Transformation of N', N'-dimethyl-N-(hydroxyphenyl) ureas by laccase from the white rot fungus Trametes versicolor

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Abstract

Transformation of N',N'-dimethyl-N-(hydroxyphenyl)ureas was assayed in the presence of purified laccase produced by the fungus *Trametes versicolor*. The *para*- and *ortho*-hydroxyphenyl derivatives were enzymatically transformed, whereas the *meta* derivative was not. The performance of laccase-mediated transformation depended on the pH with an optimum for the *para*-derivative degradation rate at pH 5. The pH also influenced the nature of the reaction products. The chemical was exclusively oxidised into *p*-benzoquinone at pH 3 and into mainly N',N'-dimethyl-N-[(2,5-cyclohexadiene-1-one)-4-ylidene]urea at pH 6. The *ortho*-derivative was transformed essentially into insoluble purple compounds, probably appearing as polymers resulting from coupling of the parent compound.

Introduction

Laccases (EC-1-10-3-2) are copper-containing oxidases widely distributed in woody plants (Mayer, 1987) and fungal species which carry out the delignification process, such as white rot fungi (Hatakka, 1994; Thurston, 1994). Typically, the enzyme catalyses the one-electron oxidation of phenolic substrates or aromatic amines. It has been also shown that in the presence of appropriate low-molecular-weight compounds called mediators, laccase was able to oxidise a wide range of other aromatic compounds. Mediators were identified among compounds naturally synthesised by the fungus (Eggert *et al.*, 1996) and among xenobiotics (Bourbonnais *et al*., 1997) such as 2,2'-azinobis(3-ethylbenz-thiazoline-6-sulfonic acid)(ABTS). Laccase catalyses oxidations, leading in most cases to coupling reactions and the formation of conjugates or polymers.

Phenylurea herbicides are widely used for pre- and post-emergence selective control of grasses and broad-leaved weeds in winter cereal crops. These chemicals undergo oxidation reactions in soil, plant and microbial systems, including N-dealkylation, aryl or ring substituent hydroxylation (Gross *et al.*, 1979; Ryan *et al*., 1981). Most of these reactions are followed by sugar conjugation, in spite of free oxidised compounds being detected. Enzymes principally involved in phenylurea transformation were identified among P450 mono-oxygenases (Mougin *et al*., 1990) and glucosyl transferases (Hatzios and Penner, 1982).

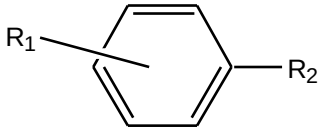
Hydroxylated metabolites formed by plant and microorganism metabolism appear as phenolic compounds and behave like substrates of laccases. In this paper, we report the results of our studies on the transformation of hydroxylated metabolites of N',N'-dimethyl-N-phenylurea - known as fenuron - by partially purified laccases of the white rot fungus *Trametes versicolor*.

Materials and Methods

Chemicals

Unless otherwise stated, all chemicals and reagents used for synthesis were purchased from Sigma (St Quentin Fallavier, France). Solvents used for HPLC analysis came from Carlo Erba (Val de Reuil, France).

Synthesis of model compounds



N',N'-dimethyl-N-(2-hydroxyphenyl)urea (2-HF) **1** [R_1: 2-OH, R_2: NH-CON$(CH_3)_2$]

The synthesis procedure for 2-HF was adapted from Hadjieva *et al.* (1988). Triethylamine (25 mL) diluted in ethanol (25 mL) was added dropwise to a stirred solution of benzoxasolone (2.7 g, 0.02 mol) and dimethylamine hydrochloride (10 g, 0.123 mole) in 50 % aqueous ethanol. The resulting mixture was heated at 80°C under reflux for 6 hours. After cooling to room temperature, diluted aqueous hydrogen chloride was added until neutralisation and evaporated under reduced pressure. The product was purified by flash chromatography on a silica gel column using ethyl acetate as a mobile phase. Yield: 585 mg; 15 %.

N',N'-dimethyl-N-(3-hydroxyphenyl)urea (3-HF) **2** [R_1: 3-OH, R_2: NH-CON$(CH_3)_2$]

Dimethylamine hydrochloride (1.6 g, 20 mmoles) was added to a solution of 3-hydroxyphenylurea (2.5 g, 15 mmoles) in water (200 ml) and heated under reflux for 5 hours. After cooling to room temperature, the clear mixture was saturated with sodium chloride, extracted with ethyl acetate (4 x 100 ml) and the combined organic layers dried over sodium sulfate. The solvent was then removed *in vacuo* and 500 mg of the crude residue (total: 2.35 g)

was chromatographied over a silicagel column using a mixture of cyclohexane and ethyl acetate 2/1 as eluant to afford pure **2**. Yield: 123 mg, 21.5%.

N',N'-dimethyl-(4-hydroxyphenyl)urea (4-HF) **3** [R_1: 4-OH, R_2: NH-CON$(CH_3)_2$]

N-(4-hydroxyphenyl)hydrazide **3a** [R_1: 4-OH, R_2: CONH-NH_2]. Ethyl 4-hydroxy-benzoate (15.4 g, 0.1 mole) was added portionwise to an excess of hydrazine hydrate (50 g, 1 mole) under stirring over a period of one hour. The solution was allowed to stand overnight at room temperature. The resulting heterogeneous mixture was diluted with cold absolute ethanol and filtered using a glass filter. The white residue was washed first with cold ethanol (4 x 20 ml), then with diethyl ether (3 x 20 ml) and dried *in vacuo* at room temperature. Yield: 14 g; 91%.

Azide **3b** [R_1: 4-OH, R_2: CON_3]. Pure acetic acid (8.4 ml, 0.14 mole) was added dropwise to a stirred mixture of **3a** (10 g, 0.066 mole) and sodium nitrite (10 g, 0.145 mole) in water (200 ml) over a period of 30 min. The solution was maintained at 0°C in an ice bath. The resulting mixture was then allowed to stand under stirring at 0°C for 3 hours, warmed to room temperature and filtered using a glass filter. The crude residue was mixed with diethyl ether (50 ml), while the insoluble residual starting material was separated by filtration. The solvent was removed under reduced pressure and the azide **3b** dried *in vacuo* at room temperature. Yield: 10.2 g; 95 %.

Protected azide **3c** [R_1: 4-$OCOCH_3$, R_2: CON_3]. Acetyl chloride (2.85 g, 36.5 mmoles) in diethyl ether (10 ml) was added dropwise to a stirred solution of **3b** (5 g, 30.5 mmole) and triethylamine (3.7 g, 36.5 mmole) in dry diethyl ether (50 ml) at room temperature over a period of 30 min. The resulting heterogeneous mixture was then allowed to stand under stirring at room temperature for 2 hours. The insoluble triethylamine hydrochloride was filtered using a glass filter. The filtrate was treated first with a 2% aqueous potassium hydrogen carbonate solution (4 x 40 ml) followed by water until neutralisation. The organic layer was dried over sodium sulphate. The solvent was removed under reduced pressure and the residue dried *in vacuo* at room temperature to afford the protected azide as a white solid substance. Yield: 5.2 g; 83%.

Protected isocyanate **3d** [R_1: 4-$OCOCH_3$, R_2: NCO]. A stirred suspension of the crude protected azide **3c** (5.2 g, 25.4 mmol) in toluene (50 ml) was heated to 80°C until nitrogen evolution had ceased (4 hours). The solvent was then removed *in vacuo* and the oily residue mixed with pentane to afford the protected isocyanate as a white solid substance. Yield: 4 g; 89%.

Protected dimethylphenylurea **3e** [R_1: 4-$OCOCH_3$, R_2: $NHCON(CH_3)_2$]. Triethylamine (11.4 g, 0.113 mol) in dichloromethane (50 ml) was added dropwise to a suspension of dimethylamine hydrochloride (9.2 g, 0.113 mol) and **3d** (4 g, 22.6 mmol) in dichloromethane (100 ml). The mixture was heated at 40°C for 3 hours. After cooling to room temperature, it was treated in turn with a 5% aqueous hydrogen chloride solution (4 x 20 ml), a 5% aqueous sodium hydrogen carbonate solution (4 x 20 ml), and water until neutralisation. The organic layer was then dried over sodium sulfate and the solvent removed *in vacuo*. Yield: 3.06 g; 61%.

N',N'-dimethyl-(4-hydroxyphenyl)urea **3**. A mixture of the crude protected dimethylphenylurea **3e** (1.7 g, 7.65 mmol) and potassium hydroxyde (1.5 g, 26.8 mmol) in 1% aqueous methanol (100 ml) was heated at 40°C for one hour. The solvent was then removed *in vacuo* and the oily residue neutralised to pH 5 with HCl acidified water. The water was evaporated and ethanol was added to the residue. The insoluble part was removed by filtration and the solvent evaporated. The product was then purified by precipitation in diethyl ether, filtered and dried. Yield: 1.06 g; 77 %.

The overall yield of the multi-step procedure was 30%.

Enzyme Production and Purification

The organism used for laccase production was *Trametes versicolor*. It was obtained from the culture collection of the Unité de Phytopathologie (INRA Versailles, France).

For biomass production, the fungus was pregrown on the medium previously described (Lesage-Meesen *et al*., 1996) using glucose (5 g l^{-1}) as a carbon source. Biomass production was achieved

in Roux flasks containing 200 mL culture medium, developed from five agar disks (5mm), and cultured at 25°C for 7 days. Then, three mat mycelia were filtered from the growth medium and resuspended in sterile, deionized water to a final volume of 300 mL. The resulting mycelia suspensions were fragmented for 1 minute with an Ultra Turrax homogeniser (10 000 rpm). The pounded mats were used to inoculate the bioreactor.

The air-lift bioreactor used for laccase production contained 5 litres of the mineral culture medium described by Collins *et al*. (1996). The culture, shaken by continuous air bubbling, was grown for a further 7-day period. Laccase production was induced with 2,5-xylidine (0.2 mM) at the beginning of the culture. The laccase-containing exocellular culture fluid was filtered through glass-wool or a cheese cloth and frozen at -80°C. The solution was then filtered successively on miracloth and glass filters (Whatman) with decreasing pore diameters (2.7 to 0.7 μm) in order to eliminate polysaccharides. A final filtration step was performed on a 0.22 μm Durapore (Millipore) membrane before concentration by ultrafiltration on an YM10 Amicon membrane. The crude enzyme concentrate (CE) was stored at -80°C.

The CE was then applied on a DEAE52 ion-exchange column (Whatman) equilibrated with a 10 mM citrate/phosphate buffer pH 6. The laccase was eluted with a linear gradient up to 0.75 M NaCl. The active fractions were pooled and concentrated by ultrafiltration on an YM10 Amicon membrane and ultradialized against 20 mM sodium phosphate buffer pH 7 before a further purification step by size-exclusion chromatography on a Sephacryl S200HR column (Pharmacia) in the same buffer. This partly-purified enzyme solution (PE) was stored at -20°C in 30 % w/v glycerol.

Enzyme Assay

Laccase activity was measured as the oxidation of 2,2'-azinobis-(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS, ε_{420} = 36 000 M^{-1} cm^{-1}) method (Wolfenden and Wilson, 1982) using a

Kontron UV-visible 860 spectrophotometer. The assay was conducted at 30°C in 1 mL of a solution containing 1 mM ABTS in 100 mM phosphate-citrate buffer pH 3. International units (μmol min^{-1}) of enzyme activity were used.

Enzymatic transformation of dimethyl-(hydroxyphenyl)ureas

Enzymatic transformation of (dimethyl-hydroxyphenyl)ureas was performed in 8 mL test tubes containing 4 mL of solution prepared as follows: ethanolic solutions of the three synthesised ureas (10 mg mL^{-1}) were dissolved to final concentrations varying from 0.7 mM to 0.9 mM in 100 mM citrate/phosphate buffer at a chosen pH. All the experiments were carried out using air-saturated buffer solutions. The reaction started by adding PE laccase solution. Laccase titres in the assays were adjusted to 0.4 unit. Enzyme was omitted in controls containing the dimethyl-(hydroxyphenyl)urea at the same pH and concentration. The samples were stirred on an orbital table and temperature was kept constant at 30°C. Specific reaction conditions are detailed in the legends of the figures.

The enzymatic reactions were stopped by heating at 100°C for 5 min. The samples were cooled and used for HPLC and/or UV spectral analysis. Control samples were also heated to 100°C and analysed to check any modification of the substrate during incubation and heating.

Isolation of the reaction products

In order to identify the 4-HF reaction products, transformation of 5.9 10^{-2} moles (11 mg) was performed at 30°C in 10 mL in the presence of 5U laccase at two pH values. Medium pH was set to 3 with phosphoric acid to avoid any interference from citrate protons in the NMR analysis. For pH 6, a 20 mM phosphate buffer was used. After 20 hours incubation, the reaction media was supplemented with NaCl to reach a 2M concentration, and then acidified to pH 1.5-2 with concentrated HCl. Organic compounds were extracted with dichloromethane (3 x 10 mL). HPLC

analysis showed that the extraction efficiency had reached 95 %. The organic solvent was dried over Na_2SO_4 and evaporated under reduced pressure. The crude extract was then purified by chromatography on a silica column using ethyl acetate as an eluant. The main product was analysed by NMR and mass spectroscopy.

Isolation of the insoluble product formed from 2-HF was achieved by filtration over a GV 0.22 µm filter (Millipore). The insoluble part was dried at 60°C for 24 hours and dissolved in 2 mL dichloromethane.

High Performance Liquid chromatography (HPLC) separation

The concentrations of the dimethyl-(hydroxyphenyl)urea derivatives were determined using an HPLC system (Waters Associates, Milford, USA) equipped with a 486 UV detector and a 600E system controler. Ten-microlitre samples were analysed using a Nova Pack C_{18} column (150×3.9 mm, Waters). The mobile phase consisted of water (solvent A) and acetonitrile (solvent B) at a flow rate of 1 mL min^{-1}. The following mobile phase gradient method was used: 100% A for 3 min, linear increase from 0% B to 50% B in the next 15 min, brought to 100% B in 2 min and kept constant for 7 min. The column was equilibrated to initial conditions for 13 min before a further injection. The total run time was 40 min. The samples were detected at 244 nm. The acquired data were analysed using the Waters chromatography system manager Millenium v2.00.

Spectroscopic methods

Mass spectrometry (MS) - The mass spectra of the separated products were obtained by electron impact (EI) mass spectroscopy analysis (70 eV) using a Ribermag R10-10 quadrupole mass spectrophotometer, with sample introduction by direct insertion probe. The molecular weight was checked by chemical ionisation (CI) using CH_4 or NH_3 as reactant gases.

GC/MS was also used. Gas chromatography was performed with a HP 5989A chromatograph fitted with a RTX 5MS column (length 30 m, 0.25 mm diameter, 0.5μm deepth film, Restek). The temperature of the injector was 260°C. The oven ramped from 80 to 300°C at 5°C per minute.

Nuclear magnetic resonance (NMR) - Proton ^{1}H NMR spectra of the analysed compounds dissolved in $CDCl_3$ or DMSO were obtained using a Bruker AC-200 NMR spectrometer at 200 MHz. TMS was used as a reference compound.

Results

Synthesis of model compounds

Spectroscopic data for synthesised 2-, 3- and 4-HF are shown in Table 1. Molecular weight of the three synthesised ureas (determined by CI-NH_3) was 180. Fragment ions for 2- and 4-HF were consistent with dimethyl-(hydroxyphenyl)urea structures. NMR data definitively confirmed the position of the hydroxyl group. Synthesis of 2-HF and 3-HF using one-step procedures gave rather low yields (15.0 and 21.5%, respectively), mostly because of the presence of by-products. No attempt was made to optimise them since the amounts obtained were sufficient to undergo the degradation assays in the presence of laccase.

We attempted to use the transamination method outlined above for the synthesis of 4-HF. The reaction between aminophenol and potassium cyanate leading to the corresponding ureidophenol was assayed according to King *et al*. (1992). Unfortunately, high amounts of the corresponding amide were formed due to the presence of acetic acid in the reaction mixture. The overall yield (including the transamination) was therefore very low. As a consequence, we turned to another multi-step method adapted from the preparation of putrescine hydrochloride, as reported by Smith (1963). The overall yield for the 6-step synthesis procedure was about 30 %, and provided enough material to successfully carry out degradation experiments.

After the final purification procedure, the purity of each dimethyl-(hydroxyphenyl)urea was at least 95%, as checked by NMR and HPLC.

Preliminary transformation experiments

The ability of the laccase from *Trametes versicolor* to transform the three dimethyl-(hydroxyphenyl)ureas was tested in assays performed at 30°C with pH from 3 to 8 and with different incubation times. Incubation of 2-HF and 4-HF with the enzyme resulted in the decrease

of their initial concentrations and the formation of new products, as shown on the chromatograms in Fig. 1. The concentrations of the 2- and the 4-substituted derivatives were reduced to 43% and 58 % of their initial value, respectively after 15-min incubation. Conversely, 3-HF was not transformed in the presence of the laccase at the pH generally known to be optimum for laccase activity. No transformation products were detected in the controls run without enzyme.

Influence of pH on N',N'-dimethyl-N-(4-hydroxyphenyl)urea degradation

The pH had a marked influence on the kinetics of 4-HF degradation. Firstly, laccase activity exhibited a bell shape as a function of the pH with a maximal transformation (91%) at pH 5. Laccase catalysis decreased rapidly when the pH was raised above the optimum. At pH 6, only 12.2 % of the 4-derivative was converted after 1 hour in the presence of enzyme. At pH 7, no change in the initial chemical concentration could be detected by HPLC in incubation media over 6 hours. However, evidence for biological transformation of the *para*-derivative occurred between 6 and 96 hours of incubation, as 84 % of the chemical had degraded. Conversely, laccase remained active at acidic pH, as 40% of 4-HF had been transformed at pH 3. Whatever the pH value, the concentration of 4-HF remained unchanged in the controls without enzyme during 96-hour periods.

Another important aspect of the pH effect was the nature of the degradation products, as seen in Fig. 2. At pH 3 and 4, the main degradation product (called compound I) was more polar than the parent compound (RT 5.6 min versus 12.0 min). At pH 6, the 4-HF was mostly transformed into the apolar compound II (RT 12.9 min) plus a significant amount of compound I. Between these two pH values, there were equal amounts of compound I and II.

Isolation and identification of the N',N'-dimethyl-N-(4-hydroxyphenyl)urea transformation products

Degradation of 4-HF was accompanied by the formation of about seven products, as revealed by HPLC analysis. However, compounds I and II were predominant (Fig. 1A). The identification of the latter was based on the analysis of degradation experiments performed at pH 3 and 6, respectively. We took advantage of the pH specificity of the reaction to minimise the purification steps needed to prepare enough samples used to carry out the identification procedures. After a 20-hour incubation, the reaction solution colour was orange at pH 3 and yellow at pH 6. The products extracted with methylene chloride and purified by liquid chromatography were then analysed through MS and NMR (Table 1). CI-CH_4 analysis of compound I revealed a molecular weight of 108. Its NMR spectrum only exhibited a singlet at 6.4 ppm, indicating a symmetric molecule. From these data, compound I was identified as *p*-benzoquinone. As further confirmation, HPLC and spectroscopic analysis (EI mass spectra and NMR data) of authentic *p*-benzoquinone showed similar results.

GC/MS spectrum of compound II suggested a molecular weight of 178 (Table 1). CI analysis gave no results, either with NH_3 or CH_4 as reactant gases. The NMR spectrum of compound II exhibited two doublets at 6.66 and 7.16 ppm, characteristic of *para*-substituted aromatic protons. A singlet at 2.90 ppm could be assigned to the dimethyl protons of the substutited urea. These data as well as fragment ions obtained by EI suggested that compound II was N',N'-dimethyl-N-[(2,5-cyclohexadiene-1-one)-4-ylidene]urea.

Study of N',N'-dimethyl-N-(2-hydroxyphenyl)urea transformation products

Several transformation products of 2-HF were detected (Fig 1B). The solution rapidly became purple in the presence of laccase and a precipitate appeared. HPLC analysis of the filtered soluble fraction revealed eight different peaks with low intensity indicating small concentrations of these soluble products. The low concentrations prevented any identification of the metabolites by MS and NMR. All the peaks were eluted after the initial parent compound. The composition of the

insoluble fraction checked by HPLC varied depending on the incubation time in the presence of laccases. After 15-min incubations, one main peak was detected whose retention time was very close to the void volume of the column (Fig. 3). Its area remained constant regardless of the duration of the experiment. Three hours after the beginning of the transformation, two additional peaks appeared. They were more retained by the column and their proportions were inverted after 3 days of incubation. The intermediate retention time product was extensively reduced (almost 5 times), while the product with the higher retention time became the main conversion product (a ten-fold increase). UV-visible spectra of the insoluble fraction of 2-HF degradation products established the appearance of an intermediate product absorbing at λ= 417 nm. However, none of these products could be identified. No interpretable results were obtained either by GC/MS analysis of a dichloromethane extract, nor through MS by direct insertion probing of the samples collected following HPLC.

Discussion

The three hydroxyphenyl derivatives of fenuron were treated with partially purified laccase from *Trametes versicolor*. Under our experimental conditions, 3-HF was not transformed by fungal laccase whereas 2- and 4-HF were converted to several products. This paper clearly establishes for the first time, to our knowledge, that laccases are able to catalyse the direct attack of herbicide metabolites.

Enzyme activity towards hydroxyphenyl ureas followed this order: *3-HF<<4-HF<2-HF*. The correlation between the amount of substrate transformed at the optimal pH and the position of the substitutents groups on the phenolic ring is reported by Shuttelworth *et al.* (1986), who showed that 3-substituted cresol was poorly oxidised, while 4-cresol was oxidised to the greater extent. Previously, Alberti and Klibanov (1981) had shown that 3-chlorophenol was not efficiently removed by peroxidase while 2- and 4-chlorophenol were clearly removed. The absence of reaction of 3-substituted phenols could be due to the reaction mechanism. The initial step involves the formation of an aryloxy radical by the removal of one electron and one hydrogen atom from the hydroxyl group. The aryloxy radical generates resonance structures, which are stabilised in *para* and *ortho* positions, whereas a *meta* substitutent does not allow stabilisation, which implies a low reactivity of the *meta*-substituted phenols.

Our results also show that dimethyl-(hydroxyphenyl)urea transformation greatly depended on the pH of the medium, with respect to the kinetics of degradation and the nature of the metabolites. The enzymatic transformation of 4-HF by laccase was greater at acidic pH (3 to 5) than at higher values. The bell-shaped dependence has been observed with different phenolic substrates such as 2,6-dimethoxyphenol, syringic or vanillic acid. Recently, Xu (1997) explained such a pH-activity

profile as the balance of two opposite effects. Due to the deprotonation of their hydroxyl group, the apparent electrochemical potential of phenols decreased with pH, by enhancing the difference with the electrochemical potential of the T1 copper centre of the laccase. The oxidation reaction of the substrate was thus thermodynamically promoted. At higher pH, the activity of the enzyme decreased. This was due to the inhibition generated by the bonding of the hydroxide anion to the coppers of laccase. However, this physico-chemical description of the pH influence on enzyme activity could not explain the preferential *p*-benzoquinone formation at lower pH.

The pH also determined the nature of the reaction product. At pH 3, laccase catalysed both the oxidation of the hydroxyl group and the breakdown of the C_{aryl}-C_{alkyl} bond. *p*-Benzoquinone was the main product formed, whereas at pH 6 the mixture was mainly composed of N',N'-dimethyl-N-[(2,5-cyclohexadiene-1-one)-4-ylidene]urea. The formation of quinones from different substituted phenols such as benzoic and cinnamic acids by laccase from *Trametes versicolor* has been previously reported (Leonowicz *et al*., 1984; Katase, 1991; Lacki, 1998). This could also be expected with fenuron derivatives. The effect of pH on the nature of the product formed is not as well documented. Leonowicz *et al*. (1984) showed that the oxidation of vanillic acid by laccase from *Rhizoctonia praticola* led to the corresponding 1,4-benzoquinone at pH < 6, whereas the same quinone was formed with syringic acid in a pH range of between 3 and 8.

With 2-HF showing two substitutents groups in the *ortho* position, a purple flocculating precipitate was observed in the reaction medium. Unfortunately, the products could not be identified due to analytical difficulties. Nevertheless, their low solubility in water seemed to indicate a polymerisation process between the *o*-quinonic products.

Bound and conjugated pesticide residues are formed in plants and soils (Khan, 1982). These are mainly oligomers of the parent compound or the degradation products of the latter. They can also

result from a coupling with naturally occurring compounds. Phenylurea herbicides are degraded to give phenolic intermediates or amines. They give bound and/or conjugated residues in soils (Gaillardon and Sabar, 1994) and plants (Gross *et al*., 1979). This paper shows that laccases allow the formation of insoluble compounds, probably as polymers of fenuron metabolites. These results might partly explain some of the reactions involved in bound residue formation in plants and soils. Moreover, laccases may also find a possible application in wastewater treatment (Maloney *et al*., 1986). Particular attention should be paid to their effectiveness in removing soluble phenolic contaminants and transforming them into insoluble compounds.

Acknowledgments

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Fig. 1: HPLC analysis of incubation media containing 4-HF and 2-HF at the beginning of the incubations (panels A and C, respectively) and after 15-min assays in the presence of laccase (panels B and D).

Reaction conditions: 0.1 M CPB buffer pH 5; concentration of fenuron derivatives: 0.75 mM; laccase activity: 0.1 U per mL medium.

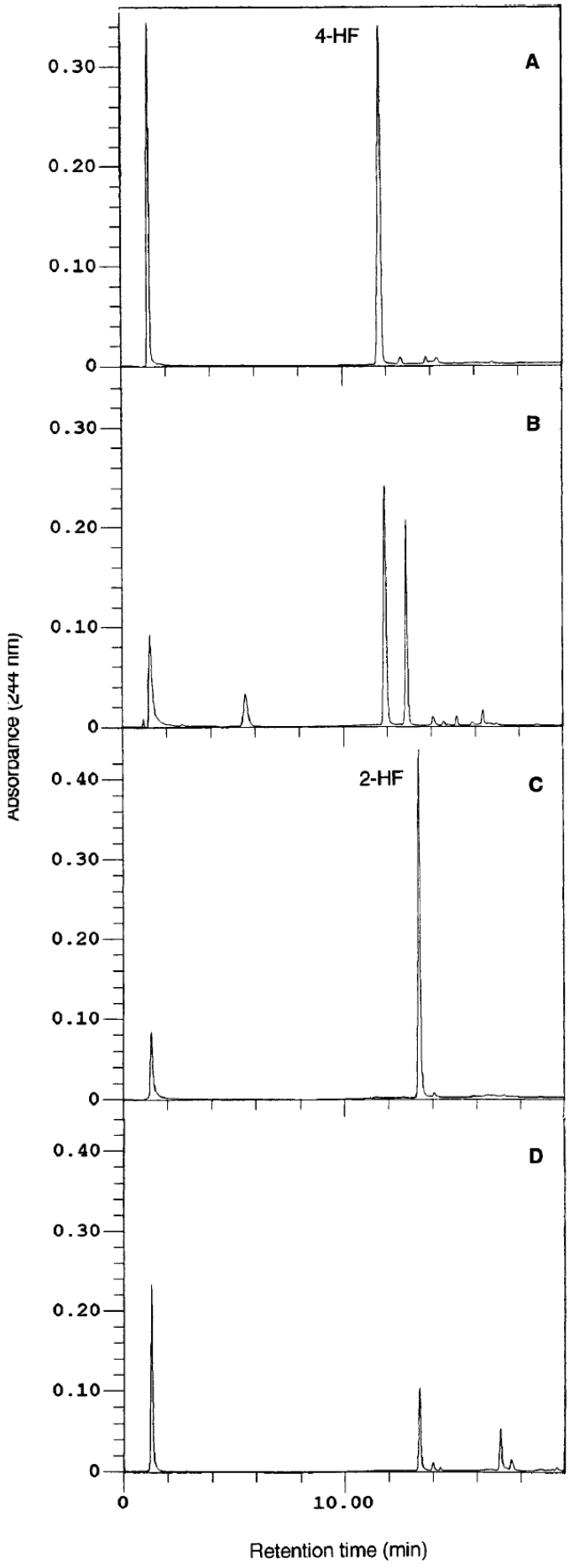
4-HF
A
B
2-HF
C
D
0.30
0.20
0.10
0
0.40
0
10.00
Absorbance (244 nm)
Retention time (min)

Fig. 2: Effect of pH on the formation of compound I (●) and compound II (■) from 4-HF in the presence of laccase.

Reaction conditions: 0.1 M CPB buffer pH 3 to 6; 4-HF concentration: 0.89 mM; laccase activity: 0.1 U per mL medium.

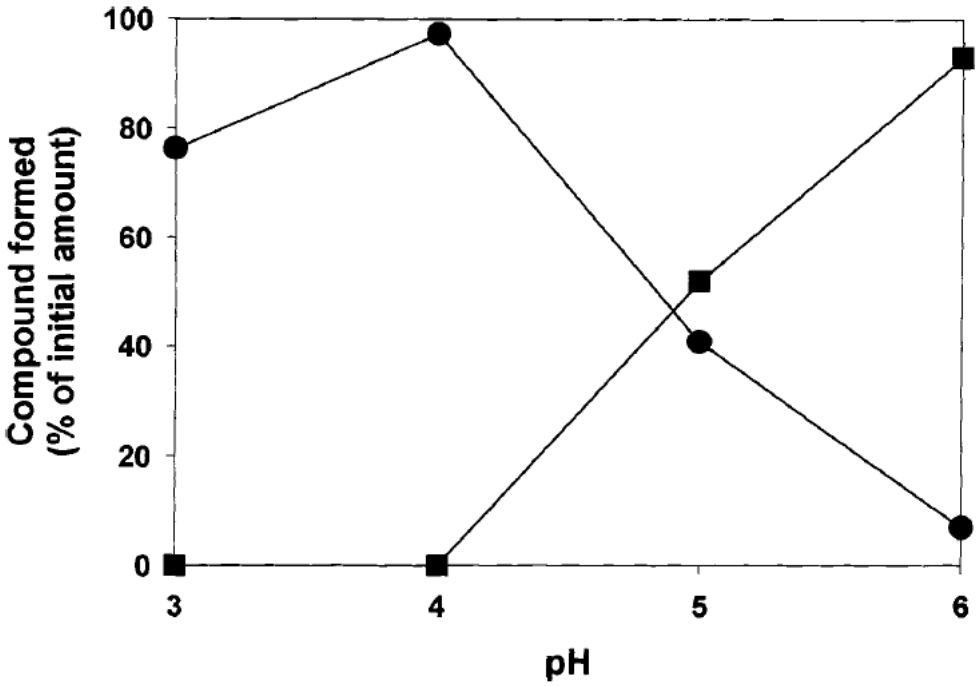

Fig. 3: HPLC separation of the insoluble fraction formed from 2-HF in the presence of laccase.

HPLC analysis was performed with an isocratic mixture of methanol/water 60/40 at 0.5 mL/min. Detection wavelength was 254 nm.

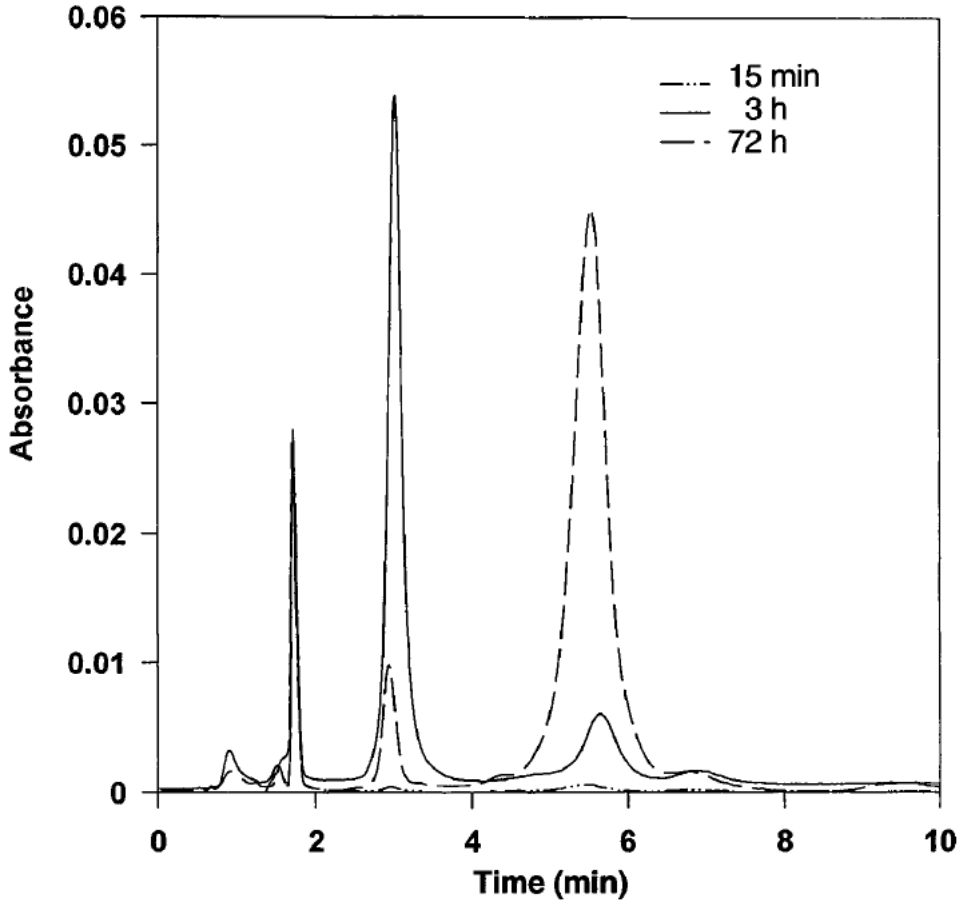

Table 1. Spectroscopic data for synthesised N', N'-dimethyl-(hydroxyphenyl)ureas and transformation products of 4-HF by laccase from *Trametes versicolor*

Identification	Abbrev.	*m/z* of fragment ions (% relative intensity)	^{1}H chemical shift
N',N'-dimethyl-N-(2-hydroxyphenyl)urea	2-HF	180 (M^+, 8), 135 (12), 72 (100), 52 (16), 45 (41)	9.55 (s, 1H), 7.05 (m, 2H), 6.86 (m, 2H), 6.44 (s, 2H), 3.06 (s, 6H)
N',N'-dimethyl-N-(3-hydroxyphenyl)urea	3-HF		9.17 (s, 1H), 8.11 (s, 1H), 6.94 m, 3H), 6.33 (d, 1H), 2.90 (s, 6H)
N',N'-dimethyl-N-(4-hydroxyphenyl)urea	4-HF	180 (M^+, 23), 135 (9), 72 (100), 52 (8), 44 (16)	8.00 (s, 1H), 7.15 (d, 2H), 6.63 (d, 2H), 2.88 (s, 6H)
p-benzoquinone	I	108 (M^+, 85), 82 (43), 54 (100)	6.4 (s)
N',N'-dimethyl-N-[(2,5-cyclohexadiene-1-one) -4-ylidene]urea	II	178 (M^+, 26), 134 (13), 106 (9), 72 (100), 44 (15)	7.16 (d, 2H), 6.66 (m, 2H), 2.90 (s, 6H)