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Mapping the hydrolytic and synthetic selectivity of a type C feruloyl esterase (StFaeC) from *Sporotrichum thermophile* using alkyl ferulates

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Abstract—The active site of *Sporotrichum thermophile* type C feruloyl esterase (StFaeC) was probed using a series of C_1–C_4 alkyl ferulates. The affinities for straight and branched alkyl ferulates were demonstrated by the K_m values of 1.64–0.51 and 0.19–0.1, respectively. Comparison of k_{cat} and k_{cat}/K_m values shows that the enzyme hydrolyzed *n*-propyl ferulate faster and *iso*-propyl ferulate more efficiently. Alkyl ferulates were applied also for substrate selectivity mapping of feruloyl esterase to catalyze feruloyl group transfer to L-arabinose, using as a reaction system a ternary water–organic mixture consisting of *n*-hexane, *t*-butanol and water. Lengthening the aliphatic side chain was the most significant factor causing lower synthetic activity of the enzyme. The reaction parameters affecting the feruloylation rate and the conversion of the enzymatic process, such as the temperature and substrate concentration have been investigated. Under identical reaction conditions, the enzyme feruloylated other monosaccharides such as D-arabinose, D-glucose, D-xylose, D-mannose, D-fructose, D-galactose, D-ribose and model substrates such as 4-nitrophenyl α-L-arabinofuranoside and 4-nitrophenyl α-L-arabinopyranoside.
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1. Introduction

Over the last 10 years, feruloyl esterases (FAE, E.C. 3.1.1.73) responsible for cleaving the ester link between the polysaccharide main chain of xylans and monomeric or dimeric ferulic acid have been purified and partially characterized.[1–6] These enzymes act synergistically with xylanases to hydrolyze ester-linked ferulic acid (FA) from cell wall material.[5–7] Feruloyl esterases have now been classified into four types (A–D) based on their specificity towards mono- and di-ferulates, for substitutions on the phenolic ring, and on their amino acid sequence identity.[8] The nomenclature of feruloyl esterases follows both the source of the enzyme and the type of feruloyl esterases (e.g., the type C feruloyl esterase from *Sporotrichum thermophile* is termed StFaeC).

In spite of numerous reports on esterases and lipases acting as biosynthetic catalysts, enzymatic esterification of phenolic acids has been rarely reported, as in general, enzymatic esterification offers an alternative to the poor selectivity of chemical synthesis.[9] Recently, the potential use of ternary water–organic solvent mixtures,[5,6,10] or oil-in-water microemulsions[11] as a reaction system for the esterification or transesterification of various phenolic acids catalyzed by feruloyl esterases has been reported. The direct esterification of phenolic acids with aliphatic alcohols catalyzed by various lipases in organic media has also been reported, but the reaction rate and yield was low.[12–16]

It has been reported that the synthetic activity pattern of feruloyl esterases for the transesterification of various methyl esters of cinnamic acids is similar to that of their hydrolytic action.[6,10,17] The active sites of feruloyl esterases from mesophilic and thermophilic sources were probed using methyl esters of phenylalkanoic acids.[18] Type B feruloyl esterases were found to be

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Scheme 1. Transesterification of methyl ferulate with L-arabinose.

appropriate biocatalysts for the synthesis of hydroxylated phenolic compounds while type A feruloyl esterases are suitable for the synthesis of methoxylated phenolic compounds. The type C feruloyl esterase StFaeC demonstrated maximum hydrolytic activity against 4-hydroxy-3-methoxy cinnamate, indicating that it may be the most promising type of feruloyl esterase as a biocatalyst for the enzymatic feruloylation of aliphatic alcohols and oligo- or polysaccharides. Recently, it was reported that StFaeC catalyzed the transfer of the feruloyl group to L-arabinose (Scheme 1) in a ternary water–organic mixture consisting of *n*-hexane, *t*-butanol and water system, with about a 40% conversion of L-arabinose to its feruloylated derivative was achieved.[17] This work was the first example of esterification of a sugar with unsaturated arylaliphatic acids like methoxylated or hydroxylated derivatives of cinnamic acids (such as ferulic acid). Lipases are not able to catalyze such a reaction due to an electronic and/or steric effect.[12,16,19]

Phenolic acid sugar esters have demonstratable antitumoric activity and have the potential to be used to formulate antimicrobial, antiviral and/or anti-inflammatory agents.[20–22] As esters based on unsaturated arylaliphatic acids, such as cinnamic acid and its derivatives, are known to display anticancer activity,[19] specific feruloyl esterases could be employed in the tailored synthesis of such pharmaceuticals.

In the present study, factors including reaction temperature, and the type of alkyl ferulates used as feruloyl donors were investigated to evaluate their effect on initial rate and conversion of feruloyl arabinose. Furthermore, the correlation between hydrolytic and synthetic activity of the esterase against different types of straight and branched C_1–C_4 alkyl ferulates has been investigated.

2. Results and discussion

2.1. Choice of solvents

The enzyme synthetic activity was tested in a *n*-hexane/*t*-butanol/water (53.4:43.4:3.2 v/v/v) ternary system monitoring the transesterification of ferulic acid methyl ester with L-arabinose. This condition was found to be the best and the synthetic reaction reached 40% conversion after 160 h.[18]

2.2. Hydrolytic and synthetic specificity of feruloyl esterase for alkyl ferulates

The feruloyl esterase was tested first for activity against a range of alkyl ferulates (Table 1). A series of seven alkyl ferulates were tested as substrates in order to determine the specificity of the feruloyl esterase for straight and branched C_1–C_4 aliphatic chains. The affinities for straight and branched alkyl ferulates were demonstrated by the K_m values of 1.64–0.51 and 0.19–0.1, respectively. The comparison of k_{cat} and k_{cat}/K_m values shows that the enzyme hydrolyzed faster *n*-propyl ferulate and more efficiently *iso*-propyl ferulate.

Comparison of the specificity profiles obtained for transesterification with those for hydrolysis shows notable differences. StFaeC shows strong preference for short chain length alkyl ferulates and especially for methyl ferulate. It has been reported that the synthetic activity pattern of StFaeC for the transesterification of various methyl esters of cinnamic acids is similar to that of their hydrolytic action.[17] The type C feruloyl esterase StFaeC demonstrated maximum hydrolytic activity against ferulate esters. Maintaining the ferulate structure but altering the alkyl substitutions of the carboxyl group the esterase demonstrated maximum synthetic activity against the methyl ester showing the requirement of short alkyl chain length for the effective esterification of L-arabinose. It seems that increasing the steric congestion around the ester group by having a more sterically demanding alkyl group increases both the affinity for the alkyl ferulate and the efficiency of the hydrolysis. This is in agreement with the fact that feruloylated carbohydrates such as 4-nitrophenyl 5-*O*-*trans*-feruloyl-α-L-arabinofuranoside, which is sterically of similar size to those obtained with *iso*-propyl and *iso*-butyl esters hydrolyzed by StFaeC 11 times more efficiently than methyl ferulate.[17] Other feruloyl esterases have been shown to de-esterify ferulic acid at a greater efficiency if it is attached to a sugar than a short alcohol,[23] and the catalytic efficiency of the hydrolysis of 5,5′-diferulic acid by AnFaeA was 20-fold greater when the diferulate was ester linked to sugars than ethyl groups.[24,25]

2.3. Effect of temperature

In equilibrium-controlled synthesis, the reaction temperature is the most critical factor to determine the conversion. As shown in Figure 1, the initial rates increased with the increase of temperature up to 45 °C, while

Table 1. Specificity of feruloyl esterase (StFaeC) for alkyl ferulates

Structure	Name	Hydrolysis		Transesterification conversion (%)
	Methyl ferulate	K_m k_{cat} k_{cat}/K_m	1.64 (0.10) 156 (5) 95 (7)	30.0
	Ethyl ferulate	K_m k_{cat} k_{cat}/K_m	0.51 (0.05) 88 (4) 171 (13)	6.3
	n-Propyl ferulate	K_m k_{cat} k_{cat}/K_m	0.51 (0.04) 326 (12) 636 (59)	3.8
	n-Butyl ferulate	K_m k_{cat} k_{cat}/K_m	0.73 (0.05) 260 (22) 357 (23)	3.4
	iso-Propyl ferulate	K_m k_{cat} k_{cat}/K_m	0.1 (0.01) 99 (2) 1016 (96)	3.3
	2-Butyl ferulate	K_m k_{cat} k_{cat}/K_m	0.16 (0.01) 53 (1) 328 (24)	2.7
	iso-Butyl ferulate	K_m k_{cat} k_{cat}/K_m	0.19 (0.01) 96 (2) 450 (34)	4.2

Kinetic constants (K_m and k_{cat}) were determined using GraFit program module, which also gives an estimate of the standard error (numbers in parentheses). K_m is expressed as mM and k_{cat} as kat/mol enzyme.

optimum conversion was achieved at 35 °C. The esterase exhibits highest hydrolytic activity around 55 °C, but at temperatures above 45 °C enzyme deactivation also takes place after 6 h of incubation Figure 2.

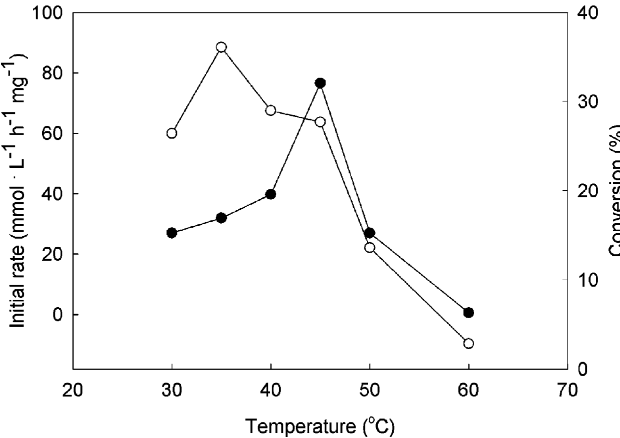

Figure 1. Effect of temperature on initial rate (●) and conversion (○). The reactions were performed in *n*-hexane/*t*-butanol/water (53.4:43.4:3.2 v/v/v) ternary system using 100 mM methyl ferulate, 30 mM L-arabinose and 0.037 nM enzyme for five days.

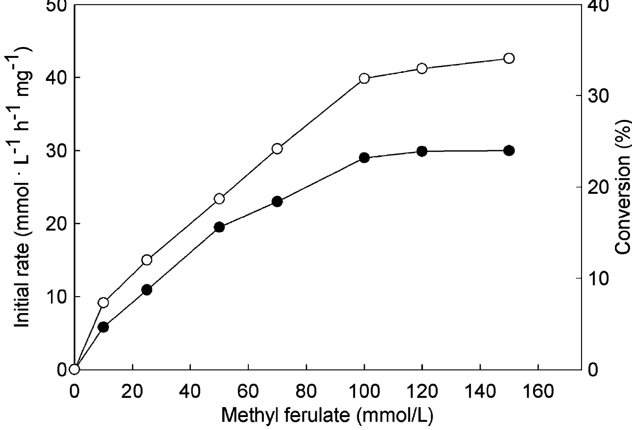

Figure 3. Effect of methyl ferulate concentration on initial rate (●) and conversion (○). The reactions were performed in *n*-hexane/*t*-butanol/water (53.4:43.4:3.2 v/v/v) ternary system, 30 mM L-arabinose and 0.037 nM enzyme at 35 °C for five days.

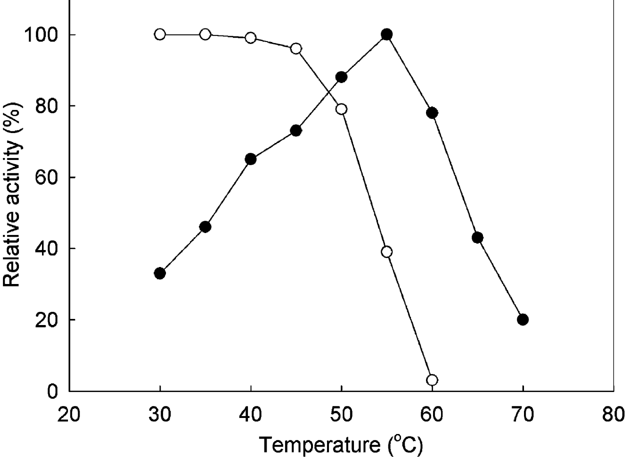

Figure 2. Effect of temperature on hydrolytic activity (●) and stability (○). For stability, the enzyme solution in pH 6 was incubated for 6 h at various temperatures, and then the residual enzyme activities were assayed. For activity, the enzyme activity was assayed at various temperatures by the standard assay method.

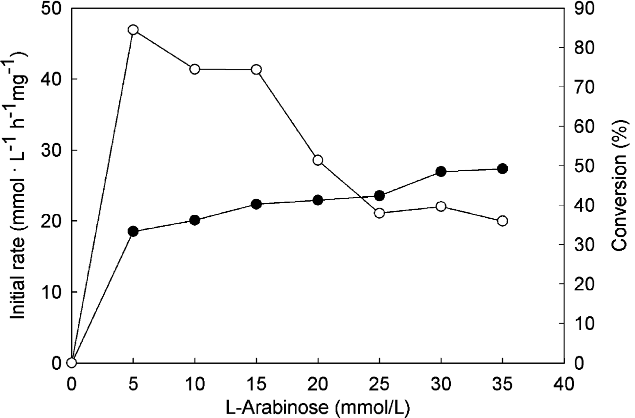

Figure 4. Effect of L-arabinose concentration on initial rate (●) and conversion (○). The reactions were performed in *n*-hexane/*t*-butanol/water (53.4:43.4:3.2 v/v/v) ternary system using 100 mM methyl ferulate, and 0.037 nM enzyme at 35 °C for five days.

2.4. Effect of substrate concentration on the transesterification reaction

Under the reaction conditions (35 °C, esterase 0.037 nM; methyl ferulate as feruloyl donor) the effect of L-arabinose and methyl ferulate concentration on the L-arabinose feruloylation rate was studied. The dependence of the initial rates (Figs. 3 and 4) as a function of the substrate concentrations were used to calculate apparent K_m values for both substrates. As it can be seen from Figures 2 and 3 the feruloylation reaction follows Michaelis–Menten kinetics. The calculated apparent K_m and V_{max} values for L-arabinose and methyl ferulate are: $K_{m\ (\text{L-arabinose})} = 33.2$ mM, $K_{m\ (\text{methyl ferulate})} = 68.9$ mM, $V_{max\ (\text{L-arabinose})} = 28.2$ mmol $L^{-1}\ h^{-1}\ mg^{-1}$, $V_{max\ (\text{methyl ferulate})} = 45.5$ mmol $L^{-1}\ h^{-1}\ mg^{-1}$.

2.5. Structural characterization of feruloyl transfer products on L-arabinose

The feruloylated L-arabinose formed was isolated by preparative TLC and identified by ^1H NMR. The chemical shifts were similar to those published for 5-*O*-(*trans*-feruloyl)-L-arabinofuranose isolated from wild rice (*Zizania aquatica*).[26] In agreement with the assigned structure, FTIR analysis of the purified product indicated the presence of an ester band at 1725 cm^{-1} and analysis by electrospray mass spectrometry operating in the negative ion mode gave an [M−1] peak at *m*/*z* 325.

2.6. Feruloylation of other carbohydrates

Results of screening for different carbohydrate feruloyl acceptors showed that the enzyme had broad acceptor specificity (Fig. 5). The saccharides having either a pyranose or a furanose ring in their chemical structure are

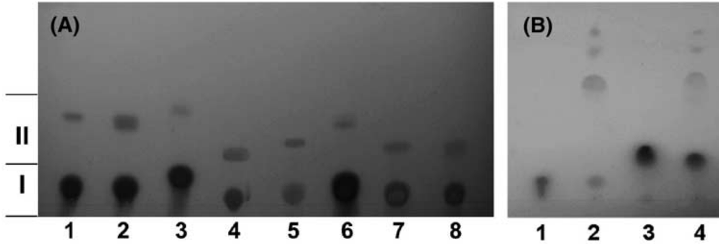

Figure 5. *S. thermophile* feruloyl esterase StFAEC catalyzed feruloylation of various monosaccharides (A) and *p*-nitrophenyl glycosides of arabinose (B) in *n*-hexane/*t*-butanol/water (53.4:43.4:3.2 v/v/v) using methyl ferulate as feruloyl donor. (A) Monosaccharides after five days incubation with 0.037 nM StFAEC: L-arabinose (1); D-arabinose (2); D-glucose (3); D-xylose (4); D-mannose (5); D-fructose (6); D-galactose (7); D-ribose (8). (I) Carbohydrates, (II) feruloylated carbohydrates. (B) *p*-Nitrophenyl arabinopyranoside (1); *p*-nitrophenyl arabinopyranoside after five days incubation with 0.037 nM StFAEC (2); *p*-nitrophenyl arabinofuranoside (3); *p*-nitrophenyl arabinofuranoside after five days incubation with 0.037 nM StFAEC (4); sugar components were detected on dried chromatograms by the aniline–hydrogen phthalate reagent (A) and ethyl acetate–benzene–*iso*-propanol reagent (B).

acceptors and this was further confirmed by the feruloylation of both 4-nitrophenyl α-L-arabinofuranoside and 4-nitrophenyl α-L-arabinopyranoside. Furthermore, stereoselectivity for feruloylation of D- and L-enantiomers of arabinose was not observed. A protease from *Streptomyces* sp. also esterified D- and L-enantiomers of arabinose to synthesize vinyl arabinose esters but enzymatic transesterification proceeded without transesterification of arabinopyranose.[27] It was reported that oligosaccharides containing C-5 modified D-arabinofuranosyl residues such as the D-feruloylated arabinose, are of interest as potential inhibitors of the α-(1→5)-arabinosyltransferase involved in the assembly of mycobacterial cell-wall arabinan.[28,29] Mycobacterial infections and most notably tuberculosis has long been a cause of morbidity and mortality worldwide. Over the past two decades, there has been an increased interest in developing new drugs to fight this deadly disease.

From these results, the esterification of a broader range of carbohydrates seems reasonable and feruloyl esterases could be employed in the tailored synthesis of phenolic sugar esters. There has been a recent report on starch chemically esterified with ferulic acid (starch ferulate).[30] This polymer showed lower viscosity, higher water-holding capacity and much less retrogradation during low temperature storage compared to native maize starch. The authors found that starch ferulate served as a better source of ferulic acid than wheat bran (dietary fibre) in the colon, where it was degraded by microbiotica. The potential of using feruloyl esterases for the synthesis of feruloylated oligomers or polymers opens the door to design probiotics and modified biopolymers with altered properties and bioactivities.

3. Experimental

3.1. Enzyme

The esterase described here was purified to homogeneity from culture supernatants of *S. thermophile* grown on wheat straw, as described previously.[17] The esterase activities were assayed using methyl esters of various alkyl ferulates (Table 1) and the release of ferulic acid measured by HPLC as previously described.[18] All assays were prepared and analyzed in duplicate, with <10% standard error for each set of results. The amount of free acid released was quantified against standard curves. One unit of activity (1 U) is defined as the amount of enzyme (mg) releasing 1 μmol of free acid per minute under the defined conditions. The relative molecular mass for the feruloyl esterase StFaeC was 46 kDa (homodimers of 23 kDa).[17] Kinetic constants (k_{cat}, K_m) were determined from Michaelis–Menten equation, using Lineweaver–Burk double reciprocal plots. Values were estimated using a non-linear regression model (GraFit) that also gives an estimate of the standard error of each parameter.[31]

The effect of temperature on enzyme hydrolytic activity and stability was measured on methyl ferulate (MFA; 2 mM) using HPLC for detection of FA. The optimum temperature was determined by assaying the enzyme activity at various temperatures (30–70 °C) for 15 min in 0.1 M MOPS buffer, pH 6.0. The thermostability was determined by measuring the residual activity at 50 °C and pH 6.0, after incubation of the purified esterase between 30 and 70 °C and pH 6.0, for 6 h.

3.2. Reaction solution

The surfactantless microemulsions were prepared by mixing *n*-hexane, *t*-butanol and 20 mM buffer piperazine–HCl pH 6.0 in the required volumes, followed by vigorous shaking for several seconds until a stable transparent solution was obtained. Alkyl ferulates were diluted in the mixture of *n*-hexane and *t*-butanol. Enzyme, L-arabinose and other carbohydrates were introduced in the form of concentrated stock solution in buffer. Enzymic transesterification was carried out in sealed flask at different temperatures without stirring.

Aliquots (5 μL) of reaction mixtures were spotted on aluminium sheets coated with Silica gel 60 (Merck, Germany). The solvent system that was used for the resolution of the feruloylated product formed by the transesterification of purified StFaeC was chloroform/methanol/water (65:15:2, v/v/v). TLC plates were visualized under a UV lamp. Sugar components were detected

on dried chromatograms by the aniline–hydrogen phthalate reagent.[32] Transesterification of 4-nitrophenyl α-L-arabinofuranoside and 4-nitrophenyl α-L-arabinopyranoside was examined in the solvent system ethyl acetate/benzene/*iso*-propanol (2:1:0.1, v/v/v). TLC plates were visualized under a UV lamp. Sugar components were detected on dried chromatograms by *N*-(1-naphthyl)ethylenediamine dihydrochloride reagent.[33]

Qualitative analysis of samples was made by HPLC on a C_{18} Nucleosil column (250 mm × 4.6 mm) (Macherey Nagel, Dren, Germany). Detection was achieved by a Jasco UV-975 detector set at 300 nm based on calibration curve prepared using standard solutions of the product in water/methanol 50:50 (v/v). The reaction mixture (100 μL), was diluted with a solution of water/methanol 50:50 (v/v) (100–1000 μL) before analysis. Elution was conducted with acetonitrile/water/formic acid (1.5:7:1) as mobile phase at a flow rate of 1.0 mL min^{-1} and at ambient temperature. Yields for the synthesis of phenolic sugar ester were calculated from the amount of L-arabinose having reacted compared to the initial quantity of the sugar. No L-arabinose consumption was observed in the absence of esterase preparation.

The enzymatic synthetic reactions were performed with 0.037 nM StFaeC.

3.3. Structural characterization of phenolic sugar ester

Proton NMR spectroscopy was performed in $CDCl_3$ with a Bruker DRX 400 spectrometer, equipped with a 5 mm $^1H/^{13}C$ dual inverse broad probe at 400.13 MHz.

Mass spectrometry was done using an Agilent 1100 MSD ion trap. The L-arabinose and the reaction product were dissolved in 50/50 methanol/water with 0.2% formic acid. The mass spectrum of L-arabinose is obtained in positive mode and the mass spectrum of the reaction product is collected in negative mode.

FTIR (Nicolet Magna-IR 560) analysis of reaction product was performed in order to detect the ester bond. FTIR spectra were obtained at 4 cm^{-1} resolution with 200 scans. The spectrometer was equipped with a DTGS KBr detector and a DRIFT accessory. The solid samples used for the FTIR-DRIFT studies were compression-moulded with KBr powder.

3.4. General procedure for the synthesis of alkyl ferulates

Except for methyl ferulate, which was purchased from Apin Chemicals Ltd (Abingdon, UK), a solution of ferulic acid (10.0 g, 0.05 mol) in the appropriate alcohol (ethanol, *n*-propanol, *iso*-propanol, *n*-butanol, *iso*-butanol, 2-butanol, 200 mL) in a 500 mL round-bottomed flask was flushed with nitrogen and immersed in a waterbath at room temperature. Acetyl chloride (40 mL, 0.5 mol) was added slowly under nitrogen and the reaction stirred for a further 16 h at ambient temperature.

Reverse phase TLC of the alkyl ferulates was performed using RP-18 F_{254} silica on aluminium backing (Merck), developed using mixtures of methanol and water containing 0.1% H_3PO_4 and visualized using UV fluorescence quenching at 254 nm.

The alcohol was removed under reduced pressure. The product was dissolved in dichloromethane (120 mL), washed with saturated aqueous $NaHCO_3$ (3 × 150 mL) and saturated aqueous NaCl (150 mL), dried ($MgSO_4$) and concentrated under reduced pressure to afford the crude ester (95–97%). The esters were recrystallized from ethyl acetate/hexanes. Proton and ^{13}C NMR spectroscopy was done in $CDCl_3$ with a Bruker DRX 400 spectrometer, equipped with a 5 mm $^1H/^{13}C$ dual inverse broad probe at 400.13 MHz (^1H) and 100.62 MHz (^{13}C).

3.4.1. Ethyl ferulate. ^1H NMR, δ, ppm: 1.32 (3H, t, $J = 7.0$ Hz, CH_2CH_3), 3.91 (3H, s, OCH_3), 4.25 (2H, q, $J = 7.0$ Hz, CH_2CH_3), 5.93 (1H, s, OH), 6.27 (1H, d, $J = 15.9$ Hz, CHCHCOO), 6.89–7.07 (3H, m, aromatic), 7.60 (1H, d, $J = 15.9$ Hz, CHCHCOO). ^{13}C NMR, δ, ppm: 14.4 (CH_2CH_3), 55.9 (OCH_3), 60.3 (CH_2CH_3), 109.3–147.9 (aromatic and CHCHCOO), 167.3 (CHCHCOO).

3.4.2. *n*-Propyl ferulate. ^1H NMR, δ, ppm: 0.99 (3H, t, $J = 7.4$ Hz, $CH_2CH_2CH_3$), 1.72 (2H, m, $CH_2CH_2CH_3$), 3.89 (3H, s, OCH_3), 4.15 (2H, t, $J = 6.7$ Hz, $CH_2CH_2CH_3$), 6.14 (1H, s, OH), 6.29 (1H, d, $J = 15.9$ Hz, CHCHCOO), 6.90–7.06 (3H, m, aromatic), 7.60 (1H, d, $J = 15.9$ Hz, CHCHCOO). ^{13}C NMR, δ, ppm: 10.8 ($CH_2CH_2CH_3$), 22.4 ($CH_2CH_2CH_3$), 56.2 (OCH_3), 66.4 ($CH_2CH_2CH_3$) 109.8–148.3 (aromatic and CHCHCOO), 167.8 (CHCHCOO).

3.4.3. *iso*-Propyl ferulate. ^1H NMR, δ, ppm: 1.29 (6H, d, $J = 6.1$ Hz, $CH(CH_3)_2$), 3.86 (3H, s, OCH_3), 5.12 (1H, septet, $J = 6.1$ Hz, $CH(CH_3)_2$), 6.25 (1H, d, $J = 16.0$ Hz, CHCHCOO), 6.29 (1H, s, OH), 6.88–7.04 (3H, m, aromatic), 7.58 (1H, d, $J = 16.0$ Hz, CHCHCOO). ^{13}C NMR, δ, ppm: 22.3 ($CH(CH_3)_2$), 56.2 (OCH_3), 68.0 ($CH(CH_3)_2$), 109.8–148.3 (aromatic and CHCHCOO), 167.3 (CHCHCOO).

3.4.4. *n*-Butyl ferulate. ^1H NMR, δ, ppm: 0.94 (3H, t, $J = 7.4$ Hz, $CH_2CH_2CH_2CH_3$), 1.40 (2H, m, $CH_2CH_2CH_2CH_3$), 1.65 (2H, m, $CH_2CH_2CH_2CH_3$), 3.88 (3H, s, OCH_3), 4.18 (2H, t, $J = 6.7$ Hz, $CH_2CH_2CH_2CH_3$), 6.23 (1H, s, OH), 6.27 (1H, d, $J = 15.9$ Hz, CHCHCOO), 6.88–7.05 (3H, m, aromatic), 7.59 (1H, d, $J = 15.9$ Hz, CHCHCOO). ^{13}C NMR, δ, ppm: 14.1 ($CH_2CH_2CH_2CH_3$), 19.4 ($CH_2CH_2CH_2CH_3$), 31.0 ($CH_2CH_2CH_2CH_3$), 56.2 (OCH_3), 64.7 ($CH_2CH_2CH_2CH_3$) 109.8–148.4 (aromatic and CHCHCOO), 167.9 (CHCHCOO).

3.4.5. *iso*-Butyl ferulate. ^1H NMR, δ, ppm: 0.97 (6H, d, $J = 6.7$ Hz, $CH_2CH(CH_3)_2$), 2.00 (1H, m, $CH_2CH(CH_3)_2$), 3.91 (3H, s, OCH_3), 3.98 (2H, d, $J = 6.7$ Hz, $CH_2CH(CH_3)_2$), 6.12 (1H, s, OH), 6.30 (1H, d, $J = 15.9$ Hz, CHCHCOO), 6.90–7.07 (3H, m, aromatic), 7.61 (1H, d, $J = 15.9$ Hz, CHCHCOO). ^{13}C NMR, δ, ppm: 19.5 ($CH_2CH(CH_3)_2$), 28.1 ($CH_2CH(CH_3)_2$), 56.3 (OCH_3), 71.0 ($CH_2CH(CH_3)_2$)

108.9–148.3 (aromatic and *C*H*C*HCOO), 167.8 (CHCH*C*OO).

3.4.6. Butyl ferulate. ^{1}H NMR, δ, ppm: 0.92 (3H, t, $J = 7.5$ Hz, $CH(CH_3)CH_2CH_3$), 1.25 (3H, d, $J = 6.3$ Hz, $CH(CH_3)CH_2CH_3$), 1.60 (2H, m, $CH(CH_3)CH_2CH_3$), 3.86 (3H, s, OCH_3), 4.95 (1H, m, $CH(CH_3)CH_2CH_3$), 6.26 (1H, d, $J = 15.9$ Hz, CHC*H*COO), 6.32 (1H, s, O*H*), 6.87–7.04 (3H, m, aromatic), 7.57 (1H, d, $J = 15.9$ Hz, C*H*CHCOO). ^{13}C NMR, δ, ppm: 10.1 ($CH(CH_3)CH_2CH_3$), 19.9 ($CH(CH_3)CH_2CH_3$), 29.3 ($CH(CH_3)CH_2CH_3$), 56.2 (OCH_3), 72.5 ($CH(CH_3)CH_2CH_3$) 109.8–148.3 (aromatic and *C*H*C*HCOO), 167.5 (CHCH*C*OO).

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