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Highly Efficient Preparation of Lipophilic Hydroxycinnamates by Solvent-free Lipase-Catalyzed Transesterification

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Various medium- or long-chain alkyl cinnamates and hydroxycinnamates, including oleyl *p*-coumarate as well as palmityl and oleyl ferulates, were prepared in high yield by lipase-catalyzed transesterification of an equimolar mixture of a short-chain alkyl cinnamate and a fatty alcohol such as lauryl, palmityl, and oleyl alcohol under partial vacuum at moderate temperature in the absence of solvents and drying agents in direct contact with the reaction mixture. Immobilized lipase B from *Candida antarctica* was the most effective biocatalyst for the various transesterification reactions. Transesterification activity of this enzyme was up to 56-fold higher than esterification activity for the preparation of medium- and long-chain alkyl ferulates. The relative transesterification activities found for *C. antarctica* lipase were of the following order: hydrocinnamate > cinnamate > 4-hydroxyhydrocinnamate > 3-methoxycinnamate > 2-methoxycinnamate ≈ 4-methoxycinnamate ≈ 3-hydroxycinnamate > hydrocaffeate ≈ 4-hydroxycinnamate > ferulate > 2-hydroxycinnamate > caffeate ≈ sinapate. With respect to the position of the hydroxy substituents at the phenyl moiety, the transesterification activity of *C. antarctica* lipase B increased in the order meta > para > ortho. The immobilized lipases from *Rhizomucor miehei* and *Thermomyces lanuginosus* demonstrated moderate and low transesterification activity, respectively. Compounds with inverse chemical structure, that is, 3-phenylpropyl alkanoates such as 3-(4-hydroxyphenyl)propyl oleate and 3-(3,4-dimethoxyphenyl)propyl oleate, were obtained by *C. antarctica* lipase-catalyzed transesterification of fatty acid methyl esters with the corresponding 3-phenylpropan-1-ols in high yield, as well.

KEYWORDS: Alkyl hydroxycinnamates; oleyl coumarates; palmityl ferulate; oleyl ferulate; 3-(4-hydroxyphenyl)propyl oleate; lipase B; *Candida antarctica*; solvent-free; transesterification

INTRODUCTION

Hydroxycinnamic acids and their analogues such as 4-hydroxycinnamic (*p*-coumaric), 4-hydroxy-3-methoxycinnamic (ferulic), 3,4-dihydroxycinnamic (caffeic), and 4-hydroxy-3,5-dimethoxycinnamic (sinapic) acids (**Figure 1**) including their medium- or long-chain alkyl esters, are common plant constituents that are synthesized via the phenylpropanoid pathway (*1*−*9*). The beneficial effects of hydroxycinnamic acids and other plant phenolics on health have been attributed to their antioxidant capacity, particularly against oxidative attacks by their radical-scavenging activity (*10*). Both antioxidant capacity and biological availability of several of these compounds may be further improved by increasing their lipophilicity (*11*−*14*), and the range of applications of such lipophilic antioxidants may be extended by their possible use as additives for food and technical applications (*14*−*18*). In addition, similar compounds with inverse chemical structure such as hydroxycinnamyl alkanoates were isolated from apple fruits (*19*, *20*). Such "retro" compounds also have antioxidant properties as they are known for alkyl hydroxycinnamates.

Enzymatic esterification of hydroxycinnamic acids may be of advantage over chemical esterification for the preparation of lipophilic hydroxycinnamic acids esters, particularly for food use. Until now, enzymatic esterification and transesterification procedures requiring organic solvents or using the alcohol component as the solvent have been reported for the preparation of various alkyl esters of hydroxycinnamic acids including alkyl ferulates (*21*−*30*). Recently, we have described a procedure for the lipase-catalyzed esterification of hydroxycinnamic acids with medium- or long-chain alcohols, which was performed at moderate temperatures without solvents or drying agents using reduced pressure to remove reaction water (*31*). This method was much more efficient than other enzymatic esterification procedures described earlier (*14*). The aim of the present work was further optimization of this simple and environmentally friendly lipase-catalyzed preparation of lipophilic medium- and long-chain alkyl hydroxycinnamates by using transesterification of methyl or ethyl hydroxycinnamates with medium- or long-chain alcohols—instead of esterification of the corresponding hydroxycinnamic acids (**Figure 2**). Similarly, compounds with

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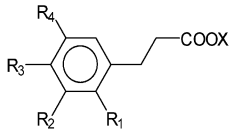
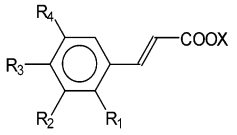
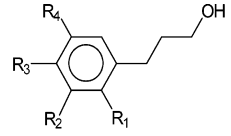


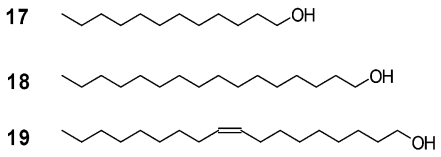
1 R_1, R_2, R_3, R_4 = H; X = C_2H_5
2 R_3 = OH; R_1, R_2, R_4 = H; X = CH_3
3 R_2, R_3 = OH; R_1, R_4 = H; X = CH_3

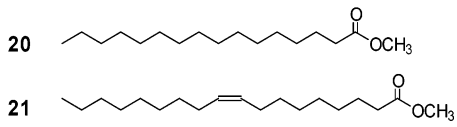


4 R_1, R_2, R_3, R_4 = H; X = C_2H_5
5 R_1 = OCH_3; R_2, R_3, R_4 = H; X = CH_3
6 R_2 = OCH_3; R_1, R_3, R_4 = H; X = CH_3
7 R_3 = OCH_3; R_1, R_2, R_4 = H; X = CH_3
8 R_1 = OH; R_2, R_3, R_4 = H; X = CH_3
9 R_2 = OH; R_1, R_3, R_4 = H; X = CH_3
10 R_3 = OH; R_1, R_2, R_4 = H; X = CH_3
11 R_2 = OCH_3; R_3 = OH; R_1, R_4 = H; X = CH_3
12 R_2 = OCH_3; R_3 = OH; R_1, R_4 = H; X = C_2H_5
13 R_2, R_3 = OH; R_1, R_4 = H; X = C_2H_5
14 R_2, R_4 = OCH_3 ; R_3 = OH; R_1 = H; X = CH_3



15 R_3 = OH; R_1, R_2, R_4 = H
16 R_2, R_3 = OCH_3; R_1, R_4 = H





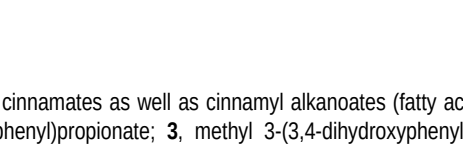


Figure 1. Substrates used for the preparation of medium- and long-chain alkyl cinnamates as well as cinnamyl alkanoates (fatty acid cinnamyl esters): **1**, ethyl hydrocinnamate (ethyl 3-phenylpropanoate); **2**, methyl 3-(2-hydroxyphenyl)propionate; **3**, methyl 3-(3,4-dihydroxyphenyl)cinnamate (methyl hydrocaffeate); **4**, ethyl *trans*-cinnamate; **5**, methyl 2-methoxycinnamate; **6**, methyl 3-methoxycinnamate; **7**, methyl 4-methoxycinnamate; **8**, methyl 2-hydroxycinnamate (methyl *o*-coumarate); **9**, methyl 3-hydroxycinnamate (methyl *m*-coumarate); **10**, methyl 4-hydroxycinnamate (methyl *p*-coumarate); **11**, methyl 4-hydroxy-3-methoxycinnamate (methyl ferulate); **12**, ethyl 4-hydroxy-3-methoxycinnamate (ethyl ferulate); **13**, ethyl 3,4-dihydroxycinnamate (ethyl caffeate); **14**, methyl 4-hydroxy-3,5-dimethoxycinnamate (methyl sinapate); **15**, 3-(4-hydroxyphenyl)propan-1-ol; **16**, 3-(3,4-dimethoxyphenyl)propan-1-ol; **17**, 1-dodecanol; **18**, 1-hexadecanol; **19**, *cis*-9-octadecen-1-ol (oleyl alcohol); **20**, methyl palmitate; **21**, methyl oleate.

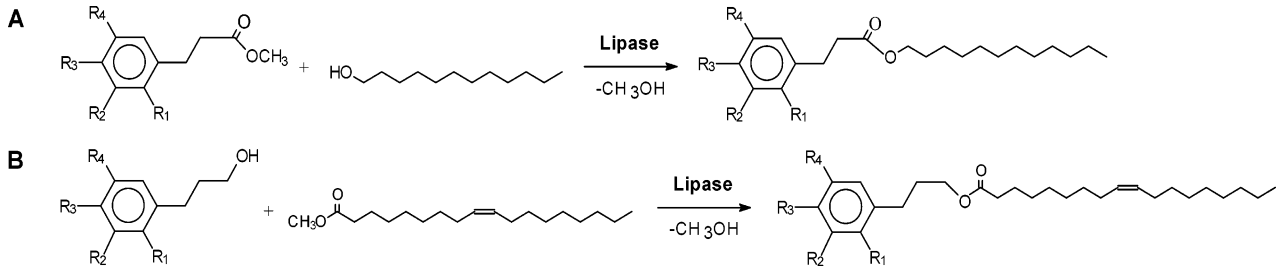


Figure 2. Transesterification of (**A**) short-chain cinnamoyl esters with 1-dodecanol and (**B**) methyl oleate with cinnamyl alcohols (R_1, R_2, R_3, R_4 as given in **Figure 1**).

inverse chemical structures such as 3-(4-hydroxyphenyl)propyl alkanoates that may be intermediates of lignin formation in plants (*32*) were prepared by transesterification of fatty acid methyl esters with hydroxy-substituted hydrocinnamyl alcohols such as 3-(4-hydroxyphenyl)propan-1-ol (hydro-*p*-coumaryl alcohol) (**Figure 2**).

MATERIALS AND METHODS

Materials. 1-Dodecanol, 1-hexadecanol, and *cis*-9-octadecen-1-ol (oleyl alcohol) as well as methyl and ethyl *trans*-cinnamate, ethyl hydrocinnamate (ethyl 3-phenylpropanoate), ethyl *trans*-2-hydroxycinnamate, ethyl 4-hydroxy-3-methoxycinnamate (ethyl ferulate), methyl 3-(4-hydroxyphenyl)propionate, and 3-(4-hydroxyphenyl)propan-1-ol were obtained from Sigma-Aldrich-Fluka (Deisenhofen, Germany). Methyl 3-(2-hydroxyphenyl)propionate, methyl 4-hydroxy-3-methoxycinnamate (methyl ferulate), and ethyl 3,4-dihydroxycinnamate (ethyl caffeate) were products of Alfa Aesar (Karlsruhe, Germany). Free carboxy groups of cinnamic acid derivatives such as 4-hydroxy-3,5-dimethoxycinnamic acid (sinapic acid), 3,4-dihydroxyhydrocinnamic acid (hydrocaffeic acid), 3-hydroxycinnamic acid, and 2-, 3-, and 4-methoxycinnamic acids (all from Sigma-Aldrich), as well as 4-hydroxycinnamic acid (from Alfa Aesar), were methylated using a solution of diazomethane in diethyl ether (*31*). The methyl esters formed were purified by column chromatography on silica gel using isohexane−diethyl ether mixtures for the elution as described recently (*31*). Immobilized lipase preparations from *Candida antarctica* (lipase B, Novozym 435), *Rhizomucor miehei* (Lipozyme RM IM), and *Thermomyces lanuginosus* (Lipozyme TL IM) were kindly provided by Novozymes, Bagsvaerd, Denmark.

Lipase-Catalyzed Reactions. As a typical example, methyl 4-hydroxycinnamate (53.4 mg, 0.3 mmol) was transesterified with *cis*-9-octadecen-1-ol (80.4 mg, 0.3 mmol) in the presence of 50 mg of immobilized Novozym 435 lipase by magnetic stirring in a screw-capped reaction tube. The tube was placed in a 100-mL Schlenk reaction vessel (made by the authors) under partial vacuum (~80 kPa) at 80 °C in the dark for periods up to 72 h with water-trapping in the gas phase using potassium hydroxide pellets. This moderate vacuum (~80 kPa) was used to prevent substantial loss of substrates. Samples of the reaction products were withdrawn at various intervals, extracted with diethyl ether, and filtered through a 1.0 μm PTFE syringe filter to separate the biocatalyst. An aliquot of the filtrate was analyzed as given below. Similar reaction conditions were used for the preparation of phenylpropyl alkanoates with inverse chemical structure. As a typical example, methyl oleate (88.8 mg, 0.3 mmol) was esterified with 3-(4-hydroxyphenyl)propanol (45.6 mg, 0.3 mmol) under conditions identical

to those described above for the transesterification of hydroxylated cinnamic acid derivatives using Novozym 435 lipase preparation as biocatalyst.

In one set of experiments, transesterifications of methyl ferulate (62.4 mg, 0.3 mmol) were carried out with *cis*-9-octadecen-1-ol (80.4 mg, 0.3 mmol) catalyzed by Novozym 435 (100 mg) under partial vacuum at 80 °C for 8 h. Thereafter, the biocatalyst was extracted twice with diethyl ether, dried in vacuo (20 min, 40 °C), and used repeatedly for nine consecutive reactions under identical conditions using fresh substrate mixture each time. Loss of Novozym 435 enzyme preparation was replaced by fresh catalyst.

Enzyme units were calculated from the initial rates (0.5, 1, or 4 h) of transesterification of methyl or ethyl (hydroxy)cinnamates with fatty alcohols. Similarly, enzyme units were determined for the transesterification of fatty acid methyl esters with 3-phenylpropanol analogues. One unit of enzyme activity was defined as the amount of enzyme (grams) that produced 1 μmol of the respective alkyl or phenylpropyl ester per minute.

Thin-Layer Chromatography (TLC). Aliquots were withdrawn from the reaction mixtures, and free carboxy groups of compounds were methylated (20 min, ~5 °C) using a ~0.2 M solution of diazomethane in diethyl ether. The conversion by transesterification was checked by TLC on 0.3 mm layers of silica gel H (VWR International, Darmstadt, Germany), and spots were located by iodine staining and, if required, by charring after spraying with 30% (v/v) sulfuric acid followed by heating (200 °C). Isohexane−diethyl ether (1:1) was used as solvent system as described previously (*31*). Similarly, 0.5 mm layers of silica gel H were used for the separation of reaction products by preparative TLC. Reaction mixtures containing phenolic hydroxy compounds were separated as described above. The various fractions were scraped off the plates and extracted from silica gel using water-saturated diethyl ether.

Purification. Alkyl 3-phenylpropanoates and 3-phenylpropyl alkanoates were extracted from the immobilized biocatalysts with diethyl ether and purified by chromatography on a silica gel 60 (VWR International) column (25 × 2 cm i.d.), using mixtures of isohexane−diethyl ether as described previously (*31*). The purification of oleyl ferulate is given as an example. Around 350 mg of the reaction mixture dissolved in 1.5 mL of diethyl ether was applied to the column and eluted first with 30 mL of isohexane and then with 30 mL portions of various isohexane−diethyl ether mixtures (95:5, 9:1, 8:2, 7:3). Elution with 30 mL of isohexane−diethyl ether (7:3) yielded ~30 mg of a mixture of oleyl alcohol and oleyl ferulate, whereas 270 mg of oleyl ferulate was obtained by elution with 30 mL of isohexane−diethyl ether (1:1). In addition, the reaction products were purified by crystallization from isohexane or mixtures of isohexane−diethyl ether (1:1).

Gas Chromatography (GC). Aliquots of esterification products were removed from the reaction mixture, dissolved in diethyl ether, and filtered through a 1.0 μm syringe filter to remove the lipase catalysts. The filtrate was concentrated in a stream of nitrogen at 40−50 °C, dissolved in diethyl ether, and treated with an ethereal solution of diazomethane to convert small proportions of hydrolyzed carboxy groups to the corresponding methyl esters. The resulting mixtures of hydroxylated cinnamic acid methyl esters, unreacted alkanols, and 3-phenylpropanols as well as medium- and long-chain alkyl hydroxycinnamates and 3-phenylpropyl alkanoates were analyzed by GC. The phenolic hydroxy group of 2-hydroxycinnamic acid derivatives was methylated to the 2-methoxy compounds by treatment with an ethereal solution of diazomethane in the presence of catalytic amounts of silica gel (*33*) to avoid coumarin formation during high-temperature GC. Similarly, phenolic hydroxy groups of hydrocaffeic and caffeic acid derivatives were (partially) methylated to improve FID response. A Hewlett-Packard (Böblingen, Germany) HP-5890 series II gas chromatograph equipped with a flame ionization detector was used. Separations were carried out on a 15 m × 0.25 mm i.d., 0.1 μm Quadrex 400-1HT fused silica capillary column (Quadrex Corp., New Haven, CT) using hydrogen as the carrier gas (column pressure = 50 kPa). The following temperature program was used to separate the various compounds of reaction mixtures: initially at 120 °C for 2 min, followed by linear programming from 140 to 180 °C at 5 °C/min, then from 180 to 250 °C at 10 °C/min (5 min isothermally), then from 250 to 410 °C at 20 °C/min, and finally kept at 410 °C for 2 min. Injector and detector temperatures were maintained at 380 °C.

Lipid extracts from transesterification reactions of methyl sinapate with oleyl alcohol were separated on a 12 m × 0.22 mm i.d., 1 μm HT5 AQ fused silica capillary column (SGE, Darmstadt, Germany) with hydrogen as the carrier gas. The following temperature program was used: initially at 100 °C (2 min), followed by linear programming from 100 to 180 °C at 5 °C/min, then from 180 to 250 °C at 10 °C/min, and finally from 250 to 410 °C at 20 °C/min (6 min).

Peaks in gas chromatograms were assigned by comparison of their retention times with those of peaks from standards. Peak areas and percentages were calculated using Hewlett-Packard GC ChemStation software. For the determination of enzyme activities, small proportions of methoxy compounds, which had been formed during carboxymethylation of hydroxycinnamic acids with diazomethane, were calculated as the original hydroxy compounds.

GC-MS Analyses. The fragmentation of the various alkyl 3-phenylpropanoates or 3-phenylpropyl alkanoates, formed by lipase-catalyzed esterification reactions, was studied by GC-MS (EI mode) as described previously (*31*).

RESULTS AND DISCUSSION

Phenolics, including hydroxylated cinnamic acids, gain importance because of their antioxidant capacity and their proposed beneficial effects on human health. Predominantly, they appear as hydrophilic compounds in the hydrophilic phases of foods. Lipophilization of hydroxylated cinnamic acids such as *p*-coumaric and ferulic acids is, therefore, of great importance to extend their field of applications to fatty food phases (*14*, *34*). Lipase-catalyzed lipophilization of phenolic antioxidants, such as hydroxylated cinnamic acids, by transesterification of the corresponding methyl esters with fatty alcohols is of special interest for their application as lipophilic antioxidants in oil-based food. Similarly, compounds with inverse chemical structure may be prepared from fatty acid methyl esters and hydroxylated phenylpropan-1-ols. Recently, we have shown that various medium- and long-chain hydroxycinnamic acid esters can be efficiently prepared from hydroxycinnamic acid analogues via lipase-catalyzed esterification with fatty alcohols. This reaction is performed under environmentally friendly conditions, particularly at moderate temperature and in the absence of organic solvents and drying reagents such as molecular sieves or sodium sulfate (*31*). In continuation of the above work we have optimized this enzymatic method for the preparation of lipophilic alkyl esters of hydroxylated cinnamic acids by using transesterification of methyl or ethyl esters of hydroxylated cinnamic acids with medium- or long-chain alcohols. Similarly, compounds with inverse chemical structure such as 3-(4-hydroxyphenyl)propyl alkanoates are prepared by lipase-catalyzed transesterification of fatty acid methyl esters with 3-(4-hydroxyphenyl)propan-1-ol or 3-(3,4-dimethoxyphenyl)propan-1-ol.

The activity of three immobilized commercial lipases, that is, Novozym 435, Lipozyme RM IM, and Lipozyme TL IM, was checked for the transesterification of equimolar mixtures of various short-chain alkyl (hydroxy)cinnamates and medium- or long-chain alcohols. The conversions were performed for up to 72 h at 80 °C under partial vacuum without solvent and drying agent in direct contact with the reaction mixture using different amounts of the respective enzyme. The time course of the transesterification of methyl cinnamate with oleyl alcohol (*cis*-9-octadecen-1-ol) catalyzed by the above lipases is shown in **Figure 3**. The results of these experiments demonstrated that immobilized lipase B from *C. antarctica* (Novozym 435) was the biocatalyst with highest enzyme activity for the transesterification reactions. Lower transesterification activity was deter-

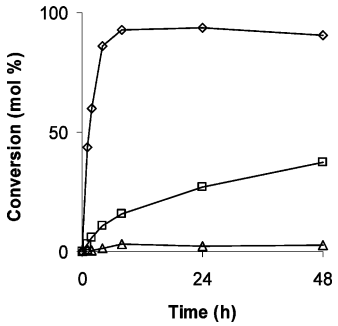


Figure 3. Time course of the formation of oleyl cinnamate by transesterification of ethyl cinnamate with oleyl alcohol catalyzed by various immobilized microbial lipases (◇, Novozym 435; □, Lipozyme RM IM; △, Lipozyme TL IM); reaction conditions: molar ratio ethyl cinnamate/oleyl alcohol (1:1; 0.3 mmol); 12.5 mg of immobilized lipase; 80 °C; 80 kPa. Values are mean of two determinations. For details see Materials and Methods.

mined for immobilized lipase from *R. miehei* (Lipozyme RM IM), whereas immobilized lipase from *T. lanuginosus* (Lipozyme TL IM) showed by far lower enzyme activity, if any. These results agree well with our previous data on the esterification activities of cinnamic and hydrocinnamic acids with oleyl alcohol using the above immobilized lipases (*31*). However, it worth noting that the transesterification activity of these lipases (**Figure 3**; **Table 1**) is far higher than their esterification activity (*31*). As an example, the following transesterification versus esterification activity was determined for the Novozym 435 catalyzed conversions of methyl hydrocinnamate and hydrocinnamic acid, respectively, with oleyl alcohol: 1049 ± 14.4 ($n = 2$) versus 195 ± 23.7 ($n = 3$) enzyme units (at 0.5 h, 80 °C, and 80 kPa; 6 mg of lipase). High differences between transesterification and esterification activities were also found for Lipozyme RM IM, whereas both transesterification and esterification activities were generally low for Lipozyme TL IM.

Figure 4 shows the time course of the transesterification of various methyl or ethyl cinnamates and hydrocinnamates with oleyl alcohol at 80 °C under partial vacuum using Novozym 435 as biocatalyst. Particularly, the formation of oleyl cinnamate and oleyl hydrocinnamate (**Figure 4A**), various oleyl hydroxycinnamates such as oleyl *o*-, *m*-, and *p*-coumarates (**Figure 4B**), and oleyl caffeate and oleyl hydrocaffeate (**Figure 4C**) as well as oleyl ferulate (4-hydroxy-3-methoxycinnamate) (**Figure 4D**) by Novozym 435 catalyzed transesterification of the corresponding methyl or ethyl cinnamates with oleyl alcohol is demonstrated over a period of 8−72 h. It is obvious from these results that (a) highest transesterification activity is observed with ethyl hydrocinnamate as the substrate (**Figure 4A**; **Table 1**); (b) introducing a >C═C< double bond to form ethyl cinnamate leads to a remarkable decrease of lipase activity (**Figure 4A**; **Table 1**) (similarly, the transesterification activity of Novozym 435 is remarkably higher for methyl 4-hydroxyhydrocinnamate and methyl hydrocaffeate than the corresponding hydroxycinnamates as is demonstrated in **Table 1** and **Figure 4C**); (c) introduction of a methoxy group or hydroxy group (**Figure 4B**; **Table 1**) leads to a remarkable loss of lipase activity; and (d) the presence of more than one methoxy and/or hydroxy group in the substrate molecule, for example, methyl ferulate, caffeate, and sinapate, results in an additional reduction of transesterification activity (**Figures 4**; **Table 1**) [similar results were recently found for the esterification of various cinnamic acid analogues with fatty alcohols using Novozym 435 as biocatalyst (*31*)].

Table 1 shows the transesterification activities of the three commercial immobilized lipases used. Moreover, maximum conversions for the transesterification of various methyl or ethyl cinnamate and hydrocinnamate analogues with fatty alcohols as well as of fatty acid methyl esters with 3-(4-hydroxyphenyl)-propanol and 3-(3,4-dimethoxyphenyl)propanol under different reaction conditions are given in **Table 1**. These data again demonstrate that immobilized lipase B from *C. antarctica* (Novozym 435) is generally superior to immobilized lipases from *R. miehei* (Lipozym RM IM) and, particularly, *T. lanuginosus* (Lipozyme TL IM), in the preparation of various alkyl hydroxycinnamates and hydroxyhydrocinnamates by transesterification. Together with the results from **Figure 4** the following relative transesterification activities are found for Novozym 435: hydrocinnamate > cinnamate > 4-hydroxyhydrocinnamate > 3-methoxycinnamate > 2-methoxycinnamate ≈ 4-methoxycinnamate ≈ 3-hydroxycinnamate > hydrocaffeate ≈ 4-hydroxycinnamate > ferulate > 2-hydroxycinnamate > caffeate ≈ sinapate. With respect to the position of the hydroxy substituents at the phenyl moiety, the transesterification activity of Novozym 435 increases in the order 3- > 4- > 2-position (meta > para > ortho) (**Figure 4B**; **Table 1**). The transesterification activity of Novozym 435 is higher for methoxy-substituted than for hydroxy-substituted cinnamates (**Table 1**). Similar relative enzyme activities were recently observed for the esterification of various methoxy- and hydroxy-substituted cinnamic acid analogues with fatty alcohols using Novozym 435 as biocatalyst (*31*). The Novozym 435 catalyzed transesterification of long-chain fatty acid methyl esters with 3-(4-hydroxyphenyl)propanol and 3-(3,4-dimethoxyphenyl)propanol leads to 3-(4-hydroxyphenyl)propyl alkanoates and 3-(3,4-dimethoxyphenyl)propyl alkanoates, respectively, in high yield (**Table 1**).

It is obvious from these results that transesterification activities of the lipases used including Novozym 435 are influenced by structural properties of the reactants such as the >C═C< double bond of the phenylpropenyl moiety or the hydroxy and methoxy substituents of the phenyl moiety. Successive introduction of such functional groups steadily leads to decreasing enzyme activities (**Figures 3** and **4**; **Table 1**). The polar and/or bulky hydroxy and methoxy substituents at the phenyl moiety may decrease the transesterification activity by sterical hindrance at the active site of the lipases. In addition, enzyme activity is influenced by the position of the substituents at the phenyl moiety. Thus, the position of the hydroxy substituent increases the transesterification activity of Novozym 435 as follows: meta > para > ortho (**Figure 4B**). A hydroxy substituent at the ortho position of the phenyl moiety particularly leads to substantial loss of enzyme activity. This may be explained by interactions of *o*-hydroxy and propenoic acid side chain of *o*-coumarates. Moreover, physicochemical factors at the oil−water interphase may be impaired by acidic groups such as phenolic hydroxy substituents of the substrates. This effect may explain the lower esterification rate of substrates containing phenolic hydroxy groups as compared to those containing methoxy groups. For the same reason, free carboxy groups of the various cinnamic acids may lead to far lower lipase activities in esterification reactions. Introducing the more unpolar carboxymethyl or -ethyl ester groups may increase, therefore, the activity of Novozym 435 in transesterifications as compared to the esterification reactions described recently (*31*). A similar correlation between the position of substituents at the phenyl moiety and the enzyme activity was demonstrated by Stamatis et al. (*34*) for the lipases from *C. antarctica* and *R. miehei*.

Table 1. Enzyme Activities of Various Lipases for the Transesterification of Methyl or Ethyl (Hydroxy)cinnamates with Fatty Alcohols and of Fatty Acid Methyl Esters with (Hydroxy)phenylpropanols

methyl or ethyl cinnamates and their analogues as well as fatty acid methyl esters	medium- and long-chain alcohols as well as 3-phenylpropanols	lipase	maximum conversion (mol %) after time [h]	enzyme activity (units/g) after time[a] [h]	± SEM
ethyl hydrocinnamate (**1**)	cis-9-octadecen-1-ol (**19**)	Novozym 435	94 [8]	1049 [0.5] [b,c]	± 14.4 ($n = 2$)
ethyl hydrocinnamate (**1**)	cis-9-octadecen-1-ol (**19**)	Novozym 435	95 [8]; 97 [24]	618 [0.5][d]	± 3.2 ($n = 2$)
ethyl hydrocinnamate (**1**)	cis-9-octadecen-1-ol (**19**)	Lipozyme RM IM	94 [8]	428 [0.5][d]	± 7.1 ($n = 2$)
ethyl hydrocinnamate (**1**)	cis-9-octadecen-1-ol (**19**)	Lipozyme TL IM	90 [24]	130 [1][d]	± 1.5 ($n = 2$)
ethyl cinnamate (**4**)	cis-9-octadecen-1-ol (**19**)	Novozym 435	93 [8]	175 [1][d]	± 10.0 ($n = 2$)
ethyl cinnamate (**4**)	cis-9-octadecen-1-ol (**19**)	Lipozyme RM IM	38 [48]	11 [4][d]	± 1.2 ($n = 2$)
ethyl cinnamate (**4**)	cis-9-octadecen-1-ol (**19**)	Lipozyme TL IM	3 [8][d]	nd[g]	– ($n = 2$)
methyl 2-methoxycinnamate (**5**)	cis-9-octadecen-1-ol (**19**)	Novozym 435	93 [8]	36 [1]	± 0.4 ($n = 2$)
methyl 2-methoxycinnamate (**5**)	cis-9-octadecen-1-ol (**19**)	Lipozyme RM IM	16 [72]	3 [1]	± 1.1 ($n = 2$)
methyl 3-methoxycinnamate (**6**)	cis-9-octadecen-1-ol (**19**)	Novozym 435	95 [8]	57 [1]	± 4.3 ($n = 2$)
methyl 3-methoxycinnamate (**6**)	cis-9-octadecen-1-ol (**19**)	Lipozyme RM IM	90 [48]	11 [1]	± 0.1 ($n = 2$)
methyl 4-methoxycinnamate (**7**)	cis-9-octadecen-1-ol (**19**)	Novozym 435	92 [8]	28 [1]	± 1.4 ($n = 2$)
methyl 4-methoxycinnamate (**7**)	cis-9-octadecen-1-ol (**19**)	Lipozyme RM IM	60 [72]	5 [1]	± 0.4 ($n = 2$)
methyl 2-hydroxycinnamate (**8**) (methyl *o*-coumarate)	cis-9-octadecen-1-ol (**19**)	Novozym 435	52 [24]	6 [1]	± 2.1 ($n = 2$)
methyl 3-hydroxycinnamate (**9**) (methyl *m*-coumarate)	cis-9-octadecen-1-ol (**19**)	Novozym 435	94 [72]	31 [1]	± 1.4 ($n = 2$)
methyl 4-hydroxycinnamate (**10**) (methyl *p*-coumarate)	cis-9-octadecen-1-ol (**19**)	Novozym 435	95 [72]	20 [1]	± 0.8 ($n = 2$)
methyl 4-hydroxyhydrocinnamate (**2**)	cis-9-octadecen-1-ol (**19**)	Novozym 435	93 [4]; 97 [72]	72 [1]	± 4.2 ($n = 4$)
methyl ferulate (**11**)	dodecan-1-ol (**17**)	Novozym 435	95 [8]; 98 [24]	25 [1][f]	± 0.4 ($n = 2$)
methyl ferulate (**11**)	hexadecan-1-ol (**18**)	Novozym 435	94 [8]; 96 [24]	25 [1][e,f]	± 1.9 ($n = 2$)
methyl ferulate (**11**)	cis-9-octadecen-1-ol (**19**)	Novozym 435	72 [8]; 95 [24]	19 [1]	± 1.5 ($n = 2$)
methyl ferulate (**11**)	cis-9-octadecen-1-ol (**19**)	Novozym 435	91 [8]; 93 [24]	15 [1][f]	± 0.5 ($n = 2$)
ethyl ferulate (**12**)	cis-9-octadecen-1-ol (**19**)	Novozym 435	89 [8]; 94 [24]	15 [1][f]	± 0.2 ($n = 2$)
methyl hydrocaffeate (**3**)	cis-9-octadecen-1-ol (**19**)	Novozym 435	82 [48]	23 [1][f]	± 0.6 ($n = 2$)
ethyl caffeate (**13**)	cis-9-octadecen-1-ol (**19**)	Novozym 435	59 [72]	1.2 [4][f]	± 0.3 ($n = 4$)
methyl sinapate (**14**)	cis-9-octadecen-1-ol (**19**)	Novozym 435	69 [72]	1.1 [4][f]	± 0.04 ($n = 2$)
methyl palmitate (**20**)	3-(4-hydroxyphenyl)propan-1-ol (**15**)	Novozym 435	84 [4]; 88 [24]	78 [1]	± 0.6 ($n = 2$)
methyl oleate (**21**)	3-(4-hydroxyphenyl)propan-1-ol (**15**)	Novozym 435	89 [4]; 92 [24]	74 [1]	± 3.2 ($n = 2$)
methyl palmitate (**20**)	3-(3,4-dimethoxyphenyl)propan-1-ol (**16**)	Novozym 435	90 [4]; 93 [24]	83 [1]	± 6.1 ($n = 2$)

[a] Standard assay conditions, if not otherwise indicated: 0.3 mmol of carboxylic acid + 0.3 mmol fatty alcohol. Immobilized lipase/assay: 50 mg; 80 °C; 80 kPa. [b] 6 mg of lipase. [c] For comparison: esterification of 0.3 mmol of hydrocinnamic acid with 0.3 mmol of *cis*-9-octadecen-1-ol, 6 mg of Novozym 435; maximum conversion, 95% [24 h]; enzyme activity (0.5 h), 192 ± 23.7 units/g ($n = 3$). [d] 12.5 mg of lipase preparation. [e] For comparison: esterification of 0.3 mmol of ferulic acid with 0.3 mmol of hexadecan-1-ol, 100 mg of Novozym 435; enzyme activity, 0.44 units/g ± 0.03 ($n = 2$), at 80 °C and 80 kPa; corresponding transesterification of methyl ferulate as shown above, enzyme activity 25 ± 1.9 units/g ($n = 2$), i.e., 56-fold increase. [f] 100 mg of lipase preparation. [g] Not determined.

Moreover, it is worth noting that high proportions of hydrocinnamic acid esters—but no cinnamic acid esters—are formed both by esterification and transesterification reactions using lipase from *Pseudomonas cepacia* (*35*).

Depending on the reaction conditions, conversions to medium- and long-chain alkyl hydroxycinnamates of up to 98 mol % are obtained by Novozym 435 catalyzed transesterification of equimolar mixtures of methyl or ethyl hydroxycinnamates and medium- or long-chain alcohols such as lauryl, palmityl, and oleyl alcohol (**Table 1**). Transesterification of long-chain fatty acid methyl esters such as methyl palmitate and oleate with hydroxylated or methoxylated phenylpropan-1-ols catalyzed by Novozym 435 lipase led to the corresponding fatty acid cinnamyl esters in high yield, as well. Moreover, the transesterification activity of Novozym 435 is much higher than the esterification activity under the conditions described (**Table 1**). As an example, the activity of Novozym 435 is 56 times higher for the transesterification of methyl ferulate with hexadecan-1-ol than for the corresponding esterification of ferulic acid (80 °C; 80 kPa; substrates 0.3 mmol, each; 100 mg of lipase). These results show that the enzyme activity of Novozym 435 is much higher when short-chain alkyl esters of hydroxy- and methoxy-substituted cinnamic acids are transesterified with fatty alcohols to obtain medium- and long-chain hydroxycinnamic acid esters (**Table 1**). The molecular ions and typical mass fragments of the transesterification products obtained by gas chromatography−EI mass spectrometry confirm the chemical structures of these compounds as demonstrated previously (*31*). These data also agree well with those given in the literature (*9*).

Figure 5 shows the percent conversion of methyl ferulate to oleyl ferulate in the transesterification with oleyl alcohol catalyzed by Novozym 435 after repeated use of the lipase catalyst as described under Materials and Methods. The reactions were carried out under partial vacuum at 80 °C for 8 h, each. From the results given in **Figure 5** it is obvious that Novozym 435 is quite stable as an enzyme catalyst at least over a period of 10 reaction cycles at 80 °C and 80 kPa, leading to ∼75 mol % average conversion of methyl ferulate to oleyl ferulate.

Under the transesterification conditions described, high yields of medium- or long-chain alkyl hydroxy- and methoxycinnamates, such as oleyl *p*-coumarate and oleyl ferulate (**Table 1**), are obtained as compared to lipase-catalyzed esterification and transesterification methods developed earlier (*21*−*30*). The procedure described above for the Novozym 435 catalyzed transesterification of various hydroxy- and methoxy-substituted methyl cinnamates is by far less time-consumptive than those reported earlier (*21*, *24*, *25*). Moreover, equimolar mixtures of reactants are used as starting materials instead of high excesses of alcohols, as described by others (*23*, *24*, *34*). Recently, we have described an efficient procedure for the lipase-catalyzed

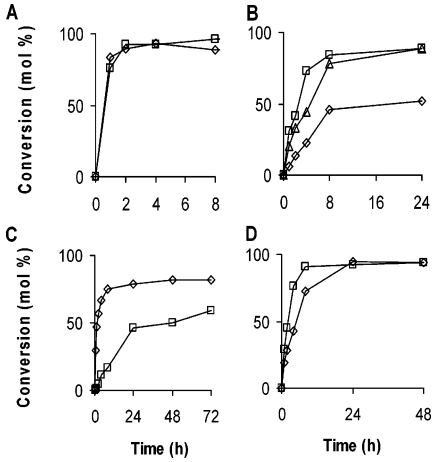


Figure 4. Time course of the formation of various oleyl cinnamate analogues by the enzymatic transesterification of methyl or ethyl esters of hydrocinnamic, cinnamic, and hydroxycinnamic acids with oleyl alcohol: (**A**) ◇, oleyl hydrocinnamate, and □, oleyl cinnamate (50 mg of Novozym 435); (**B**) oleyl hydroxycinnamates, e.g., ◇, 2-hydroxy-, □, 3-hydroxy-, and, △, 4-hydroxycinnamates (50 mg of Novozym 435); (**C**) △, oleyl hydrocaffeate, and □, oleyl caffeate (100 mg of Novozym 435). Time course of the formation of oleyl ferulate (**D**) by transesterification of methyl ferulate with oleyl alcohol using different amounts of Novozym 435 lipase (◇, 50 mg; □, 100 mg). Values are mean of two determinations. For details see Materials and Methods.

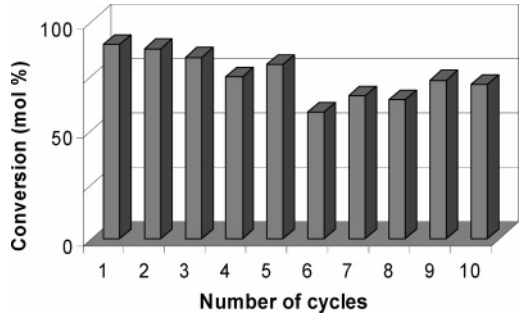


Figure 5. Repeated use of Novozym 435 (100 mg) biocatalyst for the formation of oleyl ferulate by transesterification of methyl ferulate (0.3 mmol) with oleyl alcohol (0.3 mmol). The reactions were carried out at 80 °C under partial vacuum (80 kPa) for 8 h. Values are mean of two determinations. For details see Materials and Methods.

esterification of hydroxycinnamic acids and fatty alcohols leading to medium- and long-chain hydroxycinnamic acid esters (*31*). Using this esterification procedure higher amounts of immobilized lipase B from *C. antarctica* (Novozym 435) are necessary to obtain moderate yields of medium- or long-chain hydroxylated cinnamic acid esters bearing more than one methoxy or hydroxy group within a satisfactory period of time.

In conclusion, we have developed a lipase-catalyzed transesterification procedure for the preparation of lipophilic medium- and long-chain hydroxylated cinnamic acid esters, such as alkyl coumarates and alkyl ferulates, in high yield and within a satifactory period of time. Immobilized lipase B from *C. antarctica* (Novozym 435) and, in part, immobilized lipase from *R. miehei* (Lipozyme RM IM) are found to be the most effective biocatalysts for these reactions, which are performed at moderate temperature under partial vacuum in the absence of solvents and drying agents in direct contact with the reaction mixture. Similar lipophilic antioxidants with inverse chemical structure, for example, 3-(4-hydroxyphenyl) palmitate and oleate, are also prepared in this way. The reaction products, particularly medium- and long-chain alkyl hydroxycinnamates and 3-(4-hydroxyphenyl)propyl alkanoates, such as dodecyl, palmityl, and oleyl ferulate, may be useful lipophilic antioxidants for food and feed as well as cosmetic and technical applications (*14*, *34*).

As compared to previous studies on the preparation of alkyl hydroxycinnamates (*14*, *31*), we have substantially reduced the reaction times by introducing the transesterification reaction. However, high amounts of lipase are still necessary to obtain satisfactory yields. Because costs are a major factor for the industrial use of lipases and other biocatalysts, further efforts are needed in reducing the amount of enzyme used for the preparation of hydroxycinnamates.

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