Lipase-Catalyzed Synthesis of Waxes from Milk Fat and Oleyl Alcohol

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ABSTRACT: A screening of five lipases was carried out for the synthesis of wax esters from stoichiometric amounts of oleyl alcohol and milk fat in which long-chain fatty acid content (myristic acid, palmitic acid, stearic acid, and oleic acid) represents 70% of the total fatty acid fraction. The lipases from *Alcaligenes* sp. and *Chromobacterium viscosum* both allowed for the best ester synthesis (around 60%) within 2 and 48 h, respectively. Enzeco® Lipase Concentrate gave 30% ester yield within only 2 h. During the time period of 166 h, less than 20% ester synthesis was obtained with Lipozyme™ 10,000L whereas Enzeco® Lipase XX did not catalyze the reaction. Owing to commercial availability, the food-grade Enzeco® Lipase Concentrate preparation was selected for further experiments with a view to improve wax synthesis. Wax yields were compared for three substrate molar ratios, i.e., 0.5:1, 1:1, and 1.5:1 (alcohol/fatty acid). For 0.5:1 and 1.5:1 substrate molar ratios, the addition of water increased ester yields while the effect of silica gel addition was shown to be minor. The best improvement was obtained at a substrate molar ratio of 1.5:1 with addition of water, leading to 59% wax ester synthesis.

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KEY WORDS: Alcoholysis, lipase, milk fat, wax ester.

Milk fat consumption has declined because of its association in the mind of consumers with coronary heart diseases, thus leading to a growing milk fat surplus available for new food and nonfood applications (1). Even though the long-chain fatty acid content of milk fat is lower than that of vegetable oils (70 mol% for milk fat compared to more than 95 mol% for some vegetable oils), it represents an attractive natural raw material for wax ester production with possible applications in cosmetology.

Interesterification reactions have already been demonstrated to be useful for applications in the fields of food production, cosmetology, and diesel fuel substitutes (2–9). To carry out these acyl exchange reactions, several advantages related to the use of lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) have been described. With enzymatic catalysis, the use of chemical catalysts such as sodium metal salts can be avoided, permitting mild reaction conditions with reduced energy consumption (3). In some cases selectivity toward specific fatty acids allows for production of tailored lipids, as opposed to the random products generated by chemical catalysts (5). A solvent-free system solely composed of enzyme and substrates can be implemented, leading to reductions in downstream processing (7,10).

Commercially interesting for their use as cosmetics or lubricants (11), waxes are esters of long-chain fatty acids and long-chain alcohols; besides the traditional extraction from jojoba and orange roughy, these esters can also be produced through lipase catalysis, and especially through alcoholysis (6,7).

The purpose of the present work was to increase the value of milk fat by producing waxes through lipase-catalyzed alcoholysis with oleyl alcohol. The four main wax esters (FME) studied were myristic acid oleyl ester (MAOE), palmitic acid oleyl ester (PAOE), stearic acid oleyl ester (SAOE), and oleic acid oleyl ester (OAOE), since the corresponding long-chain fatty acids represent 70% of the fatty acid content of milk fat. After a screening of five lipases, improvement of wax synthesis was studied by alteration of the substrate molar ratio and addition of water and/or silica gel in the reaction mixture.

MATERIALS AND METHODS

Chemicals. Anhydrous milk fat was obtained from Ault Foods Ltd. (Etobicoke, Canada). Oleyl alcohol and fatty acid oleyl ester standards were purchased from Sigma Chemicals (St. Louis, MO), except MAOE, which was from Nu-Chek-Prep Inc. (Elysian, MN). Acetone and acetonitrile (Omnisolv) as well as silica gel 60G were from EM Science (Gibbstown, NJ). High-performance liquid chromatography (HPLC)-grade dichloromethane was purchased from Merck (Montréal, Canada). Lipases from *Alcaligenes* sp. and *Chromobacterium viscosum* were obtained from Biocatalysts Ltd. (Mid Glamor-

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gan, United Kingdom). Enzeco® Lipase Concentrate (*Candida rugosa*) and Enzeco® Lipase XX (source not given by the manufacturer) were obtained from EDC (New York, NY). Lipozyme™ 10,000L (*Mucor miehei*) was from Novo Nordisk (Bagsvaerd, Denmark).

Reaction conditions. For the initial screening, kinetic studies were carried out for 166 h with an alcohol to fatty acid molar ratio of 1:1 (stoichiometric conditions), i.e., 140.5 mg of melted (at 80°C) anhydrous milk fat (190.6 µmol of triacylglycerols equivalent to 572 µmol of fatty acids) and 153.6 mg of oleyl alcohol (572 µmol). For subsequent experiments, reactions were stopped at 24 h with a substrate molar ratio (alcohol/fatty acid) of 0.5:1 and 1.5:1, i.e., 76.8 mg (286 µmol) and 230.4 mg (858 µmol) of oleyl alcohol added to 140.5 mg of milk fat, respectively. The effect of 15 mg silica gel and/or 20 µL water addition was also tested. Water addition resulted in water to alcohol molar ratios of 3.9 and 1.3, for alcohol to fatty acid molar ratios of 0.5:1 and 1.5:1, respectively.

For all experiments, the reaction mixtures were introduced in a closed Eppendorf tube of 1.5 mL, adjusted to 60°C before addition of 30 mg of enzyme preparation, and shaken at 1000 rpm in an Eppendorf Thermomixer (Brinkmann Instruments, Mississauga, Canada). Aliquots (5 µL) were withdrawn and diluted in 245 µL of acetone/acetonitrile/dichloromethane (45:45:10). Samples were filtered (0.45 µm) and stored at −18°C under nitrogen until HPLC analysis. Before analysis, samples were sonicated to completely dissolve solid particles which might appear during freezing. All assays were carried out in duplicate.

HPLC analysis. HPLC analysis was performed using a Lichrospher 100 RP-18 endcapped, 5 µm, column (Merck, Darmstadt, Germany). Analyses were carried out at 35°C with a mobile phase of acetone/acetonitrile (50:50) at a constant flow rate of 2.5 mL min^{-1} as described earlier (12). Elution was monitored at 206 nm. A good resolution was obtained between oleyl alcohol and the four main esters studied. Retention times in minutes were 1.6 for oleyl alcohol, 6.7 for MAOE, 7.4 for OAOE, 7.9 for PAOE, and 10.6 for SAOE. This method did not allow separation of free fatty acids, and short-chain esters were not assigned.

Quantification. Wax production was determined by HPLC according to calibration curves obtained with external standards. The initial amount of myristic acid (AMA) in the reaction mixture was calculated as follows:

$$\text{AMA (mol)} = (W_{mf}/\text{MW}_{mfTG}) \times N \times \text{PMA} \quad [1]$$

where W_{mf} is the weight of milk fat in the reaction mixture; MW_{mfTG} is the average molecular weight of milk fat triacylglycerols (MW_{mfTG} = 737) calculated from data on the fatty acid composition of anhydrous milk fat, namely, 4:0 (fatty acid), 7.8% (mol/mol); 6:0, 3.9; 8:0, 2.0; 10:0, 3.9; 12:0, 4.1; 13:0, 0.1; 14:0, 11.8; 14:1, 1.1; 15:0, 1.2; 16:0, 28.7; 16:1, 1.4; 17:0, 0.6; 18:0, 9.6; 18:1, 20.6; 18:2, 2.3; 18:3, 0.6; 20:0, 0.1 (Arul, J., personal communication); N is the number of moles of fatty acid per mole of triacylglycerol (N = 3); and PMA is the percentage (mol/mol) of myristic acid (MA) in milk fat. The same calculations were done for palmitic acid (PA), stearic acid (SA), and oleic acid (OA), taking into account their respective percentages in milk fat. FME synthesis was expressed considering the initial amount of the four main fatty acids according to the following equation:

$$\text{FME}_{FA}\ (\text{mol/mol}) = \frac{\text{MAOE} + \text{PAOE} + \text{SAOE} + \text{OAOE}}{\text{AMA} + \text{APA} + \text{ASA} + \text{AOA}} \times 100 \quad [2]$$

where MAOE, PAOE, SAOE, and OAOE correspond to the wax ester amounts; AMA, APA, ASA, and AOA correspond to initial amounts of MA, PA, SA, and OA, respectively; and FA refers to fatty acid since the yield is expressed considering the amount of the four main fatty acids of milk fat.

In a similar way, each individual ester yield (%) was calculated as the ratio of each wax ester amount to the corresponding fatty acid amount. For example, the MAOE yield (%) corresponds to the ratio of MAOE amount to AMA.

For the substrate molar ratios of 0.5:1 and 1:1, FME synthesis was also expressed considering the initial amount of oleyl alcohol, according to the following equation:

$$\text{FME}_{A}\ (\text{mol/mol}) = \frac{\text{MAOE} + \text{PAOE} + \text{SAOE} + \text{OAOE}}{\text{oleyl alcohol amount}} \times 100 \quad [3]$$

where *A* means alcohol since the yield is expressed in relation to the initial amount of oleyl alcohol.

RESULTS AND DISCUSSION

Screening of lipases for milk fat alcoholysis. Among the five lipases tested, Enzeco® Lipase Concentrate, Enzeco® Lipase XX, and Lipozyme™ 10,000L were chosen for their commercial availability and their acceptance for food use outside the United States. Lipases from *Alcaligenes* sp. and *C. viscosum* were chosen for the ability they showed in a previous work to catalyze wax synthesis from triolein and stearyl alcohol, giving an ester yield of 53% (13).

Figure 1 shows, for each lipase studied, the progress of FME_{FA} synthesis. Best results were obtained with the lipases from *Alcaligenes* sp. and *C. viscosum* (about 60%), whereas Enzeco® Lipase XX did not catalyze the reaction under our experimental conditions. Intermediate yields were obtained with Enzeco® Lipase Concentrate (30%) and Lipozyme™ 10,000L (less than 20%). Maximal yields were obtained within only 2 h for the lipase from *Alcaligenes* sp. and with Enzeco® Lipase Concentrate, whereas about 48 h was necessary with the lipase from *C. viscosum*. For Lipozyme™ 10,000L, the initial velocity was similar to that obtained with *C. viscosum* lipase, but the synthesis soon ceased, perhaps dowing to the glycerol content of the lipase preparation; glycerol could accumulate around the enzyme and/or shift the thermodynamic equilibrium by competing with oleyl alcohol. These results are in agreement with those of Decagny *et al.* (13) for alcoholysis of triolein by stearyl alcohol except that these authors showed lower yields with lipase from *C. rugosa*.

Table 1 presents the production of each of the four main esters for the five lipases. Yields in MAOE, PAOE, SAOE,

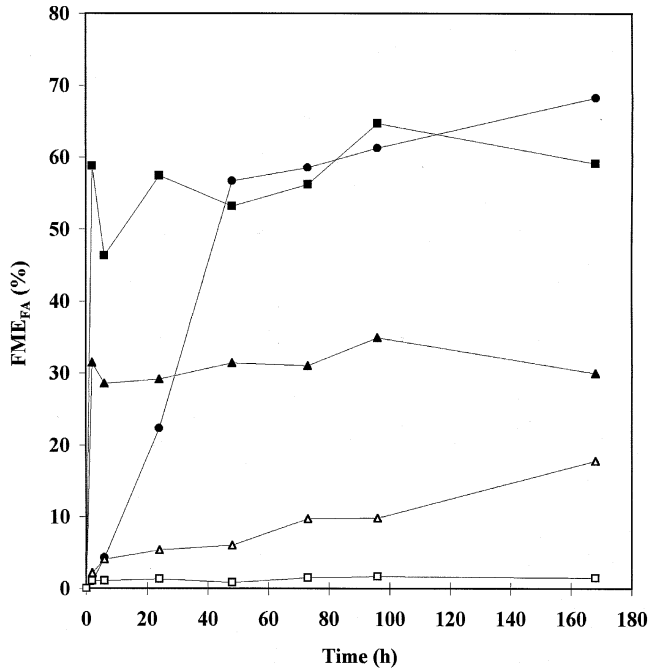

FIG. 1. Progress of wax synthesis from milk fat and oleyl alcohol for the five lipase preparations tested. (■) *Alcaligenes* sp. (Biocatalysts Ltd., Mid-Glamorgan, United Kingdom), (●) *Chromobacterium viscosum* (Biocatalysts Ltd.), (▲) Enzeco® Lipase Concentrate (EDC, New York, NY), (△) Lipozyme™ 10,000L (Novo Nordisk, Bagsvaerd, Denmark), and (□) Enzeco® Lipase XX (EDC).

and OAOE were of the same order of magnitude, indicating no lipase selectivity toward fatty acid carbon chain length, in the range of 14 to 18 carbons, and also no effect of the double bond. The relative abundance of each ester in the reaction mixture was related to the initial abundance of the corresponding fatty acid. However, Enzeco® Lipase Concentrate showed a particular behavior with a lower conversion of SA (24%) as compared to the conversion of the other fatty acids (average value of 31%), within the same time period. A lipase from *C. rugosa* has already been shown to exhibit higher specificity for unsaturated fatty acids as compared to the corresponding saturated acids, especially when the unsaturation is located on carbon 9 (14). This substrate selectivity could explain the difference observed for the conversion of oleic and stearic acids catalyzed by Enzeco® Lipase Concentrate.

Effect of substrate molar ratio. For the stoichiometric mixture (1:1), 50% (285 µmol) of the initially available alcohol content remained in the medium after 24 h of reaction (results not shown). Of the 285 µmol of consumed alcohol, only 123 µmol were used for the conversion of the four main fatty acids into wax esters. More than 50% of the alcohol consumed was presumably used for alcoholysis of triacylglycerols containing short-chain fatty acids. This hypothesis corroborates previous results showing a short-chain preference exhibited by a lipase from *C. rugosa* (14,15). However, since short-chain esters were not quantified, this point will not be further discussed.

A substrate molar ratio below stoichiometry (0.5:1) led to a slight decrease in FME_{FA} synthesis (from 30 to 25%) corresponding to a slight decrease in MAOE, PAOE, SAOE, and OAOE production (Table 2). However, if the limiting substrate, i.e., oleyl alcohol, was taken into account (Eq. 3) to express the yield, FME_A synthesis reached 36% for a substrate molar ratio of 0.5:1 and only 21% for stoichiometry. The substrate molar ratio of 0.5:1 was hence more interesting if optimization of alcohol consumption was attempted. When the substrate molar ratio was increased from stoichiometry to 1.5:1 FME_{FA} synthesis decreased from 30 to 15%. As postulated by Wehtje and Adlercreutz (16), when the alcohol concentration becomes substantial, the reaction medium cannot be called "solvent free." This decrease could hence be ascribed to modifications of medium polarity and/or enzyme activity. Our results disagree with previous works showing an increase in ester yield when the alcohol amount increases above stoichiometry (7,17).

Effect of water addition. The physical properties of lipases, and consequently their activity, have been shown to vary with their hydration state (18). Both ester yields and reaction rates can be increased by increasing the water content (19). In some cases, an optimal content is shown (depending on both lipase

TABLE 1
Wax Synthesis After 166 h of Alcoholysis Between Milk Fat and Oleyl Alcohol Catalyzed by the Five Lipase Preparations Tested[a,b]

Initial amount of fatty acid (µmol)		Ester produced	Enzeco® Lipase Concentrate	*Alcaligenes* sp.	*C. viscosum*	Lipozyme™ 10,000L	Enzeco® Lipase XX
			Wax ester[c] (µmol)				
AMA[d]	67	MAOE[e]	20 (30)	38 (57)	48 (72)	13 (19)	<2
APA	164	PAOE	53 (32)	94 (57)	115 (70)	32 (19)	<2
ASA	55	SAOE	13 (24)	32 (58)	39 (71)	10 (18)	<2
AOA	118	OAOE	37 (31)	66 (56)	76 (64)	21 (18)	<2

[a]Arul, J., personal communication.
[b]Sources: Enzeco® Lipase Concentrate, *Candida rugosa* (EDC, New York, NY); *Alcaligenes* sp. (Biocatalysts Ltd., Mid-Glamorgan, United Kingdom); *Chromobacterium viscosum* (Biocatalysts Ltd.); Lipozyme™ 10,000L (Novo Nordisk, Bagsvaerd, Denmark); Enzeco® Lipase XX, source not given by manufacturer (EDC, New York, NY).
[c]Values in parentheses correspond to yields (%).
[d]AMA, APA, ASA, and AOA are initial amounts of myristic acid, palmitic acid, stearic acid, and oleic acid, respectively.
[e]MAOE, myristic acid oleyl ester; PAOE, palmitic acid oleyl ester; SAOE, stearic acid oleyl ester; OAOE, oleic acid oleyl ester.

TABLE 2
Wax Synthesis from Milk Fat and Oleyl Alcohol Catalyzed by Enzeco® Lipase Concentrate: Effect of the Substrate Molar Ratio on Ester Yield

Substrate molar ratio (alcohol/fatty acid)	MAOE	PAOE	SAOE	OAOE	FME_{FA}[a]
	(μmol)				(%)
0.5:1	16	43	9	35	25
1:1	20	53	13	37	30
1.5:1	11	29	0	23	15

[a]FME_{FA}, four main wax ester synthesis according to Equation 2 in text. For other abbreviations, see Table 1.

and reaction conditions) beyond which a decrease is observed (20–22). However, for several authors, addition of water is clearly not beneficial to wax synthesis (6,17). The effect of water addition (20 μL) in the reaction mixture was studied for two substrate molar ratios, 0.5:1 (Table 3) and 1.5:1 (Table 4). Water addition increased the production of each of the four main esters, and consequently, FME_{FA} synthesis at all substrate molar ratios tested. This effect could be ascribed to the lubricant action of water, which enhances the conformational mobility of the enzyme, permitting a better accommodation of the substrates in the active center (18). The best improvement (from 15 to 59% FME_{FA}) was obtained with a molar ratio of 1.5:1.

Effect of silica gel addition. According to the literature, glycerol released during wax synthesis could accumulate in the microenvironment of the lipase and generate a barrier, limiting the diffusion of substrates and products to the active site (23). To avoid the formation of this barrier, Castillo *et al.* (23) and Stevenson *et al.* (24) have proposed the addition of silica gel to the reaction mixture to recover the glycerol by adsorption as it is produced. If our reactions reached completion, the glycerol concentration in our assays could reach a maximum of 1.7% (wt/vol); this concentration is of the same order of magnitude as the glycerol concentration used by Castillo *et al.* (23). The effect of 15 mg silica gel addition in the reaction mixture was assessed for 0.5:1 (Table 3) and 1.5:1 (Table 4) substrate molar ratios. Silica gel addition led to a slight increase in FME_{FA} synthesis (from 25 to 31%) at a substrate molar ratio of 0.5:1; a twofold increase (from 15 to 29%) was observed at a substrate molar ratio of 1.5:1. Thus, addition of silica gel to absorb glycerol did not provide a large improvement of our process, since FME_{FA} synthesis did not exceed 30%, even after silica gel addition at all the substrate molar ratios.

Water and silica gel addition. The combined effect of water (20 μL) and silica gel (15 mg) was investigated (Tables 3 and 4). At both substrate molar ratios, addition of both water and silica gel to the reaction mixture led to an increase in FME synthesis that was of the same order of magnitude as water only. Therefore, the effect of silica gel was minor compared to that of water.

With Enzeco® Lipase Concentrate, the best FME_{FA} synthesis (59%) was obtained for a substrate ratio of 1.5:1 with addition of 20 μL water. However, we may argue that, from an economic point of view, optimization of the use of alcohol

TABLE 3
Wax Synthesis from Milk Fat and Oleyl Alcohol Catalyzed by Enzeco® Lipase Concentrate: Effect of Water and Silica Gel Addition on Ester Yield for a Substrate Molar Ratio of 0.5:1

Addition	MAOE[a]	PAOE	SAOE	OAOE	FME_{FA}
	(μmol)				(%)
—	16	43	9	35	25
H_2O	32	74	17	64	46
Silica gel	21	55	9	41	31
Silica gel + H_2O	33	85	20	67	51

[a]See Tables 1 and 2 for abbreviations.

TABLE 4
Wax Synthesis from Milk Fat and Oleyl Alcohol Catalyzed by Enzeco® Lipase Concentrate: Effect of Water and Silica Gel Addition on Ester Yield for a Substrate Molar Ratio of 1.5:1

Addition	MAOE[a]	PAOE	SAOE	OAOE	FME_{FA}
	(μmol)				(%)
—	11	29	0	23	15
H_2O	41	99	20	79	59
Silica gel	20	53	8	38	29
Silica gel + H_2O	44	107	24	80	63

[a]See Tables 1 and 2 for abbreviations.

was achieved with a substrate molar ratio of 0.5:1 and water addition. Under these conditions, FME_A synthesis reached 65%, if oleyl alcohol yields are expressed relative to content.

For future investigations, short-chain esters may also be taken into account to get an accurate overview of the final products of the reaction and to elucidate the discrimination of the enzyme between long-chain and short-chain fatty acids. The physicochemical properties as well as the possible biological activities of each wax ester should also be determined in order to elaborate applications in the field of cosmetics.

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