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ORIGINAL ARTICLE

Lipase-catalyzed preparation of mono- and diesters of ferulic acid

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Abstract

Lipophilic and stable derivatives of ferulic acid are required to improve its efficacy in fatty foods and to optimize its use in cosmetic and pharmaceutical preparations. We report an improved synthesis of ferulic acid monoesters (ethyl ferulate and lauryl ferulate) using immobilized lipase from *Candida antarctica* B (CALB) in diisopropyl ether (DIPE). Maximum yields were 89% and 85% in 200 h for ethyl and lauryl ferulate, respectively. Ethyl ferulate was further acylated with vinyl esters to form ferulate diesters. 4-Acetoxy-ethyl ferulate was obtained with the immobilized lipase from *Alcaligenes* sp. (QLG) with 59% yield in 72 h, whereas 4-dodecanoyloxy-ethyl ferulate (a new compound) was synthesized with 52% yield in 72 h using CALB. DIPE was the best solvent for the transesterifications. Finally, the anti-inflammatory activity of the synthesized derivatives was evaluated *in vitro*; the compounds bearing a dodecyl chain showed improved anti-inflammatory activity compared with short-chain esters.

Keywords: *Ethyl ferulate, ferulic acid, food antioxidants, hydroxycinnamic acids, lipases, transesterification*

Introduction

Ferulic acid (4-hydroxy-3-methoxycinnamic acid, FA, **1**) is a dietary antioxidant found in the cell walls of many plants (Barberousse et al. 2008). There is growing interest in using FA as a bioactive ingredient in functional foods, nutraceuticals, and pharmaceutical and cosmetic products due to its abundance in agro-industrial wastes and to its biological properties—as antioxidant, antimicrobial, antiviral, anti-inflammatory, antiallergenic, and antitumor—- and for protection against UVA radiation (Srinivasan et al. 2007; Zhao & Moghadasian 2008). However, several limitations for its industrial application arise from its low solubility in organic media and its limited stability to oxidation.

One approach to overcome this is to synthesize more lipophilic and stable FA derivatives. In fatty foods, lipophilization of phenolic acids can modulate their localization in emulsions and, as a consequence, their antioxidant efficiency (Sorensen et al. 2014). Lipophilic ferulates also have great potential for use as UV-blocking agents in waterproof sunscreens (Compton et al. 2000). Ethyl ferulate (EF, **2**) is among the most studied derivatives, because it can be used as a synthon for the preparation of other FA derivatives. In addition, EF is a promising neuroprotector agent (Joshi et al. 2006; Sultana 2012), possesses anti-platelet aggregation properties (Li et al. 2009a), and has great potential as an ingredient in foods and cosmetics (Choquenet et al. 2008; Souto et al. 2005). Lauryl ferulate (LF, **3**) displays high hydrophobicity (log P 6.97) which makes it very suitable as a cosmetic ingredient (Voisin-Chiret et al. 2007). Moreover, LF displays interesting biological properties, for example antitumor.

In order to obtain such lipophilic derivatives, a biocatalytic approach is preferred over classical chemical synthesis (Gonzalez-Sabin et al. 2011), especially for food use, since phenolic acids are very sensitive to harsh reaction conditions such as high

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temperatures and extreme pH values (Munin & Edwards-Levy 2011). The esterification or transesterification of FA catalyzed by lipases is the preferred route, as lipases are versatile biocatalysts with a wide substrate acceptance and notable stability in several organic solvents (Casas-Godoy et al. 2012; Ferreira-Dias et al. 2013; Garcia-Liñares et al. 2012). However, the reported conditions to derivatize FA are not industrially economic, due to the low conversion obtained (Compton et al. 2000; Katsoura et al. 2009b), the prolonged reaction times, and the large amounts of biocatalyst needed (Weitkamp et al. 2006).

In this work, we have tried to optimize the enzymatic preparation of FA esters by appropriate selection of biocatalyst, solvent, and other experimental parameters. The synthesized monoesters were further acylated with vinyl esters in order to obtain FA diesters with significantly increased hydrophobicity, which could benefit their performance in food, pharmaceutical, and cosmetic applications.

Materials and methods

Reagents and biocatalysts

Vinyl acetate, vinyl laurate, and FA were purchased from Sigma-Aldrich. Immobilized lipase from *Candida antarctica* B (CALB, Novozym® 435, 7400 PLU/g) was kindly donated by Novozymes A/S. Immobilized lipase from *Alcaligenes* sp. (QLG, 20000 U/g) was obtained from Meito Sangyo Co. (Japan). All other reagents were of the highest available purity and were used as purchased, except for organic solvents that were dried over 3-Å molecular sieves.

Enzymatic esterification of ferulic acid

To a solution of FA (10 mg/mL, 51 mM) in the indicated solvent, alcohol (ethanol or dodecyl alcohol) and the immobilized lipase were added at different ratios. The total volume was 1 mL. The mixtures were incubated at 60°C in sealed 4-mL dark vials in an orbital incubator SI50 (Stuart Scientific) at 250 rpm. Aliquots (2 μL) were withdrawn at intervals and the progress of the reaction was analyzed by thin-layer chromatography (TLC). For the quantification of FA esters, the reactions were stopped by filtering the mixture with centrifuge tubes containing a polyvinylidene difluoride (PVDF) Durapore 0.45-μm membrane (Millipore) and the supernatants were analyzed by high-performance liquid chromatography (HPLC). In order to chemically characterize the reaction products, the reactions were scaled-up to 10 mL. The solid biocatalyst was filtered and washed with the reaction solvent (3 × 5 mL) to extract the adsorbed FA esters. The solvent was then removed under reduced pressure and the crude residue was purified by column chromatography on a column (1.5 × 30 cm) packed with silica gel 60 (230–400 mesh, Merck) using hexane:ethyl acetate 8:2 (v/v) as eluent.

Enzymatic acylation of ethyl ferulate

To a solution of EF (10 mg/mL, 44 mM) in the indicated solvent, the acyl donor (vinyl acetate or vinyl laurate) and the immobilized lipase were added at different ratios. The total volume was 1 mL. The mixtures were incubated at 60°C in sealed 4-mL dark vials at 250 rpm with orbital shaking. Aliquots (2 μL) were withdrawn at intervals and the progress of the reaction was analyzed by TLC. For the quantification of FA esters, the reactions were stopped by filtering the mixture with centrifuge tubes containing a PVDF Durapore 0.45-μm membrane and the supernatants were analyzed by HPLC. In order to chemically characterize the reaction products, the reactions were scaled-up to 10 mL. The solid biocatalyst was filtered and washed with the reaction solvent (3 × 5 mL) to extract the adsorbed FA diesters. The solvent was then removed under reduced pressure and the crude residue was purified by column chromatography on a column (1.5 × 30 cm) packed with silica gel using hexane:ethyl acetate 8:2 (v/v) as eluent.

Analytical procedure

Reactions were followed by TLC on Silica gel 60F254 aluminum sheets (0.2 mm thickness, Merck) in hexane/ethyl acetate 6:4 (v/v) with UV light (254 nm) for detection. HPLC analysis was performed using a ternary pump (model 9012, Varian) coupled to a thermostated (25°C) autosampler (model L-2200, Hitachi). The temperature of the column was kept constant at 40°C (MEF-01 oven, Analisis Vinicos, Spain). The column was a Gemini C6-Phenyl 110 Å (4.6 × 150 mm, 5 μm, Phenomenex), and the mobile phase was methanol/H_2O 60:40 (v/v) (H_2O contained 0.1% v/v of acetic acid) at 1 mL/min. Detection was performed with a photodiode array detector (ProStar, Varian), and integration was carried out using the Varian Star LC Workstation 6.41. FA and their esters were quantified at 310 nm using the purified compounds as standards. Melting points were measured in a Fisher-Johns apparatus and are uncorrected. Fourier transform infrared spectroscopy (FTIR) spectra were obtained on a Shimadzu FTIR-8300 spectrophotometer. ^{1}H nuclear magnetic

resonance (^{1}H NMR) and ^{13}C NMR spectra were recorded in $CDCl_3$ as solvent using a Bruker AM-500 NMR instrument operating at 500.1 MHz and 125.8 MHz for ^{1}H and ^{13}C, respectively. Chemical shifts are reported in δ units (ppm) relative to tetramethylsilane set at 0 ppm, and coupling constants are given in Hz. Mass spectrometry (MS) analysis was carried out using a mass spectrometer (model QSTAR pulsar, Applied Biosystems) with a hybrid quadrupole time-of-flight or QTOF analyzer. Ionization was performed by electrospray in positive mode. High-resolution MS or HRMS was recorded with a Thermo Scientific EM/DSQ II-DIP spectrometer. The results are within ±0.02% of the theoretical values.

Ethyl-(E)-3-(4-hydroxy-3-methoxyphenyl)-prop-2-enoate (2)

Yield 93%, white solid, melting point 39–41°C (lit. mp, 39°C) (Nonnenmacher et al. 1983). FT-IR (film) ν_{max} = 3403, 2980, 1698, 1633, 1593, 1515 cm^{-1}.^{1}H NMR: δ 1.33 (t, $\mathcal{J}$ = 7.0 Hz, 3H); 3.93 (s, 3H); 4.27 (c, $\mathcal{J}$ = 7.0 Hz, 2H); 6.29 (d, $\mathcal{J}$ = 16.0 Hz, 1H); 6.96 (d, $\mathcal{J}$ = 10.0 Hz, 1H); 7.08 (d, $\mathcal{J}$ = 2.0 Hz, 1H); 7.12 (dd, $\mathcal{J}$ = 10.0; 2.0 Hz, 1H);7.61 (d, $\mathcal{J}$ = 16.0 Hz, 1H). ^{13}C NMR: δ 14.4; 55.9; 60.4; 109.3; 114.7; 115,7; 123.0; 127.0; 144.6; 146.7; 147.9; 167.3. HR-ESI-MS: $[M + H]^+$ = 223.0954; $[M + Na]^+$ = 245.0806.

Dodecyl-(E)-3-(4-hydroxy-3-methoxyphenyl)-prop-2-enoate (3)

Yield 85%, white solid, melting point 36–37°C. FT-IR (film) ν_{max} = 3400, 2985, 1694, 1625, 1592, 1510 cm^{-1}.^{1}H NMR: δ 0.88 (t, $\mathcal{J}$ = 7.0 Hz, 3H); 1.26–1.36 (m, 16H); 1.57 (m, 2H), 1.70 (m, 2H); 3.93 (s, 3H); 4.19 (t, $\mathcal{J}$ = 7.0 Hz, 2H); 6.29 (d, $\mathcal{J}$ = 16.0 Hz, 1H); 6.91(d, $\mathcal{J}$ = 10.0 Hz, 1H); 7.04 (d, $\mathcal{J}$ = 2.0 Hz, 1H); 7.10 (d, $\mathcal{J}$ = 2 Hz, 1H); 7.11 (dd, $\mathcal{J}$ = 10.0; 2.0 Hz, 1H); 7.64 (d, $\mathcal{J}$ = 16.0 Hz, 1H). ^{13}C NMR: δ 14.1; 22.7; 25.7; 28.7; 29.2; 29.3; 29.4: 29.5; 29.6; 32.8; 55.9; 64.6; 109.3; 114.7; 115,6; 123.0; 127.0;144.6; 146.8; 147.9; 163.8. HR-ESI-MS: $[M + H]^+$ = 363.2525; $[M + Na]^+$ = 385.2351.

(E)-Ethyl 3-(4-acetoxy-3-methoxyphenyl)-prop-2-enoate (4)

Yield 59%, white solid, melting point 68–69°C. FT-IR (film) ν_{max} = 2923, 2848, 1697, 1682, 1557 cm^{-1}.^{1}H NMR: δ 1.34 (t, $\mathcal{J}$ = 7.0 Hz, 3H); 2.32 (s, 3H); 3.86 (s, 3H); 4.27 (c, $\mathcal{J}$ = 7.0 Hz, 2H); 6.37 (d, $\mathcal{J}$ = 16.0 Hz, 1H); 7.05 (d, $\mathcal{J}$ = 10.0 Hz, 1H); 7.13 (dd, $\mathcal{J}$ = 10; 2.0 Hz, 1H); 7.64 (d, $\mathcal{J}$ = 16.0 Hz, 1H). ^{13}C NMR: δ 14.3; 20.6; 55.9; 60.6; 111.2; 118.5; 121.2; 123.2; 133.4; 141.4; 143.8; 151.4;166.6; 168.8.HR-ESI-MS: $[M + H]^+$ = 265.1061; $[M + Na]^+$ = 287.0889.

(E)-Ethyl 3-(4-dodecanoyloxy-3-methoxyphenyl)-prop-2-enoate (5)

Yield 52%, white solid, melting point 34–35°C. FT-IR (film) ν_{max} = 2930, 2850, 1698, 1685, 1560 cm^{-1}.^{1}H NMR: δ 0.88 (t, $\mathcal{J}$ = 7.0 Hz, 3H); 1.26–1.33 (m, 16H); 1.34 (t, $\mathcal{J}$ = 7.0 Hz, 3H); 1.63 (m, 2H); 1.76 (m, 2H); 2.58 (t, $\mathcal{J}$ = 7.0 Hz, 2H); 3.85 (s, 3H); 4.27 (c, $\mathcal{J}$ = 7.0 Hz, 2H); 6.38 (d, $\mathcal{J}$ = 16.0 Hz, 1H); 7.11 (dd, $\mathcal{J}$ = 10; 2.0 Hz, 1H). ^{13}C NMR: δ 14.1; 14.3; 22.7; 24.7; 29.1; 29.2; 29.3; 29.4: 29.5; 29.6; 34.0; 55.9; 60.6; 111.2; 118.4; 121,2; 123.3; 133.3; 141.6; 144.0; 151.4; 166.9; 171.7. HR-ESI-MS: $[M + H]^+$ = 405.2603; $[M + Na]^+$ = 427.2413.

Anti-inflammatory activity

Anti-inflammatory activity was measured *in* vitro as percentage of inhibition of cyclooxygenase 2 (COX2) using the Colorimetric COX Ovine Inhibitor Kit (Cayman Chemical, USA), according to the manufacturer's instructions. Stock solutions of the derivatives were prepared in dimethyl sulfoxide (DMSO). Diclofenac was used as standard.

Statistical analysis

Data are presented as mean ± standard error (SE) from at least two independent analyses. OriginPro™ software (v. 8.5.1) was used for statistical analysis.

Results and discussion

The presence of a phenolic and an acid group in a single molecule of FA (**1**) makes this compound an interesting model for enzymatic modifications. In general, alkyl monoesters of hydroxycinnamic acids including FA can be obtained by direct esterification with alcohols (Weitkamp et al. 2006) or by transesterification of methyl or ethyl FA esters with long-chain alcohols (Compton et al. 2000). The phenolic groups of the synthesized monoesters can be further acylated to produce the corresponding diesters. In this work, we investigated the behavior of different lipases in the esterification and acylation of FA, aiming to improve the productivity of the process or to synthesize novel lipophilic compounds. Figure 1 illustrates the lipase-catalyzed reactions described in the present work.

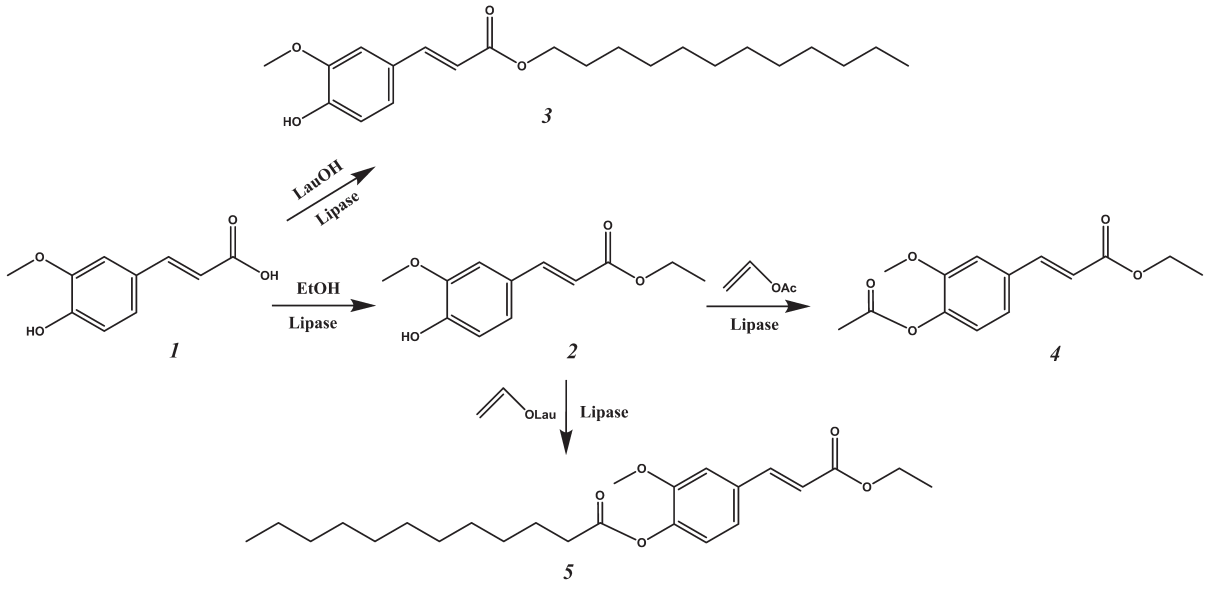

Figure 1. Scheme of the lipase-catalyzed synthesis of FA mono- and diesters.

Esterification of ferulic acid

Several immobilized lipases from bacteria (e.g., *Pseudomonas cepacia* and *Alcaligenes* sp.), fungi (e.g., *Rhizomucor miehei* and *Thermomyces lanuginosus)*, yeasts (e.g., *Candida antarctica* and *Yarrowia lipolytica*), and plants (e.g., *Carica papaya*) were evaluated in different solvents for the esterification of FA with two aliphatic alcohols: ethanol and lauryl alcohol. Reactions were carried out at 60°C using 10 mg/mL of FA, an alcohol/FA molar ratio of 10, and 100 mg/mL of biocatalyst. TLC monitoring indicated that, among the tested lipases, only the lipase from *C. antarctica* B (CALB) afforded the EF **2** and LF **3** in significant amounts. In the absence of biocatalyst no product was detected.

The corresponding ester derivatives **2** and **3** were isolated and characterized. EF (**2**) was fully identified by melting point and spectroscopic techniques, which were in accordance with reported data (Nonnenmacher et al. 1983). The molecular formula was determined as $C_{12}H_{14}O_4$, on the basis of the HR-electrospray ionization (ESI)-MS spectrum. The molecular ion was 28 Da larger than that of FA, which indicated the formation of the ethyl ester. Compared with its parent compound, the ^{1}H NMR spectrum of **2** exhibited two new signals corresponding to the ethyl group. The methylene appeared at δ 4.27 (c, J = 7 Hz) and a triplet at δ 1.33 with J = 7 Hz corresponding to the methyl group. In the ^{13}C spectrum of **2**, two new signals at δ 14.1 and 55.9 were observed corresponding to the methyl and methylene carbons, respectively.

Spectroscopic analysis of the product **3** was consistent with the formation of the lauryl ester. The molecular formula was determined as $C_{22}H_{34}O_4$, on the basis of the HR-ESI-MS spectrum. The molecular ion was 168 Da larger than that of FA, which indicated the formation of the lauryl ester. This was also established by observing the triplet at δ 0.88 corresponding to methyl group, multiplets at δ 1.26–1.36, 1.57, and 1.70 corresponding to the chain methylene groups, and a triplet at δ 4.19 (*t*, $\mathcal{J}$ = 7.0 Hz) assigned to the methylene group bonded to the carboxylic oxygen.

In order to optimize the yield of the synthesized esters we varied several reaction parameters such as the organic solvent, temperature, the molar ratio alcohol/FA, and the amount of biocatalyst.

Effect of organic solvent

It is well known that non-polar solvents are appropriate media for lipase-catalyzed reactions (Plou et al. 2002; Quintana et al. 2011). Such hydrophobic organic solvents offer several advantages, such as the enhancement of solubility of non-polar reactants, an improved stability of the enzyme, a shift in the equilibrium toward product formation, and easier separation of the enzyme from the reaction medium at the end of the process (Carrea & Riva 2008). The solvents selected for this screening were isooctane, diisopropyl ether (DIPE), and 2-methyl-2-butanol (2M2B), whose logP values are in the range 1.1–4.4, and a solvent-free system, in which the alcohol acts as bulk solvent. Table I indicates that the results were highly dependent on the solvent selected. Due to the presence of the phenol and the carboxylic group, the solubility of FA in isooctane was limited. In 2M2B

Table I. Influence of the solvent on the enzymatic esterification of FA with ethanol and lauryl alcohol.

		Esterification agent	
Solvent	logP	Ethanol	Lauryl alcohol
Solvent-free	–	–	+
2M2B	1.09	+	+
DIPE	1.40	+++	+++
Isooctane	4.37	+	+

Experimental conditions: 10 mg/mL FA; EtOH/FA ratio 4:1; LauOH/FA 1:1; 100 mg/mL CALB; 60°C; Reaction time 24 h. (+++) approx. 0.56 mmol L^{-1} h^{-1}; (–) No reaction.

it was completely soluble, but the assayed lipases were not active. In the solvent-free system only product **3** was obtained in low yield. In contrast, DIPE (whose solubility in water is less than 2 g/L) allowed the production of both esters **2** and **3** with good conversion. For this reason, DIPE was the solvent of choice to optimize the esterification reaction.

In this context, different solvents have been reported for the enzymatic esterification of hydroxycinnamic acids (Compton et al. 2000), including mixtures of miscible solvents such as hexane/butanone (Yang et al. 2012) and ionic liquids (Katsoura et al. 2009a). In some cases, the reactions have been carried out using the alcohol component as the solvent (Weitkamp et al. 2006).

Effect of alcohol/ferulic acid ratio and the amount of biocatalyst

The influence of alcohol/FA ratio on reaction yield was evaluated in the esterification of FA with ethanol (EtOH) and lauryl alcohol (LauOH) in DIPE using CALB as biocatalyst. As expected, it was observed that a molar excess of alcohol was advantageous for the reaction, with a molar ratio EtOH/FA or LA/FA 10:1 giving the best results. A higher excess of alcohol did not improve yields. Figure 2 shows the results for the synthesis of EF (**2**) at different EtOH/FA molar ratios.

The effect of the amount of biocatalyst (compared with the concentration of FA) was assessed in the enzymatic esterification of FA with ethanol, using alcohol/FA molar ratio of 10, DIPE as solvent at 60°C, and variable amounts of CALB. From the results obtained after 7 days of reaction (Table II), it can be concluded that using 10 mg/mL of FA, the optimum biocatalyst concentration was 100 mg/mL.

Effect of temperature and reaction time

The esterification of FA with ethanol was carried out between 30°C and 60°C. The other reaction parameters were set at their optimal values (100 mg/mL of

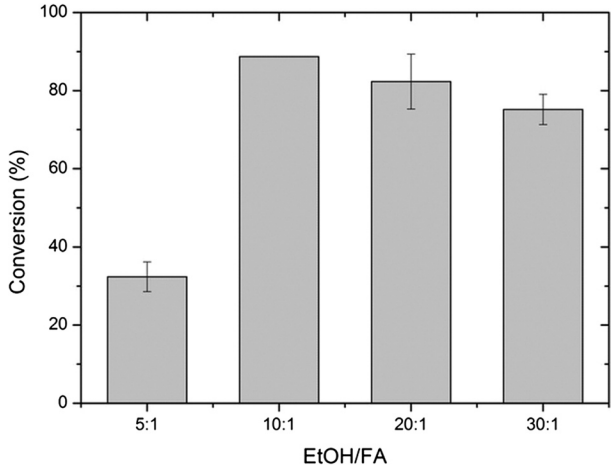

Figure 2. Effect of ethanol/FA molar ratio on CALB-catalyzed synthesis of EF. Reaction conditions: 10 mg/mL FA; Solvent: diisopropyl ether (DIPE); 100 mg/mL biocatalyst (CALB); Temperature 60°C; Reaction time: 7 days. Conversion was determined by HPLC.

CALB, DIPE, and alcohol/FA 10). Reaction yield decreased from 88.7% at 60°C to 12.2% conversion at 30°C. The optimum temperature for the reaction was set to 60°C, which correlates well with other biotransformations catalyzed by this immobilized enzyme (Stevenson et al. 2007) .

The time course of EF synthesis was studied in detail under the optimal conditions (Figure 3). We achieved an efficient enzymatic synthesis of EF (93% yield) by esterification of FA with ethanol catalyzed by CALB, in 7 days. Although the reaction was quite slow compared with other lipase-catalyzed esterifications, the rate and productivity were notably higher than those reported in previous studies. Thus, Compton et al. (2000) reached only 20% conversion of EF and 14% octyl ferulate after 12 days of reaction in 2-methyl-2-propanol. Yang et al. (2012) obtained 5% conversion of hexyl ferulate in 6 days using solvent mixtures of hexane and butanone. Katsoura et al. (2009a) reported 38.1% yield of hexyl ferulate in 3 days with an immobilized lipase from *Mucor miehei* (RMIM) employing ionic liquids. Using non-enzymatic methods, Li et al. (2009b)

Table II. Effect of the biocatalyst/FA ratio (w/w) in the enzymatic esterification of FA with ethanol.

Biocatalyst/FA (w/w)	Conversion (%)
5	29.7
10	88.7
20	77.2
30	72.4

Experimental conditions: 10 mg/mL FA; Molar ratio EtOH/FA 10/1; Enzyme: CALB; 60°C; Reaction time: 7 days. Conversion was determined by HPLC.

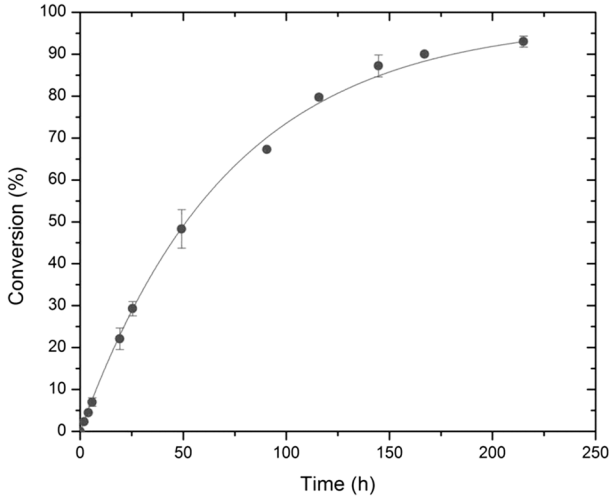

Figure 3. Time course of EF synthesis in DIPE catalyzed by CALB. Reaction conditions: 10 mg/mL FA; 100 mg/mL biocatalyst; Temperature 60°C. Conversion was determined by HPLC.

described the synthesis of EF using 10% H_2SO_4 as catalyst under reflux (8 h) or microwave irradiation (3–5 min), reporting 81% and 95% yield, respectively. However, although the product was obtained with high conversion in a shorter time than the enzymatic process, the use of a corrosive strong mineral acid such as sulfuric acid, at high concentration, is not environmentally friendly.

The optimal conditions for the enzymatic esterification of FA with ethanol were as follows: CALB biocatalyst, alcohol/FA molar ratio 10, enzyme/FA weight ratio 10, DIPE as solvent, 60°C, and 7-day reaction time.

In the esterification of FA with dodecyl alcohol, the yield of LF was 85% after 200 h, which was a higher yield than that achieved in a previous chemical synthesis (72%) using *p*-toluenesulfonic acid as catalyst (Taniguchi et al. 1999).

Acylation of ethyl ferulate acid by transesterification

The acylation of the free phenolic group in EF could lead to lipophilic diesters with enhanced hydrophobicity. In view of our previous work on enzymatic acylation of natural phenolic antioxidants such as vitamin E (Torres et al. 2008) and resveratrol (Torres et al. 2010), we decided to investigate the acylation of the 4-OH of EF, using different vinyl esters as acylating agents. We selected two chains of different lengths (C2 and C12) in order to cover a broad range of lipophilicity. It has been shown that the reactivity of vinyl esters and the tautomerization of vinyl alcohol to volatile acetaldehyde improve reaction rates and conversion in lipase-catalyzed acylations (Ferrer et al. 2000; Karmee 2009; Plou et al. 1999; Reyes-Duarte et al. 2005).

Two acyl derivatives (**4** and **5**) were obtained through transesterification of EF (**2**) with vinyl acetate and vinyl laurate, respectively. No dimers from auto-condensation of EF were observed. The reaction was examined using different immobilized lipases as described in the "Esterification of ferulic acid" section. Among them, the immobilized lipase from *Alcaligenes* sp. (QLG) gave the most satisfactory results in the acetylation of EF to afford **4,** and CALB was most efficient when vinyl laurate was used to obtain **5**. In the absence of enzymes, EF did not react at all. Some authors have suggested that phenolic alcohols inhibit lipases, in particular CALB, with inhibitory effects arising from electronic resonance between the double bond on the side chain conjugated with the *para*-hydroxyl group in cinnamic acids (Guyot et al. 1997; Stevenson et al. 2007). However, in our work, *C. antarctica* B and *Alcaligenes* sp. lipases were able to acylate the phenolic OH in the *para* position of EF. CALB was active in the synthesis of both diesters **4** and **5**, but QLG was more efficient in the case of **4**. Although *Alcaligenes* sp. lipase is typically employed in enantioselective synthesis (Chen et al. 2014), it is also able to acylate the phenolic OH in the *para* position (4′) of a related substrate (resveratrol) using vinyl esters (Torres et al. 2012).

4-Acetoxy-ethyl ferulate (**4**) was unequivocally identified by NMR and MS. The molecular formula was determined as $C_{14}H_{16}O_5$, on the basis of the HR-ESI-MS. The molecular ion was 42 Da larger than EF, indicating the addition of the acetate to the hydroxyl group. The ^{1}H NMR spectrum of **4** exhibited a new signal corresponding to the methyl group in acetate at δ 2.32, and ^{13}C NMR showed a new signal at δ 168.8 corresponding to the carbonyl carbon in acetate. Tang & Xiong (2011) described the synthesis of **4** by chemical acetylation of EF with acetic anhydride for its use in the treatment of vascular diseases.

To the best of our knowledge, the product **5** [(*E*)-Ethyl 3-(4-dodecanoyloxy-3-methoxyphenyl)-prop-2-enoate] is a new compound. It is a white solid with low melting point (34–35°C) and has been completely characterized by spectroscopic methods. The absence of the signal at about 3400 cm^{-1} in the FTIR spectrum indicates acylation of the phenol hydroxyl group in EF. In the MS spectrum, the molecular ion was 182 Da larger than EF, which indicated the formation of the lauryl ester on the phenolic group of the substrate and was in accordance with the molecular formula $C_{24}H_{36}O_5$, on the basis of the HR-ESI-MS. ^{1}H NMR spectrum showed

signals corresponding to the lauryl chain at δ 0.88, 1.26–1.33, 1.63, 1.76, and 2.58, and ^{13}C NMR spectrum showed a signal at δ 171.7 corresponding to the carbonyl carbon in lauryl group.

Optimal conditions of enzymatic transesterification

We analyzed the effect of several reaction parameters on the formation of FA diesters following the methodology described for the synthesis of monoesters **2** and **3**. Again, 60°C was the optimum temperature, with negligible conversion at 30°C. By varying the amount of biocatalyst relative to the concentration of EF, using ratios from 5 to 30 we found that a biocatalyst/EF weight ratio of 10 gave the best results for QLG and CALB. The organic solvent exerted substantial influence on the reaction yield with DIPE, again, being the best reaction medium for the transesterifications, representing a compromise between substrate solubility and enzyme activity/stability. An acyl donor/EF molar ratio of 2 gave the highest yields. In summary, we have defined the following standard conditions for the biocatalytic transesterification between EF and vinyl acetate or laurate: QLG (for **4**) and CALB (for **5**) as biocatalysts, DIPE as solvent, vinyl ester/EF = 2, biocatalyst/EF = 10, and 60°C. After 72 h of reaction, the compound **4** was obtained in 59% yield and the diester **5** was obtained in 52% yield.

Anti-inflammatory activity of the derivatives

The inhibition of the enzymes COX-1 and COX-2 is considered one of the mechanisms of anti-inflammatory action. COX enzymes are involved in the biosynthesis of prostaglandins, thromboxanes, and prostacyclins. In particular, COX-2 is responsible for the biosynthesis of prostaglandins under acute inflammatory conditions. The products **2**–**5** were evaluated as inhibitors of COX-2 using a COX-inhibitor screening assay kit. FA (**1**) was also assayed as a control. The kit measures prostanoid products of the COX-2 reaction via enzyme immunoassay, using a broad specificity antiserum that binds to all the major prostaglandin compounds (Pradelles et al. 1985). Diclofenac, a well-known non-steroidal anti-inflammatory drug, was used as reference standard.

The percentage of inhibition of COX-2 was determined and the results indicated that the FA derivatives were less active than diclofenac in inhibiting COX-2 *in vitro* (Figure 4). However, diclofenac has several undesirable effects such as hepatotoxicity (Aithal 2011). Comparing FA derivatives (Figure 4), we observed that compounds **3** and **5**, which contain a long-chain alcohol or carboxylic acid, were more active than **2** and **4** derivatives, which have only short alkylic chains. These preliminary results indicate that FA with long alkyl or acyl chains inhibited COX-2 more strongly than FA (**1**). However, further experiments are needed to evaluate the potential of FA esters in anti-inflammatory preparations. Although hepatotoxicity of new derivatives has not been tested, it is reasonable to assume that they are not toxic, because FA and EF have been reported to alleviate induced liver damage and liver cancer (Lee 2005).

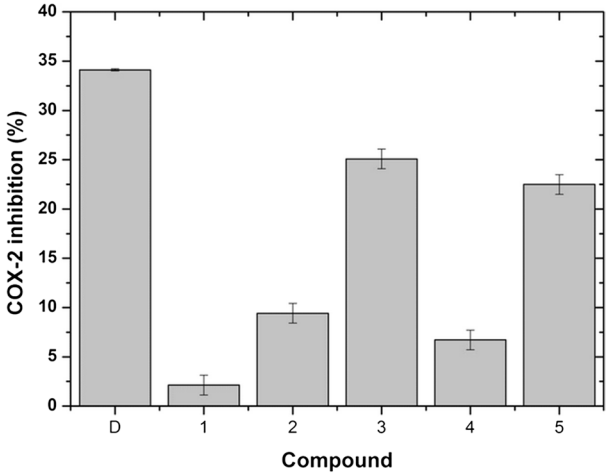

Figure 4. Anti-inflammatory activity of FA and its derivatives, measured as inhibition of COX-2 *in vitro*. Concentrations tested: Diclofenac (D), 0.1 M; FA and its mono- and diesters, 1.0 M.

Conclusions

In this work we have described an efficient enzymatic synthesis of two monoesters and two diesters of FA. The FA monoesters were obtained in higher yield and in a shorter time than in previous reports using biocatalysts. One of the synthesized diesters is a new compound. With respect to FA, the lipophilicity of the synthesized derivatives was increased to different degrees, which may modulate their behavior in emulsified systems, stability, and bioavailability. For that reason, the FA mono- and diesters here described may be useful in the food, pharmaceutical, and cosmetic industries. As a proof of concept, long-chain esters of FA exhibited better anti-inflammatory activity than their corresponding short-chain derivatives.

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