Lipase Catalyzed Alcoholysis of Sunflower Oil

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Lipase-catalyzed alcoholysis of sunflower oil under anhydrous conditions was examined. Lipases from *Pseudomonas fluorescens* and 2 immobilized enzymes from *Mucor miehei* and a *Candida sp.* gave sufficient conversion with petroleum ether as the solvent, even when methanol and ethanol were used. The overall content of tri-, di- and monoglycerides, as well as the corresponding alkyl esters, was measured. Because *Pseudomonas* lipase led to almost quantitative esterification, further studies were carried out with that enzyme varying the amounts of enzyme or the alcohols. Acceptable conversions were achieved even without solvent. Reaction rates of alcoholysis with 5 homologous alcohols, with or without the addition of water, were measured, and in all cases the reaction rates increased with higher chain length of the alcohol. In the case of methanol the highest rate was obtained without any addition of water, but a significantly higher rate was observed with 96% ethanol as opposed to absolute ethanol. The main advantages of lipase-catalyzed, nonaqueous alcoholysis as compared to classical procedures are the mild reaction conditions, the isolation of glycerin without further purification and without the formation of chemical waste, and the ability of lipases to catalyze the esterification of free fatty acids.

Alcoholysis of vegetable oils and animal fats is an important reaction that produces fatty acid alkyl esters that are valuable intermediates in oleochemistry, and methyl and ethyl esters, which are excellent substitutes for Diesel fuel (1,2). Therefore, many procedures are described in the literature that use various catalysts under different reaction conditions (3). The most commonly used catalysts are alkali hydroxides and alcoholates, both of which guarantee short reaction times and high yields. For oils and fats with a high content of free fatty acids, however, an additional step is necessary. Simultaneous alcoholysis and esterification is possible under acidic conditions, but this process requires higher reaction temperatures. A disadvantage of both alkali and acid catalyzed procedures is that the homogenous catalysts are removed with the glycerol layer after the reaction and cannot be reused. Moreover, a major economic factor in technical alcoholysis is the purification of glycerol as a secondary product; this purification is more difficult when large amounts of inorganic material have to be removed.

For most technical applications, methyl esters are produced because methanol is easily available as an absolute alcohol. But, especially for Diesel fuel substitutes, it is preferable to prepare ethyl esters because ethanol can be produced from biomass and it is less toxic than methanol. Conventional alcoholysis with 96% ethanol gives low yields, however, especially under basic conditions.

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In recent years the use of lipases as catalysts for transformations of fatty acids has been widely investigated (4). So far, the main technical application of lipases is to modify the fatty acid compositions of triglycerides by interesterification (5). But the hydrolysis of triglycerides, as well as the direct synthesis of esters, have also been described (6,7). Surprisingly, little attention has been directed to the use of lipases for the alcoholysis of triglycerides. The methanolysis of tallow using a 2-phase system (acetate-buffer/hexane) and a large excess of methanol has been published (7), and in a British patent application (8) the methanolysis of various vegetable oils using lipase from *Candida cylindracea* was performed but large amounts of catalyst, methanol and water were necessary. Because the recovery of glycerol form an aqueous solution is difficult and the activity of the enzyme quickly decreases in aqueous environment, alcoholysis should be performed in a water-free medium. The insolubility, and therefore the higher stability, of enzymes in organic media should enable an easy recovery and reuse of these catalysts. Such enzyme-catalyzed processes in entirely nonaqueous solvents were described by Klibanov *et al.* (9,10), who investigated various transesterification reactions using different lipases in polar and nonpolar organic solvents. They observed high reaction rates during the alcoholysis of tributyrin with heptanol in nonpolar solvents. The catalytic power exhibited by the lipases in organic solvents was comparable to that displayed in water.

In the present study, the lipase-catalyzed alcoholysis of sunflower oil was investigated in order to optimize conditions for the synthesis of methyl and ethyl esters.

MATERIALS AND METHODS

A refined and edible grade of sunflower oil was used and the fatty acid composition was: palmitic acid 7%, stearic acid 4%, oleic acid 25% and linoleic acid 64%. From that composition an average molecular mass of 876.4 was determined.

Alcoholysis was performed with the following lipases: *Pseudomonas fluorescens* lipase by Röhm G.m.b.H. (Darmstadt, Federal Republic of Germany), SP 382 and Lipozyme lipases by Novo Laboratories Inc. (Danbury, CT). SP 382 is based on a lipase preparation produced by a selected strain of a *Candida sp.* and is immobilized on a bead-shaped, acrylic resin. Lipozyme is a lipase from *Mucor miehei* immobilized on a macroporous anion exchange resin. As solvent, we used petroleum ether (b.p. 40-60°C) because it gave the highest enzyme activity.

Enzymatic reactions were initiated by adding a lipase powder or an immobilized preparation to a mixture of substrates and solvent. The suspension was placed in a stoppered flask and shaken on an IKA-Vibrax shaker at 200 rpm at different temperatures.

Analysis of alcoholysis mixtures. The overall content of tri-, di- and monoglycerides, as well as fatty acid alkyl esters, was determined by isocratic HPLC with density detection, which enables the simultaneous quantification of alcoholysis products without separation of individual

fatty acids (11). The following instrumentation and conditions were used: Pump, Gynkotek 300°C; Columns, waters Bondapak CN (30 cm), Waters-Styragel 100 Å+ 500 Å (30 cm each); eluent, chloroform LiChroSolv (Merck 2444, stabilized with 2-methylbutene) + 0.6% ethanol; flow rate, 1.00 ml/min, detector, density detection system DDS 70 (commercially available by A.Paar KG, Graz, Austria), consisting of a density microcell placed in a thermostatted column box, an intelligent interface DDI, coupled to an IBM-PC. Data acquisition and processing was performed using the software package CHROMA.

Isolation of glycerol after alcoholysis. In an attempt to isolate the glycerol after methanolysis of sunflower oil using *Pseudomonas* lipase, sunflower oil (20.0 g, 22.8 mmol) and methanol (2.2 g, 68.8 mmol) in 80 ml of petroleum ether were treated with 2.0 g of lipase powder and shaken for 5 hrs at 45°C. After filtration of the enzyme and washing with methanol the filtrate was concentrated to receive 2.0 g (95% of theory) of glycerol.

Esterification of free fatty acids. In an experiment to examine the ability of *Pseudomonas* lipase to esterify free fatty acids under alcoholysis conditions, sunflower oil (10.0 g, 11.4 mmol)—which contained 0.07% free fatty acids—was treated with oleic acid (0.5 g, 1.77 mmol), methanol (1.2 g, 37.5 mmol) and 1.0 g of *Pseudomonas* lipase and was shaken in 40 ml petroleum ether at 50°C. The content of free fatty acids was measured after different reaction times.

RESULTS AND DISCUSSION

Initially, a series of commercially available lipases was tested for the methanolysis of sunflower oil in petroleum ether at different temperatures without further addition of water to find the most active lipases for alcoholysis. Most of the lipases tested with methanol, even the most commonly used ones like *Candida cylindracea* and Porcine pancreatic lipases, did not give detectable amounts of methyl esters under the conditions that Zaks and Klibanov described for alcoholysis of tributyrin (10). Varying the reaction conditions or using other solvents and reaction temperatures did not lead to better results. The activity of these enzymes, however, was increased significantly when water immiscible alcohols like 1-butanol and 1-pentanol were used as reagents. This is in agreement with Zaks and Klibanov (10), who investigated the activity of enzymes in different solvents and observed that the activity could vary by almost 3 orders of magnitude, being highest in water-immiscible solvents and lowest in water-miscible ones. This could be explained by the removal of the water molecules that are bound on the enzyme surface and are necessary for enzyme activity. Such removal, favored in water-miscible solvents or reagents, leads to inactivation of the enzyme.

In our screening, only 3 lipases were found to sufficiently catalyze the methanolysis of sunflower oil. This included 1 unimmobilized lipase from *Pseudomonas fluorescens*, and 2 immobilized enzymes, 1 from *Mucor miehei* and a *Candida sp.*. These 3 enzymes were tested at different reaction temperatures with methanol and at 45°C with 96% ethanol and n-propanol; petroleum ether was the solvent. A quantity equal to 3.6 times the stoichiometric amount of alcohols was used. After 5 hrs the overall contents of tri-, di- and monoglycerides as well as the alkyl esters are given in Table 1.

It can be seen that the optimum temperature for *Pseudomonas* and *Candida* lipases was 45-50°C, and for *Mucor miehei* lipase it was 40-70°C. *Pseudomonas* lipase provided the best yield of methyl esters and, in the case of 96% ethanol, an almost quantitative esterification was observed. Reaction with 1-propanol was similar to that with methanol. The monoglyceride content was higher than that found with the other two enzymes. Overall, lower ester yields were achieved with *Candida* lipase; the yield of propyl esters was even lower than that of methyl esters. The lowest rate of conversion to methyl esters was obtained using *Mucor miehei* lipase. Although this lipase has an 1,3-specifity in interesterification reactions, this specifity was not observed in alcoholysis because a rela-

TABLE 1

Conversion Rates (% m/m) of Alcoholysis Products of Sunflower Oil

Alcohol	Temperature	Triglyceride	Diglyceride	Monoglyceride	Alkyl ester
Pseudomonas lipase					
Methanol	25	36	5	15	44
Methanol	45	11	3	7	79
Methanol	65	27	6	18	49
Ethanol	45	0	0.5	0.5	99
1-Propanol	45	11	6	2	81
Candida lipase					
Methanol	25	88	3	0	9
Methanol	45	40	4	3	53
Methanol	65	83	4	0	13
Ethanol	45	18	2	1	79
1-Propanol	45	62	2	7	29
Mucor lipase					
Methanol	25	83	3	2	12
Methanol	45	62	6	7	25
Methanol	65	67	6	4	23
Ethanol	45	7	3	8	82
1-Propanol	45	16	3	1	80

Conditions: Sunflower oil, 1.0 g, 1.14 mmol; methanol, 0.4 g, 12.5 mmol; ethanol, 0.6 g, 12.5 mmol; 1-propanol, 0.8 g, 12.5 mmol; 0.2 g lipase powder, 40 ml petroleum ether (b.p. 40-60°C); reaction time, 5 hr.

TABLE 2

Conversion Rates of Alcoholysis Without Solvent

Alcohol	% (m/m) Alkyl ester
Methanol	3
Ethanol (abs.)	70
Ethanol (96%)	82
1-Butanol	76

Conditions: Sunflower oil, 10.0 g, 11.4 mmol; methanol, 1.5 g, 46.9 mmol; abs. ethanol, 2.0 g, 43.5 mmol; 96% ethanol, 2.0 g, 41.7 mmol; 1-butanol, 3.0 g, 40.5 mmol; 1.0 g *Pseudomonas* lipase; 14 hr at 50°C.

tively low content of monoglycerides was found in the reaction mixtures. With ethanol, the content of monoglycerides was higher than that of tri- and diglycerides.

Because it appeared to give the best activity, further examinations were only made with *Pseudomonas* lipase. Alcoholysis under the same reaction conditions as described in Table 1, but with lower amounts of lipase, led to lower conversion rates. Reaction for 1 hr at 45°C with 0.1 g of lipase gave 47% ethyl esters, and with 0.05 g of lipase gave only 2%. Surprisingly, reduction in the amount of the alcohols to stoichiometric amounts led to acceptable conversion rates with 0.2 g of lipase. The yield with methanol was 72%, with absolute ethanol it was 70%, and with 96% ethanol it was 86%. Alcoholysis without any solvent might be of considerable interest, especially for technical applications, but the insolubility of triglycerides in lower alcohols requires vigorous shaking and longer reaction times. As can be seen in Table 2, reaction with methanol gave only traces of methyl esters, whereas ethanol and butanol yielded higher amounts of the corresponding alkyl esters.

We determined reaction rates using *Pseudomonas* lipase and different alcohols, with or without the addition of water. The amount of added water was 17% (m/m), corresponding to the amount of water in 96% ethanol. The results are shown in Table 3. Without the addition of water, the reaction rates increased constantly with chain length and increased lipophilicity of the alcohols. The addition of water led to even higher reaction rates with longer chain alcohols. An opposite effect was observed with methanol, leading to significantly lower conversions when water was added, a fact we have not yet been able to explain.

As mentioned in the introduction, 2 difficulties encountered in classical alcoholysis procedures are the removal and purification of glycerol and the 2-step procedure needed to esterify the free fatty acids. At optimum reaction conditions using *Pseudomonas* lipase, glycerol, free of any salts and impurities, can be obtained in 95% yield after washing the filtered enzyme with methanol.

After addition of oleic acid to the sunflower oil, treatment with methanol and *Pseudomonas* lipase reduced the content of free fatty acids from 5.0% to 1.0% after 5 hrs, and to 0.7% after 11 hrs, where it remained constant even after 21 hrs. Therefore, 80% of the free fatty acid could be transformed into fatty acid methyl esters after 5 hrs.

TABLE 3

Reaction Rates of Alcoholysis

	Reaction rate (mmol/hr.g of enzyme)	
	A without	B with
Alcohol	addition of water	
Methanol	1.35	0.42
Ethanol	1.41	7.13
1-Propanol	3.21	7.80
1-Butanol	4.27	11.71
1-Pentanol	6.38	11.62

Conditions: Sunflower oil, (5.0 g, 5,7 mmol), and 17.1 mmol alcohol were shaken with 0.2 g *Pseudomonas* lipase in 20 ml petroleum ether at 20°C. The solvent contained 0.018% (m/m) water, and the alcohols no more than 0.2% water (condition A). For condition B, 0.034 g (1.9 mmol) water was added. The remaining triglyceride concentration was measured after 1 hr by HPLC and the reaction rates were calculated from the average of at least 3 independent measurements.

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