Green Chemistry

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Enzymatic cleavage of lignin β-O-4 aryl ether bonds *via* net internal hydrogen transfer†

Cite this: *Green Chem.*, 2013, **15**, 1373

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The current greening of chemical production processes going along with a rising interest for the utilization of biogenic feedstocks recently revived the research to find new ways for the degradation of the complex lignin-backbone by means of biocatalysis and combined chemo-enzymatic catalysis. Lignin, which accumulates in 50 million t/a, is regarded as a potential substitute for phenolic and other aromatic, oil-based chemicals in the upcoming post oil age. The cleavage of the β-O-4-aryl ether linkage is the most favoured, since it accounts for approximately 50% of all ether linkages in lignin. This enzymatic cleavage was proposed to be a part of the lignin catabolism in the proteobacterium *Sphingobium* sp. *SYK6*. Three enzymes, LigD, a Cα-dehydrogenase, LigF, a β-etherase and LigG, a glutathione lyase, are supposed to be involved in lignin degradation. We cloned and recombinantly expressed these genes in *E. coli* and determined their pH and temperature optima on the lignin model substrate 1-(4-hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-1,3-propanediol **1**. Using an NAD^+ dependent glutathione reductase from *Allochromatium vinosum* (AVR) we established an efficient way to regenerate the co-substrates NAD^+ and glutathione allowing for a self-sufficient balanced enzymatic cascade with net internal hydrogen transfer (hydrogen borrowing). We showed the capability of this enzyme system to release lignin monomers from complex lignin structures coming from differently prepared real lignin substrates. This novel enzyme system could become a useful tool to release lignin monomers from complex lignin structures.

Received 10th February 2013,
Accepted 19th March 2013

DOI: 10.1039/c3gc40295a

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Introduction

Lignin is a branched polymer of high complexity that consists of substituted phenylpropane units joined by carbon–carbon and ether linkages. The mechanism of lignin biosynthesis proceeds *via* polymerization of free radical forms of the three monolignol precursors. The monomers include coniferyl, *p*-coumaryl and sinapyl alcohols that are formed by radical mediated oxidative coupling of several different interunit linkages.[1] The β-O-4 aryl ether linkages are the most abundant followed by β–β, β–5, 5–5 and 5-O-4 linkages.[2] These binding types are specifically cleaved *in vivo* by soil microorganisms that play important roles in the planet's carbon cycle. The non-phenolic enzyme cleavage distinguishes this enzymatic approach from the well investigated oxidative but unspecific laccase or peroxidase based lignin treatments. Laccases (Lac) (EC 1.10.3.2) are copper-containing enzymes mostly produced by fungi.[3] Using molecular oxygen as a final oxidant these enzymes oxidize phenolic molecules *via* single electron transfers from the aromatic compounds to enzyme bound copper(II) ions forming oxidized aromatic radicals and enzyme bound copper(I) ions. The latter are reoxidized by molecular oxygen. The oxidation capability of laccases results in breakage of aromatic and aliphatic C–C bonds and thus depolymerization of lignin.[4] These enzymatic reactions find application in a variety of technical fields including pulp and paper, textile and food industries. For instance laccases are supplemented to wood chips as biopulping agents to degrade lignin and loosen lignin structures.[5] The separation of lignin from cellulose fibers is an important step in the processing of wood for manufacturing of paper pulp.[5,6] The textile industry applies cell-free enzyme extracts originating from numerous white-rot fungi which are effective in the biodecolourization of many synthetic and industrial dyes.[7] Lignin peroxidase (LiP, EC 1.11.1.14) involves H_2O_2 as the co-substrate in addition to a mediator like veratryl alcohol to degrade lignin and other phenolic compounds. H_2O_2 is reduced to H_2O by gaining an electron from the then oxidized LiP. By acquisition of an electron from veratryl alcohol and oxidizing it to veratryl aldehyde, oxidized LiP returns to its native reduced state. Subsequently veratryl

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†Electronic supplementary information (ESI) available. See DOI: 10.1039/c3gc40295a

aldehyde gets reduced to its alcohol by gaining electrons from lignin or analogous structures such as xenobiotic pollutants.[8,9] Mangan peroxidase (MnP, EC 1.11.1.13) requires H_2O_2 as a co-substrate as well as Mn^{2+}. Oxidation of Mn^{2+} to Mn^{3+} leads to Mn^{3+} chelate-oxalate, which consecutively oxidizes the phenolic substrates forming radical cations that lead to bond breakage and depolymerization. Mn^{3+} chelate-oxalate has the ability to diffuse into areas inaccessible to the enzyme. This principle allows degradation of lignin or xenobiotic pollutants, which are not essentially available for the enzymes.[9,10] Decolourization of molasses spent wash (MSW), a by-product of sugar mills and alcohol distilleries, and black liquor was shown to be MnP-dependent in *Phanerochaete chrysosporium*.[9,11]

A new family of ligninolytic peroxidases is versatile peroxidase (VP, EC 1.11.1.16), which combine the substrate specificity of LiP and MnP thus being capable of using both Mn^{2+} as well as veratryl alcohol and other mediators like dimethoxybenzenes, different types of dyes, substituted phenols, and hydroquinones.[12,13] These established lignin degrading enzymes are characterized by their technically unresolved lack of cleavage specificity and their high predisposition to provoke the repolymerisation of previously released monolignols. A different approach using hydrolases (specifically lipases EC 3.1.1.3) to form peracids *in situ* in the presence of H_2O_2, first described by Björkling and co-workers in the early 90s, was applied to oxidize lignin and thus separate it from the cellulosic fraction.[14] Most recently Wiermans *et al.* (2013) advanced this lipase-based approach and reported on the successful oxidation of beech wood lignin into a non-aromatic lignin oil using dimethyl carbonate as a solvent and immobilized *Candida antarctica* lipase B as an enzyme.[15]

Recently Chen *et al.* showed the capability of *Novosphingobium* sp. *B-7*, a close relative of *Sphingobium* sp. *SYK-6*, to release lignin monomers with MnP and Lac from kraft lignin. The effectiveness of the β-O-4, biphenyl-, coumaryl-, pinoresinol-lignin degrading enzyme cascade of this strain could not be elucidated.[16] Specific cleavage of β-aryl ether bonds accounting for up to 50% of the ethers in lignin possibly will result in the release of monolignols and therefore be a versatile source for widely used phenolic compounds in the chemical industry. In this work, we characterize and apply a β-O-4 aryl-ether cleaving enzyme system from the proteobacterium *Sphingobium* sp. *SYK-6*, whose genome sequence was recently published, on various lignin containing substrates.[17] The three proteins involved are LigD, a Cα-dehydrogenase, LigF, a beta-etherase and LigG, a glutathione lyase. The Cα-dehydrogenase LigD oxidizes the Cα of 1-(4-hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-1,3-propanediol **1** under consumption of NAD^+ to 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-1-propanone **2**. **2** is then cleaved at its Cβ ether to 2-glutathionyl-3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-1-propanone **4** and 2-methoxy-phenol **3**, while glutathione is released from **4** by the glutathione lyase LigG into 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-1-propanone **5** and oxidized glutathione (GSSG) (Fig. 1).[18] The X-ray structure of LigG was recently published and indicates that this enzyme belongs to

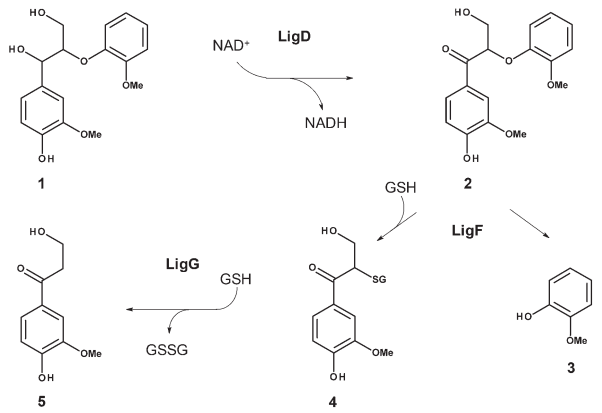

Fig. 1 Reaction mechanism of the LigDFG enzyme system. LigD oxidizes the Cα of **1** under consumption of NAD^+. LigF cleaves the intermediate **2** to **3** and **4** with attachment of glutathione at the Cβ position. Glutathione is oxidized by LigG and releases **5**.

the omega class of glutathione transferases. Its hydrophilic substrate binding site and its known ability to stereoselectively deglutathionylate **4** broadened the definition of the omega class.[19] The lignin catabolism in *Sphingomonas* sp. is stereospecific, with another three enzymes responsible for the degradation of the arylglycerol-β-aryl ether threo-isomers.[20] LigD converts just the (α*R*,β*S*)- and the (α*R*,β*R*)-isomers of **1** into the (β*S*)- and the (β*R*)-isomers of **2**, whereas the two other enzymes LigL and LigN catalyze the conversion of the (α*S*,β*R*)- and the (α*S*,β*S*)-isomers of **1** to the (β*R*)- and the (β*S*)-isomers of **2**, respectively. In the next step, the (β*S*)-isomer of **2** is specifically processed and cleaved by LigF and the (β*R*)-isomer of **2** is the stereospecific substrate for LigE and LigP.[20,21] In this work we analyzed the activity of the isolated enzyme system on the model substrate **1** and 7-(2-(4-benzyloxy)-3-methoxy-phenyl)-2-oxoethoxy)-4-methyl-2*H*-1-benzopyran-2-one **6**.[22] For the first time, we overproduced and purified recombinant LigD, LigF and LigG in *E. coli* and determined their enzymatic activities. With this knowledge we studied the capability of this enzyme system to release lignin monomers from complex lignin structures coming from differently prepared real lignin substrates, such as kraft-lignin and organosolv lignin and looked at the macromolecular size distribution after enzymatic treatment. We also demonstrate the effectiveness of an *Allochromatium vinosum* reductase (AVR) to recycle and supply this enzyme system with the expensive co-substrates NAD^+ and glutathione.

Materials and methods

Strains, plasmids and chemicals

The four genes were synthesized by Geneart GmbH (Regensburg/Germany) with codon optimization for *E. coli.*, and cloned into a pET28b vector with a C-terminal His-tag to transform *E. coli BL21 (DE3)*. Strains for all experiments were grown in LB medium with kanamycin (50 mg L^{-1}) from OD 0.1 to OD 0.6 at 37 °C and induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). All chemicals were bought from Sigma

Aldrich. **1** was purchased from TCI Japan, with 98% purity, no further data about isomer composition are available. Bagasse organosolv lignin was obtained by the Fraunhofer ICT (Germany), softwood and hardwood alkali lignin was kindly sponsored as Lignex® powder by the Chemische Werke Zell-Wildshausen GmbH (Germany).

Enzyme purification and activity assay

For the purification of enzymes, over-expressed cells were lysed in binding buffer (Tris/HCl pH 7.2, 10% (v/v) glycerol, 20 mM imidazole) in a cell disruption system (ThermoFisher, U.S.) and the cell lysate was applied to a Ni^{2+}-NTA column of the Aekta® protein purification system (GE Healthcare, U.S.) at a flow rate of 0.3 mL min^{-1} and then fractionated in elution buffer (20 mM Tris/HCl pH 7.2, 10% (v/v) glycerol and 500 mM imidazole). The elution fractions were analysed by SDS-PAGE and Coomassie staining to test the purity. The protein concentration of every fraction was determined by Bradford.[23] 5 μg of each protein was added to 0.15 mM of substrate **1**, 0.5 mM of NAD^+, 0.1 mM of glutathione in 1 mL of 20 mM Tris/HCL, pH 7.0 reaction mixture, filtered with a 0.2 μm PVTE filter and analysed with HPLC. The same reaction was used with 10 mM ammonia acetate buffer for analysing cleavage products with LC-MS (Ultimate3000, Dionex, U.S.) with a flow rate of 0.3 mL min^{-1} with ammonia acetate buffer/acetonitrile as mobile phase.

Enzyme assays with LigD

Assays were performed in microplates for the determination of pH-optima in CBP-Universal buffer (pH 4–12). Oxidation of the Cα of substrate **1** was measured spectrophotometrically by following the reduction of NAD^+ to NADH at 340 nm. The enzyme activity was analysed with different pH values in multiwell plates in a Varioscan photometer (Thermo Scientific, U.S.) with 0.5 mM of substrate **1** and 0.5 mM of NAD^+. One enzyme unit corresponds to the amount of enzyme that catalyzes the oxidation of 1 μmol of **1** per minute. The kinetics was monitored at pH 9, 45 °C pre-incubation with 0.125 mM, 0.25 mM, 0.5 mM, 1.0 mM, 1.5 mM, 1.75 mM and 2.5 mM **1** concentrations.

Synthesis of substrate 6

Synthesis of the lignin model compound **6** was performed according to Weinstein, starting from 1-(4-hydroxy-3-methoxyphenyl)ethanone. After protection of the hydroxy-function and bromination of the acyl group the compound was coupled with compound **8**.[24]

Enzyme assay with LigF

Cleavage of the reference substance **6** was analyzed fluorimetrically with an excitation at 360 nm and an emission at 450 nm.[22] 0.25 mM of **6** was cleaved in a multiwell plate at 25 °C with dilutions of LigF and the release of compound **8** was detected and calibrated with commercial compound **8** (Sigma Aldrich, U.S.) as the standard.

Kinetics of LigDFG with compound 1

The enzymes LigDFG were added with a final concentration of 0.5 mg mL^{-1} to substrate **1**, NAD^+, glutathione, each 0.1 mM in 3 mL ammonium acetate buffer pH 9. Aliquots of 250 μL were taken at 0 min, 10 min, 20 min, 30 min, 40 min, 50 min, 70 min, 100 min, 130 min and 12 h and stopped by filtration with a 10 kDa filter (VWR). Triplicates were analysed *via* HPLC (Shimadzu, CX18-column, Macherey&Nagel, Germany) with a 10 mM, pH 4.3 ammonium acetate buffer-/acetonitrile gradient. Commercially available **3** (Sigma, U.S.) and substrate **1** (TCI, Japan) samples were used for standardization.

Activity of LigG

Substrate **1**, NAD^+, glutathione, each 10 mM in 6 mL piperazine/glycylglycine buffer pH 9 were initially incubated with LigDF to produce thioether **4** and filtered. The enzyme LigG was added to the compound **4** stock solution with a final concentration of 60 μg mL_{LigG}^{-1} and to different pH probes (pH 4–12) with a final volume of 1.5 mL. Aliquots of 300 μL were taken at 0 min and 2 min, and stopped by filtration with a 10 kDa filter (VWR, Germany). Triplicates of this experiment were analysed by HPLC as described.

Co-substrate recycling by AVR

AVR was produced in *E. coli BL21 (DE3)* and purified with a His-tag. Activity was tested photometrically at 340 nm *via* decrease of NADH concentration. The enzyme system LigDFG with substrate **1**, NAD^+ and glutathione was incubated with AVR. Addition of AVR led to decrease of NAD^+ as **1** was the limiting reaction partner. HPLC samples were filtered with 10 kDa (VWR, Germany) and applied to HPLC as shown above.

Lignin analytics

The concentrations of guaiacol **3**, ferulic acid **9**, eugenol **10**, acetovanillone **11** and vanillin **12** in aqueous 200 g L^{-1} lignin samples with 1 mg LigDFG were determined using a BPX5 column (SGE, 30 m, I.D. 0 : 25 mm, film 0 : 25 μm) connected to a QP2010 Plus gas chromatograph with a Single Quad MS-detector (both Shimadzu, Japan) and helium as carrier gas. The samples were extracted with an SPME fiber (85 μm polyacrylate, Supelco/Sigma Aldrich, U.S.) on an autosampler (CombiPal, Swiss) for 30 min at 60 °C and desorbed for 5 min in an SSL-injector (290 °C) starting at 80 °C for 1 min, then heated to 170 °C at 6 °C per min, then to 280 °C at 15 °C per min and finally held for 6 min. Area integration of the triplicates was calibrated with the area averages of pure substance concentrations.[28]

GPC samples were filtered with 45 μm cellulose acetate filters (VWR, Germany) and applied to HPLC upgraded with GPC (Shimadzu, Japan) with a Mcx10 column (PSS, Germany). A 0.01 M phosphate buffer with 10% methanol was used with 99% methanol as a solvent. The isocratic method (20% methanol) had a flow rate of 0.9 mL min^{-1} at 60 °C.

Results and discussion

Cloning in *E. coli*

The four target genes from the soil proteobacterium *Sphingomonas* sp. *SYK6* were commercially synthesized with codon optimization for *E. coli*. The genes were cloned into a pET28b vector with a C-terminal His-tag under a lac promoter and used to transform *E. coli BL21(DE3)*.

Purification and proof of principle for the overall activity of the enzyme system

Subsequently, all three genes were overexpressed in *E. coli BL21(DE3)* and enzymes purified with their C-terminal His-tag on a Ni^{2+}-NTA column with an automated protein purification system. SDS-PAGE showed distinct and clean bands of all three proteins with elution fractions of 5–20 $mg_{protein}$ mL^{-1} with 33 kDa, 28.5 kDa and 33 kDa respectively (Fig. S1†). The overall activity of the enzyme system after the purification step was analyzed by detection of the cleavage products of the lignin model substrate **1** by means of HPLC and LC-MS. After one hour reaction time, an enzymatic cleavage of 37% of **1** was observed by HPLC as shown in Fig. S2.† LC-MS confirmed the mass peak of **1** and the formation of compound **5**, when cofactors and enzymes were present in the enzyme assay (Fig. S2, S3†). The recombinant enzyme system LigDFG is therefore able to cleave the beta-aryl ether of **1**.

Activity of LigDFG

Consumption of substrate **1** and formation of compound **3** of the enzyme system were determined as mentioned above. Calibration with a standard of **1** and **3** and integration of the product peak showed a decrease of the concentration of **1** in the enzyme assay probe from 78.7 μmol to 50.9 μmol within one hour. This corresponds to an enzymatic activity of 10.3 U mg^{-1}. **3**, the product of the beta-aryl ether cleavage, increased at the same time from 0.8 μmol to 27.8 μmol (Fig. 2). The maximum conversion of **1** after 10 h was *ca.* 35%. We assume LigD being unable to convert the (−)(α*S*β*S*)-isomer of **1** which is present in the racemic commercial sample of **1**. This is in accordance with the reported high substrate specificity of LigD towards the (+)(α*R*β*S*)- and the (−)(α*R*β*R*)-isomers of **1**.[21] Being such a highly specific secondary alcohol dehydrogenase LigD might be of interest for fine chemical conversions. We therefore tested LigD on six commercially important chiral alcohols (1-phenyl-2-propanol, 4-phenyl-1-butanone, 6-methyl-5-hepten-2ol, for the oxidation) and 4-propylbenzaldehyde or ketones (3,4-dimethylbenzaldehyde, acetophenone, 4-phenyl-2-butanone and 6-methyl-5-hepten-2-one) for the reduction. No activity could be detected (data not shown). The substrate and stereo-specificity of the active site of this enzyme seem to be strongly restricted.

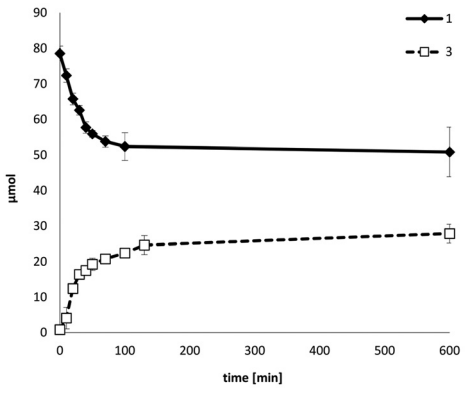

Fig. 2 Kinetics of LigDFG with the lignin model substance **1**. The triplicates were tested in parallel and analysed *via* HPLC. Concentrations of **1** and **3** were calibrated with reference substances, transformation of **1** by ligD is limited to the (+)(α*R*β*S*)- and the (−)(α*R*β*R*)-isomers of **1**. The erythro (−)(α*S*β*S*)-isomer of **1** isomer is not used by LigD.

Determination of pH and temperature optima and kinetics

The purified enzymes were tested individually to get new insights into the dependencies between enzymatic activity and pH as well as temperature.

LigD. The activity of LigD, a Cα-dehydrogenase, was determined as mentioned above. The highest activity of 11.7 U mg_{LigD}^{-1} was shown to be at pH 9, above which there is a strong decrease in activity possibly due to enzyme denaturation (Fig. 4A). The optimum temperature was determined to be at 60 °C, pH 9 with 12.0 U mg_{LigD}^{-1} and almost complete inactivation of LigD activity at 70 °C (Fig. 4B). These prerequisites allowed the determination of the kinetics of LigD. Under variation of **1** from 0.125 to 2.5 mM the $v_{max}/2$ was determined to be 3.93 U, resulting in a K_m-value of 1.10 mM. The corresponding k_{cat} was 1330 s^{-1} resulting in an efficiency (k_{cat}/K_m) of 1204.5 mM^{-1} s^{-1} (Fig. 4C).

LigF. For the activity determination of LigF, a glutathione transferase, the model substance **6** (Fig. 3) was used. The activity was determined at different pH-values and calibrated with commercial compound **8**. The highest activity of LigF was determined at room temperature with pH 10 having 5.6 mU mg_{LigF}^{-1} and increasing to 42 mU mg_{LigF}^{-1} at 60 °C (Fig. 5A and 5B). Kinetics were determined under variation of **6** from 0.0625 to 0.75 mM resulting in a $v_{max}/2$ of 0.018, in a K_m of 0.24 mM. The k_{cat} was calculated to be 0.075 s^{-1} with a k_{cat}/K_m of 3.19 mM s^{-1} (Fig. 5C).

LigG. The activity of LigG was detected indirectly *via* HPLC by following the formation of **5** (Fig. 6). **4** as a substrate for LigG is not commercially available and lacks photo- or fluorimetrical detection capability to set up an easy assay. Therefore LigG activity was tested for pH- and temperature optima *via*

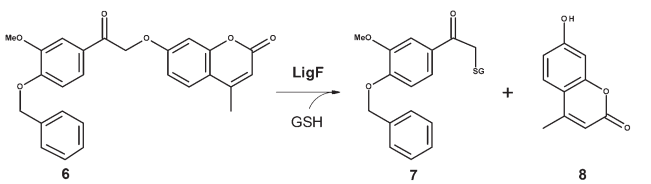

Fig. 3 Cleavage of the model substance **6**. LigF activity is determined fluorimetrically with the synthesized reference substance **6** by detection of released compound **8**.

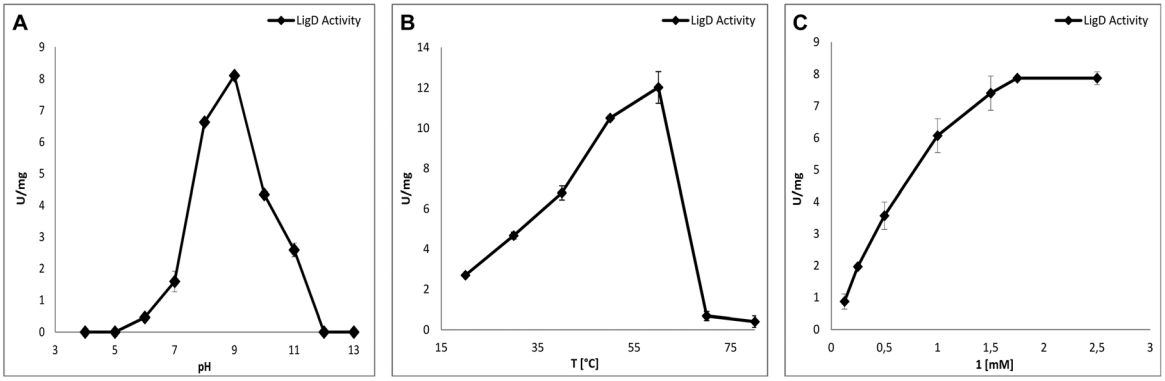

Fig. 4 Activities of LigD *via* detection of NAD^+ formation at 340 nm at different pH (**A**) and temperature (**B**) in U mg^{-1} protein at (n = 3). The enzyme activities followed Michaelis–Menten under increasing substrate conditions (n = 3) (**C**).

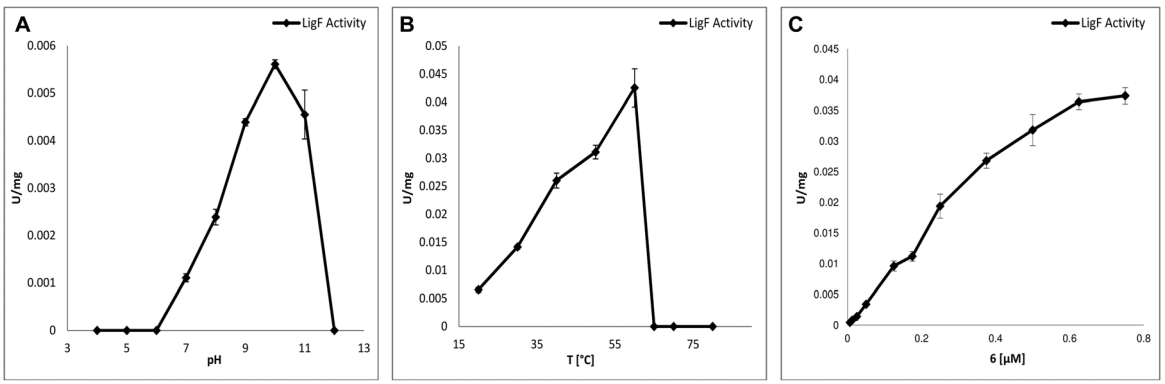

Fig. 5 Activities of LigF through cleavage of substrate **6** at Ex 360 nm, Em 450 nm at different pH (**A**) and temperatures (n = 3) (**B**). LigF kinetics with varying substrate **6** concentrations (n = 3) (**C**).

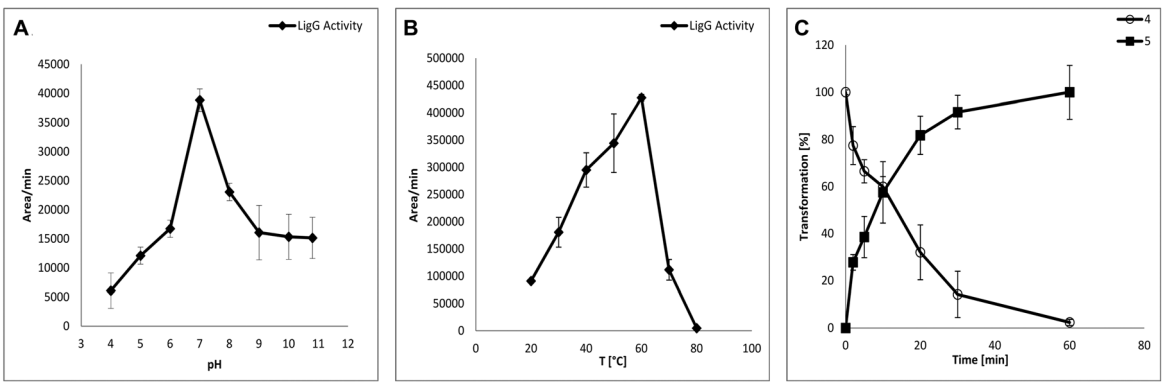

Fig. 6 Activities of LigG *via* detection of the formation of **5** with HPLC in ΔArea at different pH (n = 3) (**A**). LigG shows highest activities at 60 °C (n = 3) (**B**). Indirect LigG activity measurement *via* HPLC. The non-commercial intermediate **4** is catalysed by formation of **5** (n = 3).

HPLC without calibration. LigDF with cofactors was used to convert **1** to **4**. Enzymes were removed and this highly concentrated master mix was used for smaller pH assays (pH 4–12). Due to the broad pH-range for LigG activity the wide range buffer piperazine/glycylglycine was used. The reactions were stopped by filtering aliquots to remove LigG to analyse the initial conversion rates *via* HPLC. The kinetics shows a broad activity plateau from pH 4 to pH 11 with its optimum at pH 7 as shown in Fig. 6A. Highest activity was monitored at 60 °C with 427.424 areas min^{-1} (Fig. 6B). A more detailed quantitative analysis requires higher amounts of the thio-ether intermediate as a substrate which was not available.

Co-substrate recycling – a proof of principle

The enzyme cascade LigDFG catalyzes the reductive cleavage of an ether bond and oxidation of a secondary alcohol *via* the

conversion of the cofactors NAD^+ to NADH and two molecules of GSH to GSSG. For technical applications it is essential to efficiently recycle the cofactors to reduce the cost. We used an NADH dependent glutathione reductase from *Allochromatium vinosum* (AVR) to regenerate the cofactor(s) of the LigDFG enzyme system. AVR was first described by Chung and Hurlbert.[25] We recombinantly expressed the enzyme, characterized it and found it to accept NADH instead of the common and more expensive NADPH as a substrate (manuscript in preparation). In a first experiment we determined the cleavage of **1** by following the formation of NADH with and without AVR in the system (Fig. 7). In the initial phase of the photometrical enzyme assay (detection at 340 nm) LigDFG converts **1** under consumption of NAD^+ and glutathione to **5**. With further consumption of **1**, the reaction stopped with a significant increase of NADH concentration. In the second step of the experiment, addition of AVR decreased the previously formed NADH, reducing GSSG to glutathione *via* production of NAD^+ (Fig. 8A). To evaluate the ability of AVR to regain NAD^+ and GSH, 30 mM conversion of **1** was conducted with 3 mM initial NAD^+ and 7 mM GSSG. HPLC analysis showed significantly decreased amounts of **1** in the samples containing AVR compared to the control probes without AVR (Fig. 8B). Based on the initial concentrations of **1** and coenzyme these results clearly demonstrate the successful recycling of NAD^+ and GSH. After seven days of reaction, **1** was eventually cleaved to the previously determined maximal conversion of **1** of around 35% due to the stereospecific restriction of LigDFG (Fig. 8B).[21] With the transfer of hydrogen from NADH to GSSG the ether cleavage reaction can be regarded as having a net internal hydrogen transfer from Cα to Cβ. A similar result can also be obtained using ruthenium(II) complexes as homogenous catalysts.[26] However, much higher temperatures are required for full conversion here, in comparison to the enzymatic approach.

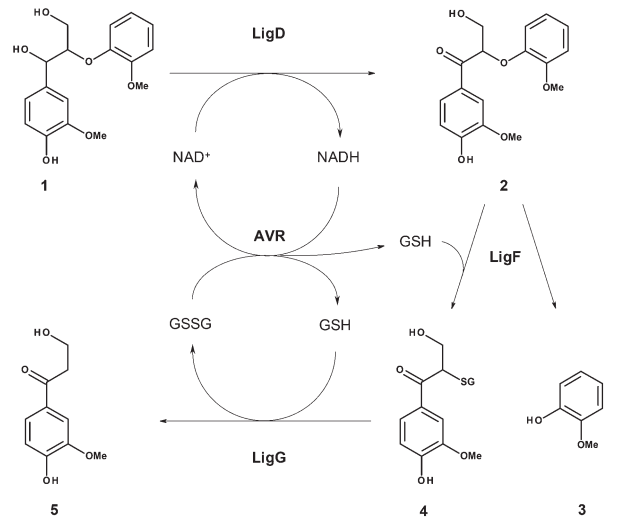

Fig. 7 Reaction of the LigDFG enzyme system with AVR. All cofactors can be recycled *via* reduction of GSSG with NADH.

Application of LigDFG on real lignin substrates

While the effect of LigDFG cell extracts on a non-natural, peroxidase polymerized substrate **6** was described previously,[27] we have advanced this concept by applying characterized and purified LigDFG on complex lignin. Therefore we used the two technical lignins, softwood/hardwood alkali-lignin and bagasse organosolv-lignin as substrates, and measured the effect of LigDFG on the monomer release and polymer distribution compared to untreated control samples. GPC analysis showed a minimal increase of the 120–200 MW area% distribution in softwood and hardwood alkali-lignin after 7 days compared to the controls by 1.8% and 2.1% respectively. A slight increase of the 300 MW fractions by 1.5 area% could also be detected in softwood alkali-lignin, whereas a decrease could be observed in hardwood alkali-lignin (Fig. S4 to S5†). GPC of bagasse organosolv showed no detectable differences between the control and the sample, which might be due to possible problems with the water solubility of the highly

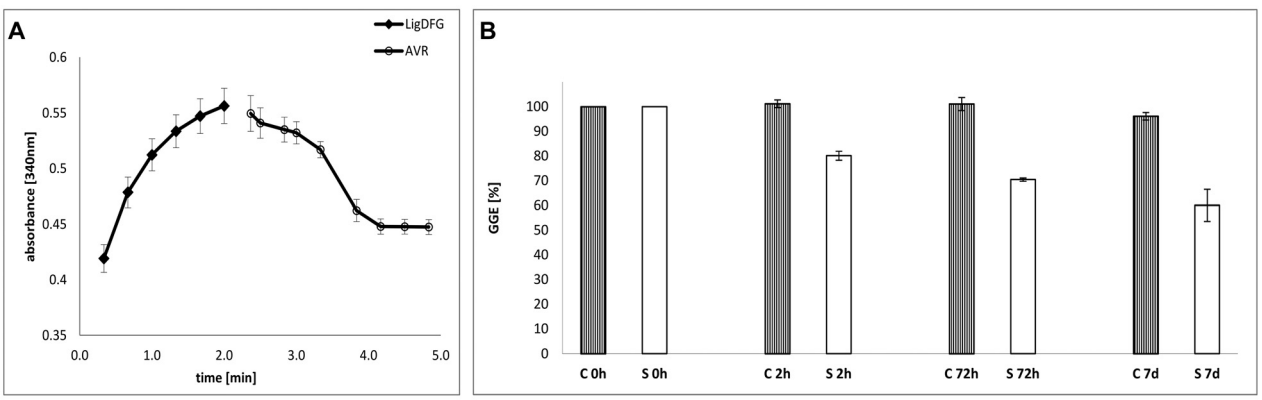

Fig. 8 Activities of AVR by detection of NADH formation at 340 nm at pH 7.5. The first reaction converted the limiting concentration of **1** under consumption of NAD^+ and formation of GSSG with LigDFG. Addition of AVR after 2 min shows re-conversion of NADH to NAD^+ (n = 3). The back reaction is diluted due to AVR addition (**A**). Recycling *via* HPLC analysis. Transformation of **1** (30 mM) was detected over seven days with (S) and without (C) AVR addition. At the beginning of the reaction 3 mM of NAD^+ were added to both probes (n = 3) (**B**).

dispersed and partly insoluble substrate. It is noteworthy that the high molecular weight fraction of all three lignin samples increased, instead of being degraded or shifted in its mass distribution. This may be addressed to on-going radical coupling under the aerobic assay conditions.

A distinct monomer releases from the complex lignin molecules after addition of LigDFG and 10 mM NAD^+ and GSH were observed in softwood- and hardwood alkali-lignin, but not in bagasse-organosolv lignin (Table 1; Fig. 9). The area ratios of the lignin monomers were calculated in mg L^{-1} by standardization with commercial references following every GC-MS SPME run according to Kolb *et al.*[28] The reaction batches contained definite amounts of free phenolic monomers including our target monomers before starting the reaction. These monomers derive from the lignin pre-treatment processes which typically consist of thermal, chemical or physical depolymerisation methods. Several additional peaks were detected but have not yet been analysed due to the lack of specific reference substances for calibration. Softwood alkali-lignin showed a significant increase of monomer release after enzyme treatment. For instance guaiacol **3** content increased by 2.14 mg L^{-1} after 50 h. Similar effects could be observed for ferulic acid **9**, being increased by 17.7 mg L^{-1} after 2 h and staying constant at that level for 50 h. Vanillin **12** increased by 58.2 mg L^{-1} within 2 h and by 89.8 mg L^{-1} after 50 h, acetovanillone **11** from 0.9 mg L^{-1} to 6.9 mg L^{-1} after 50 h. Hardwood alkali-lignin only showed a very small release of vanillin **12** from 0.47 mg L^{-1} after 2 h. Release of guaiacol **3** was even smaller (0.025 mg L^{-1} after 2 h) decreasing again to 0.0 mg L^{-1} within 50 h (Table 1). Based on the structures guaiacol **3** and ferulic acid **9** are probably originating from the aryl part of the ether. Vanillin **12** and acetovanillone **11** in contrary could be originating from either side of the ether bonded molecule. Bagasse organosolv-lignin showed no detectable enzyme induced release of phenolic monomers.

LigDFG had no major detectable effect on the polymeric fractions of all tested lignin samples. The reason for this is most likely due to the low β-aryl ether content in kraft- and organosolv lignin on the one hand and a possible inhibition of the enzymes by sulfides and/or solvent residues from the pretreatment procedures on the other.[29] In the alkali and organosolv process the β-aryl ethers are among the first bonding types being cleaved to more than 90%, while partial repolymerization occurs *via* C–C bond formation. Accordingly a complete depolymerisation of the technical substrates using LigDFG was not to be expected. It is unclear whether LigDFG serves as a lignin dimer/monomer processing tool in bacteria, or enables the bacteria together with their cocktail of phenylcoumarane-, diarylpropane biphenyl-, pinoresinol- and arylether cleaving enzymes to degrade the complex lignin polymer.[30] Applying these enzymes in addition to LigDFG could enhance polymer degradation and give rise to higher monomer yields. It was thus shown that enzymatic cleavage of ether linkages in technically pretreated lignin is only feasible with a very small monomer release by the LigDFG enzyme system. Despite the problems of enantioselectivity, substrate availability and radical coupling in the assay, these first experiments showed promising activity and can be seen as an overall proof of principle for the feasibility of the LigDFG etherase system.

Table 1 Increase of lignin monomers in aqueous complex kraft-lignin samples. The values for released concentration of the probes were first subtracted from the control probes without enzymes, and then values at 0 h were subtracted from the 2 h and 50 h values to eliminate effects of unspecific binding of released substances to the proteins. Measurements were done in triplicate with standard deviations up to 24%. All values shown in mg mL^{-1}

		2 h	50 h
Softwood	Guaiacol **3**	0.0	2.1
	Ferulic acid **9**	17.7	17.1
	Eugenol **10**	0.002	0.1
	Acetovanillone **11**	0.9	6.9
	Vanillin **12**	58.2	89.8
Hardwood	Guaiacol **3**	0.03	0.0
	Vanillin **12**	0.5	0.7

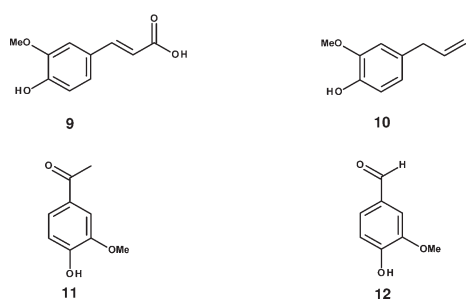

Fig. 9 Chemical structure of lignin monomers, released after LigDFG incubation; guaiacol **3**, ferulic acid **9**, eugenol **10**, acetovanillone **11**, vanillin **12**.

Conclusion

The enzymes LigD, LigF and LigG were successfully expressed and purified in *E. coli* and their proper functioning according to Sato *et al.*[20] could be confirmed. This enzyme system was able to specifically cleave the β-aryl ethers of model lignin substrates. The specific activities of every enzyme were determined. We successfully established activity assays for all three Lig enzymes. While LigD and LigF could be analysed colourimetrically (LigD spectrophotometrically *via* NADH-formation and LigF fluorometrically by cleavage of a model substance), LigG activity had to be determined by HPLC analysis. Furthermore, we were able to show that an efficient enzymatic recycling of all involved co-substrates is possible. Following the green chemistry concept of hydrogen borrowing and being able to be applied at ambient temperatures the combined (LigDFG and AVR) system could become a useful tool in lignin degradation for example for the synthesis of natural aroma compounds. Certainly the reaction cascade has to be highly improved for the conversion of complex lignin substrates to be

technically feasible. Methods of enzyme engineering should be most helpful here and will be applied. We have started to successively improve the reaction conditions, *e.g.* temperature, shaking and pH, for complex lignin enzyme assays to test the upcoming mutant enzyme libraries with their stereospecific or general process related capabilities. The crystallization of LigD, LigF and LigG as basis for further optimizations is ongoing and will most likely give new insights to improve the lignin degradation process with respect to its process related properties (overall activity, temperature stability, enantioselectivity and efficiency of recycling their co-substrates NAD^+ and glutathione). In addition the price for the co-substrates, especially for NAD^+, has to be considered. NAD^+ is very expensive and the cost should be prohibitive even though NAD^+ is recycled in the reactions. The utilization of alternative small molecules as electron carriers such as *N*-benzyl-nicotinamide derivatives can be of advantage here. These molecules are more stable than the natural cofactor, much cheaper to produce and have been shown to be accepted as alternative cofactors by enzymes from different classes.[31] In addition it has been repeatedly shown that this acceptance can be highly improved by methods of enzyme engineering.[32] That small artificial molecules can work as unnatural cofactors in lignin processing even today is demonstrated by the laccase mediator system in the pulp and paper industry.[33]

Another question is whether the application of four different enzymes could be too expensive. The production cost for enzymes is steadily decreasing[34] and such application of enzyme cascades using four and more enzymes in *in vitro* systems, free of cells and "designer bugs" is finding more and more acceptance even for the production of low value chemicals and of energy from biomass.[35] So even though it will require much more work and optimization, the lignin degrading enzyme cascade shown here might be of technical relevance sometime in the future.

Acknowledgements

Special thanks to Manuela Kaiser, Fraunhofer IGB BioCat, for synthesis of substrate **6**, to Petra Lommes, for assistance and help at the GC-MS analytics, Anja Philip for her analytical advices and Benjamin Kick, all TUM Chair of Chemistry of biogenic Resources. We also thank the Bavarian government for funding this project within the BayernFit program and both the Chemische Werke Zell-Wildshausen GmbH (Germany) and the Fraunhofer ICT (Germany), for providing lignin samples.

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