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RESEARCH ARTICLE

Comparative esterification of phenylpropanoids versus hydrophenylpropanoids acids catalyzed by lipase in organic solvent media

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Abbreviations: CAL-B: *Candida antarctica* lipase B
HPLC: High Performance Liquid Chromatography

The esterification of phenylpropanoid and hydrophenylpropanoid acids, catalyzed by *candida antarctica* lipase B (CAL-B), with several alcohols has demonstrated that the substitution pattern on the aromatic ring has a very significant influence on the reactivity of the carboxyl group due, mainly, to electronic effects, when compared to the unsaturated acids with the hydrogenated acids. It is also clear that in the saturated acids there still remain some unclear effects related to the aromatic substituents.

Phenylpropanoid acids are compounds widely present in plants, including many edible vegetable staples. Their biological properties, particularly the antioxidant activity, are well known and depend on the structural characteristics of these compounds (Nenadis et al. 2004). Because of their relative polar properties, important efforts have been made in order to increase their hydrophobicity and therefore produce amphiphilic molecules of industrial value (Figueroa-Espinoza and Villeneuve, 2005). Thus, esters of hydro-*p*-coumaric (Lee et al. 2003), hydroferulic (Hegazi

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Table 1. Products from the enzymatic esterification of phenylpropanoid acids (1 a-c) with different alcohols.

	% conv*		% conv*		% conv*
2a	98	**3a**	nd	**4a**	35
2b	93	**3b**	48	**4b**	nd
2c	91	**3c**	35	**4c**	48
2d	94	**3d**	nd	**4d**	49
2e	91	**3e**	47	**4e**	nd
2f	95	**3f**	nd	**4f**	55
2g	98	**3g**	6	**4g**	12
2h	31	**3h**	5	**4h**	nd
2i	79	**3i**	41	**4i**	53

nd: not determined.
*% of conversion determined by HPLC

and El Hady, 2002) and hydrocaffeic (Silva et al. 2000) acids, as well as alkyl coumarates (Tapia et al. 2004) and ferulates (Ou and Kwok, 2004), have been widely reported as antioxidants in food, cosmetic and pharmaceutical formulations. Interestingly, hydrocinnamic esters have been used in the synthesis of HIV-1 protease inhibitors or as precursors for the synthesis of 1,3,4,9-tetrahydropyrano[3,4-b]indole-1-acetic acid, which is used as an analgesic, inflammation inhibitor and antipyretic (Pridya and Chadha, 2003).

In spite of the remarkable industrial potential of phenylpropanoid acid esters as food antioxidants, their use in oil-based food processing has been limited, because of their high water solubility (Figueroa-Espinoza and Villeneuve, 2005). Thus, several methods have been proposed in order to obtain hydrophobic derivatives of phenylpropanoid acids. In general, biocatalytic methods are preferred over chemical methods since their mild reaction conditions avoid unwanted side reactions, and decrease the possibility of producing pollutants. For instance, the synthesis of pentyl ferulate, by a feruloylesterase from *Aspergillus niger*, was achieved with high yields in cetyl trimethyl ammonium bromide (CTAB) */n*-hexane/*n*-pentanol system, which is a water-in-oil microemulsion (Giuliani et al. 2001). However, the industrial application of micro-emulsion systems are generally limited due to the cumbersome recovery of products and biocatalysts.

As an interesting alternative, the lipase-catalyzed esterification of phenolic acids (including various hydroxycinnamic derivatives) with aliphatic alcohols in anhydrous conditions has been reported (Guyot et al. 1997). For instance, it has been reported that in lipase B from *Candida antarctica* (CAL-B), the efficiency of esterification of phenolic acids is strongly dependent on the different characteristics of arylaliphatic substrates, suggesting that hydroxycinnamic acid access to the active site of the enzyme is hindered due to reduced flexibility of the acyl residue (Otto et al. 2000).

Recently, Kermasha's group (Karboune et al. 2005; Lue et al. 2005) reported the lipase catalyzed esterification of cinnamic acid with oleoyl alcohol and mono-and diacylated glycerols in several solvent mixtures. In both cases the equilibrium was achieved after long periods of reaction, more than five days. A significant improvement in the reaction time (time for equilibration <3 days) was observed when, dihydrocaffeic acid, a saturated phenylpropanoid acid, was used as substrate under similar reaction conditions (Sabally et al. 2005).

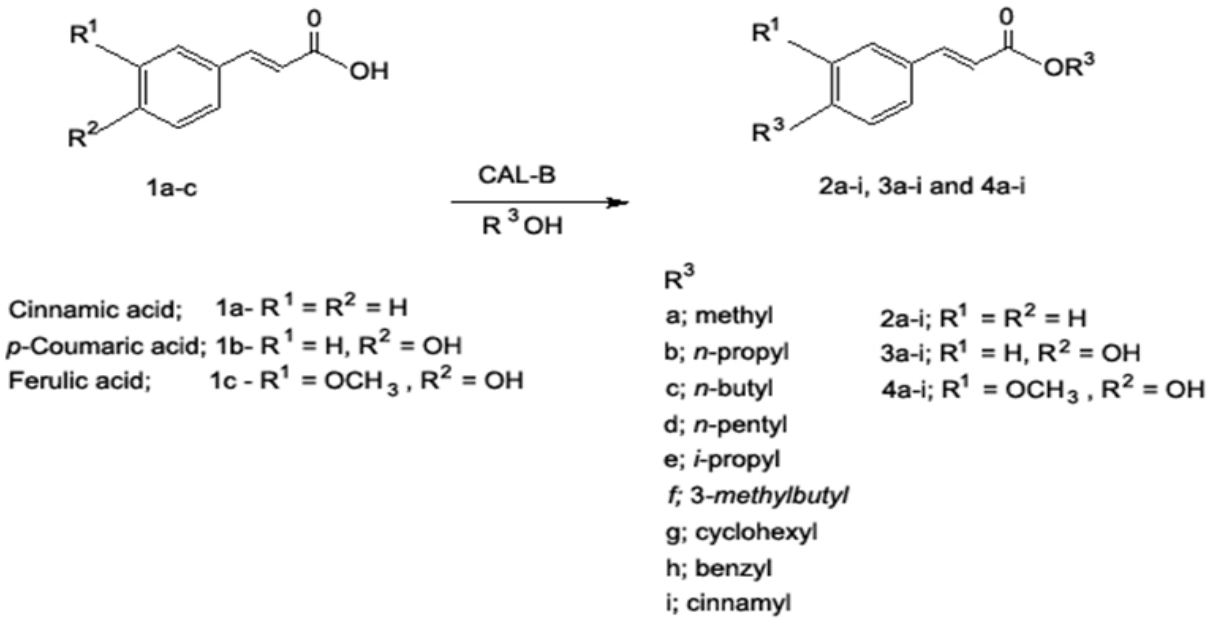

Figure 1. Direct esterification catalyzed by CAL-B of phenylpropanoid acids (1 a-c) with alcohols structurally different.

In the present study, we compared an investigation on the esterification of phenylpropanoid versus hydrophenylpropanoid acids catalyzed by lipases in organic media. Additionally, we studied the effect of the alkyl chain of the alcohol over the esterification reaction with CAL-B.

MATERIALS AND METHODS

Immobilized CAL-B (Novo Nordisk, México), Methanol, *n*-propanol, 3-methyl butanol and hexane were High Performance Liquid Chromatography (HPLC) grade (Tecsiquim, México); *n*-butanol, *n*-pentanol, isopropanol, benzyl alcohol and cinnamyl alcohol were analytical grade (J.T. Baker, México). The water content of all the reactions was set at a_w = 0.24, by equilibration of all reagents and enzymes with a saturated potassium acetate solution, for 48 hrs before de reaction. ^{1}H-NMR spectra were determined in $CDCl_3$ (Aldrich, México) using a 400 MHz spectrometer, with TMS as internal standard. Infrared Spectroscopy was determined in a Paragon 1000 spectrometer as a film or KBr disk. Reactions were monitored by HPLC using an Hypersil BDS-C18 column with detection at 280 and 260 nm, with methanol/water 80:20 as eluent, at 25ºC and a flow of 0.8 mL/min. The esters used as references were prepared by the reaction of the phenylpropanoid acids and the corresponding alcohols, with *p*-toluen sulphonic acid (PTSA) as catalyst (Taniguchi et al. 1997). Hydrophenylpropanoid acids were prepared, in quantitative yield, by hydrogenation in methanol using 5% Pd/C at 60 psi and room temperature; the products were characterized by IR and ^{1}H-NMR, and compared with literature data (Pridya and Chadha, 2003).

Procedure for enzymatic ester preparation

The corresponding alcohol (25 mmol) was added to 5 mmol of the acid dissolved in hexane (5 mL followed by 100 mg of CAL-B); the mixture was shaken for 360 hrs at 50ºC. The reaction mixture was filtered, washed with 5% sodium bicarbonate and evaporated; the residue was obtained in hexane/ethyl ether/ethyl acetate/methanol (80:20:2:3 mL) and percolated through a flash silica gel pad to removed the unreacted acid. Evaporation of the solvent produced the ester, practically pure.

Benzyl cinnamate (2 hrs). 90% yield of a yellow liquid. IR (cm^{-1}): 3029, 1730, 1495, 1450; ^{1}H-RMN δ($CDCl_3$): 7.72 (*d*, 1H, *J* = 16 Hz); 7.39 - 7.52 (*m*, 10H), 6.47 (*d*, 1H, *J* = 15.6 Hz), 5.20 (*s*, 2H) ^{13}C-RMN δ($CDCl_3$): 166.3, 144.8, 135.6, 133.9, 130.0, 128.5, 128.3, 128.2, 128.1, 128.0, 127.9, 127.8, 117.5, 66.2.

Benzyl hydrocinnamate (6 hrs). 90% yield of a yellow liquid. IR (cm^{-1}): 3030, 1731, 1604, 1496, 1454; ^{1}HRMN δ($CDCl_3$): 7.0 - 7.30 (*m*, 10H); 5.2 (*s*, 2H), 2.90 (*t*, 2H), 2.67 (*t*, 2H) ^{13}C-RMN δ($CDCl_3$): 172.3, 140.1, 135.5, 128.2, 128.2, 127.9, 127.9, 125.9, 66.1, 35.8, 30.8.

RESULTS AND DISCUSSION

It is understood that the reactive characteristics of the carboxyl group in phenylpropanoid acids are strongly influenced by their electronic conjugation with the aromatic ring and distorted by the substituents attached to this ring. In order to assess the reactive characteristics towards esterification of the carboxyl group of these compounds, we performed, as a model of study, the lipase-catalyzed esterification of three different acids: cinnamic, *p*-coumaric and ferulic with different alcohols. We tested the esterification reaction of cinnamic acid and methanol with CAL-B using a variety of solvents such as *t*-butyl alcohol, acetonitrile, toluene, THF, and *n*-hexane, obtaining the best conversions in toluene and hexane (20 and 30% conversion respectively).

With this in mind, different phenylpropanoid acids were confronted to various aliphatic and aromatic alcohols in the presence of CAL-B as described in Figure 1. All the reactions were performed in *n*-hexane as solvent using a five fold excess of the respective alcohol and an initial A_w equal to 0.24. In order to reach the equilibrium, all reactions were incubated at 50ºC under rotatory shaking for 360 hrs. Thereafter the reaction products were isolated and conversions at the equilibrium were quantified by HPLC.

Table 2. Products from the enzymatic esterification of hydrophenylpropanoid acids (5 a-c) with all the alcohols.

	% conv* (24 hrs)		% conv* (48 hrs)		% conv* (360 hrs)
6a	99	**7a**	99	**8a**	94
6b	100	**7b**	99	**8b**	98
6c	99	**7c**	99	**8c**	99
6d	100	**7d**	100	**8d**	98
6e	85	**7e**	99	**8e**	99
6f	99	**7f**	98	**8f**	99
6g	99	**7g**	90	**8g**	98
6h	99	**7h**	94	**8h**	91
6i	95	**7i**	96	**8i**	98

*Conversion % determined by HPLC.

From the results shown in Table 1, it can be stated that the number and nature of substituents on the aromatic ring have a strong influence over the catalytic behavior of the lipase. As a matter of fact, the higher conversions were obtained for reactions with cinnamic acid (1a), a phenylpropanoid acid without any substitutions in the aromatic ring, which in most cases reached conversion values higher than 90% after 360 hrs of reaction. On the other hand, conversions for the reactions with ferulic acid (1c), a phenylpropanoid acid containing two substitutions (4-OH and 3-OCH_3 groups) and *p*-coumaric acid (1b), a phenylpropanoid acid containing a 4-OH substitution, were in general lower than

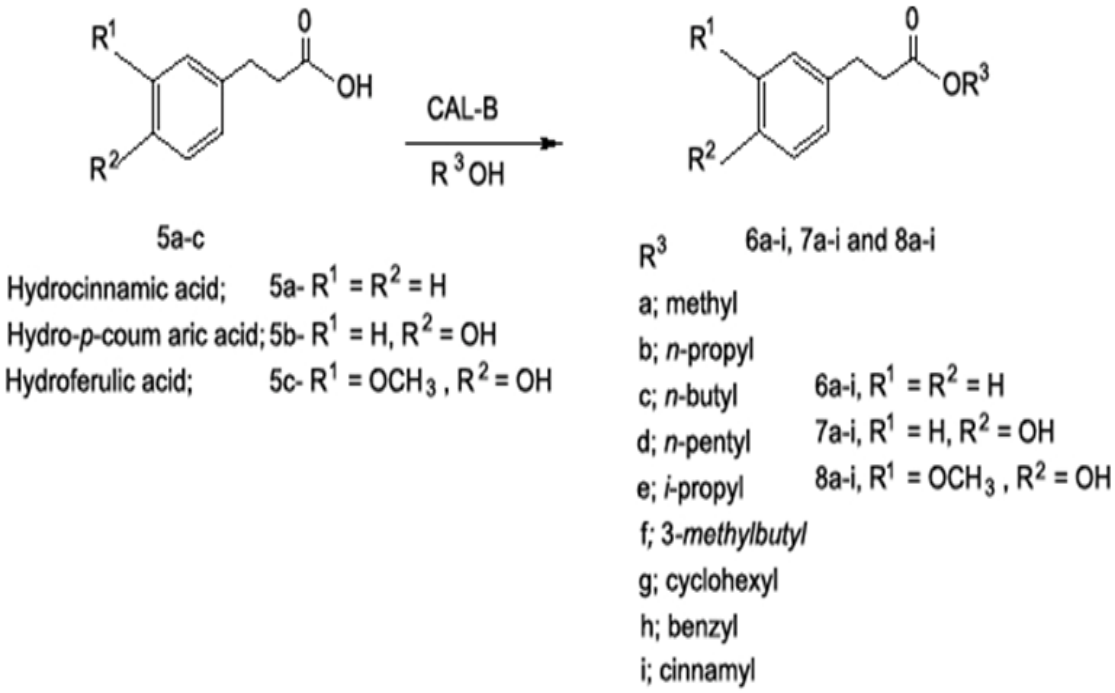

Figure 2. Direct esterification catalyzed by CAL-B of hydrophenylpropanoid acids (5 a-c) with alcohols structurally different.

50% under the same reaction conditions. It is noteworthy to mention that, for all phenylpropanoid acids, no marked effect was observed when using different alcohols, the exception being the reactions carried out with benzyl alcohol, which showed the lower conversions.

Similar reactivity of aromatic substrates containing various substituents in the phenyl ring, when using lipases as biocatalysts, has already been reported (Bornscheuer et al. 2005; Castillo et al. 2007). Both groups report that the differences of reactivity in terms of the reaction rates are due to the stronger inductive effect of the substituted phenyl ring. However, it may also be suggested that the differences arise from the different substrate interactions caused by the size of the substituents of the aryl ring on the active site of the enzyme (Smidt et al. 1996; Guieysse et al. 2003; Bornscheuer et al. 2005; Castillo et al. 2007).

It has been shown that the low reactivity of phenylpropanoid acids is influenced by the number and nature of substituents of their phenyl ring. However, it is not clear whether this behavior is only due to electronic effects, by steric hindrance, or both, from the substituents over the carboxylic group. Actually, it is generally accepted that the presence of a conjugated double bond on their vicinal acyl chain strongly influences the reactivity of this group. In order to avoid the conjugation with the aromatic ring and therefore modify the reactivity of the carboxylic group, the use of saturated phenylpropanoid derivatives as acyl-donors is proposed as an alternative. Thus, in a second set of experiments the lipase-catalyzed esterification of hydrocinnamic, hydro-*p*-coumaric and hydroferulic acids, where the chain double bond has been hydrogenated, with aliphatic and aromatic alcohols was performance in the presence of CAL-B, as described in Figure 2. All reactions were completed conditions to similar to those used in previous experiments (*n*-hexane at 50ºC, five fold excess of alcohol and initial $a_w = 0.24$).

From the results shown in Table 2, it can be observed that both the reaction time to reach equilibrium and final conversions are notably enhanced using these hydrogenated acids (5a-c), if compared with the corresponding conjugated acids (1a-c) as substrates. Indeed, conversions higher than 95% were obtained in almost all reactions and the equilibrium was reached in 24 and 48 hrs for hydrocinnamic (5a) and hydro-*p*-coumaric acid (5b) respectively. In the case of hydroferulic acid (5c) reaction times of 360 hrs were necessary to achieve such equilibrium; however, final conversions were significantly higher than those obtained for the corresponding ferulic acid (1c). From these results, it can be suggested that negative electronic effects of substituents over the carboxylic group may be diminished when the resonant double bond is eliminated. Similarly to the results observed with unsaturated phenylpropanoid acids (1a-c), the alcohols do not influence the conversions at equilibrium significantly.

CONCLUDING REMARKS

As a conclusion, we can say, in one hand, that the results confirm that the number and nature of substituents have a strong influence on the time required to reach the equilibrium and final conversions in the esterification of phenylpropanoid acids. Certainly, when unsaturated phenylpropanoid acids (1a-c) are used as acyl-donors, it is possible for the hydroxy and methoxy substituens to act as electrodonors, inducing the decrease of the electrophilic character of the carboxyl group by resonance and therefore reducing its reactivity toward the esterification. On the other hand, our results confirmed that the use of saturated phenylpropanoid derivatives (5a-c) as acyl-donors can prevent this negative electronic effects. It is noteworthy to mention, that besides the electronic effects, it has been suggested in literature that α, β unsaturations on carboxylic acids significantly reduce the flexibility of the acyl residue hindering the access of the substrate to the active site of lipases and esterases and reducing their reactivity by difficulting the fitting of the substrates on the hydrophobic crevice of the enzyme (Otto et al. 2000).

From our results, it is also clear that this inductive effect seems not to be completely eliminated by the elimination of the conjugation with the double bond present in acyl chain of phenylpropanoid acids, since the time required to reach equilibrium was significantly reduced only for the non substituted substrates. In fact, although high conversions at equilibrium are achieved for substituted hydrophenylpropanoid acids, our results and those previously reported in literature show that the nature and number of substituents on the aryl ring of saturated substrates drastically increase the time required to reach the equilibrium (Sabally et al. 2005).

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