MODIFICATION OF LIGNANS BY *TRAMETES HIRSUTA* LACCASE

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Oxidative polymerization of two isolated lignans, secoisolariciresinol, and secoisolariciresinol diglucoside, as well as the lignan macromolecule, by a high redox potential *Trametes hirsuta* laccase was studied with different analytical methods. The reactivity of laccase with the different compounds was studied by an oxygen consumption measurement. The polymerization of laccase-treated lignans was evidenced by size exclusion chromatography, reversed phase - high performance liquid chromatography, and matrix-assisted laser desorption/ionisation - time of flight mass spectrometry. The data showed that the selected substrates could be oxidised by laccase. Secoisolariciresinol and secoisolariciresinol diglucoside were polymerized by laccase to a similar extent. The lignan macromolecule reacted to a lesser extent. Polymerization of the macromolecule proceeded mainly via its secoisolariciresinol diglucoside moieties. Furthermore, it was shown that ferulic acid can be linked to polymerized secoisolariciresinol via decarboxylation by laccase. This investigation showed that lignans can be enzymatically modified by *Trametes hirsuta* laccase.

Keywords: Lignan; Laccase; Polymerization; SEC; MALDI-TOF MS; FTIR

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INTRODUCTION

Lignans are polyphenolic compounds that are commonly found in many foods such as seeds, nuts, fruits, and beans (Milder et al. 2005), in addition to different wood components (Holmbom et al. 2003; Lindsey and Tollens 1892; Bamberger 1894). High concentrations of lignans have been found especially in flax seeds (Milder et al. 2005). Although several hundreds of lignans have been identified (Mazur et al. 1996; Peňalvo et al. 2005; Liggins and Grimwood 2000; Meagher and Beecher 2000; Mazur and Adlercreutz 1998, 2000; Willför et al. 2004a, 2004b), their detailed function and biological role are still under discussion. It is assumed that lignans have an important role in plant defence as well as in the regulation of plant growth (Willför et al. 2006). Lignans may also function as natural protectants against different cancers (Thompson et al. 2005) as well as decreasing the incidence of osteoporosis (Vanharanta et al. 1999; Ward et al. 2001) due to their antioxidant properties (Prasad 2000) or their influence on estrogen metabolism (Penttinen et al. 2007).

Lignans are formed by stereoselective coupling of two coniferyl alcohols (Gang et al. 1999), followed by a series of modification reactions, after which they can occur in either aglyconic or glycosidic form (Ward 2000). In flax seed, the most important lignan, secoisolariciresinol diglucoside (SDG), as well as the flavonoid herbacetin diglucoside (HDG) are ester-linked in the lignan macromolecule (LM) via the hydroxymethyl-glutaric acid (HMGA) linker-molecule (Kamal-Eldin et al. 2001; Struijs et al. 2007). The schematic structure of the lignan macromolecule adapted from Struijs et al. (2009) is shown in Fig. 1. The hydroxycinnamic acid glucosides, *p*-coumaric acid glucoside (*p*-CouAG) and ferulic acid glucoside (FeAG), are directly ester-linked to the glucose moieties of SDG (Struijs et al. 2008). Since HDG constitutes only 6% (w/w) of the lignan macromolecule, HDG has not been included in Fig. 1. The deglycosylated form of SDG, i.e. secoisolariciresinol (SECO), has been found in *Picea abi*es and *Abies alba* knots (Willför et al. 2004a,b).

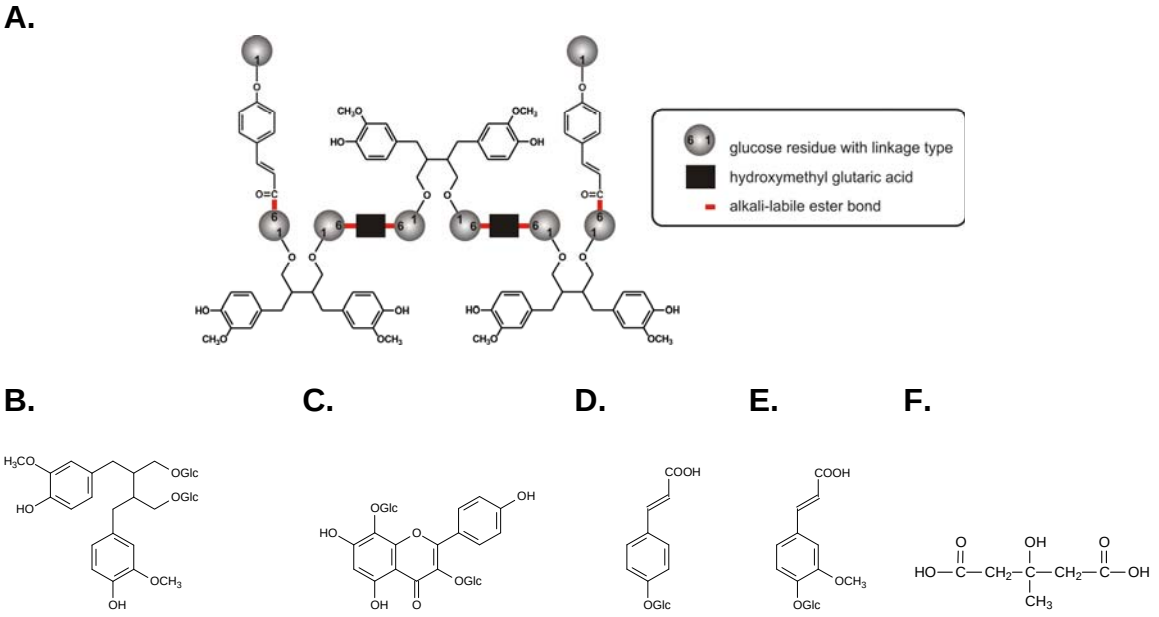

Fig. 1. Schematic structure of the lignan macromolecule (A). Below the polymer are shown structures of the constituents of lignan macromolecule: SDG (B), HDG (C), *p*-CouAG (D), FeAG (E) and HMGA (F).

Laccases are phenol-oxidizing enzymes. When acting on phenolic compounds such as lignin, they can display both degradative and polymerizing abilities. The substrate specificity range of the enzyme can be extended to cover both phenolic and non-phenolic compounds by using mediators (Widsten and Kandelbauer 2008). However, the oxidation of isolated lignans by laccase is not yet fully understood (Willför et al. 2006; Smeds et al. 2007). It has been shown that *Trametes hirsuta* laccase (ThL) can polymerize isolated heartwood spruce lignans (Buchert et al. 2002), although nearly half of the reactive groups remained intact after ThL-treatment. Oligomers containing 4 to 5 lignan units were formed in the reactions. Thus in technical processes, e.g. in the food, pulping, and paper-making industries, but also in environmental samples, where these compounds are

dissolved in water, polymerization of lignans by laccase may be a useful technique to remove lignans from waste streams, where they have undesirable characteristics.

The aim of this research was to study the polymerization of lignans by laccase and to characterize the reaction products formed.

EXPERIMENTAL

Materials

Chemicals

Ferulic acid (FeA) was purchased from Fluka (MW: 194.184 g mol^{-1}, Buchs, Switzerland) and *p*-coumaric acid (*p*-CouA) from Sigma-Aldrich, USA (MW: 164.15 g mol^{-1}, C9008). Their glycosidic forms, FeAG (MW: 356 g mol^{-1}) and *p*-CouAG (MW: 326 g mol^{-1}) as well as SDG (MW: 686 g mol^{-1}), and LM (MW = 4 – 5 kg mol^{-1}) were extracted from flax seed hulls and purified as described in Struijs et al. (2007). $SECO^1$ (MW: 362 g mol^{-1}) was obtained by dissolving 2 mg saponified LM in 1 mL 50 mM NaOAc buffer at pH 5. The reaction mixture was incubated for 24 h at 35 °C after adding 5 µL Rapidase Liq+ (DSM, Delft, The Netherlands). Rapidase Liq^+ is an enzyme preparation that was used to deglucosylate both lignan glucosides and hydroxycinnamic acid glucosides, which were liberated upon saponification of the lignan macromolecule. The enzymes were inactivated by boiling. $SECO^1$ was extracted from the reaction mixture by solid phase extraction and purified by preparative RP-HPLC as described by Struijs et al. (2007). $SECO^2$, isolated from *Araucaria angustifolia* according to Willför et al. (2003), was used for the enzymatic reaction mechanism studies. For the GC-MS analyses, silylation reagents *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) were purchased from Fluka (Buchs, Switzerland).

Methods

Enzyme reactions

Laccase from the white-rot fungus *Trametes hirsuta* was purified and characterized as described in Rittstieg et al. (2002). Activity of the enzyme was determined with 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS) as substrate, using a method developed by Niku-Paavola et al. (1988). The enzymatic reactions were typically carried out in 25 mM succinic acid at pH 4.5 (Merck, Darmstadt, Germany) at room temperature. The reaction volumes and incubation times varied between experiments, but the enzyme dosage was constantly 70 nkat per μmol^{-1} of the reactive group of the substrate. Typically, no mixing was used during the incubations. The presence of oxygen was ensured by performing the enzymatic reactions in open vessels. All enzymatic reactions were terminated (Johannes and Majcherczyk 2000; Kiiskinen et al. 2002; Murugesan et al. 2007) by adding 0.05 % (w/v) NaN_3, unless otherwise indicated.

Oxygen consumption measurement

Reactivities of ThL with 1 mM FeA, FeAG, *p*-CouA, *p*-CouAG, as well as with 0.5 mM SDG and two SECOs of different origin were measured by monitoring the consumption of dissolved oxygen during the enzymatic reaction. In the case of the less well-defined polymeric LM substrate, a concentration of 2 mg mL^{-1} was used in the measurement. Before ThL addition, the substrate solutions were stabilized at room temperature. After initiation of the reaction by addition of ThL, oxygen consumption was monitored with an oxygen electrode (FIBOX 3 fiber-optic oxygen meter, PreSens, Regensburg, Germany). The measurements were carried out under constant mixing in 1.84 mL volume in fully sealed flasks in order to avoid entry of oxygen into the reaction mixture.

SEC

Size exclusion chromatography (SEC) analyses were carried out using an alkaline elution method. A Waters size-exclusion chromatography apparatus equipped with three μHydrogel columns (pore size: 2000 Å, 250 Å and 120 Å) connected in series (Milford MA, USA) was used. The chromatographic instrument consisted of an M-2690 separation module and an M-996 diode array detector (detection range: 230 – 500 nm). Isocratic chromatography was performed at 60 ^{o}C using 50 mM NaOH as the eluent at a flow rate of 0.50 mL min^{-1}. The system was controlled and data was analysed with Empower software. For the analyses, ThL- and untreated SDG, $SECO^{2}$ (3 mM, 200 μL) and LM (2 mg mL^{-1}, 200 μL) were freeze-dried after 24 h incubation time, and dissolved in 100 mM NaOH at a concentration of 1 mg mL^{-1}. Injection volume was 50 μL.

RP-HPLC

For the reversed phase - high performance liquid chromatography (RP-HPLC) analyses, 1 mL aliquots of ThL- and untreated LM (2 mg mL^{-1}, 3 mL) after 0, 1 and 24 h incubation times were saponified with 75 mM NaOH for 24 h at room temperature and cleaned by solid phase extraction using the protocol described in Struijs et al. (2007). The eluates were analyzed on RP-HPLC, using the same analytical equipment as described in Struijs et al. (2007), equipped with an XTerra C18 MS column (3.5 μm particle size, 150 x 4.9 mm) from Waters (USA).

HPAEC

The release of glucose during incubation of *p*-CouA, *p*-CouAG, (1 mM, 3 mL) and LM (2 mg mL^{-1}, 3 mL) with 200 μL ThL was determined from aliquots of the reaction mixtures after 0, 1, 6, and 24 h incubation times. The samples were transferred to Eppendorf tubes and boiled for 5 min. The soluble and insoluble parts were separated by centrifugation (10 min, 13,000 rpm, 20 °C). The samples were then diluted 5-fold (40 μL supernatant and 160 μL of Milli-Q water) before analysis on high performance anion-exchange chromatography (HPAEC). Glucose standard was prepared by dissolving 3.5 mg glucose in 35 mL succinic acid buffer at pH 5. This solution was treated in the same way as the real samples (boiled, centrifuged, and diluted) to obtain a 20 μg mL^{-1} standard solution. The Dionex chromatographic system (USA) equipped with ICS 3000 autosampler, dual pump, and an electrochemical detector (in the pulsed amperometric

detection (PAD) mode), were used for the analyses. Dionex CarboPac PA1 (2 x 250 mm with a 2 x 50 mm guard column, 10 μm particle size) was used for separation of the compounds. The injection volume was 20 μL. During the first 30 minutes of the total running time of 73 min, 100 % Milli-Q water was used as eluent with a flow rate of 0.3 mL min^{-1}. During this period, the sugars were eluted from the column. Finally 100 mM NaOH as well as a mixture of 1 M NaOAc and 100 mM NaOH were used for cleaning and regenerating of the analytical column between the experiments. In order to facilitate detection of the sugars with PAD, 0.5 M NaOH (0.1 ml min^{-1}) was added post-column (De Ruiter et al. 1992).

MALDI-TOF MS

Matrix-assisted laser desorption ionisation - time of flight mass spectroscopy (MALDI-TOF MS) spectra of ThL- and untreated SECO samples from different origins (3 mM, 200 μL) were analysed after 30 min incubation with a Bruker Autoflex II instrument equipped with an N_2-laser (337 nm/100 μJ) (Bremen, Germany). Before the analyses, the ThL- and untreated SECO samples were mixed 1:2 (v:v) with saturated α-cyano-4-hydroxycinnamic acid matrix (Leipzig, Germany) dissolved in 50 % acetonitrile (J.T. Baker, NL) containing 0.1 % TFA (Fluka, USA). For the analyses, 1 μL of the mixture was pipetted onto the stainless steel MALDI target plate and dried at room temperature. The positive ion MALDI-TOF MS spectra were collected from the sample spots in the reflector mode. Peptide standard solution (Bruker, Germany) was used for the molecular mass calibration.

FTIR

$SECO^2$ (3 mM in Milli-Q water, 200 μL) was treated by ThL for 24 h. The enzymatic reaction was terminated by boiling to avoid NaN_3 bands in the Fourier transform infrared (FTIR) spectrum. Freeze-dried ThL- and untreated $SECO^2$ were analysed on a Bruker Equinox 55 FTIR spectrometer equipped with an IR-microscope and MCT detector (Karlsruhe, Germany). A few milligrams of dried samples were applied on a diamond cell and the transmission spectra (4000 to 600 cm^{-1}) were measured at room temperature. The spectral resolution was 4 cm^{-1} and the number of scans was typically 100. Opus software was used for baseline correction and normalisation of the FTIR spectra.

GC-MS

Relative amounts of phenolic acids, FeA and *p*-CouA, in the two SECOs, FeAG, *p*-CouAG, and SDG, were determined by gas chromatography - mass spectrometry (GC-MS). For the analysis, exact amounts of the samples, in the order of 1-5 mg were dissolved in 2 mL of methanol and mixed with 100 μL of the internal standard (heptadecanoic acid 200 mg L^{-1} dissolved in methanol). The samples were evaporated to dryness at room temperature under nitrogen flow. The dry samples were trimethylsilylated by adding 120 μL of BSTFA and 30 μL of TMCS (80 ^oC, 2 h) and analysed by GC-MS (Agilent 6890A GC and 5973N MS). The capillary column used for separation was a Nordion NB-54 (length: 15.0 m, id: 200 μm, film thickness: 0.25 μm) coated with poly(5% diphenyl / 95 % dimethyl)siloxane stationary phase (HNU-Nordion

Ltd. Oy, Finland). Split injection mode with a 50:1 ratio was used at 250 °C. The injection volume was 1.0 µL. The following temperature program for the GC oven was used: initial temperature: 100 °C (0 min), rate: 15 °C min^{-1}, final temperature: 280 °C (18 min). Helium with a flow rate of 1 mL min^{-1} was used as the carrier gas. The data were collected (ChemStation) in scan mode with a mass range of 40 - 800 Da.

RESULTS AND DISCUSSION

Oxidation of the Model Compounds

The reactivities of ThL with different substrates (FeA, *p*-CouA, FeAG, *p*-CouAG, SDG, SECO, and LM) were determined by measuring the oxygen consumption during the enzyme treatment. On the basis of the oxygen consumption curves (Fig. 2, A and B), clear oxidation of FeA, *p*-CouA, SDG, the two SECO samples, and LM by ThL was observed already in a few minutes. Oxygen consumption rate was the fastest for FeA, as expected, and the slowest for LM due to its polymeric nature. Unexpectedly, slight oxidation of FeAG and *p*-CouAG by ThL was observed. This may due to the presence of phenolic compounds in the reagents and/or deglycosylation of the compounds by a possible glycosidase side-activity present in the ThL-enzyme preparation, which may release phenolic hydroxyl groups that can be further oxidised by ThL. The oxidation of $SECO^1$ was also unexpectedly faster than that of $SECO^2$ (for composition of SECO samples see below). This was probably because of the small amount of phenolic compounds present in the isolated sample. The oxidation curve of SDG was between the two SECO curves. Although the glucosyl units are rather large groups, ThL reactivity towards freely rotating aromatic groups was not expected to be hampered by the glycosylation.

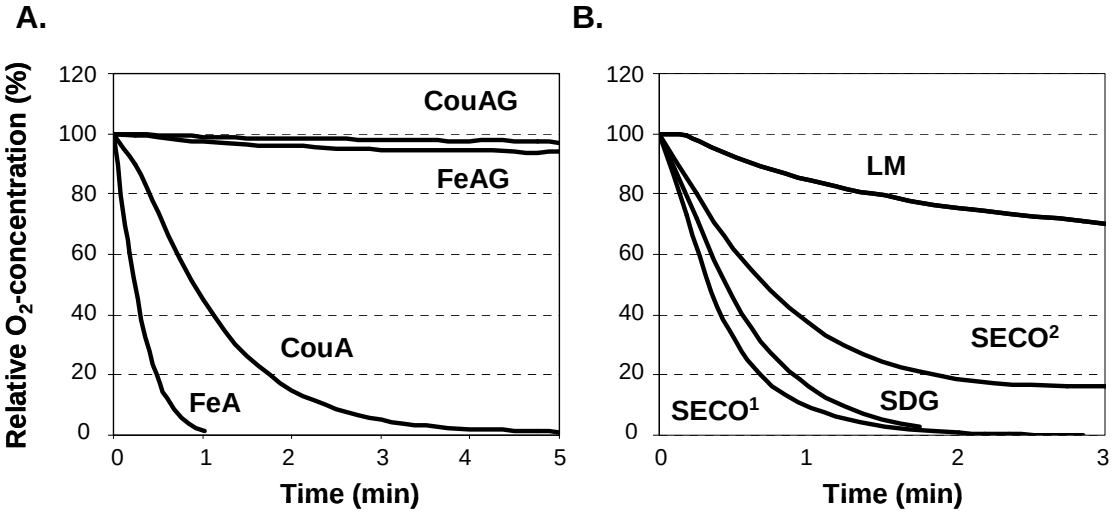

Fig. 2. Oxidation of (A) small molecules (FeA, *p*-CouA, FeAG, and *p*-CouAG, concentration 1 mM) as well as (B) lignans (SDG, $SECO^1$, $SECO^2$, and LM, concentration: 0.5 mM) by ThL as followed by oxygen consumption measurements

The presence of possible glycosidase side-activity in the ThL-enzyme preparation was determined by HPAEC, using *p*-CouAG as substrate by monitoring the release of glucose after ThL-treatment as a function of time. Before the experiments it was verified that the ThL-enzyme preparation did not contain any glucose as impurity. Only the *p*-CouAG contained small amounts of free glucose and *p*-CouA (data not shown). When *p*-CouAG was treated with ThL, the amount of released glucose increased as a function of incubation time, suggesting the presence of a small amount of phenyl glycosidase side-activity in the ThL-enzyme preparation (data not shown). This side-activity also explains the oxygen consumption of FeAG. The effect of this side-activity was taken into account in the data analysis.

The relative amount of phenolic acids in the SECO samples of different origins was determined by GC-MS. SECO^1 contained ca. 10 % of FeA and ca. 3 % of *p*-CouA determined as peak areas from the total ion chromatogram. Thus the presence of small amounts of phenolic acids in the sample explained the somewhat faster oxygen consumption rate of SECO^1 compared to that of SECO^2, which did not contain phenolic acids according to the GC-MS analysis. Neither SDG nor *p*-CouAG contained any phenolic acids according to GC-MS analysis. FeAG contained ca. 11 % of FeA, consistent with the slight oxidation of FeAG (Fig. 1). The polymeric lignan macromolecule was too large to be analysed by the GC-MS method. The presence of phenolic acids in the substrates was taken into account in the data analysis.

Polymerization of Lignans (SECO and SDG) by ThL

To study the polymerization of SECO and SDG by ThL, the reaction products were analysed first by SEC. On the basis of the measured chromatograms (Fig. 3), both lignans could be polymerized by ThL in 24 h with an enzyme dosage of 70 nkat per µmol^{-1} of the reactive group of the substrate. To simplify the interpretation of the data, only SECO^2 was used in the experiment.

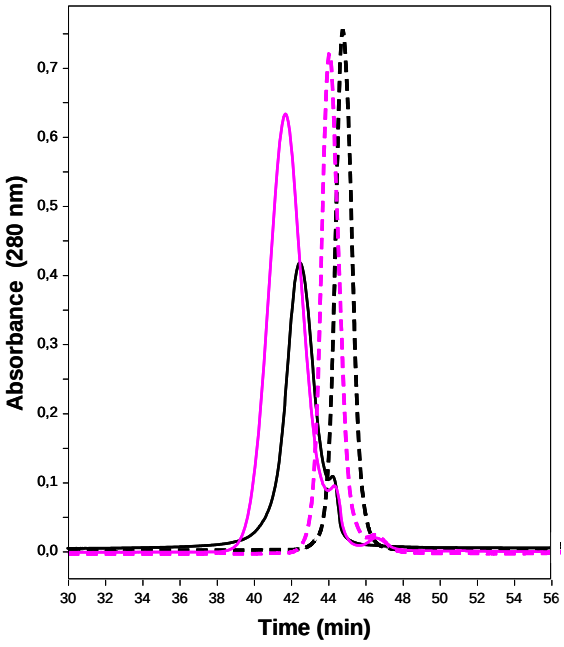

Fig. 3. Size exclusion chromatograms measured at 280 nm and at room temperature from ThL-treated (reaction time: 24 h) and untreated SECO^2 (black) and SDG (pink). The control samples are shown with dotted lines using the corresponding colors.

Positive-mode MALDI-TOF mass spectroscopy was used to study the structures of the SECO polymers in detail. The mass spectra were measured directly from the reaction mixtures of ThL-treated SECO[2] and SECO[1] (Figs. 4 A and B, respectively). Clear evidence of polymerization of the model compounds was observed in both cases after 30 min reaction time. In Fig. 4 A, $(SECO)_n$ homopolymers with up to ten units are shown. In the MALDI-TOF mass spectra of ThL-treated SECO[1] (Fig. 4 B), an additional series of unknown polymers was detected in the reaction mixture.

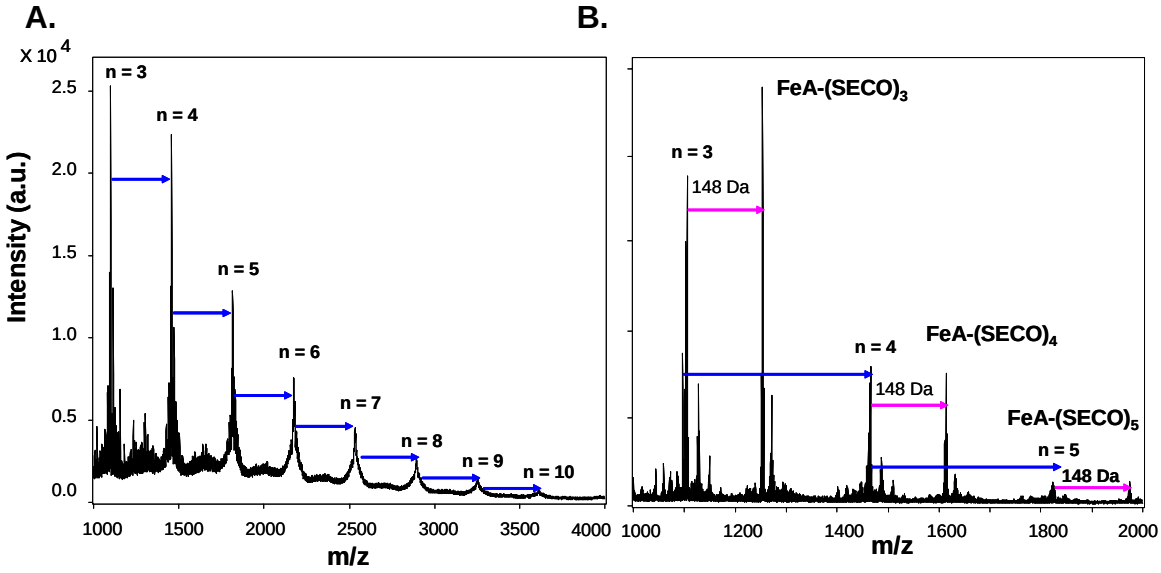

Fig. 4. Positive-mode MALDI-TOF mass spectra from ThL-treated (A) SECO[2] and (B) SECO[1]. The numbers of monomers (n) in the $(SECO)_n$ homopolymers are shown above the corresponding peaks in the spectra. The positions of the peaks for decaboxylated FeA-$(SECO)_n$ heteropolymers are also marked above the corresponding peaks.

The covalent bond formation between the SECO monomers in the homopolymers was due to elimination of two hydrogen atoms according the equation: $[nMM - (n - 1)2H + Na]^+$, where n is the number of monomers and MM is the molecular mass of the substrate. Furthermore, during the MALDI-TOF MS experiment, the SECO polymers were ionized as sodium salts. The observed experimental m/z values of the polymerization products corresponded to the predicted values, within the error of the method (< 0.1 Da). The m/z values of the SECO homopolymers are shown in Table 1 (n = 3-5).

When the masses of unknown series of polymers in Fig. 4 B were analysed in detail, the m/z difference between the unknown polymers and the closest $(SECO)_n$ homopolymer was always 148 Da, suggesting polymerization of SECO and attachment of FeA via a decarboxylation reaction mechanism (Ralph et al. 2008). The formation of decarboxylated FeA-$(SECO)_n$ heteropolymers is expected to start by dehydrogenative coupling as in the case of SECO homopolymers, followed by addition of FeA and elimination of the carboxylic acid group [$MM(FeA) - MM(CO_2) = 194.06$ Da – 43.99 Da $-2H^+ = 148$ Da], as shown in Fig. 5. The masses of the decarboxylated FeA-$(SECO)_n$ heteropolymers are also shown in Table 1, when n is 3 – 5.

Table 1. Assignment of the m/z Values of the $SECO_n$ Homopolymers and Heteropolymers Resulting from Incubation of $SECO^2$ and $SECO^1$ with ThL

Compound	Observed mass (Da)	Predicted mass (Da)	Mass difference (Da)
$[(SECO)_3]Na^+$	1105.66	1105.51	-0.10
$[(SECO)_4]Na^+$	1465.70	1465.68	0.03
$[(SECO)_5]Na^+$	1825.70	1825.85	0.18
$[FeA\text{-}(SECO)_3]Na^+$	1253.67	1253.57	-0.15
$[FeA\text{-}(SECO)_4]Na^+$	1613.71	1613.74	-0.02
$[FeA\text{-}(SECO)_5]Na^+$	1973.73	1973.91	0.15

(The corresponding MALDI-TOF mass spectrum is shown in Fig. 4 A) and Decarboxylated FeA-$(SECO)_n$; the corresponding MALDI-TOF mass spectrum is shown in Fig. 4 B); The difference between observed and predicted masses is shown in the last column.)

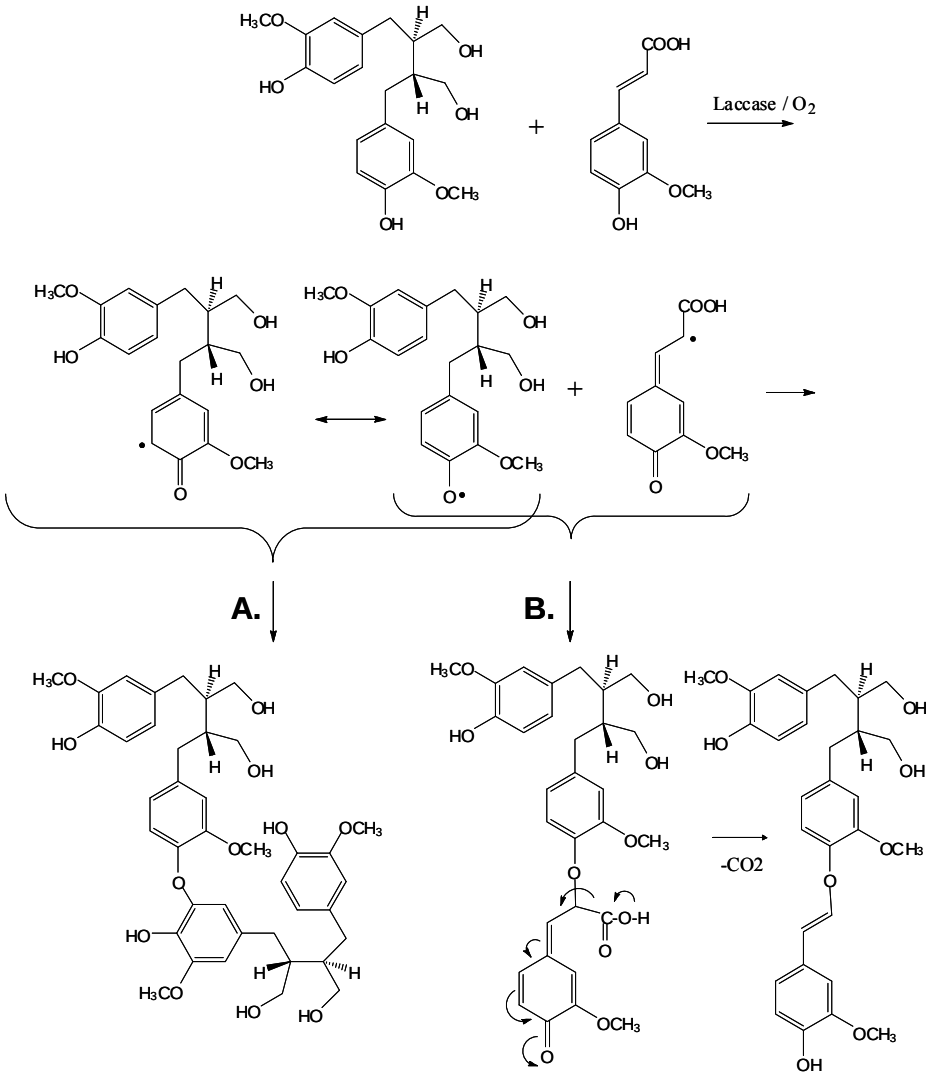

Fig. 5. Proposed reaction mechanism of ThL-catalysed oxidation of SECO with and without FeA. (A) Ether bond formation between two SECO molecules in the absence of FeA. (B) Linking of FeA to SECO via a decarboxylation reaction mechanism (Ralph et al. 2008). Other types of cross-links may be formed in a similar way.

Finally, to verify that monomers in $(SECO)_n$ homopolymers were linked to each other partly *via* ether (C-O-C) bonds, ThL-treated and untreated $SECO^2$ samples were analysed by FTIR spectroscopy (Fig. 6). When the reference spectrum (black) was compared to the spectrum (blue) measured from the ThL-treated $SECO^2$, a C-O-C band at ca. 1142 cm^{-1} showed polymerization of SECO via an ether bond. The corresponding band was recently detected directly from the reaction mixture when tyrosine-containing peptides were incubated with ThL with and without FeA (Mattinen et al. 2005). Another striking difference between the spectra of the ThL- and untreated SECO is a band at 1657 cm^{-1}. However, this band most likely was due to the moisture presence in the ThL-treated sample.

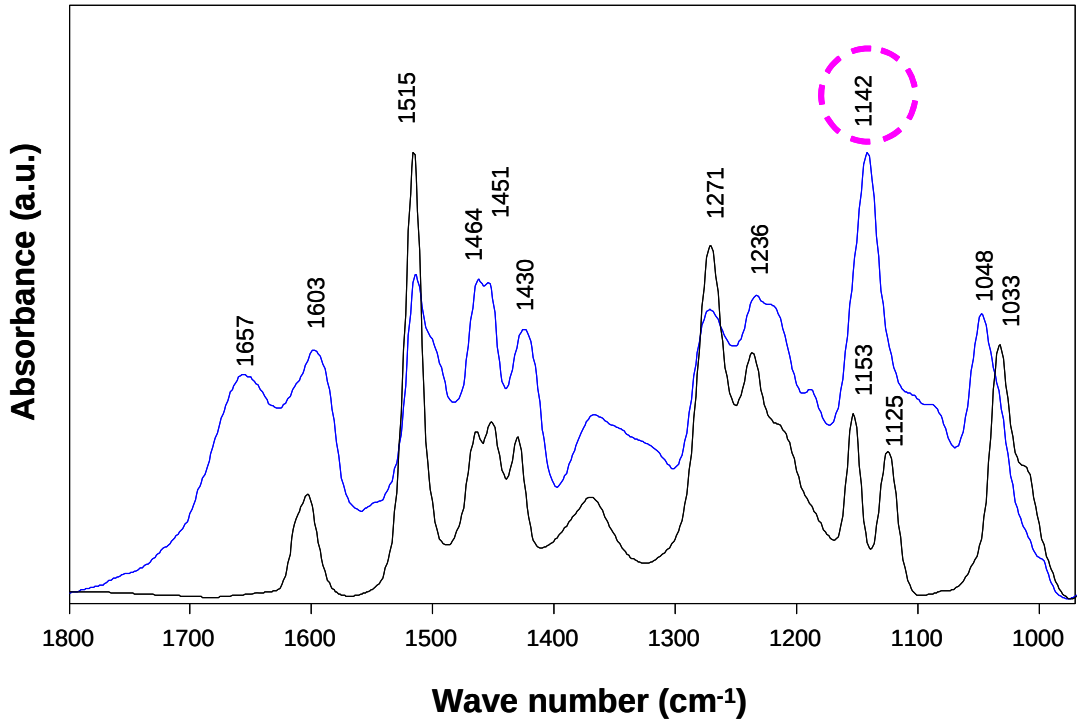

Fig. 6. FTIR spectra measured from ThL-treated (blue) and untreated (black) $SECO^2$. The position of the C-O-C band (1142 cm^{-1}) is highlighted with a pink circle.

Reactivity of Lignan Macromolecule (LM)

The reactivity of ThL with LM was first confirmed by oxygen consumption measurement (Fig. 2 B). The polymeric lignan macromolecule could be oxidised to some degree already within 12 minutes, suggesting oxidation and polymerisation of LM via its reactive phenolic hydroxyl groups.

To confirm the polymerization of LM by ThL, the reaction products were analyzed by SEC after 24 h incubation time. For the SEC analyses, the samples were solubilized in NaOH. Under these alkaline conditions, the ester-linkages of LM were hydrolyzed and thus the SEC profiles actually reflect the changes in the saponified samples (Fig. 7).

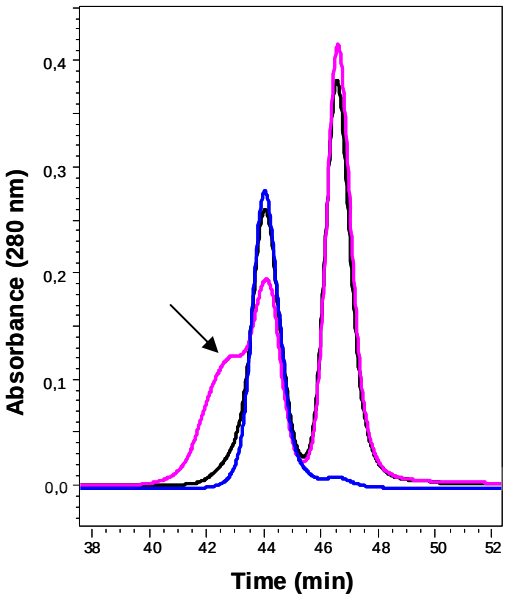

Fig. 7. Size exclusion chromatograms measured from ThL-treated and untreated saponified LM (pink and black chromatograms, respectively), after 24 h incubation at 280 nm and at room temperature. The band of the polymerization product is highlighted by the arrow. The SEC-profile of SDG is shown with blue for comparison.

On the basis of the measured SEC profiles shown in Fig. 7, polymerization of LM by ThL is clearly shown. A novel band of the reaction products eluting after ca. 43 minutes was formed concomitantly with decrease of the band eluting at ca. 42 - 45 minutes, indicating polymerization of LM. In contrast to polymerization of SDG alone, during the polymerization of LM, linkages between two SDG moieties might have been formed, as well as between SDG and *p*-CouAG or FeAG after deglucosylation (as discussed above). To determine the polymerization sites of LM, ThL-treated LM samples after 1, 6, and 24 h incubation were saponified and purified before the analysis by RP-HPLC. The chromatograms of an untreated sample and one of the samples treated for 24 h are shown in Fig. 8.

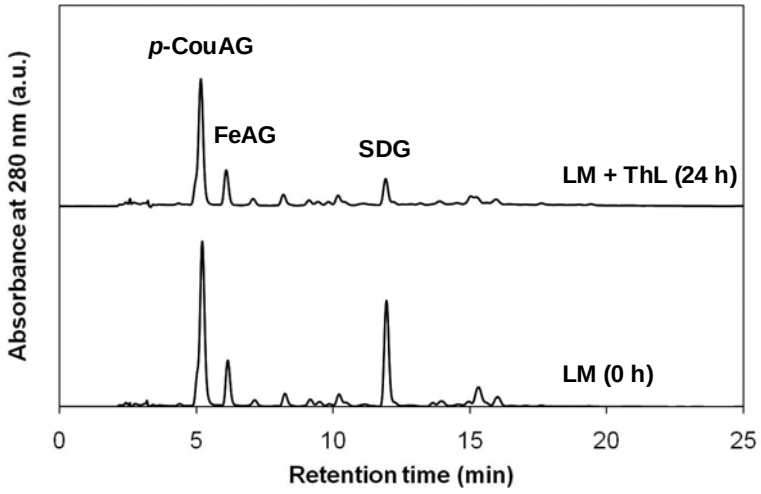

Fig. 8. RP-HPLC profile of ThL-treated LM after 24 h incubation times as well as the control sample (after 0 h) measured at 280 nm and at room temperature after saponification. Identification of the main bands is shown above the corresponding peaks.

The RP-HPLC profiles clearly show a decrease in peak area (Table 2) of SDG, demonstrating its participation in the polymerization reaction. Also the peak areas of *p*-CouAG and FeAG are lower after ThL-treatment compared to the untreated LM, suggesting that *p*-CouAG and FeAG have also reacted. FeAG and *p*-CouAG and are not laccase substrates, as the reactive phenolic hydroxyl groups are blocked via glucosylation. However, in this study phenolic hydroxyl groups could have become available for polymerization by ThL due the deglucosylating side-activity presence in the ThL enzyme preparation (as discussed above). All the controls behaved as expected.

Table 2. Decrease of the RP-HPLC peak area (%) of *p*-CouAG, FeAG and SDG components of ThL-treated LM after saponification. The peak areas were normalized by the corresponding peak areas determined from LM (0 h) sample.

Reaction mixture	*p*-CouAG (%)	FeAG (%)	SDG (%)
LM + ThL (24 h)	69	60	24
LM + ThL (6 h)	72	62	29
LM + ThL (1 h)	85	74	36
LM (0 h)	100	100	100

The most striking phenomenon observed from Table 2 is that most of the ThL-catalyzed reaction occurred during the first hours, although the saponification experiment indicated that there was still some of the substrate left in the reaction mixture. This is likely due to the limited accessibility of the reactive groups in the polymerized LM, which hindered further polymerization.

CONCLUSIONS

1. All of the selected model lignans SECO and SDG could be efficiently oxidized and polymerized by ThL. In addition, polymerization of LM by ThL via the SDG constituent was observed.
2. In the presence of a small amount of FeA, heteropolymers between SECO and FeA were formed via a decarboxylation mechanism, in addition to the homopolymer $(SECO)_n$ formation.
3. The results presented in this paper with different types of lignans from various plant materials show that lignans as well as lignins can be enzymatically modified.

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