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Enzymatic synthesis of hydrophilic and hydrophobic derivatives of natural phenolic acids in organic media

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Abstract

The enzymatic esterification of natural phenolic antioxidants such as cinnamic acid and benzoic acid derivatives, with aliphatic alcohols, monosaccharides as well as alkylglucosides, using various lipases and esterases in non-aqueous media, was investigated. Reaction rate and esterification yield seems to be linked to the structural characteristics of the substrates (aromatic acids and alcohols or sugars) used.

Keywords: Cinnamic acids; Esterification; Lipase; Phenolic antioxidants; Organic media

1. Introduction

There are many naturally occuring phenolic, non-flavonoid compounds, which have biological and physiological properties. A major portion of the antioxidant activity of oil-seeds and oil-seed flours and concentrates is attributed to phenolic acids [1]. Phenolic acids, including hydroxylated (*p*-coumaric acid, caffeic acid) or methoxylated (ferulic acid) derivatives of cinnamic acids, are present in soybeans, cottonseeds and peanuts in free forms or as carbohydrate esters [1,2]. Due to their phenolic nucleus and an extended side chain conjugation, they can readily form resonance-stabilized phenoxy radicals, which account for their antioxidant performance [3–5]. It is well recognized that phenolic acids or phenolic acid esters exhibit antioxidative avtivity [6], peroxy radical scavenging activity [6], and they are inhibitors of carcinogenesis [7]. Moreover, sugar esters of various phenolic acids isolated from plants as well as phenolic alkylglucoside esters are pharmacologically active compounds and are known to have antimicrobial and/or antiviral activity [8].

Many of the natural phenolic acids exhibit low solubility and low stability in various solvent systems. For example, the hydrophilic character of phenolic acids reduces their antioxidant effectiveness in stabilizing fats and oils and has been reported as a

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serious disadvantage if an aqueous phase is also present [9]. It is thus important to improve the solubility of these compounds in order to enhance their usefulness as food antioxidants. Therefore, the modification of these compounds via esterification with aliphatic alcohols can be used as a tool to alter solubility in oil based formulae and emulsions. The chemical synthesis of benzoic and phenolic acid esters is usually carried out with basic or acidic catalysts under reflux [10]. To overcome the disadvantages of the conventional processes, the use of enzymes in non-aqueous media has opened new ways for producing many valuable products under mild conditions [11–13]. Recently, the enzymatic esterification of phenolic acids with aliphatic alcohols or alkylglucosides catalyzed by *Candida antarctica* lipase has been reported [14–16]. In this work we describe the possibilities of esterification of various cinnamic acid and benzoic acid derivatives with long-chain alcohols, monosaccharides as well as alkyl glucosides catalyzed by various lipases and esterases in organic media (see Fig. 1). The solvent used for the enzymatic esterification of phenolic acids with monosaccharides and alkyl-glucosides, such as *tert*-butanol, is non-toxic and can solubilize relatively large amounts of substrates. Various reaction parameters affecting the enzyme catalytic behavior such as the structural characteristics of substrates as well as the nature of the organic medium used have been examined Scheme 1.

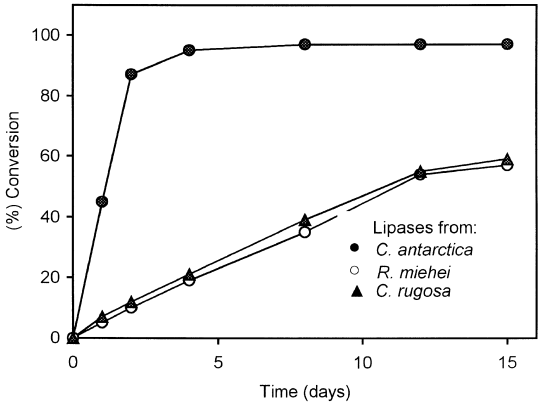
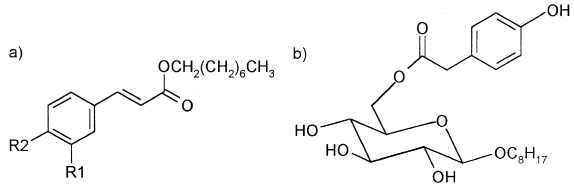


Fig. 1. Reaction progress for the esterification of *p*-hydroxyphenylpropionic acid with 1-octanol catalyzed by various lipases. Reaction conditions are as described in Materials and methods.



Scheme 1. Structure of enzymatically synthesized: (a) octyl esters of various cinnamic acids — $R_1 = R_2 = H$: cinnamic acid, $R_1 = R_2 = OH$: caffeic acid, $R_1 = OCH_3$ and $R_2 = OH$: ferulic acid, $R_1 = H$ and $R_2 = OH$: *p*-coumaric acid — and (b) 6-O-*p*-hydroxyphenylacetyl-1-*n*-octyl-β-D-glucopyranose.

2. Materials and methods

2.1. Materials

Lipase from *Rhizomucor miehei* (Lipozyme™) immobilized onto a macroporous anion-exchange resin was kindly provided by Novo Nordisk (Denmark). The enzyme activity is 25 BIU/g, (BIU = batch interesterification unit, defined as μmoles of palmitic acid incorporated into triolein per minute at 40°C). Lipase from *C. antarctica* (Novozyme 435) immobilized on a macroporous acrylic resin was also kindly offered by Novo Nordisk. Its activity is 7 PLU/mg (the enzyme activity refers to the synthesis of propyl laurate at 60°C). Lipase from *C. rugosa* (2425 U/mg; using olive oil as substrate) was purchased from Sigma and used without any further purification. Esterase from *Fusarium oxysporum* (0.24 U/mg using *p*-nitrophenyl butyrate as substrate) was prepared as described elsewhere [17]. Cutinase from *F. solani* (0.24 U/mg using triacetin as substrate) was from Unilever (NL) and immobilized on Accurel EP 100. Various phenolic acids, aliphatic alcohols, glucose, fructose, *n*-octyl glycoside, fatty acids and organic solvents were purchased from Aldrich, Merck or Sigma and were of the highest available purity.

2.2. Enzymatic esterification

The esterification of various cinnamic acids as well as benzoic acids with aliphatic alcohols was

carried out in sealed stirred flasks. In a typical experiment, the reaction mixture consisted of various amounts of aromatic acids (0.3–3 mmol) in 5 ml of alcohol and 150 mg of enzymatic preparation. The flasks were incubated in an orbital shaker at 200 rpm with the temperature fixed at 45°C. Control experiments were conducted without enzyme. Alcohols were dehydrated before use with 4 Å molecular sieves. In order to evaluate the rate of esterification, every 1 h a solution sample (10 μl) was withdrawn and subjected to HPLC analysis.

In a typical enzymatic synthesis of various phenolic acid sugar esters, 0.5–1.5 mmol of aromatic acids, 0.3 mmol of monosaccharides or *n*-octyl glycoside and 150 mg of enzymatic preparation were incubated in 5 ml of *tert*-butanol. The reaction mixture was incubated in an orbital shaker at 200 rpm at 45°C. Organic solvents were dehydrated before use with 4 Å molecular sieves. Control experiments were conducted without enzyme. Samples were withdrawn at various times to determine the concentration of substrate and product by HPLC. All reactions were carried out in the presence of 4 Å molecular sieves (20 mg/ml of organic solvent).

2.3. Analytical methods

Qualitative analysis of reaction mixtures was made by TLC on silica gel 60 plates (Merck, Germany) using a solvent mixture of $CHCl_3/CH_3OH/CH_3COOH$ (63/2/1 v/v/v) or $CHCl_3/CH_3OH/CH_3COOH$ (65/15/2 v/v/v) for the esterification of phenolic acids with alcohols and sugars, respectively. TLC plates were visualized by spraying the plates with a 5% (v/v) ethanolic solution of H_2SO_4 and heating for 10 min at 150°C or visualized under a UV lamp. Quantitative analysis of samples was made by HPLC on a C_{18} Nucleosil column, particle size 10 μm, length 300 mm, diameter 3.9 mm. Detection was achieved at 280 nm based on calibration curves prepared using standard cinnamic acids, as well cinnamic acid esters solutions in methanol. Samples were first sieved to remove the enzyme and molecular sieves. The resulting clear solution (50 μl) was diluted with methanol (950 μl) before analysis. Elution was conducted with methanol or metha:water (90:10 v/v) at a flow rate of 1 ml/min. Yields for the synthesis of various phenolic esters were calculated from the amount of phenolic acids having reacted compared to the initial quantity of phenolic acids. Reaction rates were calculated from the slope of the linear portion of plots of degrees of conversion versus time, and expressed as μmol h^{-1} g^{-1} of enzymatic preparation. All experiments were carried out in duplicate.

2.4. Phenolic acid esters purification

Alkyl esters of various cinnamic acid derivatives were obtained by extraction with diethyl ether followed by washing with saturated $NaHCO_3$ solution [18]. Sugar esters of phenolic acids were separated by silica gel chromatography as described elsewhere [16]. The presence of the ester bond on the phenolic acid esters was identified by infrared spectroscopy while the chemical structure of the esters were determined by ^{13}C-NMR in CD_3OD on a Varian-300 MHz spectrometer (Palo Alto, CA, USA). ^{13}C-NMR for 6-O-*p*-hydroxylphenylacetyl-1-octyl-β-D-glucopyranose: δ (ppm) = 14.7 (C-8*), 20.96 (C-7*), 23.6 (C-3*), 24.9(C-6*), 24.6 (C-2*, 4*, 5*), 35.0 (C-2), 58.9 (C-6′), 64.8 (C-1*), 65.7 (C-4′), 69.0 (C-2′), 69.2 (C-5′), 71.9 (C-3′), 98.3 (C-1′), 120.8 (C-4, 5, 7, 8), 125.4 (C-3), 151.5 (C-6), 170.4 (C=O).

3. Results and discussion

3.1. Synthesis of hydrophobic derivatives of various phenolic acids

The enzymatic esterification of various phenolic acids with octanol was carried out without added solvent, whose role was ensured by the excess of alcoholic substrate, e.g. 1-octanol. Table 1 shows the reaction rates as well as the conversion yields of the esterification of cinnamic, ferulic, *p*-coumaric and *p*-hydroxyphenylpropionic acid, catalyzed by lipases from *C. antarctica*, *R. miehei*, and *C. rugosa*, esterase from *F. oxysporum* and cutinase from *F. solani*. Fig. 2 depicts the esterification *p*-hydroxyphenypropionic acid catalyzed by lipases from *C. antarctica*, *R. miehei* and *C. rugosa*, respectively, in

Table 1
Effect of phenolic acids structure (0.5 mmol) on the reaction rates and conversion yields for their esterification with 1-octanol (5 ml) catalyzed by *C. antarctica* lipase (CAL), *R. miehei* lipase (RML), *C. rugosa* lipase (CRL), *F. oxysporum* esterase (FOE) and *F. solani* cutinase (FSC) at 45°C. The concentration of all enzymatic praparations used was 30 mg/ml

Phenolic acids	CAL	RML	CRL	FOE	FSC
	Reaction rate μmol h^{-1} g^{-1} (% Conversion after 12 days)				
Cinnamic	19.2 (82)	11.0 (59)	6.7 (19)	3.5 (8)	3.6 (12)
p-Coumaric	6.4 (25)	6.2 (22)	4.5 (16)	2.5 (10)	3.0 (10)
Ferulic	n.d.[a] (11)	3.7 (30)	n.d.[a] (8)	n.d.[a] (3)	n.d.[a] (5)
p-Hydroxyphenyl propionic	277.0 (97)	27.2 (57)	30.5 (59)	21.0 (34)	15.2 (29)

[a] n.d.: not determined.

1-octanol. As it can be seen from Table 1, all enzymes used are able to catalyze the esterification of cinnamic acid *p*-hydroxyphenylpropionic with relatively high rates. Lipase from *C. antarctica* was able to catalyze the esterification of aromatic acids with high reaction rate and yield, while the reaction rate for the esterification of methoxylated or hydroxylated derivatives of cinnamic acid (such as ferulic acid and *p*-coumaric acid) are very low. A similar inhibitory effect by ferulic acid and *p*-coumaric acid was also observed for all enzymes used. However, lipase from *R. miehei* seems to catalyze the acylation of ferulic acid, with relatively higher rate and yield (30% conversion after 12 days of reaction period; see Table 1) than all other lipolytic enzymes used in this work. It must be noted that when the side chain on the aromatic ring was saturated (such in the case of *p*-hydroxyphenylpropionic acid), *p*-hydroxylation had no effect on enzyme activity. The inhibitory effect of various methoxy- and hydroxy-substituted phenolic acid derivatives has also been reported by other researchers [14,15] and could be attributed to an electronic and/or steric effect. It has been proposed that the electronic donating effects deactivate the electrophilic carbon center of the carboxylic group for nucleophilic attack of the alcohol [15]. Unfortunately, the relatively low reaction rates and the solubility limitation of ferulic acid in 1-octanol make difficult an extensive kinetic study of the above esterification reaction.

The effect of the concentraof various hydroxylated derivatives of cinnamic acid (*o*- and *p*-coumaric acid) as well as *o*- and *p*-hydroxylated derivatives of phenylylacetic acid on *C. antarctica* lipase activity was studied in a solvent-free system. In this case, 1-butanol was used as substrate since this polar solvent can solubilize relatively large amounts of various aromatic acids. Fig. 2a and b shows the effect of the concentration of *o*- and *p*-coumaric

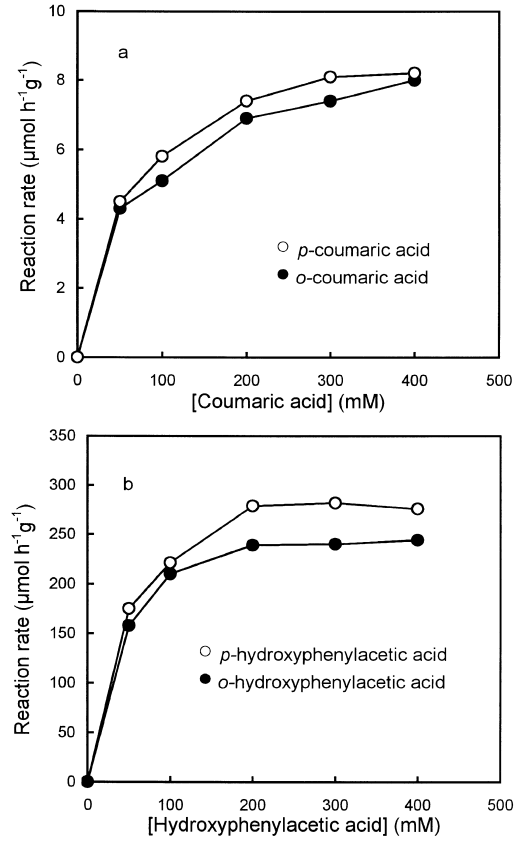


Fig. 2. Effect of the concentration of (a) *o*- and *p*-coumaric acid and (b) *o*- and *p*-hydroxylphenylacetic acid on rate of their esterification catalyzed by *C. antarctica* lipase in 1-butanol.

Table 2
Reaction yields for the esterification of various sugars with cinnamic as well as *p*-hydroxyphenylacetic acid (*p*-HPA) catalyzed by *C. antarctica* lipase in *tert*-butanol at 45°C. Reaction conditions are as described in Materials and methods

Sugars	Cinnamic acid	*p*-HPA acid	Comments
	% Conversion after 5 days of reaction		
Glucose	314	25	Only monoester was formed
Fructose	21	33	Mono- and di-ester were formed at a molar ratio 2:1
n-Octyl glucoside	45	55	Only monoester was formed

acid as well as *o*- and *p*-hydroxylphenylacetic acid on the rate of their esterification catalyzed by *C. antarctica* lipase in 1-butanol. As it can be seen, an increase in aromatic acid concentration resulted in an increased of the reaction rate. However, the rate of esterification of *p*-coumaric acid by *C. antarctica* lipase was several times lower than the rate of esterification of hydroxylated derivatives of phenylacetic acid in a similar concentration range. These results confirm that the lipase inhibiting effect of electron donating substituents conjugated to the carboxylic groups in hydroxylated derivatives of cinnamic acid is strong. Moreover, if the lateral chain is saturated, as in the case of hydroxylated derivatives of phenylacetic acid or phenylpropionic acid (Fig. 2b and Table 1), no inhibition was observed since the esterification reactions take place with very high rates.

3.2. Synthesis of hydrophilic derivatives of various phenolic acids

The esterification of various phenolic acids with monosaccharides such as glucose or fuctose as well as with *n*-octyl glycoside catalyzed by *C. antarctica* lipase in *tert*-butanol at 45°C, have been examined (see Fig. 1). Table 2 shows the conversion yields after 5 days of reaction for the esterifications studied. As it can be seen, lipase from *C. antarctica* catalyzes with relatively high yield (45–55%) the esterification of both fenolic acids with *n*-octyl glucoside. It must be noted that for the esterification of glucose or *n*-octyl glycoside, only one product was identified in all cases studied (using TLC and HPLC analysis), which indicates that this lipase-catalyzed esterification is regioselective. On the other hand, it was observed that in the esterification of fructose, monoester seems to be the dominant product, while significant amount of di-ester was proved by TLC and HPLC analysis after 12 days of reaction. The above observation is in accordance with other studies concerning the ability of *C. anatarctica* lipase to catalyze the acylation of fructose in similar reaction systems [19]. Various enzymatically synthesized sugar esters of *p*-hydroxyphenylacetic acid was purified and characterized by ^{13}C-NMR analysis. From ^{13}C-NMR data it could be proven that the acylation proceeded in a highly regioselective manner at the 6-OH group of sugars (glucose or *n*-octyl-glucoside).

From our results, it can be concluded that both hydrophobic and hydrophilic derivatives of natural phenolic acids can be easily synthesized through lipase-catalyzed esterification with aliphatic alcohols as well as sugars under mild reaction conditions using non-toxic solvents. Investigation is now in progress to test the antioxidant and antimicrobial properties of the above phenolic compounds.

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