Lipase-Catalyzed Transesterification of Rapeseed Oil and 2-Ethyl-1-Hexanol

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Lipase-catalyzed transesterification (alcoholysis) of low-erucic acid rapeseed oil and 2-ethyl-1-hexanol without an additional organic solvent was studied in stirred batch reactors. Of a number of commercially available enzymes investigated, the best results were obtained with a *Candida rugosa* lipase. The optimal transesterification conditions were an oil/alcohol molar ratio of 1:2.8, a minimum of 1.0% (w/w) added water, and with a temperature of 37–55°C. Under the optimal conditions, a nearly complete conversion was obtained in one hour with 14.6% (w/w) lipase, whereas 0.3% (w/w) lipase required 10 h for similar results. The enzyme was inactivated at 60°C.

KEY WORDS: Alcoholysis, biocatalysis, enzyme, 2-ethyl-1-hexanol, 2-ethyl-1-hexyl ester, lipase, rapeseed oil, transesterification.

Interest in lipase-catalyzed biosynthesis is rapidly increasing (1,2). Lipases have great potential in food-related lipid modifications (3,4), in the production of esters (5,6), biodegradable polyesters (7,8) and fatty acids (9,10). Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are esterases that catalyze hydrolysis and synthesis of glycerol esters. In transesterification, the acyl moiety is exchanged either between an ester and acid (acidolysis), ester and alcohol (alcoholysis) or two esters (acyl exchange) (10,11). Acyl exchange between two molecules is also called interesterification, and between two acyl groups within a molecule it is called intraesterification. Ester synthesis is favored under restricted water availability (low water activity) (12), although a minimum quantity of water is necessary for enzyme catalysis to take place (13).

Lipase-catalyzed alcoholysis in the absence of solvent is important in industrial applications, especially for food uses. Complete transesterification between one mole of triacylglycerol and three moles of alcohol yields three moles of ester and one mole of glycerol (14). Although it has been claimed that the presence of additional organic solvent may be useful, for example, in controlling water activity and microbial contaminations (15,16), the absence of solvent allows higher substrate and product concentrations (17), simplifies downstream processing (18), and improves safety (19). Zaks and Klibanov (20) were the first to study the transesterification of tributyrin with a number of primary and secondary alcohols, catalyzed by porcine pancreatic lipase, to yield an ester of butyric acid and dibutyrin. They demonstrated the importance of the quantity of water present, both to the activity and stability of the biocatalyst. Macrae (12) and Halling (21), among others, have further emphasized the importance of water relationships in lipase-catalyzed synthetic reactions. Hirata *et al.* (22) later demonstrated that water requirements in the alcoholysis of tributyrin by different lipases may vary widely.

In spite of the great technical interest of fatty acid esters (23,24), lipase-catalyzed transesterification involving high-molecular weight fatty acids has only recently been investigated. Mittelbach (25) has studied the alcoholysis of sunflower oil, both in petroleum ether as solvent and without additional solvent, to synthesize methyl and ethyl esters as diesel oil substitute. Shaw *et al.* (26) used Celite-immobilized *Pseudomonas fluorescence* lipase in the alcoholysis of olive oil. An excess of alcohol has been claimed to be beneficial in alcoholysis by suppressing the hydrolytic side reaction (17,18). Nevertheless, in such cases, disadvantages such as the removal of excess alcohol from the product should also be considered. Erucyl erucate, the main component of jojoba oil, has been produced by transesterification of high-erucic acid rapeseed oil and erucyl alcohol (18). The aim of the present work was to investigate lipase-catalyzed transesterification (alcoholysis) of rapeseed oil and 2-ethyl-1-hexanol, currently used in the chemical synthesis of a number of important compounds (27), without the use of an additional organic solvent and as a possible alternative to acid or base catalysis.

MATERIALS AND METHODS

Materials. Refined, low-erucic acid rapeseed oil and synthetic rapeseed oil 2-ethyl-1-hexylester were obtained from the Raisio Group (Raisio, Finland). The approximate fatty acid composition of the oil was 57% oleic acid, 22% linoleic acid, 12% linolenic acid, 4% palmitic acid, 1% stearic acid, 2% eicosanoic acid <1% erucic acid and 1% others. 2-Ethyl-1-hexanol was obtained from Fluka Chemie AG (Buchs, Switzerland). Mono-, di- and triolein standards were from Sigma (St. Louis, MO), and glycerol from May & Baker (Dagenham, United Kingdom).

Enzymes. The following powdered microbial lipases were obtained from Biocatalysts Ltd. (Pontypridd, United Kingdom): *Candida rugosa* (ex. *cylindracea*) (42,500 U/g; water 5.0% w/w), *Chromobacterium viscosum* (13,300 U/g; water 5.9% w/w), *Mucor miehei* (7,200 U/g; water 7.4% w/w) and *P. fluorescens* (11,900 U/g; water 3.1% w/w).

Transesterification. A preliminary study with those four lipases (10 mg; 3.3% w/w) was carried out with 0.277 mmol (*ca.* 0.2 g) rapeseed oil and 0.680 mmol (107 μL) of 2-ethyl-1-hexanol (molar ratio of 1:3) in capped 13-mL test tubes under magnetic stirring at 200 rpm with 3.0% added water. Transesterification was allowed to continue for 72 h, after which lipase was separated by centrifugation for 5 min at 2000 rpm (Martin Christ Type UJ3; Osterode, Germany), and the supernatant was pipetted into Eppendorf tubes for storage at −20°C and later analysis. Further transesterification reactions were carried out for up to 72 h with varying substrate molar ratios (rapeseed oil/ethyl hexanol from 1:1 to 1:10), *C. rugosa* lipase (from 0.3 to 14.6% w/w) and added water (from 0.25 to 50% w/w) quantities and temperatures (from 37–60°C).

Lipase activity. Lipase activity was determined according to the Biocatalysts Ltd. assay method "Lipase Assay," which is based on the hydrolysis of 50% (vol/vol) olive oil emulsion (Product No. 800-1; Sigma) as substrate at pH 7.7, and 37°C in one hour. The quantity of free fatty acids formed was titrated with 0.1M sodium hydroxide. One unit of lipase activity was defined as the quantity of

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enzyme that catalyzes the release of one μmole of free fatty acid from olive oil in one minute under those conditions.

Analytical methods. Qualitative analyses were carried out by thin-layer chromatography (TLC). Samples were diluted 1:10 (vol/vol) with ethanol, and 0.01 mL of the diluted samples were used for TLC analysis. Hexane/diethyl ether/acetic acid (80:20:1) was used as solvent on Kieselguhr 60 F_{254} plates (E. Merck, Darmstadt, Germany) with one hour running time. Slightly dried plates were sprayed with 0.1% 2′,7′-dichlorofluorescein (Aldrich-Chemie, Steinheim, Germany) in 99.5% ethanol (Alko Ltd., Rajamäki, Finland) for detecting the spots at 254 and 360 nm.

Rapeseed oil conversion (% rapeseed oil used) and ester yield (% of theoretical) were determined by reversed-phase high-performance liquid chromatography (HPLC), as modified from El-Hamdy and Perkins (28) and Forssell *et al.* (3), with a Perkin-Elmer (Norwalk, CT) 4 pump module, ISS-100 sampler, and 101 oven, Novapack C18 3.9 × 150 mm column with 4 μm silica particles, HP 1047A refractive index detector (Hewlett-Packard, Palo Alto, CA), PE 316 integrator and PE 7500 professional computer. Samples were diluted with acetone to 10–20 mg/mL, filtered through a Millex-LCR_4 disposable filter with 0.5 μm porosity (Millipore, Bedford, United Kingdom), and 0.02 mL of the filtrate was used for the analysis. The running solvent was acetone/acetonitrile (1:1) at 1.0 mL/min, 37°C, 30 min. Residual 2-ethyl-1-hexanol could not be determined by the HPLC method because the alcohol overlapped with the acetone used as the diluent. Consequently, any excess 2-ethyl-1-hexanol was determined by TLC as described above. Moisture content of the enzyme preparations was determined by drying about 4-g samples overnight at 105°C.

RESULTS AND DISCUSSION

Lipase. To identify the most suitable enzyme for subsequent transesterification trials, preliminary experiments were carried out with the most promising commercial lipases of a total of 25 previously screened for *n*-butyl oleate biosynthesis (5). A substrate molar ratio of 1:3, 3.3% (w/w) of lipase and 3.0% (w/w) of added water were used. Figure 1 shows that in 24 h the use of *C. rugosa* lipase as biocatalyst resulted in the highest ester production, with no detectable residual rapeseed oil and little by-product. A 98% conversion of rapeseed oil was obtained in 24 h. Also, a superior cost/benefit ratio has been reported previously for this lipase in the direct biocatalytic synthesis of *n*-butyl oleate (4). The use of *P. fluorescens* and *C. viscosum* lipases also resulted in relatively high ester production, with 96% conversion in 24 h and 97% conversion or higher in 48 h, although clearly more residual alcohol and by-products could also be seen. The poorest results were obtained with *M. miehei* lipase. Consequently, *C. rugosa* lipase was chosen for further studies.

Substrate molar ratio. One of the aims was to obtain a maximum rapeseed oil conversion with no or little residual 2-ethyl-1-hexanol. When an alcohol excess was used, rapeseed oil conversion was always low, and the product mixture contained large quantities of residual alcohol and, in some cases, residual oil. The relative ester yield decreased with an increase in the alcohol molar excess.

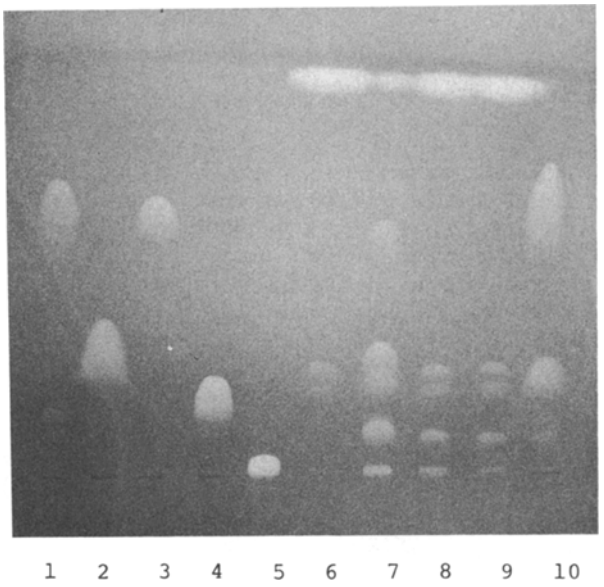

FIG. 1. Thin-layer chromatograms with different lipases (rapeseed oil/2-ethyl-1-hexanol substrate molar ratio 1:3; 3.5%, w/w, lipase; 3.0%, w/w, added water; reaction time 24 h). 1, Rapeseed oil; 2, 2-ethyl-1-hexanol; 3, triolein; 4, diolein; 5, monoolein; 6, *Candida rugosa* lipase; 7, *Mucor miehei* lipase; 8, *Pseudomonas fluorescence* lipase; 9, *Chromobacterium viscosum* lipase; 10, blank.

Consequently, the use of alcohol excess was not further investigated.

About 50% rapeseed oil conversion was reached in one hour, with a nearly complete conversion in 10 h when the substrate molar ratio was between 1:2.8 to 1:3.0, lipase quantity was 3.3% (w/w) and the added water 3.0%. It could be concluded from several replicate transesterifications that the highest ester yield with the least residual alcohol was obtained with the substrate molar ratio of 1:2.8, although the differences with different substrate molar ratios, down to 1:2.5, were small. Consequently, the molar ratio of 1:2.8 was used in most of the subsequent trials.

Lipase quantity. As could be expected, an increase in lipase quantity markedly increased the rapeseed oil conversion during the first few hours, but after seven hours the differences had almost leveled off. Figure 2 illustrates, as an example, the rapeseed oil conversion as the function of time with 0.3, 2.3 and 14.6% (w/w) lipase, substrate molar ratio of 1:2.8 and 3.0% (w/w) added water. The reaction was nearly complete (the maximum theoretical conversion under the conditions used is 93.3%) in one hour with the highest lipase quantity used, whereas with only 0.3% (w/w) lipase, the conversion in one hour was only about 20%. Nevertheless, a nearly complete conversion was obtained in 10 h, even with the lowest lipase quantity used which, in addition to the stability of the enzyme, is important in considering costs. Interestingly, Goldberg *et al.* (29) reported that an increase in the quantity of powdered *C. rugosa* lipase results in a decrease in the apparent enzyme activity in the production of heptyl oleate, owing to an increase in diffusion limitation, a problem which may be minimized in large-scale experiments by optimal biocatalyst and reactor design.

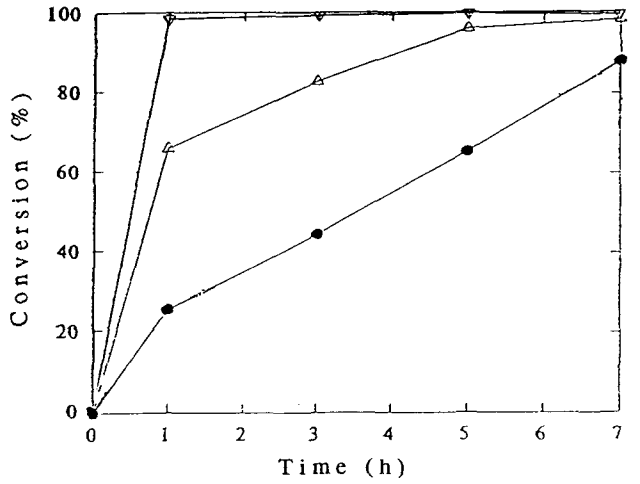

FIG. 2. Effect of *Candida cylindracea* lipase (●, 0.3%; △, 2.3%; ▽, 14.6%; w/w) quantity on transesterification (substrate molar ratio 1:2.8; 3.0%, w/w added water).

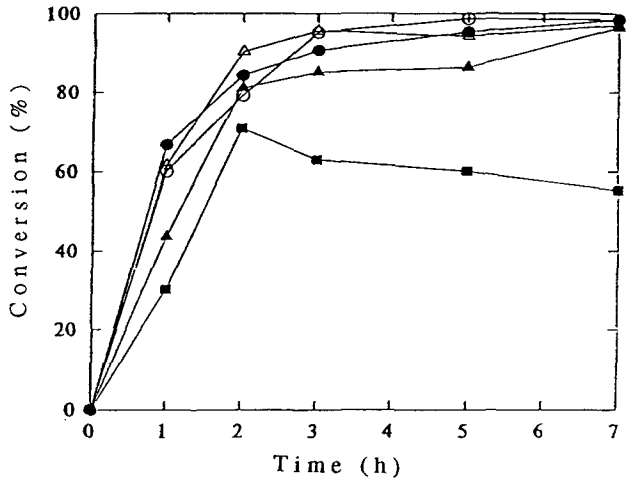

FIG. 4. Effect of temperature (●, 37°C; ○, 45°C; ▲, 50°C; △, 55°C; ■, 60°C) on transesterification (substrate molar ratio 1:2.8; 3.3%, w/w, lipase; 3.0%, w/w added water).

Added water. The importance of the control of water content (and of water activity) in lipase-catalyzed esterifications has been frequently emphasized. Figure 3 shows the effect of added water on transesterification when the substrate molar ratio was 1:2.8 and the lipase quantity was 3.3% (w/w). No phase separation took place with up to 5% (w/w) of added water. At higher water quantities, the organic phase was used for HPLC analyses. The water (*ca.* 5% w/w of lipase) present in the lipase preparation was insufficient, and only about 25% conversion was reached in seven hours without added water. With 0.25% (w/w) of added water, about 60% conversion was reached in one hour, but an increase in time did not bring about a further increase in rapeseed oil conversion. With a minimum of about 1.0% added water, about 50% conversion was reached in one hour, and a nearly complete conversion in five hours. Little difference was observed between 1.0 and 3.0% added water. Additional increases in added water did not result in further improvements. Interestingly, the reaction proceeded nearly identically with 50% (w/w) added water as with 3.0%. Although direct lipase-catalyzed ester synthesis may not be directly compared with transesterification, it is of interest to note that 90% or higher butyl oleate yields have been reported in the presence of excess water (6,30).

Temperature. Figure 4 illustrates the effect of temperature on the time course of rapeseed oil conversion. There was little difference within the temperature range of 37–55°C, with about 90% conversion reached in 2–3 h and nearly a complete conversion in 7 h. However, at 60°C lipase was clearly inactivated under the experimental conditions. The results agreed well with those of Mittelbach (25), according to whom the optimal temperature for *Candida* sp. lipase-catalyzed sunflower oil alcoholysis is 45–50°C. Hirata *et al.* (22) also reported 50°C as the optimal temperature for the transesterification of tributyrin and 1-octanol with *C. rugosa* lipase.

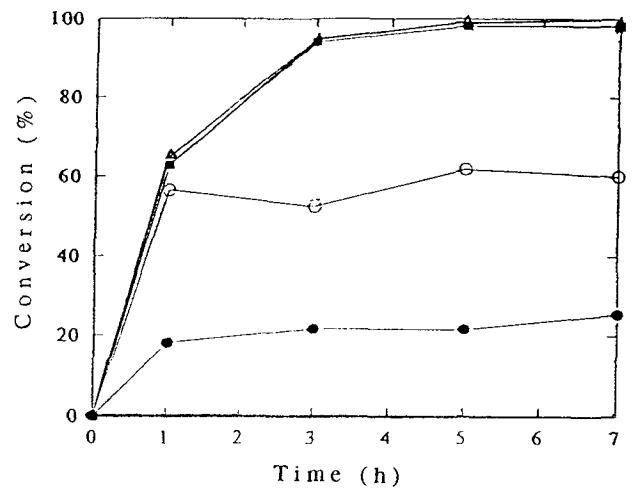

FIG. 3. Effect of added water (●, 0%; ○, 0.25%; ■, 3.0%; △, 50%) on transesterification (substrate molar ratio 1:2.8; 3.3%; w/w, lipase).

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