Exploring the biochemical and biocatalytic properties of bacterial DyP-type peroxidases
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Chapter 5:

Creating oxidase-peroxidase fusion enzymes as toolbox for cascade reactions

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Abstract
In this work we prepared a set of bifunctional oxidase-peroxidases by fusing four distinct oxidases to a peroxidase. While such fusion enzymes have not been observed in nature, they could be expressed and purified with good yields. Characterization revealed that the artificial enzymes retained the capability to bind the two required cofactors and were catalytically active as oxidase and peroxidase. The peroxidase fusions of alditol oxidase and chitooligosaccharide oxidase could be used for selective detection of xylitol and cellobiose with a detection limit in the low µM range. The peroxidase fusions of eugenol oxidase and 5-hydroxymethylfurfural oxidase could be used for dioxygen-driven one-pot two-step cascade reactions to convert vanillyl alcohol into divanillin and eugenol into lignin oligomers, respectively. The designed oxidase-peroxidase fusions represent attractive biocatalysts that allow efficient biocatalytic cascade oxidations that only require molecular oxygen as oxidant.
Introduction
In nature most enzymes take part in metabolic pathways in which each formed product is a substrate for the next enzymatic reaction. For optimizing the efficiency of such intricate biocatalytic cascades the enzymes are often brought together to form enzyme complexes, e.g. the pyruvate dehydrogenase complex, the microbial type I fatty-acid synthase complex and the cellulosome.\textsuperscript{1–3} The cellulosome is found in anaerobic microorganisms and consists of a scaffolding protein which brings together the required hydrolytic enzymes to degrade cellulosic biomass.\textsuperscript{3} In some cases, this has even led to the fusion of two or more enzymes creating a bi/multifunctional protein\textsuperscript{4, e.g. pyrroline-5-carboxylate synthase which features both glutamate kinase and γ-glutamyl phosphate reductase activities or the pentafunctional AROM complex from \textit{Aspergillus nidulans} that is involved in aromatic amino acid biosynthesis.\textsuperscript{5,6}

Inspired by the latter observation, various artificial enzyme fusions have been created in recent years in order to engineer efficient multifunctional biocatalysts. The first artificial bifunctional fusion enzyme, a histidinol dehydrogenase/ aminotransferase, was published in 1970.\textsuperscript{7} Several fusion enzymes have been made since.\textsuperscript{4,8,9} For example, a fusion between a fatty acid decarboxylase cytochrome P450 (OleTJE) and alditol oxidase (AldO) was made to fuel the reactions of OleTJE with hydrogen peroxide produced by the oxidase.\textsuperscript{10} For enabling efficient cofactor regeneration we have shown that various NAD(P)H-dependent monooxygenases can be produced fused to phosphite dehydrogenases which efficiently regenerates NAD(P)H.\textsuperscript{11,12}

Fusion enzymes have several advantages over separate enzymes. They are cheaper and less labor intensive concerning their production, since only one enzyme needs to be expressed and purified. Another advantage is the close proximity of the catalytic sites enabling substrate channeling.\textsuperscript{4,9,13,14} Substrate channeling circumvents diffusion of the intermediate product in the solution, and hence increases the combined reaction rate.

We were particularly inspired by the interplay between oxidases and peroxidases as is found in nature. Oxidases and peroxidases are often co-expressed as oxidases produce hydrogen peroxide which again is a substrate for peroxidases. Well-known examples of such interplay between oxidases and peroxidases are found in fungi.\textsuperscript{15,16} Many fungi secrete specialized peroxidases (e.g. lignin peroxidase and manganese peroxidase) that aid in biomass degradation.\textsuperscript{15} Except for secreting these heme-containing enzymes, these fungi also secrete various oxidases (e.g. pyranose oxidase and aryl alcohol oxidase) to serve as hydrogen peroxide producing enzymes to fuel the peroxidases.\textsuperscript{15,16} The consecutive reactions of oxidases and peroxidases are also applied in enzyme
activity screening approaches and biosensors. Numerous assays and biosensors are based on the combination of an oxidase and a peroxidase, for instance for the detection of glucose or uric acid levels in blood serum.\textsuperscript{17–19} The activities of various oxidases were studied in a coupled assays in which typically horseradish peroxidase (HRP) is employed.\textsuperscript{20–23} HRP, however, is still extracted from horseradish because of the difficulties in the heterologous expression of this plant peroxidase.\textsuperscript{24} Therefore, for this study we selected a recently discovered bacterial peroxidase, SviDyP, which is easily produced using \textit{Escherichia coli}.\textsuperscript{25,26}

Fusion enzymes between oxidases and peroxidases were not made before while they form catalytically logical combinations as the oxidase-formed hydrogen peroxide will drive the fused peroxidase (Fig. 1).

![Diagram](image)

\textbf{Figure 1.} Fused oxidase-peroxidases (P-oxidases) enable O\textsubscript{2}-driven oxidative cascade reactions. The cascade reaction starting from vanillyl alcohol to divanillin is shown as example.

In this work we fused a bacterial peroxidase (SviDyP from \textit{Saccharomono- spora viridis} DSM 43017, EC 1.11.1.19) to four different bacterial oxidases (EC 1.1.3.x).\textsuperscript{25,26} SviDyP belongs to the family of DyP-type peroxidases which are known for their activity on dyes and phenolic compounds.\textsuperscript{27–30} The peroxidase has been shown to be easily expressed in a bacterial host while it is also a very robust enzyme. SviDyP was fused to two FAD-containing oxidases that are active towards sugars: alditol oxidase (HotAldO) from \textit{Acidothermus cellulolyticus} 11B and chitooligosaccharide oxidase (ChitO) from \textit{Fusarium graminearum}.\textsuperscript{20,23} In this study, a ChitO triple mutant Q268R/G270E/S410R (ChitO*) was used because of its increased catalytic efficiency towards glucose, lactose, cellobiose and maltose. Besides these oxidases, SviDyP was fused to two other flavoprotein...
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oxidases that feature a partially overlapping substrate/product scope to SviDyP: eugenol oxidase (EugO) from *Rhodococcus* sp. strain RHA1 and 5-hydroxymethylfurfural oxidase (HMFO) from *Methylovorus* sp. strain MP688. This overlap in substrate/product scope, with both fusion partners active on phenolic compounds, would allow one-pot cascade reactions. In such a way we were able to produce four novel bifunctional fusion biocatalysts that can either serve a role in biosensing or act as a catalyst for one-pot two-step cascade reactions.

**Results and discussion**

**Expression and UV-vis analysis of the fusion enzymes**

The four DyP-type peroxidase/oxidase fusion enzymes (which we termed P-oxidases) were made by cloning the genes of the individual oxidases ChitO*, EugO, HMFO and HotAldO C-terminally to the gene encoding for His-tagged SviDyP. The resulting fusion enzymes were overexpressed and subsequently purified by affinity chromatography yielding 26-60 mg of enzyme per liter culture broth medium. The fusion enzymes displayed an intense red-brown color indicative for binding of the heme and flavin cofactors. Analysis by UV-vis absorbance spectroscopy revealed absorbance maxima at 280 nm (protein) and 406 nm (heme) for all enzymes. The Reinheitszahl (Rz-value) of the fusion enzymes varied between 0.61 and 0.97 and suggests effective incorporation of the heme cofactor. The typical absorbance maxima of FAD, around 350-385 and 440-460 nm, could not be observed due to the high absorbance of the heme cofactor. To confirm binding of the FAD cofactor, the purified ChitO*, EugO and HotAldO fusion enzymes were analyzed for in-gel fluorescence after SDS-PAGE. This revealed that all three fusion enzymes contained a covalently bound flavin cofactor. Such analysis was not feasible for the HMFO fusion enzyme as this flavoprotein oxidase contains a dissociable FAD. Yet, activity measurements (vide infra) confirmed that also this fusion enzyme was functional as oxidase confirming the presence of the flavin cofactor.

**Oxidase and peroxidase activities of the fusion enzymes**

In order to verify that the prepared fusion enzymes are fully functional, activities of both fusion partners were measured (Table 1). The observed peroxidase activities for all fusion enzymes were in good agreement with the $k_{cat}$ determined for the isolated peroxidase. From this it can be concluded that the activity of the peroxidase was unaffected by fusing it to the oxidases. Also, the oxidases displayed activity when fused to SviDyP, although the activities were somewhat lower than the activities of the non-fused enzymes. Oxidase activities of 15-43% were observed for the fused oxidases ChitO*, EugO, HMFO and HotAldO.
can be partly explained by that the activity was measured at a fixed substrate concentration \( (k_{\text{obs}}) \) which will yield lower rates when compared with \( k_{\text{cat}} \) values taken from literature. Another explanation of the lower observed rates may lay in incomplete flavin cofactor incorporation. Yet, prolonged incubation of the fusion enzymes did not result in higher activities. The somewhat lowered oxidase activities may also be caused by structural effects of bringing the enzymes together. Nonetheless, it can be concluded that both fusion partners of the created fusion enzymes show significant activities. Therefore we started to explore their use as bifunctional biocatalysts.

<table>
<thead>
<tr>
<th>Fusion enzyme</th>
<th>Peroxidase ( k_{\text{obs}} ) ( (\text{s}^{-1}) )</th>
<th>Oxidase ( k_{\text{obs}} ) ( (\text{s}^{-1}) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-ChitO*</td>
<td>7.7 (6.6)</td>
<td>1.0 (6.5 [23])</td>
</tr>
<tr>
<td>P-EugO</td>
<td>5.0 (6.6)</td>
<td>2.5 (12 [22])</td>
</tr>
<tr>
<td>P-HMFO</td>
<td>7.1 (6.6)</td>
<td>9.0 (21 [21])</td>
</tr>
<tr>
<td>P-HotAldO</td>
<td>8.6 (6.6)</td>
<td>0.43 (1.9 [20])</td>
</tr>
</tbody>
</table>

Table 1. Peroxidase and oxidase activities of the fusion enzymes.\(^a\)

\(^a\) The peroxidase activity was measured using Reactive Blue 19 as substrate at pH 4.0. Oxidase activity of P-EugO and P-HMFO towards vanillyl alcohol was measured at pH 7.5 and 8.0 respectively. Activity of P-HotAldO towards xylitol was measured at pH 7.5 and activity of P-ChitO* towards cellobiose was measured at pH 7.6. The value in brackets indicates the \( k_{\text{cat}} \) values of the separate enzymes as determined for SviDyP (see SI) or as reported in literature.

**Biosensors: sugar detection by P-ChitO and P-HotAldO**

There are numerous applications in which the combined use of a peroxidase and oxidase is exploited for detection purposes. One known application that uses such an oxidase-peroxidase couple is the combined use of glucose oxidase and HRP in biosensors to determine the glucose level in blood.\(^{17}\) Glucose oxidase oxidizes glucose to gluconic acid in the presence of molecular oxygen, and the formed hydrogen peroxide is subsequently used to translate the oxidase activity into a readout. Here we explored SviDyP-oxidase fusion enzymes for their use in detecting sugars. SviDyP is a representative of a newly discovered class of peroxidases, the DyP-type peroxidases, which have the advantage over HRP that they are typically easily overexpressed and purified from a heterologous host, such as *E. coli*.\(^{24,27}\) First, we produced and probed native SviDyP for its performance, and found it to be mainly active at pH 3–7 at ambient temperature, with an optimum for activity towards Reactive Blue 19 at pH 4.0. SviDyP showed to
be active towards 4-aminoantipyrine (AAP) and 3,5-dichloro-2-hydroxybenzene-sulfonic acid (DCHBS) which are commonly used as chromogenic substrates in peroxidase assays (AAP/DCHBS assay). The P-ChitO* and P-HotAldO fusion enzymes were tested with the AAP/DCHBS assay for their use in detecting sugars. ChitO is active towards mono-, di- and oligosaccharides and is the only oxidase known to be able to oxidize N-acetylated carbohydrates. Various ChitO mutants have been engineered that display distinct preferences for different carbohydrates. This would allow generating dedicated oxidase-peroxidase fusions for detection of specific mono- and oligosaccharides. HotAldO is mainly active on alditols, such as xylitol and sorbitol, which would allow its use for xylitol or sorbitol sensing. For testing the fusion enzymes a pH of 6 was used as this is where the pH optima of the oxidases and the peroxidase overlap. With saturating concentrations of test sugars (24 mM cellobiose for P-ChitO* and 1.4 mM xylitol for P-HotAldO) a clear and rapid color developed with a rate of 0.3 s\(^{-1}\) for both sugars. In fact, the rate of color formation was close to the observed rate when native SviDyP was tested in the AAP/DCHBS assay (0.4 s\(^{-1}\)). This indicates that at the employed conditions the peroxidase is rate-determining in the assay. A more detailed analysis of the sensitivity of P-ChitO* and P-HotAldO revealed that the fusion enzymes are able to detect low levels of cellobiose (25 µM) and xylitol (10 µM) respectively (Supporting Information Fig. S3). When using Amplex Red as fluorogenic peroxidase substrate, we could even lower the detection limit by one order of magnitude (Supporting Information Fig. S4). This shows that such peroxidase-oxidase fusion enzymes are perfectly suited for sensing purposes by harboring the full catalytic arsenal for an oxygen-driven biosensor.

One-pot two-step cascade reactions by P-HMFO and P-EugO

For the generated P-HMFO and P-EugO fusion enzymes, we explored their use in fully linked cascade reactions. We imagined that, except for the use of the oxidase-generated hydrogen peroxide, also the aromatic product formed by the oxidases could be used as substrate for the fused peroxidase. DyP-type peroxidases have been shown to act on various aromatic compounds while HMFO and EugO are, among other substrates, active on monophenolic compounds. This overlap in substrate/product scope is perfect for one-pot cascade reactions. In earlier work we showed that another DyP-type peroxidase, TfuDyP, dimerizes vanillyl alcohol, vanillin and vanillyl acetone. Dimerization of phenolic compounds is a known reaction for peroxidases and laccases and involves oxidative phenolic coupling and keto-enol tautomerization. Divanillin is a desired taste/flavor enhancer and was reported to give an impression of creaminess to food and to mask the sense of bitterness. In this work we examined whether P-EugO
and P-HMFO could produce divanillin from vanillyl alcohol, a cascade reaction in which vanillyl alcohol is oxidized to vanillin by an oxidase and subsequently dimerized to divanillin by SviDyP (Fig. 1). P-EugO and P-HMFO were incubated with vanillyl alcohol at pH 5.5, and the reaction mixtures were subsequently analyzed by LC-MS (Supporting Information Fig. S5-S14). Both P-EugO and P-HMFO were found to convert vanillyl alcohol. After 21 hours P-HMFO had oxidized 90% of vanillyl alcohol into vanillin (69%) and divanillin and related oligomers (21%). Using the same conditions, P-EugO converted 92% of vanillyl alcohol into vanillin (53%) and a higher amount of oligomers (39%) of which the most dominant product was divanillin (see Supporting Information). These results demonstrate that the fusion enzymes are suitable for the production of taste enhancer divanillin.\(^{33}\) Besides being recognized as flavors, vanillin and divanillin are also considered as renewable building blocks for the production of biobased plastics.\(^ {34-36}\) Furthermore, divanillin and related phenolic dimers were reported to have an antimetastatic potential.\(^ {37}\) Recently, we developed a one-pot two-step cascade reaction in which EugO and HRP or SviDyP were combined to produce low molecular weight lignin-like oligomers from eugenol.\(^ {38}\) The created fusion enzyme P-EugO simplifies this newly developed approach to synthesize lignin oligomer starting from eugenol. HPLC analysis revealed that incubation of eugenol with P-EugO gave the same lignin products: phenyl coumaran, pinoresinol, coniferyl alcohol, dieugenol and a lignin tetramer (Supporting Information Fig. S15).

**Conclusions**

In this work we made four active fusion enzymes of DyP-type peroxidase SviDyP and four different oxidases which we termed P-oxidases. All designed fusion enzymes could be overexpressed by E. coli as a soluble protein. SviDyP proved to be a good substitute for HRP in the horseradish peroxidase coupled assay and could be applied at an acidic pH. This SviDyP-assay could be applied to explore the substrate scope of oxidases or as biosensor for the detection of for instance sugars. SviDyP has an overlapping substrate/product scope with multiple oxidases, which is perfect for cascade reactions. Fusion enzymes P-HMFO and P-EugO were used in one-pot two-step cascade reactions. P-HMFO could be used to prepare divanillin as main product while P-EugO could be used for the synthesis of lignin oligomers. For future work it would be interesting to shift the pH optima of the oxidase and peroxidase closer together. The pH-optima of several enzymes were shifted through site directed mutagenesis in the past.\(^ {39-41}\) By optimizing these artificial fusions of redox enzymes, novel effective bifunctional biocatalysts can be developed.
Materials and methods

Chemicals, reagents and enzymes

Chemicals, media components and reagents were obtained from Sigma(-Aldrich), Merck, BD, Acros Organics, TCI, Alfa Aesar, Thermo Fisher and Fisher Scientific. Amplex Red (Amplisyn Red) was obtained from SynChem. Oligonucleotides and horseradish peroxidase were obtained from Sigma. Restriction enzyme HindIII was obtained from New England Biolabs. The *Pfu*Ultra Hotstart PCR master mix was from Agilent Technologies, and the In-Fusion HD EcoDry cloning kit was obtained from Clontech.

Cloning

The genes of oxidases ChitO* (ChitO triple mutant, Q268R/G270E/S410R), EugO, HMFO and HotAldO were amplified and cloned C-terminal to the *SviDyP* gene in vector pBAD His-*SviDyP*²⁶, original and new plasmids (Supporting Information Table S1). pBAD His-*SviDyP* contains C-terminal to the *SviDyP* gene a stop codon, a HindIII restriction site and another stop codon. The vector was linearized using restriction enzyme HindIII. The gene of HotAldO (including a C-terminal 6xHis-tag, without the first codon for methionine) was cloned into pBAD His-*SviDyP* using restriction free cloning.⁴² The obtained plasmid contained a stop codon between the genes of *SviDyP* and *HotAldO*. The stop codon was mutated to serine by QuikChange PCR yielding vector pBAD His-*SviDyP*-HotAldO-His. The above mentioned stop codon was mutated to serine before the cloning of the other oxidase genes. The oxidase genes were subsequently amplified and cloned into the obtained plasmid by In-Fusion cloning (In-Fusion HD EcoDry cloning kit, Clontech). The HindIII restriction site was retained on both sides of the oxidase genes. *E. coli* strain TOP10 (Invitrogen) was transformed by the obtained plasmids.

Culture growth and enzyme purification

Precultures were grown on 5 mL Luria-Bertani (LB) medium at 37 °C, 135 rpm, overnight. To inoculate 400 mL Terrific Broth (TB) medium, 1:100 preculture was added. These cultures were grown at 37 °C, 135 rpm until OD₆₀₀ ~0.4-0.6, after which they were induced by 0.02% L-arabinose and grown at 17 °C, 135 rpm for 70 hours. All cultures were supplemented with 50 μg/mL ampicillin. Cells were harvested by centrifugation at 6700xg and 4 °C for 20 minutes (Beckman Coulter, Avanti JE centrifuge, JLA 10.500 rotor). Pellets were washed with buffer A (50 mM potassium phosphate, 0.5 M NaCl, pH 8.0), harvested by centrifugation (3000xg, 4 °C, 40 minutes, Eppendorf centrifuge 5810R) and stored at -20 °C till use. Prior to enzyme purification the pellets were thawed and resuspended in
buffer A supplemented with 0.1 mM PMSF. Cells were disrupted by sonication (70% amplitude, 5 min total on time with cycles of 5 sec on and 10 sec off) and the cell-free extract was obtained by centrifugation at 16,000xg, 4 °C for 15 minutes (VWR, Micro Star 17R centrifuge). The enzymes were purified from the cell-free extract using a 5-mL His-Trap HP column (GE Health care). The columns were washed with buffer A and buffer A supplemented with 6, 12 and 24 mM imidazole. The enzymes were eluted with 300 mM imidazole in buffer A. Subsequently the buffer was exchanged to buffer B (20 mM potassium phosphate, 150 mM NaCl, pH 7.5) using a 10-mL Econo-Pac 10 DG desalting column (BioRad). The purified enzymes were flash frozen with liquid nitrogen and stored at -20 °C. UV-vis absorbance spectra of the enzymes were recorded between 250-800 nm at ambient temperature (V-660 spectrophotometer, Jasco). The protein concentrations were determined using Lambert Beer’s law and the predicted molecular extinction coefficients (ExPASy ProtParam tool\[43\]): ε_{280 nm} = 48,470 M⁻¹ cm⁻¹ for SviDyP, ε_{280 nm} = 124,915 M⁻¹ cm⁻¹ for P-ChitO* (SviDyP-ChitO*, in case it contains one disulfide bond), ε_{280 nm} = 127,770 M⁻¹ cm⁻¹ for P-EugO (SviDyP-EugO), ε_{280 nm} = 126,850 M⁻¹ cm⁻¹ for P-HMFO (SviDyP-HMFO), ε_{280 nm} = 116,880 M⁻¹ cm⁻¹ for P-HotAldO (SviDyP-HotAldO).

**Steady-state kinetic analysis of SviDyP**
The steady-state kinetic parameters of SviDyP were determined for Reactive Blue 19 (ε_{595 nm} = 10 mM⁻¹ cm⁻¹, \[26\]) in a 50 mM sodium citrate buffer at pH 4.0 with 100 μM H₂O₂ and 20 nM enzyme. SviDyP was added to start the reaction. Oxidation of Reactive Blue 19 was followed spectrophotometrically (JASCO V-660) at ambient temperature.

**Oxidase and peroxidase activity of the fusion enzymes**
The activities of both fusion partners were determined separately. For all reactions, a saturating substrate concentration of twenty times the K_M-value was used. For SviDyP the same reaction conditions were used as described above, with a substrate concentration of 100 μM Reactive Blue 19 (K_M = 4.6 μM). For the oxidases the same reaction mixtures and pH’s were used as described before.\[20-23\] Prior to the reactions the oxidases were incubated with 100 μM FAD for one hour at ambient temperature. Vanillyl alcohol was used as substrate for EugO\[22\] and HMFO\[21\], D-(+)-cellobiose for ChitO*\[23\] and xylitol for HotAldO\[20\]. The oxidation of vanillyl alcohol was followed spectrophotometrically at 340 nm (vanillin, ε_{340 nm} = 14 mM⁻¹ cm⁻¹ at pH 7.5\[22\] and 8.0\[21\]). The oxidation of D-(+)-cellobiose and xylitol were followed via a horseradish peroxidase-coupled assay. In this assay the hydrogen peroxide is produced by the oxidases and used.
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by horseradish peroxidase (HRP) to couple 3,5-dichloro-2-hydroxybenzene-sulfonic acid (DCHBS) and 4-aminoantipyrine (AAP) to a pink product ($\varepsilon_{515\text{ nm}} = 26\text{ mM}^{-1}\text{ cm}^{-1}$).\textsuperscript{20,23}

**SviDyP-coupled assay for the detection of oxidase substrates**

This assay is a variant of the horseradish peroxidase-coupled assay mentioned above and makes use of the peroxidase activity of dye decolorizing peroxidase SviDyP instead of HRP. The coupled activity of fusion enzymes P-ChitO* and P-HotAldO were determined at pH 5 (50 mM sodium citrate buffer) and pH 6 (50 mM potassium phosphate buffer). The reaction mixtures contained 0.1 mM AAP, 1.0 mM DCHBS and 23.8 mM (20x $K_M$) D- (+)-cellobiose for ChitO* or 1.4 mM xylitol (20x $K_M$) for HotAldO. The formation of the pink product was followed spectrophotometrically at ambient temperature ($\varepsilon_{515\text{ nm}} = 26\text{ mM}^{-1}\text{ cm}^{-1}$). To determine whether the oxidase or the peroxidase was the limiting factor in these reactions, the reactions were repeated in the presence of 100 μM $\text{H}_2\text{O}_2$ to determine the optimal reaction rate of SviDyP.

**Analysis of the sensitivity of the SviDyP-assay**

The sensitivity of the coupled-assay was studied by determining the lower concentration limit for substrate detection, as described before.\textsuperscript{44} Reaction mixtures of 200 μL contained 0.1 mM AAP, 1.0 mM DCHBS, 150 nM fusion enzyme and varying substrate concentrations (0.5 μM - 1 mM) in 50 mM potassium phosphate buffer pH 6.0. D- (+)-cellobiose and xylitol were used as substrates for P-ChitO* and P-HotAldO, respectively. The enzymes were added to start the reaction. Reactions were performed in triplicate and absorbance at 515 nm (pink product, $\varepsilon_{515\text{ nm}} = 26\text{ mM}^{-1}\text{ cm}^{-1}$) was followed at ambient temperature for 15 minutes by a SynergyMX (BioTek) plate reader. The obtained values after 15 minutes were corrected for both the path length and the blank. For comparison, the sensitivity of the coupled-assay was also determined using 60 μM Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine, Amplisyn Red) instead of AAP/DCHBS. A stock of 6.0 mM Amplex Red was prepared in DMSO. The oxidation of Amplex Red was followed by measuring the fluorescence of the product resorufin (excitation 530 nm, emission 590 nm) for 15 min at ambient temperature.

**One-pot cascade reaction for synthesis of divanillin and related dimers and oligomers**

Vanillyl alcohol was dissolved in water at a concentration of 50 mM. Reaction mixtures of 2.0 mL contained 2 mM vanillyl alcohol and 1.0 μM SviDyP, P-HMFO
or P-EugO in 50 mM sodium citrate buffer pH 5.5. In case of SviDyP 500 μM H₂O₂ was added. Reactions were incubated in 15 mL closed tubes at 30 °C, 100 rpm for 21 hours. Control reactions were prepared without enzyme. After 2, 3 and 21 hours samples were taken. Enzymes were heat inactivated at 95 °C for 10 min after which the samples were centrifuged for 5 min at 13,200 rpm. Reaction products were analyzed by reverse phase HPLC using a Jasco HPLC system. Samples of 10 μL were injected on a Grace Altima HP C18 column (5 μm, 2.1x150 mm, with a 1.0 cm precolumn of the same material). Solvents used: A, water with 0.1% formic acid and B, acetonitrile. HPLC method: 2 min 10% B, 2-20 min gradient to 70% B, 20-23 min 70% B, 23 min 10% B followed by 7 min re-equilibration. Detection by a UV-detector at 280 nm and flow rate of 0.5 mL/min. LC-MS analysis was performed on Surveyor HPLC-DAD coupled to LCQ Fleet detector using scanning for both positive and negative mode. Samples were injected on a Grace Altima HP C18 column (3 μm, 2.1x100 mm, with 1.0 cm precolumn of the same material), flow rate 0.3 mL/min. Solvents used: A, water with 0.1% formic acid and B, acetonitrile with 0.08% formic acid. LC-MS method: 2 min 100% A, 2-32 min gradient to 80% B, 32-37 min 80% B, 37-38 min 100% A, 38-48 min 100% A re-equilibration.

One-pot cascade reaction for the synthesis of lignin-like oligomers from eugenol

The activity of P-EugO towards eugenol was assayed as described before. Reaction mixtures of 2.0 mL contained 1.0 μM P-EugO, 10 mM eugenol and 5% DMSO (v/v) in 20 mM potassium phosphate buffer pH 6.0. A stock solution of 300 mM eugenol was prepared in DMSO. For comparison a reaction mixture containing 1.0 μM SviDyP and 1.0 μM EugO was assayed. All reactions were performed in duplicate and compared to a reaction without enzyme. Reaction mixtures were incubated at 30 °C and 50 rpm in 20-mL pyrex tubes with a headspace to volume ratio of 10:1. Samples of 200 μL were taken after 24 and 96 hours. These samples were heat treated and analyzed by reverse phase HPLC as described above for the production of divanillin and related oligomers.
Acknowledgements
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Supporting information
The supporting information includes Tables S1-S4 and Figures S1-S15. This information can be found online, connected to the publication: DOI: 10.1002/cbic.201700478
References

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