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Calculated ionisation potentials determine the oxidation of vanillin precursors by lignin peroxidase

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Abstract In view of the biocatalytic production of vanillin, this research focused on the lignin peroxidase (LiP) catalysed oxidation of naturally occurring phenolic derivatives: *O*-methyl ethers, *O*-acetyl esters, and *O*-glucosyl ethers. The ionisation potential (IP) of a series of model compounds was calculated and compared to their experimental conversion by LiP, defining a relative IP threshold of approximately 9.0 eV. Based on this threshold value only the *O*-acetyl esters and glucosides of isoeugenol and coniferyl alcohol would be potential LiP substrates. Both *O*-acetyl esters were tested and were shown to be converted to *O*-acetyl vanillin in molar yields of 51.8 and 2.3%, respectively.

Key words: Lignin peroxidase; Vanillin; Ionization potential

1. Introduction

Producing vanillin from natural precursors using enzymatic or fermentative processes is of growing interest. Many natural precursors like eugenol, coniferaldehyde, and ferulic acid have been considered to produce the aromatic aldehyde [3,9,10]. All of these have an alkyl side chain which has to be cleaved to form vanillin.

Fungal lignin peroxidase (LiP) can cleave similar alkyl side chains in lignin and in lignin model compounds [8,14,16] and may therefore have potential applications in the production of vanillin. Incubation of underivatised phenolic vanillin precursors like coniferyl alcohol [13] with LiP and H_2O_2 yield unwanted lignin-like polymers. When the phenolic group is protected, as in *O*-ethyl isoeugenol, C_α-C_β cleavage occurs yielding the corresponding benzaldehyde [12]. This suggests that protection of the phenolic OH-group makes polymerisation a less important event in the overall reaction mechanism.

LiP's ability to oxidise a series of methoxybenzenes to the corresponding radical cations has been shown to be limited by the experimentally determined ionisation potential (IP) [7]. The IP value is a measure of the ease with which one electron is abstracted from the highest occupied molecular orbital (HOMO). Above a certain IP threshold value the methoxybenzenes were no longer oxidised by LiP [7].

Here we use calculated IP values as a tool to predict the susceptibility of phenolic derivatives: *O*-methyl ethers, *O*-acetyl esters, and glucosides to oxidation by LiP. Especially the *O*-acetyl esters and glucosides are interesting potential vanillin precursors since the acetyl and glucosyl moieties can be enzymatically removed.

2. Materials and methods

2.1. Lignin peroxidase

LiP-2 was purified from the extracellular culture broth of *Bjerkandera* sp. strain BOS55 according to ten Have et al. [6].

2.2. Incubation mixtures

The incubation reactions were performed in a HPLC vial and contained: 100 mM sodium succinate pH 3.0, 0.5 mM substrate, 1.0 mM H_2O_2, 2.0 mM 1,4-dimethoxybenzene, and LiP 1000 U/l (V_{tot} = 250 μl). The mixture was incubated for 10 min at 20°C. Subsequently 500 μl acetonitrile was added which stopped the enzymatic activity completely. Samples were capped and analysed with HPLC according to ten Have et al. [5].

2.3. Commercially available chemicals

O-Methyl isoeugenol, 3,4-dimethoxyphenylacetone and 3,5-dimethoxycinnamic acid were obtained from Aldrich (Milwaukee, WI, USA). Homoveratryl alcohol, *O*-methyl eugenol, *O*-methyl ferulic acid, cinnamyl alcohol, homoveratric acid, 3-methoxycinnamic acid, cinnamaldehyde and cinnamic acid were obtained from Acros (Geel, Belgium). *O*-Acetyl isoeugenol and *O*-acetyl eugenol were obtained from Roth (Karslruhe, Germany).

2.4. Synthesised chemicals

GC-MS analyses were carried out on a HP5973 quadrupole MS coupled to a HP6890 gas chromatograph equipped with a fused silica capillary column (HP-5MS, 30 m × 0.25 mm i.d., film thickness: 0.25 μm). Carries gas and flow: He at 1.0 ml/min. Injector temperature 220°C; temperature program: 70–250°C at 7°C/min, hold 5 min. EIMS were obtained at 70 eV. ^{1}H-NMR spectra and ^{13}C-NMR spectra were recorded on a Bruker AC-E 200 spectrometer at 200 MHz and 50 MHz, respectively in $CDCl_3$.

O-Methyl coniferyl alcohol was synthesised from *O*-methyl ferulic acid according to the literature [1,2]. EIMS *m/z* 194 $[M]^+$ (58), 165 (10), 151 (100), 138 (59), 119 (16), 107 (13), 91 (36), 77 (29), 65 (12), 55 (15), 39 (7). ^{1}H-NMR δ 1.67 (1H, br. s), 3.86 (3H, s), δ 3.87 (3 H, s), 4.29 (1H, d, J = 5.5 Hz), 6.22 (1H, dt, J = 15.9, 5.8 Hz), 6.53 (1H, d, J = 15.8 Hz), 6.85 (1H, d, J = 8.7 Hz), 6.93 (1H, s). ^{13}C-NMR δ 55.8 (q), 55.9 (q), 63.8 (t), 108.7 (d), 111.0 (d), 119.6 (d), 126.5 (d), 129.7 (s), 131.1 (d), 2x 149.0 (s).

O-Methyl coniferyl aldehyde was synthesised from *O*-methyl coniferyl alcohol according to the literature [1]. EIMS *m/z* 192 $[M]^+$ (88), 177 (22), 161 (100), 149 (24), 133 (14), 121 (24), 103 (19), 91 (35), 77 (43), 63 (15), 51 (10). ^{1}H-NMR δ 3.88 (3H, s), 3.89 (3 H, s), 6.57 (1H, dd, J = 7.8, 15.7 Hz), 6.87 (1H, d, J = 8.3 Hz), 7.04 (1H, d, J = 2.0 Hz), 7.13 (1H, d, J = 8.3 Hz), 7.38 (1H, d, J = 15.8 Hz), 9.62 (1H, d, J = 7.8 Hz). ^{13}C-NMR δ 55.9 (q), 56.0 (q), 109.8 (d), 111.1 (d), 123.5 (d), 126.7 (d), 127.0 (s), 149.3(s), 151.9 (s), 152.9 (d), 193.6 (d).

O-Acetyl coniferyl alcohol was synthesised from *O*-acetyl eugenol (ICN, Zoetermeer, The Netherlands) according to the literature [15]. EIMS *m/z* 222 $[M]^+$ (6), 180 (74), 152 (8), 137 (100), 124 (63), 103 (9), 91 (20), 77 (14), 65 (9), 55 (9), 43 (27). ^{1}H-NMR δ 2.18 (1H, br. s),

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Table 1
The calculated ionisation potential (in eV ± S.E.M.) of various commercially available compounds and the qualitive outcome of the incubation of these compounds with LiP

Compound	Substrate (±)	IP (eV)
O-Methyl isoeugenol	+	8.41 ± 0.04
O-Methyl coniferyl alcohol	+	8.58 ± 0.05
O-Methyl eugenol	+	8.64 ± 0.07
Homoveratryl alcohol	+	8.66 ± 0.03
3,4-Dimethoxyphenylacetone	+	8.84 ± 0.05
O-Methyl ferulic acid	+	8.87 ± 0.05
O-Methyl coniferaldehyde	+	8.92 ± 0.11
Homoveratric acid	+	8.99 ± 0.08
Cinnamyl alcohol	−	9.00 ± 0.04
3,5-Dimethoxycinnamic acid	−	9.16 ± 0.03
3-Methoxycinnamic acid	−	9.29 ± 0.03
Cinnamaldehyde	−	9.39 ± 0.02
Cinnamic acid	−	9.53 ± 0.05

A + indicates that the compounds is transformed by LiP. For incubation conditions see Section 2.

2.28 (3 H, s), 3.79 (3H, s), 4.25 (1H, d, J = 3.5 Hz), 6.25 (1H, dt, J = 15.9, 5.5 Hz), 6.52 (1H, d, J = 15.8 Hz), 6.97 (1H, m). ^{13}C-NMR δ 20.7 (q), 55.8 (q), 63.4 (q), 110.1 (d), 119.1 (d), 122.8 (d), 129.0 (d), 130.1 (d), 135.9 (s), 139.2 (s), 151.1 (s), 169.2 (s).

O-Acetyl coniferyl aldehyde was synthesised from *O*-acetyl coniferyl alcohol according to the literature [1]. EIMS *m/z* 220 $[M]^+$ (3), 178 (100), 161 (20), 147 (42), 135 (31), 118 (14), 107 (17), 89 (10), 77 (19), 63 (8), 51 (14), 43 (43). ^{1}H-NMR δ 2.32 (3H, s), 3.86 (3 H, s), 6.65 (1H, dd, J = 8.1, 11.6 Hz), 7.14 (3H, m), 7.43 (1H, d, J = 15.9 Hz), 9.68 (1H, d, J = 7.6 Hz). ^{13}C-NMR δ 20.7 (q), 56.0 (q), 111.4 (d), 121.9 (d), 123.5 (d), 128.7 (d), 132.9 (s), 142.4 (s), 151.6 (s), 151.9 (d), 168.7 (s), 193.5 (d).

2.5. Calculation of ionisation potentials

Ionisation potentials were calculated on a Silicon Graphics Indigo computer using Spartan version 5.0 (Wave function, Inc., Irvine, CA, USA). A semi-empirical molecular orbital method applying the AM1 Hamiltonian was used. Closed shell calculations were performed using the Restricted Hartree Fock method. Geometries were optimised for all bond lengths, bond angles and torsion angles. These calculated ionisation potentials are those for molecules in a vacuum.

The IP strongly depends on the three dimensional structure of a molecule. Therefore, the IPs of at least four and maximally eight conformations have been calculated. The average value of these are presented with the standard error of the mean (S.E.M.).

3. Results

3.1. Determination of the ionisation potential threshold

Several non-phenolic compounds, characterised by an aromatic ring and an alkyl side chain, were incubated with LiP. Table 1 shows the observed relationship between the substrate consumed and the calculated IP. The results presented show that compounds with a calculated IP lower than 9.0 eV were all converted by LiP, whereas all model compounds with an IP above this threshold value were not.

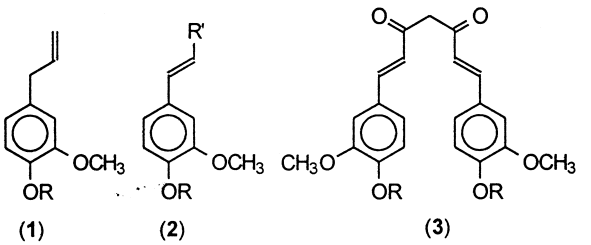

Fig. 1. Structural formulas of eugenol (1, R = H), isoeugenol (2, R = H, R′ = CH_3), coniferyl alcohol (2, R = H, R′ = CH_2OH), coniferaldehyde (2, R = H, R′ = CHO), ferulic acid (2, R = H, R′ = COOH), and curcumin (3, R = H). In *O*-methyl ethers R = methyl, for *O*-acetyl esters R = acetyl, and for glucosides R = glucosyl.

3.2. Natural phenolics and their derivatives

Direct incubation of phenolics with LiP and H_2O_2 yield unwanted polymers [4,13,17]. Literature data on *O*-ethyl isoeugenol show that the latter is cleaved by LiP to the corresponding benzaldehyde [12], whereas it was observed that isoeugenol was polymerised to a white precipitate. Therefore, we studied protected phenolic derivatives: *O*-methyl ethers, *O*-acetyl esters, and glucosides (see Fig. 1). Table 2 presents the calculated IP values for the various phenolics and derivatives, showing that the IP values for the *O*-acetyl esters and the glucosides are significantly higher than those of the phenolics and the *O*-methylated analogues. This trend was found in all cases. Protection of the phenolic OH-group with an acetyl or a glucosyl moiety increases the IP of the compounds beyond 9.0 eV, suggesting that they are not substrates for LiP. Only the *O*-acetyl esters and glucosides of isoeugenol and coniferyl alcohol have IP values below 9.0 eV.

3.3. C_α-C_β cleavage of O-methyl ethers and O-acetyl esters

The results presented so far allow us to predict which compound will be a LiP substrate. The first step in the C_α-C_β cleavage reaction is the removal of one electron, forming a radical cation intermediate. This reactive species may undergo spontaneous addition of water or loss of a proton which are essential for the C_α-C_β cleavage reaction required to obtain the benzaldehyde derivatives. The C_α-C_β cleavage was studied by measuring the formation of 3,4-dimethoxybenzaldehyde (VAD) from five *O*-methyl ethers. Table 3 shows that these LiP substrates were significantly cleaved to VAD by LiP, suggesting that predicted LiP substrates with an alkyl side chain of three carbon atoms in general undergo C_α-C_β cleavage. This hypothesis is further evidenced by the results in Table 4 showing that both *O*-acetyl isoeugenol and coniferyl alcohol are consumed and converted to *O*-acetyl vanillin to varying extents in the presence of 1,4-dimethoxybenzene (1,4-DMB). It should be noted that the cofactor 1,4-DMB was required for extensive LiP oxidation of the *O*-acetyl esters. Without

Table 2
The calculated ionisation potential (± S.E.M. in eV) of several phenolic compounds and their derivatives

Compound	Phenol	*O*-Methyl ether	*O*-Acetyl ester	Glucoside
Eugenol	8.73 ± 0.06	8.64 ± 0.07	9.17 ± 0.07	9.14 ± 0.05
Isoeugenol	8.46 ± 0.04	8.41 ± 0.04	8.81 ± 0.06	8.76 ± 0.02
Ferulic acid	8.95 ± 0.05	8.87 ± 0.05	9.33 ± 0.06	9.23 ± 0.06
Coniferyl alcohol	8.60 ± 0.06	8.58 ± 0.05	8.91 ± 0.05	8.92 ± 0.04
Coniferaldehyde	8.86 ± 0.05	8.92 ± 0.11	9.21 ± 0.07	9.15 ± 0.05
Curcumin	8.78 ± 0.02	8.64 ± 0.04	9.17 ± 0.05	no data

Table 3
The average molar VAD yield (%), calculated as the amount of VAD formed per mol substrate converted, obtained after incubation of different substrates (0.5 mM)

Substrate	Conversion (%)	Molar VAD yield (%)
O-Methyl ferulic acid	60.3 ± 0.3	54.3 ± 3.2
O-Methyl isoeugenol	100 ± 0.0	53.2 ± 3.0
O-Methyl coniferaldehyde	12.1 ± 1.0	24.7 ± 1.6
O-Methyl coniferylalcohol	100 ± 0.0	11.7 ± 0.1
O-Methyl eugenol	100 ± 0.0	2.6 ± 0.1

For incubation conditions see Section 2.

Table 4
The conversion (%) of various *O*-acetyl esters and the yield of *O*-acetyl vanillin (product, %), calculated as mol product per initial substrate amount, formed during the LiP catalysed reaction

Substrate	Conversion (%)	Molar *O*-acetyl vanillin yield (%)
O-Acetyl isoeugenol	97.3	51.8
O-Acetyl coniferyl alcohol	59.8	2.3
O-Acetyl eugenol	0.0	0.0
O-Acetyl coniferaldehyde	0.0	0.0

Values are the average of two duplicate run incubations. For incubation conditions see Section 2.

1,4-DMB the conversion of *O*-acetyl isoeugenol and coniferyl was 3.8 and 6.5%, respectively. Such a stimulating effect was not observed in the case of the *O*-methyl ethers (data not shown). The other acetyl esters, *O*-acetyl eugenol and coniferaldehyde, were not converted by LiP (Table 4). This was as expected based on the calculated IP values which are above the relative threshold value of 9.0 eV.

4. Discussion

It has been shown that the experimentally determined IP of small methoxybenzenes correlates with the susceptibility to oxidation by LiP [7]. The results of the present study extend this model with quantum-mechanically calculated IP values. Using non-phenolic aromatic compounds with an alkyl side chain an IP threshold value of 9.0 eV was obtained which allowed us to screen potential vanillin precursors.

The calculated IP values of the *O*-acetyl esters and glucosides were significantly higher than those of the corresponding phenolics and the *O*-methyl ethers, probably due to the electron withdrawing effect of the *O*-acetyl and *O*-glucosyl groups, decreasing the electron density in the aromatic ring.

In contrast to the *O*-methyl ethers, the conversion of the *O*-acetyl esters of isoeugenol, and coniferyl alcohol was significantly enhanced in the presence of 1,4-DMB, suggesting that both *O*-acetyl esters were poor substrates for LiP compound II. Without 1,4-DMB LiP compound II accumulates and may react with H_2O_2 to form the inactive LiP compound III [11]. In the presence of 1,4-DMB (IP = 8.55 eV) compound II is effectively reduced back to native LiP, completing the catalytic cycle and thus enhancing the total turnover of the enzyme.

The conversion of a series of phenolic derivatives, *O*-methyl ethers, to the C_α-C_β cleavage product 3,4-dimethoxybenzaldehyde by LiP allowed us to hypothesise that similar compounds with an IP < 9.0 eV would undergo the same C_α-C_β cleavage reaction upon incubation with LiP. *O*-Acetyl isoeugenol and coniferyl alcohol both were indeed converted to *O*-acetyl vanillin. Renganathan et al. [12] have proposed that the C_α-C_β cleavage mechanism of *O*-ethyl isoeugenol occurs via a diol intermediate. The oxidation of the double bond in the alkyl side to a diol might also occur in the case of the *O*-acetyl esters studied here. There are, however, strong indications that superoxide also plays an important role in the C_α-C_β cleavage mechanism, suggesting the formation of instable hydroperoxides during LiP catalysis [5].

Future research will concentrate on studying the C_α-C_β cleavage mechanism in more detail in order to explain the difference in yield of 3,4-dimethoxybenzaldehyde between the *O*-methyl ethers of isoeugenol and coniferyl alcohol. This could give ideas how to increase the low molar *O*-acetyl vanillin yield of 2.3% from *O*-acetyl coniferyl alcohol.

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