Synthesis of Monobutyrylglycerol by Transesterification with Soluble and Immobilized Lipases

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ABSTRACT

Transesterification between ethyl butyrate and glycerol using very different lipase preparations (native, modified, and immobilized) in a two-phase system—no extra solvent added—has been investigated. Optimal conversion was obtained with the presence of 5% water in the reaction mixture. Only monobutyrin was produced in all conditions tested. The best enzyme preparations were native *Candida cylindracea* lipase, *Mucor miehei* lipase immobilized on a phenol-formaldehyde exchange resin (Lipozyme™), and *C. cylindracea* lipase immobilized on Celite.

Index Entries: Monobutyrin; ethyl butyrate, transesterification of; glycerol esterification; *Candida cylindracea* and pancreatic lipases; lipases immobilized on Celite and on agarose; polyethylene glycol modified-lipase.

INTRODUCTION

Lipases (EC 3.1.1.3) compose a group of enzymes whose biological function is to catalyze the hydrolysis and synthesis of triacyglycerols (*1*). Since the demonstration that enzymes can catalyze reactions in organic solvents (*2*), lipases have received growing attention, owing to their selectivity of action. When using lipases (and other hydrolases) as syn-

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thetic catalysts, it is absolutely necessary to decrease water concentration in the reaction mixture by the inclusion of suitable organic solvents (*3*). Many strategies have been used to stabilize lipases in organic solvents, including immobilization on supports and chemical modification of the protein.

As regards their ability to hydrolyze triacylglycerols, lipases have been classified as unspecific, 1,3-specific, and fatty-acid-specific, these various specificities being exploited in industry (*4*). Therefore, it may be interesting to investigate the synthesis of acylglycerols with lipases in order to see if the specificity of action is the same in hydrolysis and synthesis (i.e., if a 1,3-lipase yields 1,3-diglyceride but no triglycerides in the synthetic reaction). On the other hand, Cesti et al. (*5*) used pancreatic lipase for the transesterification of diols with ethyl carboxylates: as a result, only primary monoesters were produced. In the present work, the transesterification between ethyl butyrate and glycerol, using different lipase preparations (soluble, immobilized, modified), has been investigated. The acylation has proven to be very selective: only monobutyrin has been produced.

MATERIALS

Candida cylindracea (now named *C. rugosa*) Type VII lipase and porcine pancreas Type II lipase (containing, respectively, 700 and 160 U/mg powder and using olive oil as substrate) were purchased from Sigma (St. Louis, MO), and *Rhizopus arrhizus* lipase (14000 U/mg protein, using olive oil) from Boehringer Mannheim (Mannheim, FRG) was used. *Mucor miehei* lipase immobilized on Duolite 568, a macroporous weakly basic anion exchange resin (Lipozyme™) (*6*), was donated by Novo Industri A/S (Bagsvaerd, Denmark) and contained 375 μg of enzyme/g of support.

Monomethoxypolyethylene glycol (PEG, MW 5000) was ordered from Sigma; Celite for gas-liquid chromatography (30-80 mesh [0.18–0.59 mm]), from BDH (Poole, UK); 2,3-epoxy-1-propanol, from Merck (Darmstad, FRG); and ethyl butyrate, from Scharlau (Barcelona, Spain). Trichloroethylbutyrate was synthesized from butyryl chloride and dichloroethanol (*7*), both Aldrich (Darmstad, FRG) products.

METHODS

Immobilization of *C. cylindracea* Lipase

Covalent immobilization of the enzyme through its amino groups on Sepharose CL-6B (from Pharmacia, Sweden) activated with 2,3-epoxy-1-propanol was carried out as previously described (*8*). After 30 min of reaction at 25°C between enzyme and support, reduction was effected

with sodium borohydride. The immobilized preparation contained 31 mg lipase/mL of agarose gel.

For adsorption of the enzyme on Celite, 200 mg of lipase powder were dissolved in 10 mL of 0.1*M* phosphate buffer, pH 7.2, 0.1*M* NaCl. After stirring for 1 h at room temperature, 600 mg of the support was added, and the stirring continued during 2 h. Then, 10 mL of cold (−15°C) acetone were slowly added, with stirring. The immobilized enzyme was filtered, washed with 2×10 mL of acetone, dried 30 min *in vacuo*, and stored in a closed vial at 4°C.

Chemical Modification with Polyethylene Glycol

Candida lipase was modified following the procedures of Inada and collaborators (*9*). Preparation of the modifier, 2,4-*bis* (*o*-methoxypolyethylene glycol)-6-chloro-*s*-triazine, and subsequent binding to amino groups of *Candida* lipase were carried out as reported (*9*). Twenty-five percent of the lipase amino groups, measured with trinitrobenzene sulfonate (*10*), disappeared after the covalent attachment of polyethylene glycol.

Hydrolytic Activity

The hydrolytic activity of lipases was evaluated following the hydrolysis of tributyrin at 40°C in 1 m*M* Tris buffer, with 0.1*M* NaCl, and 0.1*M* $CaCl_2$. The acid released was continuously titrated to pH 7.2 with the aid of a pH-stat from Radiometer (Copenhagen, Denmark), model TTT80.

Synthesis of Butyrylglycerols

To 12.3 mmol (0.9 mL) (except otherwise noted) of glycerol were successively added 0.1 g of lipase; a variable amount of 0.1*M* phosphate buffer, pH 7.2, containing 0.1*M* NaCl; and 7 mL (6.15 g) of ethyl butyrate. The reaction vial was maintained at 40°C (unless otherwise specified), with stirring; at intervals, aliquots were drawn from the top layer (ethyl butyrate) in order to evaluate the progression of the reaction using a Shimadzu gas chromatograph provided with FID. Liquid (0.1 μL) was injected into a 3.2 mm×1.8 m methyl silicone column (3% SP-2100 on 100/120 Supelcoport, from Supelco, Inc. (Gland, Switzerland)); the temperature was raised to 220°C at 10°C/min after 5 min at 65°C.

RESULTS

Esterification Using Soluble Native *Candida cylindracea* Lipase

Figure 1 shows the rate of transesterification, at 40°C, of glycerol with ethyl butyrate as a function of the amount of buffer added to the reaction

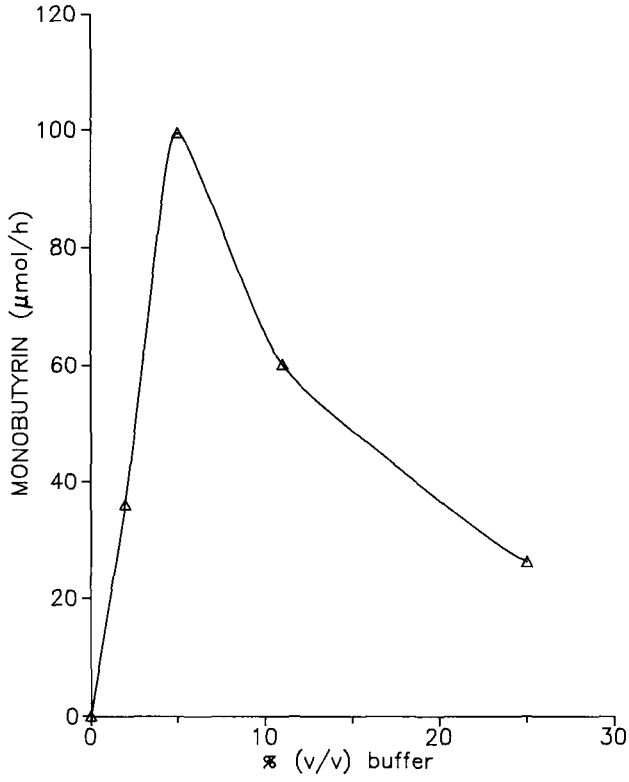


Fig. 1. Rate of monobutyrin formation vs percentage of buffer present in the reaction. Reaction conditions: 12.3 mmol glycerol; 0.1*M* phosphate buffer, pH 7.2, 0.1*M* NaCl; 0.1 g *Candida* lipase; 40°C.

vial. The rate corresponds to the formation of monobutyrylglycerol, since in these conditions the conversion to dibutyrin and tributyrin was negligible. The highest conversion was obtained with 5% (v/v) of aqueous phase. In a control experiment carried out without enzyme and in the presence of 2% (v/v) buffer, the conversion to acyglycerols was $<0.1\%$, even after 8 d of reaction.

The effect of the pH of the buffer on the conversion was studied at the optimal aqueous concentration—5%. Figure 2 presents the results obtained at two temperatures. The optimal pH, 7.0 at 20°C, shifts to 7.2 at 40°C.

To investigate the dependence of the conversion on the amount of biocatalyst present in the reaction, two experiments were carried out at 30°C using 0.5 or 1 g of lipase, at the optimal pH and percentage of buffer. The amount of glycerol was one-quarter that used in Fig. 1, and therefore the enzyme/glycerol ratio was 20- or 40-fold larger than in Fig. 1. After 20 h of reaction, 460 and 920 μmol monobutyrin were obtained, which indicates that with large lipase concentration the rate of synthesis is proportional to the amount of catalyst.

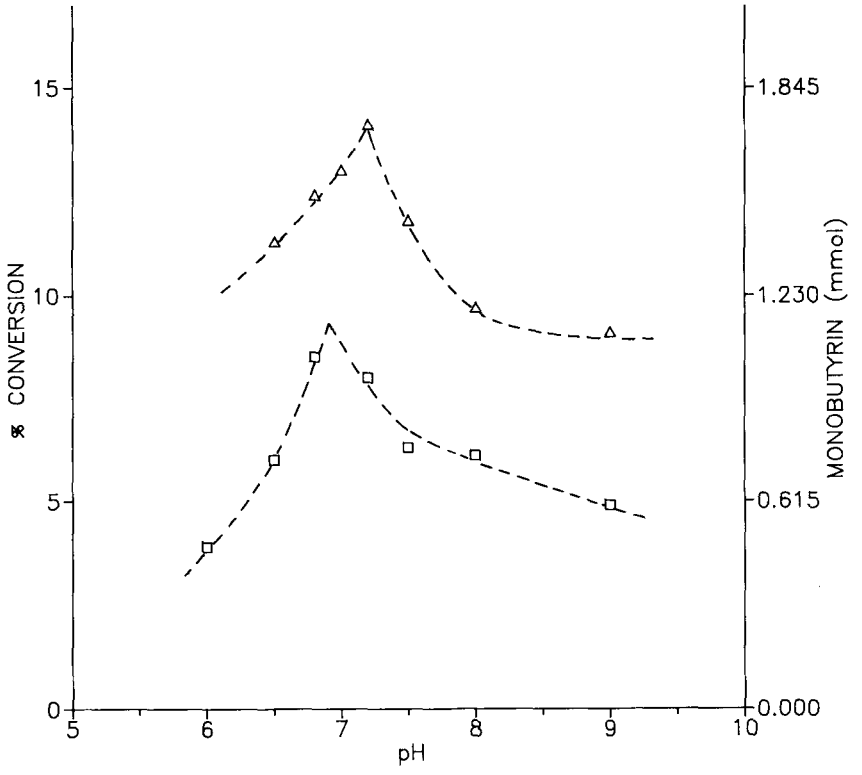


Fig. 2. Percent of conversion (to monobutyrin) vs pH of the 0.1*M* buffer added (final concentration, 5%), acetate, pH 6.0; phosphate, pH 6.8–8.0; bicarbonate, pH 9.0. Reaction conditons: 12.3 mmol glycerol; 0.1 g *Candida* lipase; 20 h of reaction. □, 20°C; △, 40°C. The right ordinate indicates the amount of monobutyrin formed.

The dependence of the reaction rate on temperature is depicted in Fig. 3. An Arrhenius plot (not shown) of the data in the linear region (20–45°C) yields an activation energy of 6.0 kcal/mol (25.1 kJ/mol), which corresponds to a Q_{10} (enhancement of reaction rate corresponding to an increase of 10°C) value of 1.5. At ≥50°C, the enzymatic activity strongly decreases.

Time-Course of the Conversion to Monobutyrin

The percentage of glycerol converted to monobutyrylglycerol at two different temperatures is shown in Table 1. As expected, the synthesis is much faster at 40°C than at 20°C (cf Fig. 3). At 20°C and after 19 d, a plateau (25% conversion) is attained, which is not affected by the addition of fresh enzyme. This indicates that we have come to an equilibrium. At 40°C, a 30% plateau is reached (after 28 d). After long reaction times (>36

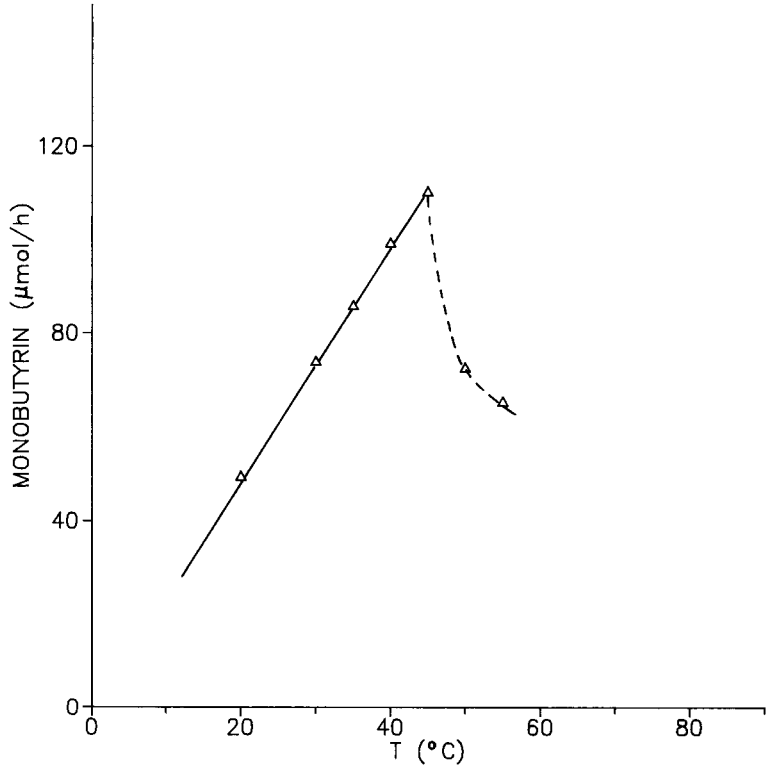


Fig. 3. Rate of formation of monobutyrin at different temperatures. Reaction conditions: 12.3 mmol glycerol; 5% buffer, pH 7.2; 0.1 g *Candida* lipase.

Table 1
Time Course of the Formation of Monobutyrin (MB)[a]

Time, h	20	116	212	452	668	812	857	870
% MB, at 20°C	5.5	16.3	22.5	24.4	24.5	25.7[b]	25.0	25.0
% MB, at 40°C	14.2	21.4	24.9	27.5	30.5	–	–	–

[a]Reaction conditions: 6.1 mmol glycerol, 5% buffer, 0.5 g lipase.
[b]After this measurement, 1.25 g of lipase were added.

d), a small amount (<3%) of tributyrin was observed at both temperatures; no significant amounts of dibutyrin were detected.

Esterification Using Soluble Pancreatic Lipase

The extent of esterification after 20 h with pancreatic lipase, at 2 and 11% buffer (0.1*M* Tris, pH 8.5, 0.1*M* NaCl) concentration, was 120 and 100 μmol monobutyrin (as opposed to values of 720 and 1200 μmol monobutryrin, respectively, using *Candida* lipase in the same optimal conditions). Table 2 presents the time-course of reactions in the 11% aqueous phase. It is clear that the pancreatic enzyme is not a good catalyst for this synthesis.

Table 2
Time-Course of the Synthesis Using Soluble Enzymes[a]

	Enzymes from	
Time, h	*Candida*	*Pancreas*
	mmol monobutyrin	
17	1.02	0.074
186	2.19	0.25

[a]Reaction conditions: 12.3 mmol glycerol; 11% buffer (at optimal pH for each enzyme); 0.1 g lipase; 40°C.

Table 3
Transesterification at 40°C with Several Lipase Preparations

Biocatalyst	Hydrolytic Activity nkat	Monobutyrin after 20 h of reaction, %[a]
Mucor/resin	6.5/mg biocatalyst	16.7
Candida/agarose	110/mL biocatalyst	0[b]
Candida/Celite	9.0/mg biocatalyst	11.1
Pancreatic	394/mg powder	3.0[c]
Rhizopus	28600/mg protein	0.59
PEG-*Candida*	8700/mg protein	0
Candida	108/mg powder	15.0
		21.7[d]
		24.9[e]

[a,b,c]Reaction conditions: 12.3 mmol glycerol; 5% buffer; variable amount of biocatalyst: [a]10^4 nkat, [b]10^2 nkat, and [c]40×10^4 nkat.
[d,e]280 and 560 mg of trichloroethyl butyrate, respectively, were also included in the reaction mixture.

Transesterification with Several Biocatalysts

Several very different enzyme preparations, native, soluble, and heterogenized, have been tested to compare their monobutyrin yields and selectivities, the percentage of conversion to monobutyrin for each appearing in Table 3, where the hydrolytic activities using tributirin as substrate are also given. For comparative purposes, the syntheses were each carried out with an amount of biocatalyst equivalent to 10^4 nanokatals (hydrolytic), except for *Candida* lipase immobilized on agarose gel (in order not to exceed the optimal 5% aqueous phase) and pancreatic lipase (which was found to be a poor catalyst for this synthesis; *see* Table 2). *Mucor*/resin and *Candida* are clearly the best biocatalysts, and *Candida*/Celite a fair one. In the case of *Candida*/agarose gel, it is difficult to arrive at a compromise between adding more units of enzyme activity and increasing the water

concentration too much, so, unless a very high loading of the gel with enzyme is achieved, this type of support will not be suitable for syntheses.

DISCUSSION

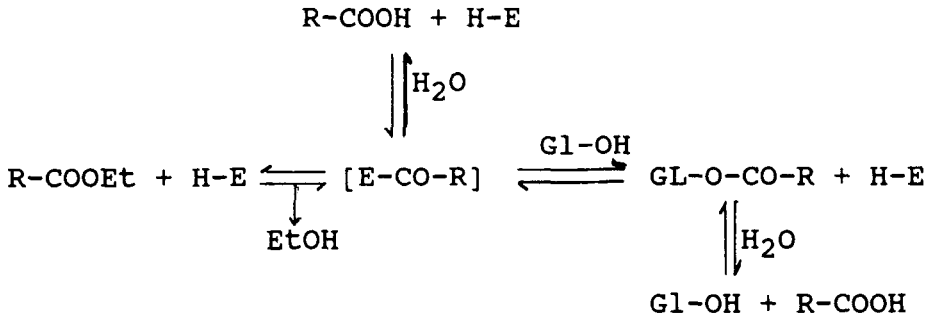
In the last four years, many investigations using hydrolases in organic media as synthetic catalysts have been carried out, usually with lipases, owing to their good activity, broad specificity, and low cost. Hoq et al. (*11*) studied several lipases for continuous glyceride synthesis: in 97% glycerol solution, *C. cylindracea* lipase was quickly inactivated, whereas the *M. miehei* enzyme yielded a fair conversion to mono- and diglycerides. Kawamoto et al. (*12*) screened fifty different hydrolases for high esterification activity of citronellol, and found that the lipase from *C. cylindracea* was the best one.

In this paper, the interesterification of a trialcohol—glycerol—with butyric acid ethyl ester in a two-phase system has been tested, using lipases from several sources: pancreas, *M. miehei, R. arrhizus,* and *C. cylindracea* (the first three are 1,3 specific; the fourth, unspecific). In all cases the reaction proceeded to the formation of monoacylglycerol. Of the native lipases tried, only the *Candida* enzyme had good activity, whereas a well-purified preparation from *Rhizopus* gave negligible conversion. The pancreatic enzyme, although used in a 40-fold excess and tested at its optimal pH (1), showed only 3% conversion; certainly, this low-cost commercial preparation is not suitable for this transesterification reaction.

The attachment of PEG to enzymes has been pioneered by Inada et al. (*9*) as a way to make them soluble in organic solvents. Thus, *C. cylindracea* lipase modified with PEG has been successfully employed in benzene in the synthesis of esters of α- or β-substituted carboxylic acids (*13*). In our studies, PEG-*Candida* lipase was inactive (cf Table 3) in the synthesis of glycerides in a two-phase system. This could result from low stability of the modified enzyme in the organic solvents during the long times required for the reaction. In fact, it has been recently found (*14*) that PEG-*C. cylindracea* lipase, although 10× more stable in water than the native enzyme, was twofold less stable in benzene.

Ester synthesis in organic solvents catalyzed by lipase immobilized on different supports has been reported from many laboratories. Lipase adsorbed on Celite with good activity has been described in several publications: Marlot et al. (*15*), for the synthesis of geranyl esters; Ison et al. (*16*), for the interesterification of fats; and Kawase and Tanaka (*17*), for the esterification of menthol. Ergan et al. (*18*) have reported the synthesis of glycerides by Lipozyme, using stoichiometric amounts of glycerol and oleic acid; the timing of addition of molecular sieves (to scavenge the water formed in the esterification reaction) controlled the triglyceride/diglyceride ratio.

In this work we have immobilized the *Candida* enzyme on agarose and on Celite; in addition, we have used the Lipozyme preparation. In



Scheme 1. Competition between glycerol (Gl-OH) and water for the butyryl-enzyme intermediate [E-CO-R]. R-COOEt, ethyl butyrate; H-E, enzyme.

our reaction conditions—two phases, no solvent addition—Lipozyme yielded the higher conversion to monobutyrin, with *Candida*/Celite also showing good activity. The immobilization of the enzyme on a matrix as hydrophilic as agarose (Sepharose 6) was not, in our hands, a good procedure for synthesis of the glyceride, owing to the large amount of water (approximately 94%) present in the added agarose gel; however, this type of support yields good biocatalysts for quick hydrolysis to glycerol (*19*).

The importance of water content in synthetic reactions in organic solvents has been stressed recently (*20*). Our data in Fig. 1 showed that 5% water was the optimal concentration for the reaction. Omar et al. (*21*) also found that the lipase from *Humicola lanuginosa* required 7% added water for maximal esterification of lauric acid. The optimal amount of water present in a synthetic reaction represents a compromise between activity of the enzyme and too much hydrolysis. Since it is well known that this type of enzymic reaction proceeds through the formation of an acyl-enzyme intermediate (*1*), water will compete with glycerol as acyl acceptor (*see* Scheme 1). The amount of water that gives optimal synthesis activity always must be found experimentally for each reaction system.

Cesti et al. (*5*) found in the enzymatic transesterification of diols that primary alcoholic functions were more reactive; therefore, primary monoesters of glycols were produced. In our present investigations, monobutyrin was also produced by the biocatalysts. In the reaction of glycerol and oleic acid catalyzed by purified lipase from *Pseudomonas fragi*, monoolein and 1,3-diolein were the main products (*22*), which indicates that this enzyme has 1,3-position specificity in glyceride synthesis. However, with either 1,3-specific or unspecific lipases, we have not found this specificity, since dibutyrin was not formed in our experiments.

In the synthesis of an ester, the two possibilities, esterification or transesterification, must be considered. In the first case, the production of water during the reaction will favor hydrolytic steps; in the second; the biocatalyst must withstand the presence of alcohol. In this work, as in others (*5*), the use of an acyl carboxylate has proved to be very convenient.

The yield of monobutyrylglycerol obtained in the presence of *Candida* lipase was substantially enhanced when, in addition to ethyl butyrate, a better acyl donor (trichloroethyl butyrate) (*5*) was also included in the reaction mixture. We are now trying to increase the yield of monobutyrin by other approaches (i.e., reaction engineering).

ACKNOWLEDGMENTS

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