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Laccase-catalyzed dimerization of ferulic acid amplifies antioxidant activity

Oluyemisi E. Adelakun[a], Tukayi Kudanga[a,*], Ayesha Parker[a], Ivan R. Green[b], Marilize le Roes-Hill[a], Stephanie G. Burton[a]

[a] Biocatalysis and Technical Biology Research Group, Cape Peninsula University of Technology, Bellville Campus, Symphony Way, P.O. Box 1906, Bellville 7535, Cape Town, South Africa
[b] Chemistry Department, University of the Western Cape, Modderdam Road, Private Bag X17, Bellville 7530, South Africa

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ABSTRACT

Enzymatic modification can be used to enhance the bioactive properties of phenolic compounds. The present study employed laccase from *Trametes pubescens* to catalyze the modification of ferulic acid in a monophasic or biphasic system, as a way of enhancing its antioxidant capacity. Two dimeric products (m/z 385.1) were purified and characterized as the β-5 and β–β dimers. In the monophasic system, the β-5 dimer was preferentially formed in dioxane while the β–β dimer formation was enriched in ethanol as co-solvent. In the biphasic system, formation of the dimers increased as the concentration of ethyl acetate was increased from 80% to 95%. The β-5 dimer showed higher antioxidant capacity than the substrate as demonstrated by standard antioxidant assays (DPPH and TEAC). These results demonstrate that alteration of reaction conditions influences the laccase-mediated oxidation of ferulic acid to form dimers with higher antioxidant capacity than the substrate.

1. Introduction

Plant phenols and phenolic acids are increasingly becoming a subject of intensive research due to their bioactive properties which include antioxidant, anti-mutagenic, anti-viral and anti-inflammatory activities [1–4].

Among the phenolic acids, ferulic acid, 3-(4-hydroxy-3-methoxy-phenyl)-acrylic acid, is the most abundant hydroxycinnamic acid in the plant world [5], constituting 5 g kg^{-1} in wheat bran, 9 g kg^{-1} in sugar beet pulp [6,7], 15–28 g kg^{-1} of rice bran oil [8] and 25 g kg^{-1} in corn kernel [9]. It is one of the major phenolic lignin monomers found in woods and grasses, and is widely distributed in cereals, fruits and vegetables [10,11]. Some of these sources are used to provide ferulic acid as a substrate for conversion into value added chemicals such as guaiacol, vanillin, vanillic acid and protocatechuic acid [12]. Ferulic acid is found as the free acid, low molecular weight conjugates, esters with cell wall heteroxylans, or covalently bound to lignin and other biopolymers. Ferulic acid is one of the active ingredients of many Chinese traditional medicines used in the prevention and treatment of various diseases [13–15]. It shows many physiological functions, including antioxidant, antimicrobial, anti-inflammatory, anti-thrombosis, and anti-cancer activities [16,17] and can be easily absorbed and metabolized in the human body [18]. It has been reported to also increase sperm viability, lower cholesterol in serum and liver and protect against angina, hypertensive and coronary diseases [19]. However, it has been most widely investigated for its antioxidant properties which are important in the prevention of lipid oxidation in food and also in the putative prevention of free-radical-induced diseases such as cancer and atherosclerosis, or aging caused by oxidative tissue degeneration [20,21]. Antioxidant properties are ascribed to its structure (Fig. 1) and specifically to its phenolic nucleus coupled to an extended conjugated side chain which facilitate the formation of a resonance-stabilized phenoxy radical [22]. Other important structural properties include electron-donating groups on the benzene ring (3-methoxyl and, more importantly, 4-hydroxyl) which give the additional property of terminating free radical chain reactions; the carboxylic acid group with an adjacent unsaturated C–C double bond which can provide additional attack sites for free radicals thus preventing them from attacking membranes; and the carboxylic acid group which acts as an anchor group which binds it to the lipid bilayer, providing some protection against lipid peroxidation [20]. Despite having these attractive properties, the antioxidant capacity of ferulic acid is generally low compared to conventional antioxidants and other hydroxyl-cinnamic acids [23,24]. Sánchez-Moreno et al. [25] also reported that ferulic acid has a low free radical scavenging activity, but showed higher activity in inhibition of lipid oxidation and in the protection against hydroxyl and peroxyl radical oxidation in synaptosomal and neuronal cell culture systems *in vitro* [26]. Recently there have been a number of attempts to enzymatically modify phenolic molecules as a way of improving antioxidant prop-

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* Corresponding author. Tel.: +27 21 953 8497; fax: +27 21 953 8494.
E-mail addresses: tikudanga@yahoo.co.uk, kudangat@cput.ac.za (T. Kudanga).

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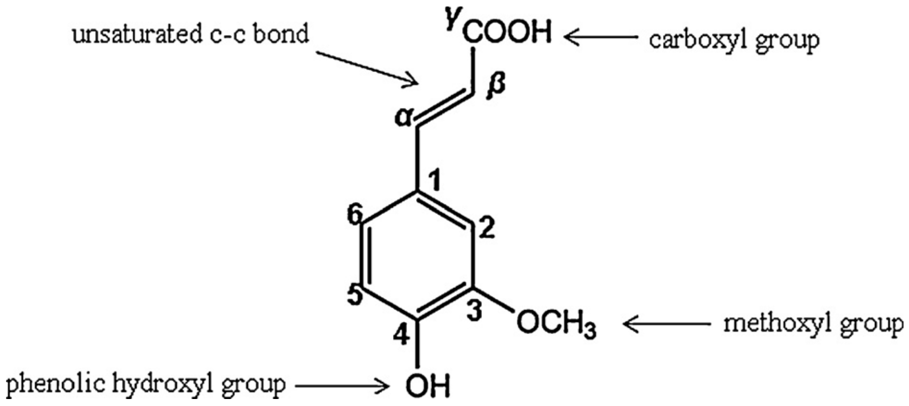

Fig. 1. Structure of ferulic acid showing the important features for antioxidant activity.

erties. Laccases are among the enzymes that are currently being investigated.

Laccases (EC 1.10.3.2) are enzymes that are capable of catalyzing the one-electron oxidation of phenols to produce phenoxy radicals and concomitantly reduce molecular oxygen to water [27]. Oligomerization or polymerisation of the radicals to form oligomers or polymers with higher antioxidant properties than the starting materials has been reported [28–31]. Consequently, research interest in enzymatically transforming ferulic acid is now increasing with some researchers having reported the transformation of ferulic acid by laccase [32–34]. Although possible structures of the oxidation products have been proposed [33], none of the researchers has conclusively elucidated the structures nor reported the antioxidant capacity of the products. This prompted us to investigate the modification of ferulic acid by laccase produced from *Trametes pubescens* (strain CBS 696.94) as a way of enhancing the antioxidant capacity of ferulic acid. To the best of our knowledge, our work for the first time conclusively elucidates the structure of two dimers formed from laccase-mediated oxidation of ferulic acid in organic media, one of which has higher antioxidant capacity than the substrate.

2. Materials and methods

2.1. Chemicals and enzyme

Ferulic acid and other chemicals were purchased from Sigma–Aldrich, South Africa. An airlift reactor was used to grow *T. pubescens* (strain CBS 696.94) for the production of laccase [29] which was purified according to the method of Ryan et al. [35].

2.2. Enzyme activity

Laccase activity was determined spectrophotometrically by monitoring the oxidation of 2,2′-Azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS, $\varepsilon_{420} = 36{,}000\,M^{-1}cm^{-1}$) as the substrate [36]. The reaction mixture contained 0.330 mL ABTS (5 mM), 2.5 mL 0.1 M sodium acetate buffer (pH 5.0) and 0.17 mL laccase. Oxidation of the ABTS was monitored by measuring the increase in absorbance at 420 nm. One unit of laccase activity was defined as the amount of enzyme required to oxidise 1 μmol of ABTS min^{-1} at 25 °C.

2.3. Oxidation of ferulic acid

The oxidation reactions were carried out either in a biphasic system comprising buffer with ethyl acetate as co-solvent or in a monophasic system with miscible solvents (dioxane, methanol, ethanol or acetone) as co-solvents. For the biphasic system the reaction mixture contained ferulic acid (10 mM), laccase (10 U) in 100 mM sodium acetate buffer (pH 5.0) and ethyl acetate at various concentrations (80, 85, 90, 95 and 96%, v/v). For the monophasic system the miscible solvents were used at 80% (previously determined as the optimum for product yield and reduced side reactions). The reactions were carried out for 24 h at 28 °C with shaking on an orbital shaker at 180 rpm. The reactions were monitored by Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC) as described below (Sections 2.4 and 2.5).

2.4. Chromatographic separation of reaction products

TLC analysis was performed on aluminium-backed silica gel 60 F_{254} (Merck) plates using toluene: dioxane: acetic acid (10:2.5:0.2, v/v/v) or ethyl acetate: dioxane: acetic acid (6:0.2:0.05, v/v/v) as the mobile phase. The compounds were then visualized by exposure to UV light at 254 nm.

2.5. High performance liquid chromatography (HPLC)

Prior to HPLC analysis, the enzyme was precipitated out of the reaction solution by the addition of an equal volume of ice-cold methanol (only when miscible solvents were used; in biphasic system, the enzyme was readily separated from the product). The mixture was incubated at 0 °C for 20 min and then centrifuged at 0 °C for 15 min at 14,000 × g. The supernatant (1.5 mL aliquots) was transferred into clean vials and analyzed by HPLC. HPLC analysis was carried out using a Hitachi LaChrom HPLC system from Merk (Merck, Hitachi, Germany). Separation of the reaction products was carried out on a reversed phase LUNA 5 μ PFP(2) 100A, 250 mm × 4.60 mm column under isocratic conditions using acetonitrile: water: acetic acid (25:75:0.1, v/v/v) as the mobile phase at a flow rate of 1 mL min^{-1}, with 1 h running time. The products were detected at 270 nm. Alternatively gradient elution using 0.1% formic acid (solvent A) and acetonitrile (solvent B) was used in order to reduce the running time to 23 min. The gradient set up was as follows: 98% A to 0% A (20 min); 0% A to 98% A (20–21 min); 98% A (21–23 min). Peaks were analyzed using HPLC Manager, Merck Hitachi model D 700 data software.

2.6. Purification of reaction products

The reaction products were purified by Flash Chromatography. The miscible solvents containing products were evaporated using a rotary evaporator and the product extracted with ethyl acetate followed by separation using a separation funnel. The aqueous phase was washed twice and monitored for the absence of product.

The organic phase was dried using a rotary evaporator (Heidolph, Germany). For the biphasic system, the organic phase was separated using a separation funnel and the aqueous phase washed twice with ethyl acetate. The organic phase was evaporated under reduced pressure with a rotary evaporator and the crude residue purified by silica Flash Chromatography using ethyl acetate: dioxane: acetic acid (6:0.2:0.05,v/v/v) as mobile phase for purifying product 1 (P1) or toluene: dioxane: acetic acid (10:2.5:0.2, v/v/v) as mobile phase for purifying product 2 (P2). The pure fractions were dried using a rotary evaporator and the products sequentially washed with acetone, methanol and then acetone again to remove the acetic acid.

2.7. *Characterization of products*

The purified products (P1 and P2) were characterized by mass spectrometry and nuclear magnetic resonance (NMR) analysis.

2.7.1. *Liquid chromatography–mass spectrometry (LC–MS)*

LC–MS was performed on a Dionex HPLC system equipped with a binary solvent manager and autosampler coupled to a Brucker ESI Q-TOF mass spectrometer. The products were separated using the same linear gradient of acetonitrile (solvent B) and 0.1% formic acid (solvent A) as described in Section 2.5 above, at a flow rate of 1 mL min^{-1}, using an injection volume of 10 μL and an oven temperature of 30 °C. MS spectra were acquired in negative mode and electrospray voltage was set to +3500 V. Dry gas flow was set to 9 L min^{-1} with a temperature of 350 °C and nebulizer gas pressure was set to 35 psi.

2.7.2. *Nuclear magnetic resonance (NMR) analysis*

Nuclear magnetic resonance (NMR) spectra were recorded using a VARIAN 200 spectrometer (^{1}H, 200 MHz; ^{13}C, 50 MHz). The spectra were determined at ambient temperature in deuterated chloroform ($CDCl_3$) and methanol solutions, with $CHCl_3$ at δ 7.26 for ^{1}H NMR spectra and chloroform (δ 77.00) for ^{13}C NMR spectra as internal standards. In the NMR spectra, assignments of signals with the same superscripts are interchangeable. Splitting patterns are designated as "s", "d", "t", "q", "m" and "bs". These symbols indicate "singlet", "doublet", "triplet", "quartet", "multiplet" and "broad singlet".

2.8. *Antioxidant activity determination*

2.8.1. *DPPH (2,2′-diphenyl-1-picrylhydrazyl) scavenging effect*

Antioxidant capacity was determined by measuring DPPH radical-scavenging activity [37]. Briefly, 3.9 mL of DPPH dissolved in methanol (0.025 mg/mL) was added to 0.1 mL sample (dissolved in methanol) at various concentrations. The mixture was shaken vigorously and incubated at room temperature in the dark for 60 min, and the decrease in absorbance at 517 nm determined using a spectrometer. The remaining concentration of DPPH in the reaction medium was then calculated from a calibration curve obtained with DPPH at 517 nm.

The percentage of remaining DPPH ($DPPH_R$) was calculated as follows:

$$\%\,\mathrm{DPPH_R} = \left[\frac{(\mathrm{DPPH})_T}{(\mathrm{DPPH})_{T=0}}\right] \times 100$$

where $(\mathrm{DPPH})_T$ is the concentration of DPPH at time 60 min and $(\mathrm{DPPH})_{T=0}$ is the concentration of DPPH at time zero (initial concentration). The percentage of remaining DPPH against the sample/standard concentration was plotted to obtain the amount of antioxidant (mM) necessary to decrease the initial concentration of DPPH by 50% (EC_{50}).

2.8.2. *TEAC (Trolox equivalent antioxidant capacity) assay*

The ABTS radical scavenging activity of ferulic acid and the two products were determined according to the method described by Re et al. [38]. The trolox equivalent antioxidant capacity (TEAC) method is based on the ability of antioxidant molecules to quench $ABTS^{\bullet+}$, a blue–green chromophore with characteristic absorption at 734 nm, compared with that of Trolox, a water soluble vitamin E analog. The addition of antioxidants to the preformed radical cation decolourizes the $ABTS^{\bullet+}$ as it is reduced to ABTS. $ABTS^{\bullet+}$ solution was prepared 12–16 h before use by mixing ABTS salt (7 mM) with potassium persulfate (2.45 mM) and then stored in the dark until the assay was performed. The $ABTS^{\bullet+}$ solution was diluted with methanol to give an absorbance of 0.70 ± 0.002 at 734 nm. Each sample (100 μL) prepared at different concentration was mixed with 1100 μL $ABTS^{\bullet+}$ solution and the absorbance was read after 30 min incubation at 25 °C.

3. Results and discussion

3.1. *Oxidation of ferulic acid in organic solvents*

Biocatalysis is conducted in organic media for a number of reasons, mainly poor solubility of some compounds in aqueous media; ability to carry out new reactions which are impossible in water because of kinetic or thermodynamic restrictions; relative ease of product recovery from organic solvents as compared to water; and the insolubility of enzymes in organic media, which permits their easy recovery and reuse (thus eliminating the need for immobilization) [39,40]. Ferulic acid is not soluble in aqueous media, thus organic solvents were employed for this study in either a monophasic or biphasic system. Both the biphasic system and monophasic systems produced three main oxidation products which were designated as P1 ($t_R = 10.535$); P2 ($t_R = 12.202$); P3 ($t_R = 13.446$) (Fig. 2). LC–MS analysis of the oxidation products in negative mode showed dominant signals at *m/z* 385.1143 (P1) and *m/z* 385.1077 (P2) (Fig. 3) which suggested that these oxidation products were dimers of ferulic acid (exact mass [M] 386.1). When the mass spectrometer was set to run in positive mode $[M+H]^+$ ion signals were observed at *m/z* 387.1, while signals at *m/z* 409.1 indicating Na adducts, confirmed that the molecules were indeed dimers. LC–MS results of the product P3 (*m/z* 579.2154) suggest oligomerization to form a trimer. In addition, a tetramer (*m/z* 769.1948; $t_R = 13.7$) was observed with the more sensitive MS detector (not observed with HPLC). After purification, the yields of P1 and P2 were 11.190% and 38.189%, respectively. Work is currently underway to purify product P3. Significant amounts of insoluble polymeric products were also observed.

The NMR results indicated that P1 is a dimer of two ferulic acid monomers covalently bound through a β-5 linkage while the results for P2 suggest dimerization through a β–β linkage (Fig. 4).

The symmetry of the molecule P2 made assignment easy due to the signals being clear and separate from each other. In the ^{1}H NMR spectrum 2-H appeared as a single peak at δ 3.56 while the methoxy group appeared as a three proton singlet at δ 3.91. The phenolic OH appeared as a single peak at δ 5.86 in close proximity to 3-H which appeared as a single peak at δ 5.69. Coupling between 2-H and 3-H was not observed due to the dihedral angle being in the vicinity of ~90°. The aromatic region displayed a typical pattern of a 1,3,4-trisubstituted system viz., a 2-proton multiplet at δ 6.79 assigned to 2′-H and 6′-H while 5′-H appeared as an *ortho* coupled doublet at δ 6.93 with $J = 8.8$ Hz. In the ^{13}C NMR spectrum all signals could be assigned according to the structure with C2 at δ 48.5, the methoxy group at δ 56.3 and C3 at δ 82.0. Three strong signals at δ 107.6, 115.1 and 117.5 were assigned to C2′, C5′ and C6′ respectively while C1′ appeared at δ 130.0. The more deshielded carbon signals

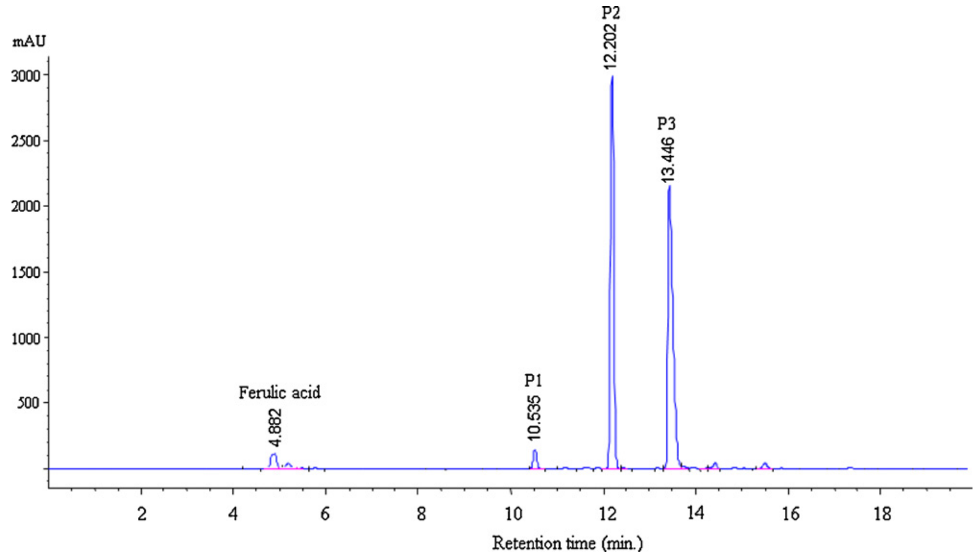

Fig. 2. HPLC chromatogram of products formed during laccase-catalyzed oxidation of ferulic acid. P1, P2 and P3 – oxidation products.

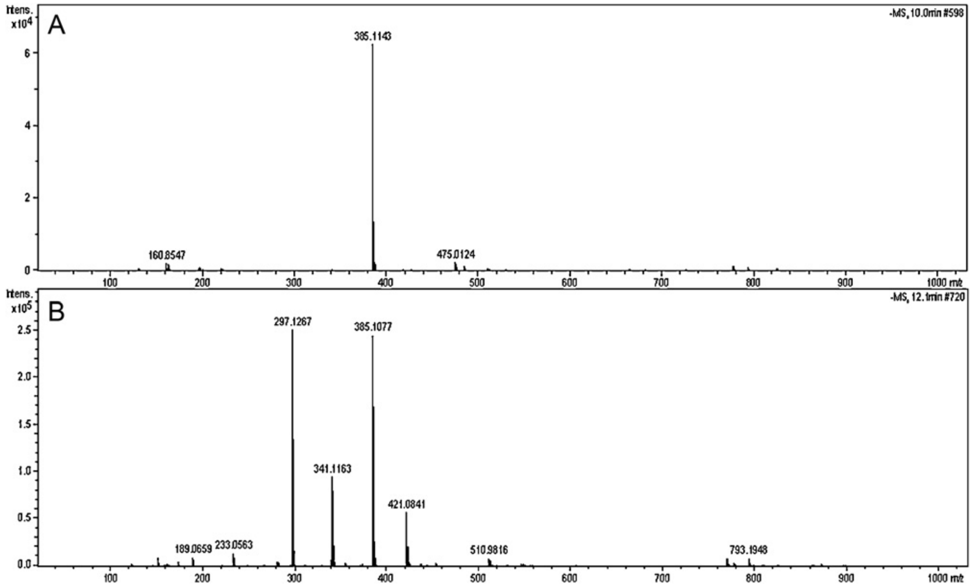

Fig. 3. Mass spectrum of Product P1 (A) and P2 (B) formed during laccase-mediated oxidation of ferulic acid.

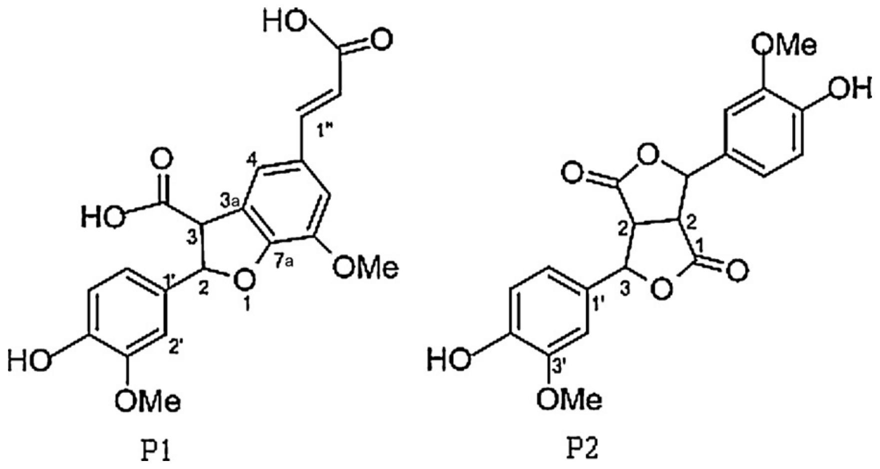

Fig. 4. Dimers (P1 and P2) formed during laccase-catalyzed oxidation of ferulic acid.

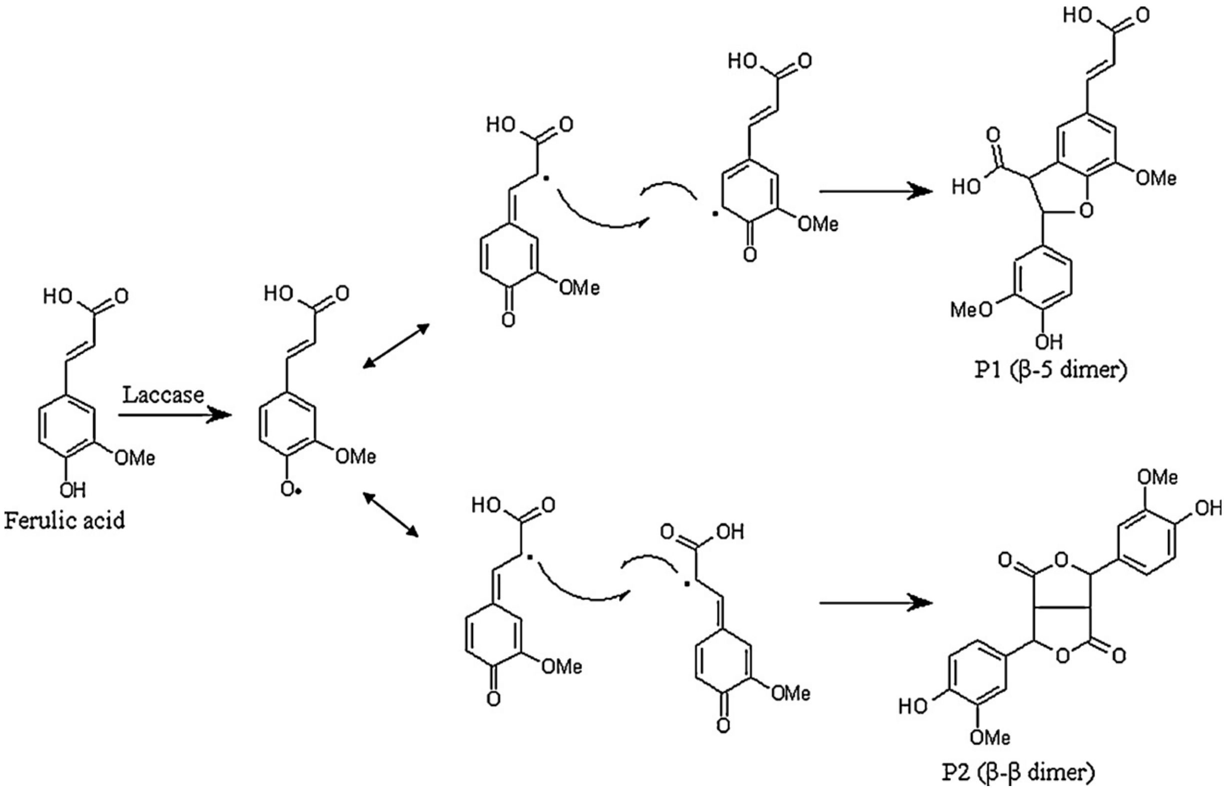

Fig. 5. Laccase-catalyzed oxidation of ferulic acid to produce β–β and β-5 dimers.

of C3′ and C4′ appeared at δ 146.5 and 147.2. Finally the lactone carbonyl appeared as expected at δ 175.0.

As expected the spectra were more complicated for P1 due to a lack of symmetry in the compound and the spectrum was run in deuteromethanol. The two methoxy groups appeared as 3-proton singlets at δ 3.83 and 3.91 while 3-H appeared as a sharp doublet at δ 4.01 (J = 2.6 Hz). A clear doublet at δ 5.61 (J = 2.6 Hz) was assigned to 2-H. A rather broad multiplet centred around δ 6.70 integrated for 5 protons and assigned to 6-H, 5′-H, 1″-H, 2″-H and 4′-OH. A double doublet at δ 7.01 (J = 8.0 and 2.0 Hz) was assigned to 6′-H while the corresponding doublet at δ 7.07 (J = 2.0 Hz) was assigned to 2′-H. A downfield doublet at δ 7.57 (J = 1.8 Hz) was assigned to 4-H. The two COOH hydrogen were not observed due to exchange with protons in the deuteromethanol solvent. The ^{13}C spectrum was assigned as follows: A broadish signal at δ 55.7 is due to the two methoxy carbons as well as C3. The signal at δ 81.2 is assigned to C2. Strong signals in the aromatic region have been assigned as follows: δ 108.3(C2′), 112.7(C8), 115.1(C5′), 118.0(C4), 118.7(C6′). The sp^2 carbons of the ethylene side chain were assigned as follows: δ 115.2(C2″) and 146.3(C1″). The ring junction carbons C3a and C7a were assigned as follows: δ 125.5 and 148.9 respectively. C5 and C1′ were assigned to the signals at δ 125.9 and 131.2 respectively. The deshielded carbon atoms attached to the oxygens were assigned as: C7(δ 140.8), C4′(δ 147.5) and C3′ (δ 148.9). Finally the two carbonyl signals at δ 169.2 and 172.6 were assigned to the two carboxylic acid groups.

Based on the results from LC–MS and NMR, the scheme shown in Fig. 5 is proposed as the possible reaction pathway for the dimerization of ferulic acid to form a β-5 and β–β linkage [33]. As depicted in Fig. 5, laccase initiates the reaction by oxidizing the para-hydroxyl group resulting in a radical. Through resonance stabilization, the unpaired electron can occupy different positions on the radicalized molecule [27]. The radicals are stabilized through coupling with other radicals to form dimers. Therefore coupling of an unpaired electron at the β position with another at position C5 of the next radical yields a β-5 linked dimer. Similarly covalent coupling of radicals both with unpaired electrons at the β position produces the β–β linked dimer. While there were several possibilities for coupling due to the large number of mesomeric forms, it appears that our reaction conditions favored the formation of β-5 and β–β linkages due to the stability of C–C bonds [41] and the lower heat of formation of β-5 linkages [42]. We have observed similar findings in which C–C bonds predominated in the coupling of functional molecules to lignin models, though mostly 5–5 linkages were formed due to the absence of unsaturated side chains [43,44]. In addition, recent reviews agree that in dimerization of a similar molecule, coniferyl alcohol, the radicals favor coupling at their β positions, resulting essentially in only the β–β, β-O-4, and β-5 dimers [45,46] although the β-O-4 are the predominant linkages in polymerization reactions during lignin synthesis [47].

3.2. *Effect of organic solvents*

The effect of the nature of the organic solvent and the concentration of the solvent was studied in order to obtain the best reaction conditions for product formation.

In the biphasic system, there was an increase in the formation of P2 (β–β dimer) as the concentration of ethyl acetate was increased from 80% to 90% after which a decline in product formation was observed (Fig. 6). A similar pattern was observed during the formation of P1 (β-5 dimer) except that a decline was observed after the composition of organic solvent was increased beyond 95% (Fig. 6).

In the monophasic system, four co-solvents namely acetone, dioxane, ethanol and methanol were investigated. As shown in Fig. 7 most of the substrate was converted to products in ethyl acetate: a biphasic system. In the monophasic system, the order of substrate conversion was ethanol > acetone > dioxane > methanol. However, for product formation, β–β dimer formation was

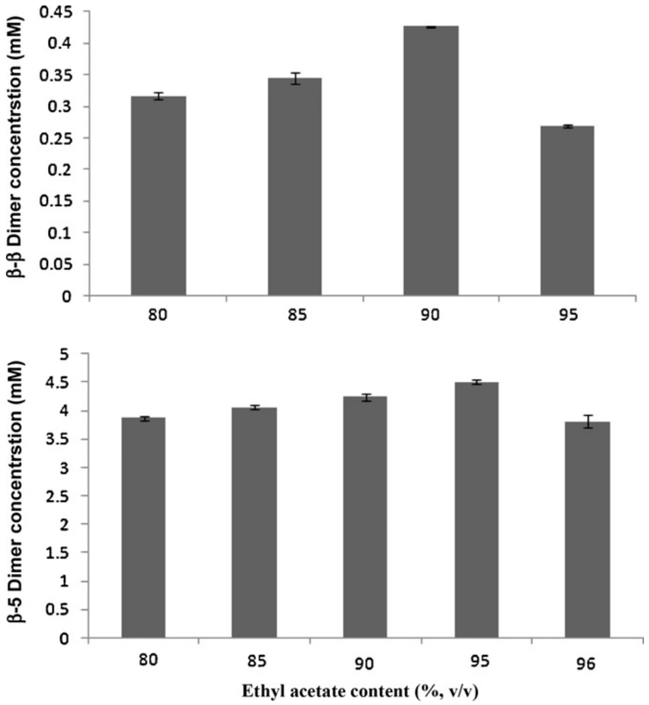

Fig. 6. Effect of ethyl acetate content on the formation of β–β and β-5 dimers in a biphasic system with sodium acetate buffer pH 5.0 as co-solvent. All results are means ± standard deviation (SD) of three replicate determinations.

Table 1
Antioxidant activity of ferulic acid and its dimers.

Molecule	Molecular weight	EC_{50} DPPH[a]	TEAC[b] value
Ferulic acid	194.18	1.837 ± 0.012	0.682 ± 0.005
P1 (β-5 dimer)	386.11	1.310 ± 0.016	0.653 ± 0.006
P2 (β–β dimer)	386.11	2.320 ± 0.057	0.700 ± 0.013

[a] EC_{50} parameter is defined as the concentration (mM) of substrate that brings about 50% loss of the DPPH• [37]. Values are means ± SD of three replicate determinations.

[b] The Trolox equivalent antioxidant activity (TEAC) of the antioxidant is defined as the concentration of antioxidant giving the same percentage inhibition of absorbance of the radical cation ($ABTS^+$) at 734 nm, as 1 mM of Trolox, at specific points [38].

facilitated by the use of ethanol as co-solvent followed by methanol, ethyl acetate, dioxane and acetone. Ethyl acetate, which was the best solvent for combined product formation, promoted the formation of the β-5 dimer and product formation of this product decreased in the order of ethyl acetate > dioxane > methanol > ethanol > acetone. For the water miscible organic solvents employed in this study, solvents with higher polarity index favored the formation of the β–β dimer: ethanol (5.2) > methanol (5.1) > dioxane (4.8). However, the reverse was observed for the β-5 dimer: dioxane (4.8) > methanol (5.1) > ethanol (5.2); only acetone did not follow a particular order for both dimers. The effect of different solvents on enzyme catalysis allows 'medium engineering' in order to modulate and possibly improve, the regioselective outcome of reactions [48]. However, some miscible solvents tended to inactivate the enzyme over time. For example, only 0.12% or 2.8% residual activity was observed after 3 h when methanol or dioxane was used, respectively, which explains the poor substrate conversion in these solvents. Therefore, meticulous optimisation is required in order to convert the substrate quickly within the first few hours; the second phase of the reaction (resonance stabilisation and coupling) is non-enzymatic. In contrast, the enzyme was more stable in ethyl acetate which probably explains the higher conversion of substrate to products. In related work, Ma et al. [49] reported the enhancement of catechol-laccase catalysis in less polar organic solvents as compared with highly polar media, due to the hydrophobicity of the end product. It should be noted that both dimers are less polar than the substrate (ferulic acid) as demonstrated by the retention times which were observed to be longer than that of ferulic acid (Fig. 2).

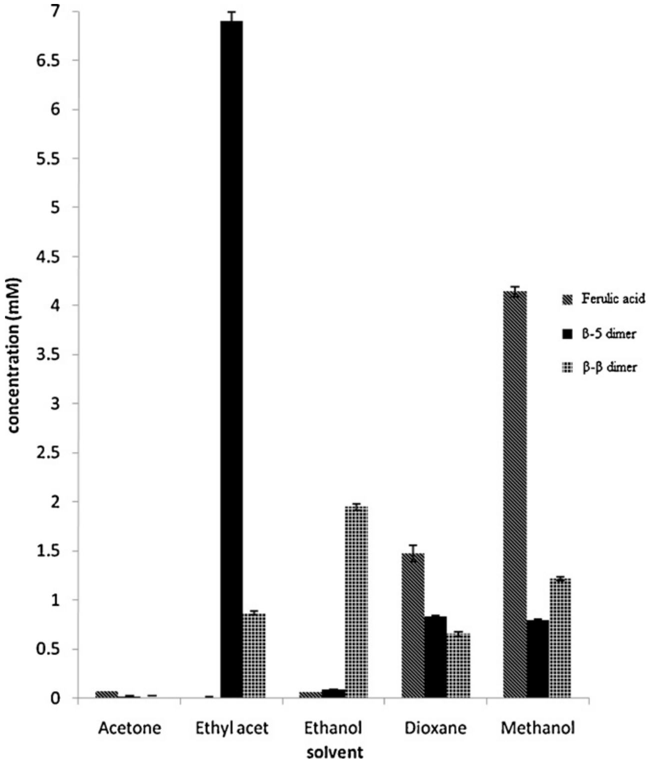

Fig. 7. Effect of organic co-solvent on laccase-catalyzed oxidation of ferulic acid to form β-5 and β–β dimers. All results are means ± SD of three replicate determinations.

3.3. Antioxidant activity determination

Antioxidant activities of the synthesised dimers were evaluated in relation to that of ferulic acid. Interestingly, the DPPH scavenging effect and Trolox equivalent antioxidant capacity (TEAC) of the products showed that the β-5 dimer exhibited higher antioxidant activity than ferulic acid (Table 1). However, the β–β dimer showed lower antioxidant activity than ferulic acid. The antioxidant activity of phenolic compounds depends on various structural features such as the O–H bond dissociation energy, resonance delocalisation of the phenoxyl radical and the steric hindrance due to bulky groups substituting hydrogen in the aromatic ring [50]. The increase in antioxidant capacity of the β-5 dimer could be attributed to an increase in electron donating groups after dimerization [51] and the carboxylic acid group with an adjacent unsaturated C–C double bond which can provide additional attack sites for free radicals [20]. In the β–β dimer, the unsaturated C–C bonds and carboxyl groups are lost during the dimerization process which could explain the reduction in antioxidant capacity.

4. Conclusions

This study has, for the first time, resulted in the elucidation of the structures of two isolated dimers formed during laccase-catalyzed oxidation of ferulic acid in organic media. By altering the nature of the organic solvent used, it was possible to facilitate the formation of the β-5 dimer which is more potent than ferulic acid in terms of antioxidant capacity. This new product may find useful application in the health and cosmetic industries.

Acknowledgements

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