 V.

2.3. Oxidation of lignin-related compounds

Our initial study on *Tfu*DyP already revealed that the substrate scope is not restricted to dyes. Also activity on phenolic compounds was observed by identifying guaiacol, 2,6-dimethoxyphenol and veratryl alcohol as substrates.[9] As part of the current study we explored some more simple and complex phenolic compounds. Activity on several other monophenols could be confirmed: catechol, acetosyringone, syringaldehyde, vanillin, vanillyl alcohol and vanillyl acetone. Activities towards these small phenolic substrates were rather low with observed rates of 0.1–0.7 s^{-1}. Yet, activity on these compounds may hint to a role of *Tfu*DyP in delignification of plant biomass as such phenols are often described as natural mediators that are used by laccases and peroxidases.[17]

For vanillin and vanillin-related compounds, vanillyl alcohol and vanillyl acetone, product analysis was performed. LC–MS analysis revealed the appearance of one dominant product upon *Tfu*DyP-catalyzed oxidation of vanillin. The formed compound could be identified as divanillin with a mass of 301.46 Da (negative mode, see Supplementary data). Formation of divanillin from vanillin has been proposed to result from oxidative phenolic coupling and keto-enol tautomerisation to give the final product.[18] For vanillyl alcohol and vanillyl acetone, also dimerization products were observed (see

Supplementary data). The selective oxidation of vanillin into divanillin may open up new avenues for the application of DyP peroxidases. Divanillin is valued as taste enhancer and efficient methods to prepare this food flavor are in demand.[18,19] In addition to flavor production, vanillin oxidation may be used for polymer production because renewables-based monomers, such as furfural, 2,5-furandicarboxylic acid, and vanillin, are currently considered as polymer precursors.[20]

As several recent papers hint at a role of DyPs in oxidizing lignin or lignin-derived complex molecules,[21,22] we also investigated the activity of DyP on more complex aromatic molecules. Analysis of lignin degradation can be extremely complex. Therefore, lignin model compounds are often used to identify targets of enzyme action. In this work two model lignin dimers were tested: guaiacylglycerol-β-guaiacyl ether and veratrylglycerol-β-guaiacyl ether (Fig. 2). Testing these substrates allows to discriminate between two possible degradation pathways: (1) oxidation of the phenoxy group, or (2) oxidative cleavage of the β-ether linkage, which constitutes up to 50% of the bonds in lignin. Interestingly, no peroxidase activity was detected for veratrylglycerol-β-guaiacyl ether. In contrast, for guaiacylglycerol-β-guaiacyl ether 50% substrate depletion was measured. Only the latter lignin model compound contains a phenolic moiety which suggests that *Tfu*DyP acts on this part of the lignin dimer, using the phenolic group as electron donor. This is in contrast to the observation that a DyP from *Rhodococcus jostii* (DyPB) degrades lignin dimers by acting on the β-ether bond.[22,23] An explanation of the observed difference in reactivity may be the fact that the two respective DyPs are representatives from two different DyP subgroups, with *Tfu*DyP being an A-type DyP and DyPB a B-type DyP. The sequence clustering of these DyP subgroups may reflect differences in the type of reaction they catalyze. As the DyPB-catalyzed conversion of lignin results in the liberation of vanillin,[22,23] we set out to determine which products are formed upon *Tfu*DyP-catalyzed conversion of guaiacylglycerol-β-guaiacyl. As vanillin itself is also a substrate for *Tfu*DyP, we did not anticipate formation of this monophenol.[18] Indeed, upon incubation of 0.5 mM guaiacylglycerol-β-guaiacyl ether and 1.0 mM H_2O_2 with *Tfu*DyP, no vanillin nor divanillin was observed as product. This again confirms that *Tfu*DyP does not cleave the ether bond in lignin-derived compounds but oxidizes the phenolic moiety. Our LC–MS analyses reveal that, similar to vanillin, *Tfu*DyP catalyzed the oxidative coupling of the guaiacylglycerol-β-guaiacyl ether resulting in several products with higher mass. Mainly dimers and trimers of guaiacylglycerol-β-guaiacyl ether were formed (masses of 661.44 and 979.62 Da in positive mode, 637.58 Da and 955.76 Da in negative mode). The obtained mass spectra are shown in the Supplementary data.

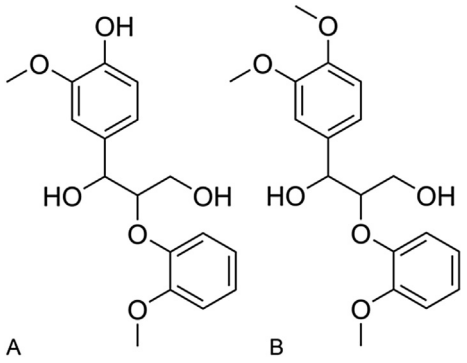

Fig. 2. Lignin model dimers: guaiacylglycerol-β-guaiacyl ether (A) and veratrylglycerol-β-guaiacyl ether (B).

As our study reveals that *Tfu*DyP has a preference for acting on phenolic moieties, we also tested whether several proteins are substrates. Other oxidative enzymes have been shown to oxidize tyrosyl residues of proteins which trigger formation of cross-linked proteins. Such biocatalytic protein cross-linking methods are highly valuable in the food industry as it introduces new properties in protein-based food.[24] Two model proteins were tested: β-lactoglobulin (18 kDa) and lactalbumin (14 kDa). However, incubation of these proteins with hydrogen peroxide and *Tfu*DyP did not result in any protein cross-linking. While a small fraction of naturally occurring dimers were observed in both beta-lactoglobulin and lactalbumin preparations, *Tfu*DyP did not promote formation of additional cross-linked proteins as judged by SDS-PAGE and gel permeation analysis.

3. Conclusions

DyPs represent a relatively poorly explored group of biocatalysts that have only been recently identified. While DyPs have been shown to act on a variety of dyes, this study provides a better view on the biocatalytic potential of a DyP. The studied *Tfu*DyP was found to be active on a wide variety of dyes. Furthermore, it shows activity on phenolic compounds ranging from monophenols to lignin model compounds. This shows that, except for the degradation of dyes, DyPs can be used for selective polymerization reactions (e.g., production of divanillin) and modification of lignin-derived compounds.

4. Experimental section

4.1. Chemicals and reagents

Lignin model dimers were supplied by TCI Europe. Hydrogen peroxide was obtained from Merck. Reactive Blue 19, Neutral Red and indigo carmine were supplied by Acros Organics and resorufin by TCI Europe. *Tert*-butyl hydroperoxide (Luperox), copper phthalocyanine-3,4′,4″,4‴-tetrasulfonic acid tetrasodium salt and all other dyes were obtained from Sigma–Aldrich.

4.2. Enzyme purification and growth conditions

*Tfu*DyP was expressed and purified as described before,[5] with some modifications. *Tfu*DyP was expressed in *E. coli* TOP10 (Invitrogen) using Terrific broth medium for cultivation, in order to achieve a higher cell density. An initial culture in Luria–Bertani medium was grown to saturation at 37 °C overnight. This preculture was 1:100 diluted in 1.6 L Terrific broth medium and grown at 37 °C. At OD_{600}=0.6 the culture was induced with 0.02% L-arabinose and grown to saturation at 30 °C overnight. All cultures were supplemented with 50 μg/mL ampicillin. Purification was performed using Ni-Sepharose obtained from GE. The enzyme was eluted using a sodium-acetate buffer of pH 4.5 to avoid possible inhibition of the peroxidase by imidazole.

4.3. Thermal stability assays

The ThermoFluor method was used to determine the apparent melting points of *Tfu*DyP using an enzyme concentration of 0.5–1.0 mg/ml. This method is based on the fluorescence increase upon binding of SYPRO Orange to hydrophobic protein surfaces that become exposed upon thermal protein unfolding or multimer dissociation. The fluorescence of the SYPRO Orange dye was monitored using a RT-PCR machine (CFX-Touch, Bio-Rad). The temperature was increased with 0.5 °C per step, starting at 25 °C and ending at 99 °C, using a 10 s holding time at each step. The temperature at the maximum of the first derivative of the observed fluorescence was taken as the apparent melting temperature. Stability was assayed in 100 mM sodium acetate buffers pH 3.0–5.0, 100 mM MES buffer pH 6.0 and 100 mM Tris–HCl buffers pH 7.0–8.0.

4.4. Kinetic analyses of dyes oxidation

The activity of *Tfu*DyP towards seven different classes of dyes (anthraquinone, azo, arylmethane, heterocyclic, xanthene, indigoid and phthalocyanine dyes), three carotenoid pigments and hemin was measured spectrophotometrically (Jasco V-660). Reaction mixtures containing 50 mM citrate buffer pH 3.0, 4.0 or 5.0, supplemented with 50 μM dye, 30–300 nM purified enzyme and 100 μM H_2O_2 were used. In case the absorbance of a dye at λ_{max} was too high, a lower concentration of 25 or 10 μM was used. The enzyme was added to start the reaction. First the initial rate of oxidation was measured at the corresponding wavelength maximum for each dye (Table 1.). Reactions were subsequently incubated for 1 h at ambient temperature after which spectra between 350 and 750 nm were taken to estimate the level of degradation, calculated as percentage compared to the starting solution. Control reactions were included without enzyme. The two dyes Disperse Blue 1 and resorufin, the two pigments β-carotene and curcumin are poorly water soluble. Therefore, stock solutions were prepared in DMSO and added to the reaction mixtures to a final concentration of 2.5% or 10% DMSO. The initial activity of *Tfu*DyP in the presence of 10% DMSO was tested using Reactive Blue 19 as substrate and revealed that the enzyme remained 40% of its activity. The stock solution of porphyrin hemin was obtained by dissolving hemin to a concentration of 5.0 mM in 0.2 M NaOH and used at a final concentration of 50 μM in buffer.

4.5. Cyclic voltammetry

Redox potentials of the dyes (when soluble without DMSO) were measured using cyclic voltammetry (CH-Instruments, Electrochemical analyzer CHI630B, USA). A Saturated Calomel Electrode (SCE) (BAS Inc, Japan) was used as reference electrode, glassy carbon as working electrode and a platinum wire as counter electrode. Scans were taken between −0.4 and 1.1 V at ambient temperature. Dyes were dissolved in a 0.1–0.2 M citric acid buffer of pH 3–5 to a concentration of 0.25–1.0 mM. The redox potential of a dye was analyzed at the pH at which *Tfu*DyP was most active towards that dye.

4.6. Oxidation of small phenolics

Catechol, sinapic acid, syringaldehyde and acetosyringone were tested in different concentrations and oxidation was followed spectrophotometrically. Oxidation of vanillin, vanillyl alcohol and vanillyl acetone was performed in 1 mL reactions consisting of 90 nM *Tfu*DyP, 0.5 mg/mL substrate and 1.0 mM H_2O_2 in 100 mM citric acid buffer pH 4.0. Reactions were incubated at 30 °C for 150 min and analyzed by HPLC and LC–MS.

4.7. *Tfu*DyP catalyzed lignin and lignin model compounds degradation

Lignin model compounds, guaiacylglycerol-β-guaiacyl ether and veratrylglycerol-β-guaiacyl ether, were dissolved in DMSO at a concentration of 10 mM. Reaction mixtures contained 2.0 μM *Tfu*DyP, 0.20 mM lignin dimer and 0.10 mM H_2O_2 in 100 mM Na-acetate buffer pH 3.5. Reactions were incubated at 30 °C for 1 h. Control reactions were made with H_2O_2 and buffer mixed with substrates and also with enzyme and substrate without H_2O_2. The reactions were incubated for 150 min at 30 °C, 500 rpm and samples were then centrifuged for 10 min at 13,000 rpm. Products of reaction of *Tfu*DyP with lignin model compounds and other small phenolics were analyzed by reverse phase HPLC using Jasco HPLC system. 10 μL samples were injected on a Grace Altima HP C18 column (3 μm, 4.6×100 mm, with precolumn of same material). Solvents used: A water with 0.1% formic acid and B acetonitrile. HPLC method: 2 min 15% B, 2–16 min 80% B, 14–16 min 80% B, 16 min 15% B followed by re-equilibration. Detection with UV detector at 254 nm and flow rate of 0.5 mL/min. LCE–MS analysis was performed on Surveyor HPLC-DAD coupled to LCQ Fleet detector.

4.8. Cross linking of proteins

Beta-lactoglobulin and lactalbumin (final concentration 1 mg/mL) were tested as model proteins for *Tfu*DyP mediated cross-linking in the presence and absence of syringaldehyde (0.2 mM). Reactions were carried out in 100 mM Na-acetate buffer pH 3.5 with 0.10 mM H_2O_2 at 30 °C with shaking for 4 h. Samples were analyzed by gel permeation on a Superdex 200 column and by SDS-PAGE.

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Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.tet.2015.12.078.

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