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Preparation of lipophilic alkyl (hydroxy)benzoates by solvent-free lipase-catalyzed esterification and transesterification

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Abstract Long-chain alkyl benzoates, e.g., lauryl 4-hydroxybenzoate, palmityl 4-hydroxybenzoate, and oleyl 4-hydroxy-3-methoxybenzoate, are formed in high to moderate conversion by lipase-catalyzed transesterification of the corresponding short-chain alkyl benzoates (0.3 to 1 mmol) with fatty alcohols in an equimolar ratio. The substrates are reacted in vacuo in the absence of solvents and drying agents in the reaction mixture. Immobilized lipase B from *Candida antarctica* (Novozym 435) demonstrates higher activity for the transesterification of various methyl (hydroxy)benzoates with long-chain alcohols than for the corresponding esterification reactions. For example, transesterification activity is around 25-fold higher than esterification activity for the preparation of oleyl 4-hydroxybenzoate. The relative transesterification activities of methoxy- and hydroxy-substituted methyl benzoates found for Novozym 435 are as follows: 2-methoxybenzoate≈3-methoxybenzoate>4-methoxybenzoate>3-hydroxybenzoate≈2-hydroxybenzoate>4-hydroxybenzoate≈4-hydroxy-3-methoxybenzoate≈3-hydroxy-4-methoxybenzoate>>3,4-dihydroxybenzoate. With respect to the position of the substituents at the phenyl moiety of methyl benzoates, transesterification activity of Novozym 435 increases in the order ortho≈meta>para. Compounds with inverse chemical structure, e.g., (methoxy)benzyl alkanoates, are formed in much higher rates both by esterification and transesterification than the analogous alkyl benzoates. Purification by deacidification, crystallization, or vacuum distillation yielded 74% to 89% of the reaction products.

Keywords Oleyl 4-hydroxybenzoate · Oleyl 4-hydroxy-3-methoxybenzoate (Oleyl vanillate) · 3-Hydroxybenzyl oleate · Immobilized lipase B from *Candida antarctica* (Novozym 435) · Esterification · Transesterification

Introduction

Hydroxybenzoic acid as well as other ω-(hydroxyphenyl) alkanoic and ω-(hydroxyphenyl)alkenoic acids such as hydroxyphenylacetic and hydroxycinnamic acids are common constituents of plant tissues (Mattila et al. 2006; Paterson et al. 2006). Small proportions of alkyl hydroxybenzoates and other phenolic acid esters have also been isolated from plants (Walker et al. 2003; Baderschneider and Winterhalter 2001). Beneficial effects on health have been attributed to the antioxidant capacity of plant phenolics, particularly against oxidative attacks by radical-scavenging activity (Paterson et al. 2006). For example, short-chain alkyl 4-hydroxybenzoates (*p*-hydroxybenzoates; PHB esters) and alkyl 3,4,5-trihydroxybenzoates (alkyl gallates) are known as antioxidants for food and feed (Stöckmann et al 2000; Soni et al. 2005). 2-Hydroxybenzoic (salicylic) acid is a plant-derived phenolic acid demonstrating biological effects on animals, plants, and microorganisms (Paterson et al. 2006; Kupferwasser et al. 2003; Vasyukova and Ozeretskovskaya 2007). Antioxidant capacity, biological availability, and

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physiological activity of several rather hydrophilic hydroxybenzoic acid derivatives may be further improved by increasing their lipophilicity via esterification with medium- and long-chain alcohols (Buisman et al. 1998; Figueroa-Espinoza and Villeneuve 2005; Stamatis et al. 1999; Stöckmann et al 2000). Moreover, the range of applications of such lipophilic alkyl hydroxybenzoates may be extended by their possible use as additives for foods, cosmetics, flavor ingredients, and technical applications. Similar compounds with inverse chemical structure, i.e., acyl esters of (hydroxy)benzyl alkohols, may also show antioxidant and biological effects as they are known for alkyl (hydroxy)benzoates.

Various methods are known for the chemical esterification of benzoic acid derivatives, predominantly esterification in the presence of acid catalysts such as sulfuric acid or *p*-toluenesulfonic acid using an excess of the corresponding alcohol (CIR 1984; Soni et al. 2005). Sulfuric acid is removed by washing with water and nonreacted (hydroxy) benzoic acid by deacidification with sodium carbonate. The final alkyl benzoates are purified by crystallization. The esterification of *p*-hydroxybenzoic acid with *n*-butanol is improved under microwave irradiation using $ZnCl_2$/sulfuric acid as a catalyst (Liao et al. 2002). Particularly, benzyl salicylate is prepared by reacting sodium salicylate with benzyl chloride (Kohrman et al. 1983). Enzymatic esterification of ω-(hydroxyphenyl)alkanoic acids and transesterification of methyl ω-(hydroxyphenyl)alkanoates, respectively, may be of advantage over chemical esterification and transesterification for the preparation of lipophilic ω-(hydroxyphenyl)alkanoic acid 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 ω-(hydroxyphenyl)alkanoates including alkyl (hydroxy)benzoates (Buisman et al. 1998; Larios et al. 2004; for review, see Figueroa-Espinoza and Villeneuve 2005). Recently, we have described procedures for the lipase-catalyzed esterification and transesterification of (hydroxy)cinnamic acids and methyl (hydroxy)cinnamates, respectively, with medium- or long-chain alcohols using equimolar mixtures of nonactivated reactants at moderate temperatures. The reactions were performed without solvents or drying agents in the reaction mixture using reduced pressure to remove reaction water or methanol (Vosmann et al. 2006; Weitkamp et al. 2006). This method was much more efficient than various other enzymatic esterification and transesterification procedures described earlier (Figueroa-Espinoza and Villeneuve 2005). The aim of the present work was to extend this simple and environmentally friendly lipase-catalyzed process to the preparation of lipophilic medium- and long-chain alkyl (hydroxy)benzoates as well as (hydroxy)benzyl alkanoates by using esterification and transesterification reactions (Fig. 1).

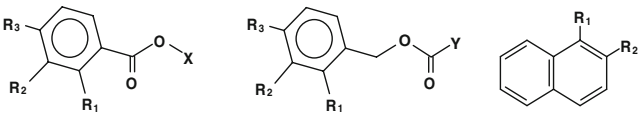

Fig. 1 Chemical structure of various lipophilic phenolic esters prepared by lipase-catalyzed esterification and/or transesterification such as **a** benzoic acid alkyl esters (alkyl benzoates): oleyl benzoate (R_1, R_2, R_3=H; X=oleyl), oleyl 2-methoxybenzoate (R_1=OCH_3, R_2, R_3=H; X=oleyl), oleyl 3-methoxybenzoate (R_2=OCH_3, R_1, R_3=H; X=oleyl), oleyl 4-methoxybenzoate (R_3=OCH_3, R_1, R_2=H; X=oleyl), oleyl 2-hydroxybenzoate (R_1=OH, R_2, R_3=H; X=oleyl), oleyl 3-hydroxybenzoate (R_2=OH, R_1, R_3=H; X=oleyl), oleyl 4-hydroxybenzoate (R_3=OH, R_1, R_2=H; X=lauryl, palmityl, and oleyl), oleyl 3-hydroxy-4-methoxybenzoate (R_2=OH, R_3=OCH_3, R_1=H; X=oleyl), oleyl 4-hydroxy-3-methoxybenzoate (R_3=OH, R_2=OCH_3, R_1=H; X=oleyl), oleyl 3,4-dihydroxybenzoate (R_2, R_3=OH, R_1=H; X=oleyl); **b** alkanoic acid benzyl esters (benzyl alkanoates): benzyl oleate (R_1, R_2, R_3=H; Y=oleoyl), 2-methoxybenzyl oleate (R_1=OCH_3, R_2, R_3=H; Y=oleoyl), 3-methoxybenzyl oleate (R_2=OCH_3, R_1, R_3=H; Y=oleoyl), 4-methoxybenzyl oleate (R_3=OCH_3, R_1, R_2=H; Y=oleoyl), 3-hydroxybenzyl oleate (R_2=OH, R_1, R_3=H; Y=palmitoyl and oleoyl), 3-hydroxy-4-methoxybenzyl oleate (R_2=OH, R_3=OCH_3, R_1=H; Y=oleoyl), 4-hydroxy-3-methoxybenzyl oleate (R_3=OH, R_2=OCH_3, R_1=H; Y=oleoyl), **c** naphthoic acid alkyl esters (alkyl naphthoates): oleyl 1-naphthoate (R_1=$C_{18}H_{35}O$–CO–, R_2=H), oleyl 2-naphthoate (R_1=H, R_2=$C_{18}H_{35}O$–CO–), and **d** alkanoic acid naphthalenemethyl esters (naphthalenemethyl alkanoates): 1-naphthalenemethyl oleate (R_1=$C_{17}H_{33}$–COO–CH_2–, R_2=H); 2-naphthalenemethyl oleate (R_1=H; R_2=$C_{17}H_{33}$–COO–CH_2–)

Materials and methods

Materials

Benzoic acid, benzyl alcohol, and methyl oleate were purchased from VWR International, Darmstadt, Germany. Methyl 3-methoxybenzoate and *i*-butyl 4-hydroxybenzoate were obtained from Alfa Aesar (Karlsruhe, Germany). All other benzoic acid and benzyl alcohol derivatives, naphthoic acid and naphthalenemethanol derivatives, as well as fatty acids, fatty acid methyl esters, and fatty alcohols were products of Sigma-Aldrich-Fluka (Deisenhofen, Germany). Methyl 1-naphthoate was prepared from the corresponding carboxylic acid by the reaction with diazomethane. 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-hydroxybenzoate (45.6 mg, 0.3 mmol) was transesterified with *cis*-9-octadecen-1-ol (80.4 mg, 0.3 mmol) in the presence of 12.5 to 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 under partial vacuum

(~80 kPa) at 80°C for periods up to 72 h with water trapping in the gas phase using potassium hydroxide pellets. This moderate vacuum was used to prevent substantial loss of substrates. Samples of the reaction products were withdrawn at various intervals, extracted, and filtered (0.45 μm polytetrafluoroethylene [PTFE] syringe filter) as described earlier (Weitkamp et al. 2006). An aliquot of the filtrate was analyzed as given below. Similar reaction conditions were used for the preparation of various benzyl alkanoates. Blanks were performed under standard assay conditions without lipase. Enzyme activities were calculated from the initial rates (0.5, 1, or 4 h) of esterification or transesterification reactions (1 U=1 μmol alkyl (hydroxy)benzoate or (hydroxy)benzyl alkanoate per g enzyme×min).

Purification

Generally, diethyl ether extracts of reaction mixtures (~250 mg) were purified by chromatography on a silica gel 60 (VWR International) column (25×2 cm inner diameter) using mixtures of *i*-hexane-diethyl ether as described previously (Vosmann et al. 2006; Weitkamp et al. 2006). Similarly, an experiment with higher amounts of starting materials was performed as follows. Benzoic acid (3.66 g) and lauryl alcohol (5.58 g), 30 mmol, each, were esterified as described above (conversion ~93% after 48 h as checked by gas chromatography [GC]). The reaction products were extracted with diethyl ether (3×10 mL) and filtered. Aqueous sodium carbonate solution (8 mL; 2% *w*/*v*) was added to the ether extract, mixed, and centrifuged. After removing the aqueous phase, the organic phase was dried over sodium sulfate and filtered, and the solvent was evaporated in vacuo yielding ~9.0 g of extract. Distillation in vacuo at 0.04 kPa and 130°C for 15 min led to 7.66 g lauryl benzoate (yield 88.0%, purity 98.3%).

Gas chromatography

Aliquots of esterification and transesterification products were removed from the reaction mixture, extracted, filtered (0.45 μm PTFE syringe filter) and treated with an ethereal solution of diazomethane to convert carboxylic acids to the corresponding methyl esters. The resulting mixtures of (hydroxy)benzoic acid methyl esters, unreacted medium- or long-chain 1-alkanols, and alkyl (hydroxy)benzoates or of benzyl alcohols, fatty acid methyl esters, and (hydroxy) benzyl alkanoates were analyzed by GC as described previously (Vosmann et al. 2006; Weitkamp et al. 2006). The phenolic hydroxy groups of 2-hydroxybenzoates and 3,4-dihydroxybenzoates as well as vanillyl and isovanillyl alcohols were partly methylated to the corresponding methoxy derivatives by treatment with an ethereal solution of diazomethane in the presence of catalytic amounts of silica gel (Vosmann et al. 2006) to improve separation and flame-ionization detection response. Peaks in gas chromatograms were assigned by comparison of their retention times with those of peaks from standard preparations, which were identified by GC-mass spectrometry (MS). Peak areas and percentages were calculated using a Hewlett-Packard GC ChemStation software. For the determination of enzyme activities, small proportions of methoxy compounds, which had been formed during methylation of hydroxylated benzoic acids with diazomethane, were calculated as the original hydroxy compounds.

Mass spectrometry

GC-MS was performed in electron ionization mode as described previously (Vosmann et al. 2006; Weitkamp et al. 2006). Typical molecular ions and other important mass fragment ions (*m*/*z*; rel. %) were observed for the various reaction products. The results are as follows: benzoic and naphthoic acid esters: Oleyl benzoate 372 $[M]^+$ (0.9), 105 $[M–C_{18}H_{35}O]^+$ (100). Oleyl 2-methoxybenzoate 402 $[M]^+$ (0.8), 135 $[M–C_{18}H_{35}O]^+$ (100). Oleyl 3-methoxybenzoate 402 $[M]^+$ (0.7), 135 $[M–C_{18}H_{35}O]^+$ (58.9). Oleyl 4-methoxybenzoate 402 $[M]^+$ (0.8), 135 $[M–C_{18}H_{35}O]^+$ (100). Oleyl 2-hydroxybenzoate 388 $[M]^+$ (0.6), 121 $[M–C_{18}H_{35}O]^+$ (100). Oleyl 3-hydroxybenzoate 388 $[M]^+$ (0.4), 121 $[M–C_{18}H_{35}O]^+$ (80.3). Oleyl 4-hydroxybenzoate 388 $[M]^+$ (0.5), 121 $[M–C_{18}H_{35}O]^+$ (100). Oleyl 1-naphthoate 422 $[M]^+$ (0.5), 155 $[M–C_{18}H_{35}O]^+$ (66.1). Oleyl 2-naphthoate 422 $[M]^+$ (0.5), 155 $[M–C_{18}H_{35}O]^+$ (57.1). Oleyl 2-phenoxybenzoate 464 $[M]^+$ (0.6), 197 $[M–C_{18}H_{35}O]^+$ (100). Oleyl 3-phenoxybenzoate 464 $[M]^+$ (0.4), 197 $[M–C_{18}H_{35}O]^+$ (37.5). Oleyl 4-phenoxybenzoate 464 $[M]^+$ (0.5), 197 $[M–C_{18}H_{35}O]^+$ (42.9). Oleyl 4-hydroxy-3-methoxybenzoate 418 $[M]^+$ (0.6), 151 $[M–C_{18}H_{35}O]^+$ (62.5). Oleyl 3-hydroxy-4-methoxybenzoate 418 $[M]^+$ (0.7), 151 $[M–C_{18}H_{35}O]^+$ (49.5). Oleyl 3,4-dihydroxybenzoate: GC-MS as dimethoxy derivative. Benzyl and naphthalenemethyl esters: benzyl oleate 372 $[M]^+$ (0.3), 91 $[M–C_{18}H_{33}O_2]^+$ (100). 2-Methoxybenzyl oleate 402 $[M]^+$ (0.3), 121 $[M–C_{18}H_{33}O_2]^+$ (100). 3-Methoxybenzyl oleate 402 $[M]^+$ (0.5), 121 $[M–C_{18}H_{33}O_2]^+$ (100). 4-Methoxybenzyl oleate 402 $[M]^+$ (0.3), 121 $[M–C_{18}H_{33}O_2]^+$ (100). 3-Hydroxybenzyl oleate 388 $[M]^+$ (0.4), 107 $[M–C_{18}H_{33}O_2]^+$ (100). 4-Hydroxy-3-methoxybenzyl oleate 418 $[M]^+$ (0.7), 137 $[M–C_{18}H_{33}O_2]^+$ (100). 3-Hydroxy-4-methoxybenzyl oleate 418 $[M]^+$ (0.6), 137 $[M–C_{18}H_{33}O_2]^+$ (100). 1-Naphthalenemethyl oleate 422 $[M]^+$, 141 $[M–C_{18}H_{33}O_2]^+$ (100). 2-Naphthalenemethyl oleate 422 $[M]^+$, 141 $[M–C_{18}H_{33}O_2]^+$ (100). Molecular ions of the last two compounds were determined in the chemical ionization mode with methane as reagent gas.

Results

Enzyme activities and maximum conversions of three immobilized commercial lipases, i.e., Novozym 435, Lipozyme RM IM, and Lipozyme TL IM, were studied for the esterification and transesterification of equimolar mixtures of (hydroxy)benzoic acids and their short-chain alkyl esters with medium- or long-chain alcohols. Moreover, enzyme activities were determined for the esterification and transesterification, respectively, of 1- and 2-naphthoic acids as well as their methyl esters with long-chain alcohols.

Table 1 and Fig. 2 demonstrate various reaction parameters affecting the lipase-catalyzed esterification and transesterification of phenolic acids and phenolic alcohols with long-chain alcohols and alkanoic acids, respectively. It is evident from the data given in Table 1 that the enzyme activity of the immobilized lipase is at least tenfold higher for the transesterification as compared to the esterification of, e.g., methyl benzoate and benzoic acid (60 vs. 6.3 U) or methyl 4-hydroxybenzoate and 4-hydroxybenzoic acid (8 vs. 0.3 U). Moreover, maximum conversions of various benzoic acid derivatives are clearly lower than those of the corresponding methyl benzoates (Table 1).

The results of the experiments given in Table 1 also reveal that the transesterification activities of immobilized lipases from *R. miehei* (Lipozyme RM IM) and *T. lanuginosus* (Lipozyme TL IM) are far lower than that from *C. antarctica* (Novozym 435). Novozym 435 was

Table 1 Enzyme activities of various lipases for the esterification and transesterification of various benzoic and naphthoic acid derivatives and their short-chain alkyl esters, respectively, with medium- and long-chain alcohols as well as for the esterification and transesterification of fatty acids and fatty acid methyl esters, respectively, with various benzyl and naphthalenemethyl alcohol derivatives

Benzoic acids and short-chain alkyl benzoates as well as fatty acids and fatty acid methyl esters	Medium- and long-chain alcohols as well as benzyl alcohols	Immobilized Novozym 435 lipase (mg)	Maximum conversion (mol%) after [h]	Enzyme activities (units/g)±SEM after [h][a]
Esterification of benzoic and naphthoic acid derivatives				
Benzoic acid[b]	*cis*-9-Octadecen-1-ol	12.5	57 [48]	6.3±0.41 [4]
4-Hydroxybenzoic acid	*cis*-9-Octadecen-1-ol	50	12 [48]	0.3±0.06 [8]
1-Naphthoic acid	*cis*-9-Octadecen-1-ol	50	5 [72]	0.1±0.01 [8]
2-Naphthoic acid	*cis*-9-Octadecen-1-ol	50	87 [72]	2.1±0.01 [8]
2-Phenoxybenzoic acid	*cis*-9-Octadecen-1-ol	50	34 [72]	1.7±0.01 [0.5]
3-Phenoxybenzoic acid	*cis*-9-Octadecen-1-ol	50	96 [24]	7.9±0.81 [0.5]
4-Phenoxybenzoic acid	*cis*-9-Octadecen-1-ol	50	99 [48]	2.7±0.47 [0.5]; 4.0±0.01 [8]
Transesterification of short-chain alkyl benzoate and naphthoate derivatives				
Methyl benzoate[c,d]	*cis*-9-Octadecen-1-ol	12.5	72 [24]	60±11.0 [0.5]
Methyl 1-naphthoate	*cis*-9-Octadecen-1-ol	50	94 [24]	30±1.5 [0.5]
Methyl 2-naphthoate	*cis*-9-Octadecen-1-ol	50	96 [48]	17±2.6 [0.5]
Methyl 2-methoxybenzoate	*cis*-9-Octadecen-1-ol	12.5	92 [48]	108±5.8 [0.5]
Methyl 3-methoxybenzoate	*cis*-9-Octadecen-1-ol	12.5	92 [48]	86±5.5 [0.5][e]
Methyl 4-methoxybenzoate	*cis*-9-Octadecen-1-ol	12.5	90 [72]	29±2.1 [0.5][e]
Methyl 2-hydroxybenzoate	*cis*-9-Octadecen-1-ol	50	78 [72]	17±1.8 [0.5]
Methyl 3-hydroxybenzoate	*cis*-9-Octadecen-1-ol	50	89 [48]	16±1.0 [0.5]
Methyl 4-hydroxybenzoate	*cis*-9-Octadecen-1-ol	12.5	44 [48]	16±2.9 [4]
		25	89 [48]	15±1.6 [4]
		50	91 [72]	6.4±1.0 [0.5]; 8±0.6 [4]
		100	88 [24]	7±0.6 [4]
Ethyl 4-hydroxybenzoate	*cis*-9-Octadecen-1-ol	12.5	39 [72]	1.5±0.74 [4]
n-Propyl 4-hydroxybenzoate	*cis*-9-Octadecen-1-ol	12.5	24 [72]	2.9±0.31 [4]
iso-Butyl 4-hydroxybenzoate	*cis*-9-Octadecen-1-ol	12.5	14 [72]	0.5±0.09 [24]
Methyl 4-hydroxybenzoate[f]	Dodecan-1-ol	50	88 [48]	9±0.8 [4]
Methyl 4-hydroxybenzoate	Hexadecan-1-ol	50	93 [72]	8±0.2 [4]
Methyl 3-hydroxy-4-methoxybenzoate	*cis*-9-Octadecen-1-ol	50	94 [48]	9±0.4 [4]
Methyl 4-hydroxy-3-methoxybenzoate	*cis*-9-Octadecen-1-ol	50	89 [48]	6±0.7 [4]
Ethyl 3,4-dihydroxybenzoate[g]	*cis*-9-Octadecen-1-ol	100	39 [72]	0.9±0.07 [4]
Esterification and transesterification of benzyl and naphthalenemethyl alcohols				
Oleic acid	Benzyl alcohol	12.5	–	464±22.0 [0.5][h]
			94 [1]	729±10.1 [0.5][i]
Methyl oleate	Benzyl alcohol	12.5	92 [8]	383±12.0 [0.5][i]
			91 [0.5]	643±32.2 [0.5]

Table 1 (continued)

Benzoic acids and short-chain alkyl benzoates as well as fatty acids and fatty acid methyl esters	Medium- and long-chain alcohols as well as benzyl alcohols	Immobilized Novozym 435 lipase (mg)	Maximum conversion (mol%) after [h]	Enzyme activities (units/g)±SEM after [h][a]
Oleic acid	1-Naphthalenemethanol	12.5	–	21±0.5 [0.5][h]
			93 [1]	692±9.3 [0.5]
Methyl oleate	1-Naphthalenemethanol	12.5	97 [8]	278±18.8 [0.5]
Oleic acid	2-Naphthalenemethanol	12.5	–	162±5.1 [0.5][h]
			93 [0.5]	741±3.8 [0.5]
Methyl oleate	2-Naphthalenemethanol	12.5	96 [4]	595±5.1 [0.5]
Methyl oleate	2-Methoxybenzyl alcohol	12.5	91 [4]	350±17.4 [0.5]
Methyl oleate	3-Methoxybenzyl alcohol	12.5	96 [4]	606±19.6 [0.5]
Methyl oleate	4-Methoxybenzyl alcohol	12.5	90 [8]	594±6.9 [0.5]
Oleic acid[j]	3-Hydroxybenzyl alcohol	50	90 [8]	132±4.4 [0.5]
Methyl oleate	3-Hydroxybenzyl alcohol	50	80 [72]	70±2.8 [0.5]
Methyl palmitate	3-Hydroxybenzyl alcohol	50	86 [72]	71±3.7 [0.5]
Oleic acid[g]	3-Hydroxy-4-methoxybenzyl alcohol	50	33 [1]	64±3.4 [0.5]
Methyl oleate[g]	3-Hydroxy-4-methoxybenzyl alcohol	50	36 [72]	3±0.9 [4][e]
Oleic acid[g]	4-Hydroxy-3-methoxybenzyl alcohol	50	19 [72]	13±0.4 [0.5]

[a] Standard assay conditions, if not otherwise indicated: 0.3 mmol carboxylic acid or methyl ester+0.3 mmol alcohol; 80°C; 80 kPa; immobilized lipase/assay: as given in the table; number of experiments, $n=2$

[b] Blank=1.5 mol% (80°C; 48 h)

[c] Blank~0 mol% (80°C; 72 h)

[d] For comparison: Lipozyme RM IM, enzyme activity 1.0 U/g (±0.45 [$n=2$]); Lipozyme TL IM, enzyme activity 0.5 U/g (±0.06 [$n=2$]); 12.5 mg immobilized lipase/assay, each

[e] $n=4$.

[f] GC analysis: 0.25-μm HP-5 column (Agilent), 30 m×0.32 mm inner diameter; temperature program as given in "Materials and methods"

[g] After derivatization with diazomethane+silica gel (cf. "Materials and methods").

[h] At 20°C

[i] At 45°C

[j] 2- and 4-Hydroxybenzyl alcohol: no esterification and transesterification detected using 50 mg Novozym 435 or Lipozyme RM IM as the biocatalyst

used, therefore, as a biocatalyst for all subsequent esterification and transesterification reactions. Similar results were previously obtained for the esterification and transesterification of cinnamic acid derivatives using the above enzymes as biocatalysts (Vosmann et al. 2006; Weitkamp et al. 2006).

Figure 2a shows the time course of the lipase-catalyzed transesterification of methyl 4-hydroxybenzoate with oleyl alcohol at 80°C using different amounts of Novozym 435 (12.5, 50, and 100 mg) as the biocatalyst. It is evident that the conversion rate increases with increasing amounts of the immobilized lipase added to the reaction mixture. Under the reaction conditions described, the highest conversion (88 mol% at 24 h) is obtained with 100 mg of the immobilized enzyme (Fig. 2a and Table 1).

The effect of temperature on the time course of the transesterification of methyl oleate with benzyl alcohol at 45°C and 80°C, catalyzed by Novozym 435, is shown in Fig. 2b. It is obvious from Fig. 2b and further data given in Table 1 that a rise of temperature increases esterification and transesterification activities of Novozym 435 leading to higher conversion in a shorter period of time. Therefore, esterification and transesterification reactions were preferentially performed at 80°C.

Figure 3 shows the effects of various substituents on the lipase-catalyzed transesterification of short-chain alkyl benzoates. The effect of the position of methoxy and hydroxy substituents, respectively, at the phenyl moiety on the lipase-catalyzed transesterification of methyl 2-, 3-, and 4-methoxybenzoates (Fig. 3a) as well as the corresponding methyl 2-, 3-, and 4-hydroxybenzoates (Fig. 3b) with oleyl alcohol is studied using Novozym 435 as the biocatalyst. The results of these experiments demonstrate that transesterification activities depend on both the position and nature of these substituents. For example, methyl 4-methoxybenzoate is converted slower to the oleyl ester than the corresponding 2- and 3-methoxy analogues (Fig. 3a). Figure 3b shows that the maximum conversion of methyl 2-hydroxybenzoate is slower than that of the corresponding 3- and 4-hydroxy analogues, although the enzyme activity of Novozym 435 is relatively high at the beginning of the reaction. This may be caused by neighboring group effects, particularly intramolecular hydrogen bonds formed by the 2-hydroxy substituent. From

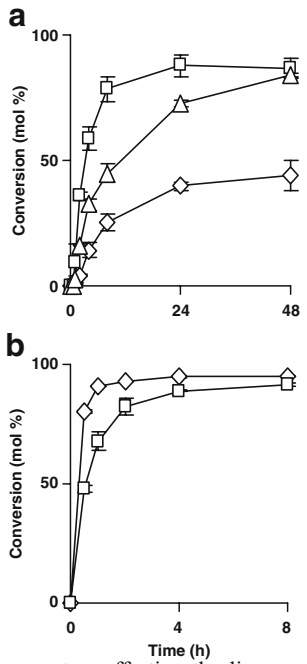

Fig. 2 Reaction parameters affecting the lipase-catalyzed esterification and transesterification of phenolic acids and phenolic alcohols with long-chain alcohols and alkanoic acids, respectively. **a** Effect of different amounts of Novozym 435 lipase on the transesterification of methyl 4-hydroxybenzoate with oleyl alcohol at 80°C (*diamonds*, 12.5 mg; *triangles*, 50 mg; *squares*, 100 mg). **b** Effect of temperature on the transesterification of methyl oleate with benzyl alcohol catalyzed by Novozym 435 (12.5 mg) at 45°C (*squares*) and 80°C (*diamonds*) for various periods (n=2, each)

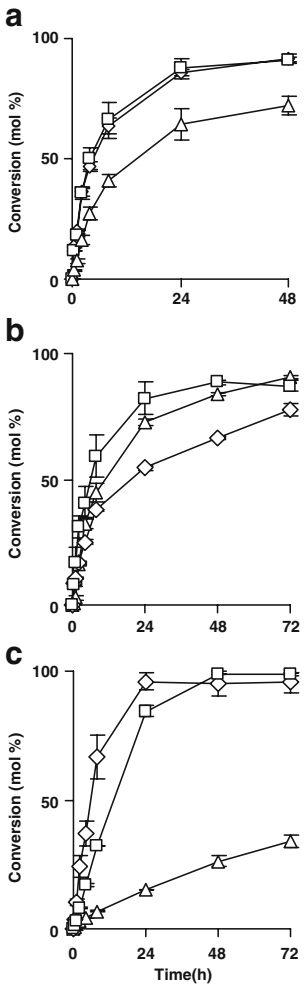

Fig. 3 Effects of various substituents on the lipase-catalyzed transesterification of short-chain alkyl benzoates. **a** Position of methoxy substituents at the phenyl moiety influencing the transesterification of methyl 2-methoxy- (*diamonds*), 3-methoxy- (*squares*), and 4-methoxybenzoates (*triangles*) with oleyl alcohol at 80°C using 12.5 mg Novozym 435. **b** Position of hydroxy substituents at the phenyl moiety influencing the transesterification of methyl 2-hydroxy- (*diamonds*), 3-hydroxy- (*squares*), and 4-hydroxybenzoates (*triangles*) with oleyl alcohol at 80°C using 50 mg Novozym 435. **c** Effect of the position of bulky (*triangle*) 2-phenoxy, (*diamond)* 3-phenoxy, and (*square*) 4-phenoxy substituents of benzoic acid) on the lipase-catalyzed esterification with olcyl alcohol at 80°C using 50 mg Novozym 435 (n=2, each)

Fig. 3a,b as well as Table 1, it is also obvious that the transesterification activity of Novozym 435 is far higher for compounds containing methoxy substituents than those containing the more polar hydroxy groups, which may be due to the specificity of the acyl-binding site of Novozym 435 (Otto et al. 2000). Low transesterification rates are found for benzoates bearing two hydroxy groups such as ethyl 3,4-dihydroxybenzoate (ethyl protocatechuate; Table 1), whereas methyl 3,4,5-trihydroxybenzoate (methyl gallate) is not transesterified at all (data not shown).

2-, 3-, and 4-phenoxybenzoic acids as well as 1- and 2-naphthoic acids and their methyl esters were reacted with oleyl alcohol using Novozym 435 as the biocatalyst to study the effect of the position of bulky substituents at the phenyl moiety on the conversion to various oleyl benzoates and naphthoates (Table 1). It is evident from Fig. 3c and Table 1 that bulky 2-phenoxy moiety strongly decreases esterification rate of 2-phenoxybenzoic acid. Table 1 shows the esterification of 1- and 2-naphthoic acids as well as the transesterification of methyl 1-naphthoate and methyl 2-naphthoate with oleyl alcohol. (1) These results again demonstrate that the conversion rates of both short-chain alkyl naphthoates are far higher than those of the corresponding naphthoic acids. (2) Obviously, esterification activity of Novozym 435 is higher for 2-naphthoic than 1-naphthoic acid. On the other hand, transesterification of methyl 1-naphthoate with oleyl alcohol is preferred over that of methyl 2-naphthoate. The latter results point at specific interactions with the (phenyl)acyl-binding site of Novozym 435 lipase rather than at steric effects.

Table 1 demonstrates the effect of various short-chain alkyl moieties, e.g., methyl, ethyl, *n*-propyl, and *iso*-butyl, of 4-hydroxybenzoate on the Novozym 435-catalyzed transesterification with oleyl alcohol. These results show that the transesterification activity of the immobilized lipase depends on length and branching of the various alkyl chains.

Obviously, high transesterification rates are observed for the methyl ester, whereas decreasing conversion rates are found for ethyl, *n*-propyl, and particularly, *iso*-butyl esters of 4-hydroxybenzoic acid. On the other hand, no distinctly different transesterification activities are found for the reactions of various medium- and long-chain alkan-1-ols such as dodecan-1-ol, hexadecan-1-ol, and *cis*-9-octadecen-1-ol with methyl 4-hydroxybenzoate (Table 1).

In addition to the esterification and transesterification of benzoic or naphthoic acid derivatives, various benzyl and naphthalenemethyl alcohols are reacted with oleic acid and methyl oleate, respectively, to prepare benzyl or naphthalenemethyl oleates. These compounds have an inverse chemical structure as compared to the alkyl benzoates or naphthoates described above. It is obvious from the results given in Table 1 that high conversion rates are obtained for both Novozym 435-catalyzed esterification and transesterification of various analogous methoxybenzyl alcohols or 1- and 2-naphthalenemethanols with oleic acid and methyl oleate, respectively. In contrast to the enzyme activities observed for benzoic acids and methyl benzoates, Novozym 435 activity is higher for the esterification of the various benzyl and naphthalenemethyl alcohol derivatives than for the corresponding transesterification reactions. However, Novozym 435 shows low enzyme activities, if any, for both the esterification and transesterification of hydroxybenzyl alcohols (data not shown), with the exception of 3-hydroxybenzyl alcohol (Table 1). In addition to particular electronic and mesomeric effects of the hydroxy groups in ortho- and para-position, these results may be caused by the specific assembly of active groups of the Novozym 435 (phenyl)alkyl-binding site, which is quite different from the (phenyl)acyl-binding site (Otto et al. 2000).

Discussion

Plant phenolics such as hydroxybenzoic acids having antioxidant and antimicrobial activities are important ingredients for food processing. Moreover, they are gaining importance because of beneficial effects on human health. Hydroxylated benzoic acids are polar compounds that predominantly appear as hydrophilic compounds in the aqueous phases of foods. Lipophilization of plant phenolics such as 4-hydroxybenzoic and 4-hydroxy-3-methoxybenzoic acids is of great current interest due to the possibility to extend their field of applications to fatty food phases; particularly, they may be applied as lipophilic antioxidants in oil-based food (Buisman et al. 1998; Figueroa-Espinoza and Villeneuve 2005; Stamatis et al. 1999; Stöckmann et al 2000). (Hydroxy)benzyl alkanoates with an inverse chemical structure also have lipophilic and antioxidant properties and may be used for similar applications.

Recently, we have demonstrated that various hydroxycinnamic acid derivatives are efficiently esterified and transesterified with fatty alcohols to form medium- and long-chain alkyl hydroxycinnamates using immobilized microbial lipases under environmentally friendly conditions, particularly at moderate temperature in the absence of organic solvents and drying agents such as molecular sieves or sodium sulfate (Vosmann et al. 2006; Weitkamp et al. 2006). Moreover, nonactivated reactants in an equimolar ratio were employed as starting materials for the above experiments, and reduced pressure was used for the removal of reaction water or methanol. This lipase-catalyzed process was superior to most other biocatalytic preparations reported earlier utilizing high excess of substrates, chemically reactive reactants, long reaction times, drying agents, and in part, toxic solvents (Figueroa-Espinoza and Villeneuve 2005; Buisman et al. 1998; Larios et al. 2004; Kobayashi et al. 2003). In continuation of the above work, we have applied this enzymatic method to the preparation of lipophilic alkyl esters of benzoic acid derivatives by using esterification and transesterification, respectively, of various benzoic acids and their short-chain alkyl esters with medium- or long-chain alcohols. High to moderate conversions are obtained using equimolar mixtures of carboxylic acids or their methyl esters and alcohols, which reduce costs and simplify purification.

C. antarctica lipase B (Novozym 435) is generally used as a biocatalyst for the various esterification and transesterification reactions (Table 1). In studies with arylaliphatic glycolipids and their aglycons, it was demonstrated that this enzyme has two different binding sites for alkyl and acyl moieties including phenylalkyl and phenylacyl groups owing to active groups that are highly specific for the various phenolic substrates (Otto et al. 2000). This may explain the order of transesterification activities found for the various hydroxy- and methoxy-substituted methyl benzoates (2-methoxybenzoate≈3-methoxybenzoate>4-methoxybenzoate>3-hydroxybenzoate≈2-hydroxybenzoate >4-hydroxybenzoate≈4-hydroxy-3-methoxybenzoate≈3-hydroxy-4-methoxybenzoate>>3,4-dihydroxybenzoate) as well as the very different esterification and transesterification activities observed for the various analogous benzoate and benzyl alcohol substrates (Table 1). Our results also show that—in contrast to many chemical esterification procedures (Sefkow and Kaatz 1999)—the primary aliphatic hydroxy group of (hydroxy)benzyl alcohols is regioselectively esterified using Novozym 435 as a biocatalyst. Moreover, 3-hydroxybenzyl alcohol is exclusively esterified and transesterified, whereas the corresponding 2- and 4-hydroxy derivatives do not react at all (Table 1).

Electronic delocalization and mesomeric effects occurring in phenolic acids and alcohols, particularly phenylpropenoic (cinnamic) and benzoic acid derivatives, may affect enzyme activity of Novozym 435 leading to

decreasing esterification and transesterification rates (Otto et al. 2000; Leopoldini et al. 2004). The influence of steric hindrance by hydroxy and methoxy groups at the 2-position of the phenyl moiety seems to be rather low for benzoic acid derivatives because relatively high Novozym 435 activities are found for, e.g., 2-hydroxy- and 2-methoxybenzoates as compared to other hydroxy- and methoxy-substituted benzoates (Table 1). However, a bulky 2-phenoxy group clearly decreases enzyme activity as observed for the esterification of 2-phenoxybenzoic acid with oleyl alcohol (Fig. 3c). Intramolecular hydrogen bonds of hydroxy groups, particularly in the 2-position, may also affect the rates of lipase-catalyzed esterification and transesterification. Effects of chain length and unsaturation of acyl moieties of phenolic acids on the enzyme activity of Novozym 435 have been observed by others, as well (Otto et al. 2000; Weitkamp et al. 2006; Chang and Wu 2007).

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