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Review

Synthesis of triglycerides of phenylbutyric acids by lipase-catalyzed glycerolysis in a solvent-free system

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ABSTRACT

Triphenylbutyrin, a triglyceride prodrug of 4-phenylbutyric acid, has potential for use in drug delivery systems. Immobilized *Candida antarctica* lipase B catalyzed the synthesis of triphenylbutyrin by glycerolysis of 4-phenylbutyrate with glycerol in a solvent-free system. The use of 4-phenylbutyate with a leaving alcohol moiety in the acyl-transfer reaction can shift the equilibrium of the reaction toward the synthesis of triphenylbutyrin, since the alcohol by-product can be removed by vacuum. A 97% yield of triphenylbutyrin was achieved in a solvent-free system at a reaction temperature of 75 °C by combination of molecular sieves to limit the hydrolysis side reaction and a vacuum system to shift the reaction equilibrium in a solvent-free system.

Contents

1. Introduction

4-Phenylbutyrate (4-PBA) has a variety of biological functions. Sodium phenylbutyrate is a hyperammonemia agent used for the chronic management of patients with urea cycle disorders [1,2]. Histone-deacetylase (HDAC) inhibitors are an exciting new class of chemotherapeutic drugs that can reactivate gene expression and inhibit the growth and survival of tumor cells. 4-PBA is an HDAC inhibitor that affects the dynamics of chromatin folding during gene transcription, resulting in anti-proliferative, differentiation-inducing and apoptosis-enhancing effects [3–5]. A large number of diseases result from protein misfolding and aggregation. 4-PBA possesses chemical chaperone activity that alleviates protein misfolding and mislocalization under conditions of endoplasmic reticulum stress [6–8]. There are currently only two commercial dosage formulations of sodium phenylbutyrate: a 500 mg tablet and

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a granular powder that has a very unpleasant taste and is difficult to swallow at the recommend daily dosage [9,10].

The pharmaceutical industry has displayed increasing interest in the natural, triglyceride form of fatty acids. As prodrugs, triglyceride molecules can increase the *in vivo* half-life and stability of the active component of a drug, allowing it to be maintained at a constant level in plasma over a long period of time. For example, different chain lengths of triglycerides of valproic acid were used to evaluate the oral absorption characteristics of phospholipid prodrugs for drug delivery [11]. Pharmacokinetic studies of tributyrin, a triglyceride prodrug of butyric acid, have shown it to be a more potent inhibitor of cell growth and inducer of differentiation than natural butyrate, whose metabolism is too fast to reach pharmacologic concentrations in neoplastic cells [12,13]. Similarly, triphenylbutyrin, a triglyceride prodrug of 4-phenylbutyric acid that is slowly hydrolyzed by cellular lipases and esterases, has been shown capable of maintaining higher serum levels for longer periods of time than those observed using phenylbutyrate salts. The use of triphenylbutyrin over sodium phenylbutyrate has several additional advantages; triphenylbutyrin has a higher molecular weight, does not release an offensive odor, and contains glycerol, a normal product of metabolism, instead of sodium, which can help many patients stay within their daily dosage limit of sodium [9].

Several lipase-catalyzed glycerolysis reactions have been studied. Enzymatic synthesis of glycerides from DHA-enriched PUFA ethyl ester under vacuum pressure can push the reaction equilibrium towards a high conversion and prevent the degradation of the oil [14]. Weber and Mukherjee investigated the production of diacylglycerols for nutritional purposes using Lipozyme TL and Novozym 435 to catalyze the glycerolysis of rapeseed oil triacylglycerols under vacuum pressure at 60 °C, and they found that the of diacylglycerol content did not exceed 50% [15]. In our previous study, a lipase-catalyzed esterification process in a solvent-free system at 65 °C using molecular sieves for the elimination of water was developed for the synthesis of triphenylbutyrin with a 98% yield [16]. However, it is difficult to remove water continuously since molecular sieves are not easy to reuse or scale up. Haraldsson et al. obtained a 99% yield of homogeneous triglycerides from a solvent-free system at 65 °C by maintaining the reaction pressure between 0.1 Torr and 0.01 Torr with a vacuum system to control water levels [17]. Here we develop an enzymatic process for the production of triphenylbutyrin through lipase-catalyzed glycerolysis of phenylbutyrate in a solvent-free system using *Candida antarctica* B, as shown in Scheme 1. We selected phenylbutyrates with a leaving alcohol moiety containing a 2,2,2-trifluoroethyl or an ethyl group, whose by-products, 2,2,2-trifluoroethanol or ethanol, are expected to vaporize under vacuum pressure with a diaphragm pump because of the lower boiling point of alcohol compared to water. We also investigated several reaction parameters for the synthesis of triglycerides including temperature, method of water removal, and effect of the leaving alcohol moieties on phenylbutyrate.

2. Materials and methods

2.1. Materials

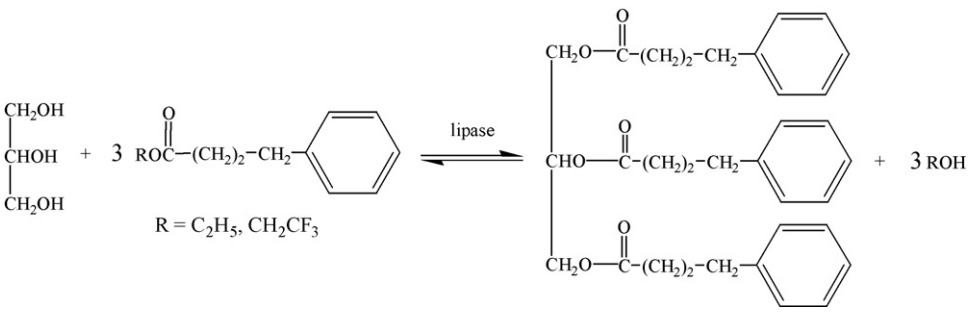
The immobilized lipase, Novozym 435, was immobilized *C. antarctica* lipase B which was purchased from Novo Nordisk A/S. 4-Phenylbutyric acid was purchased from Fluka (Buchs, Switzerland). Other chemicals of analytical grade were commercially available.

2.2. General procedure for the synthesis of ethyl 4-phenylbutyrate and 2,2,2-trifluoroethyl 4-phenylbutyrate

One hundred milliliters of toluene was added to 75 mmol phenylbutyric acid and 150 mmol thionyl chloride and heated to 90 °C with reflux for 4.5 h. The resulting solution was concentrated under reduced pressure, and then 150 mL of toluene, 75 mmol of anhydrous pyridine and 150 mmol of trifluoroethanol or anhydrous ethanol were added. The reaction mixture was refluxed at 90 °C for 4 h. It was then cooled to room temperature and extracted four times with 250 mL NaOH solution (pH 12.5) and twice with 500 mL of deionized water. The organic layer was separated and dried over $MgSO_4$, centrifuged and concentrated under reduced pressure. The resulting oil was purified by silica gel liquid chromatography with a mobile phase of n-hexane:ethyl acetate (5:1) and concentrated under reduced pressure. The yields of ethyl 4-phenylbutyrate and 2,2,2-trifluoroethyl 4-phenylbutyrate were 75.47% and 85.2%, respectively. The desired esters were confirmed by ^{1}H NMR spectra recorded at 400 MHz on a Bruker AMX-400 spectrometer in solutions of deuteriochloroform. ^{1}H NMR ($CDCl_3$) data for ethyl 4-phenylbutyrate and 2,2,2-trifluoroethyl 4-phenylbutyrate were reported as follows: ethyl 4-phenylbutyrate: δ 1.23 (3H, t, J = 7.1 Hz), 1.94 (2H, quin, J = 7.5 Hz), 2.29 (2H, t, J = 7.5 Hz), 2.63 (2H, t, J = 7.5 Hz), 4.10 (2H, q, J = 7.1 Hz), 7.16 (3H, m), 7.25 (2H, t, J = 7.8 Hz); 2,2,2-trifluoroethyl 4-phenylbutyrate: δ 1.98 (2H, quin, J = 7.5 Hz), 2.41 (2H, t, J = 7.4 Hz), 2.65 (2H, t, J = 7.4 Hz), 4.43 (2H, q, J = 8.4 Hz), 7.18 (3H, m), 7.28 (2H, t, J = 7.2 Hz).

2.3. Analysis of reaction substrates and products

Glycerolysis was monitored by HPLC using a LiChrospher 100 RP-8, end-capped column 25 cm × 0.4 cm i.d., 5 μm (Merck, Germany) capable of separating a internal standard of 2-nitrotoluene, mono-, di- and tri-glycerides of phenylbutyric acids with the retention times and gradient conditions listed in Table 1. 2.5 μL samples were withdrawn at various time intervals from reactive system and were diluted in 5 mL acetonitrile. The HPLC method was modified by using a solvent gradient to enhance the separation of mono-, di- and tri-glycerides of phenylalkanoic acids. Detection of UV absorbance at 216 nm was used for quantification and maintained the column temperature at 27 °C.



Scheme 1.

Table 1
Retention times of 2-nitotoluene (internal standard), substrates, and products with different gradient conditions.

Compound	Retention time (min)	Gradient condition[a]		
		Solvent A (%)	Solvent B (%)	Flow rate (mL/min)
Monophenylbutyrin	5.47	100	0	0.5
4-Phenylbutyric acid	6.56	100	0	0.5
2-Nitrotoluene	9.09	100	0	0.5
Ethyl 4-phenylutyrate	11.49	100	0	0.5
Diphenylbutyrin	11.87	100	0	0.5
2,2,2-Trifluoroethyl 4-phenylbutyrate	12.48	100	0	0.5
Triphenylbutyrin	25.31	70	30	1

[a] Solvent A (acetonitrile:water:trifluoroacetic acid = 65:35:0.0001) and solvent B (acetonitrile).

2.4. Reaction apparatus and enzymatic reaction

Three reaction apparatuses were used herein: (i) open system: reactants were mixed in a 5 mL uncapped glass vial to allow free evaporation of the alcohols formed during the lipase-catalyzed reaction; (ii) small closed system: lipase-catalyzed reactions were performed in a 5 mL capped glass vial; (iii) large closed system for water removal: lipase-catalyzed reactions were performed in a 5 mL uncapped glass vial put into a 10 mL wide-mouth capped glass vial that contained sufficient molecular sieves to maintain a low water content in the enzyme reaction. Since the molecular sieves maintained the water activity under 0.01, the water removal system did not need to be pre-equilibrated with the molecular sieves which would strip the essential water from the enzyme.

All lipase-catalyzed reactions were performed in 5 mL glass screw-cap bottles. Unless otherwise specified, 3 mmol 2,2,2-trifluoroethyl 4-phenylbutyrate or ethyl 4-phenylbutyrate dissolved in 1 mmol glycerol was added to 3.6% (w/w) Novozym 435 lipase. The reaction apparatus were placed in a temperature-controlled water bath with a magnetic stir bar.

3. Results and discussion

3.1. Effect of enzyme content on the reaction course

We studied the effect of enzyme content on the time course of the yield of triphenylbutyrin (X_{tri}) in the open solvent-free system. We found that the time for equilibrium conversion was the same for both 10% (w/w) and 3.6% (w/w) of the enzyme, as shown in Fig. 1. In terms of the development of process, using a lower amount (3.6%) of the enzyme reduces the operation cost.

3.2. Effect of temperature on the synthetic rate of triphenylbutyrin

Fig. 2 compares rates of synthesis of triphenylbutyrin (V_{tri}) by glycerolysis or esterification at various temperatures in an open system. The maximum V_{tri} by glycerolysis and esterification was obtained at 75 °C and 65 °C, respectively. The V_{tri} of esterification sharply decreased when the reaction temperature exceeded the optimal temperature of 65 °C. This decrease may have resulted from the production of water as a side-product of the esterification process, leading to the deactivation of the enzyme. This observation explains why the thermal stability of enzyme in a glycerolysis reaction is apparently superior to that in an esterification reaction for the synthesis of triphenylbutyrin. Glycerolysis can effectively enhance the thermal stability of the enzyme in the lipase-catalyzed reaction, creating favorable conditions for the development of industrial processes. The equilibrium conversion

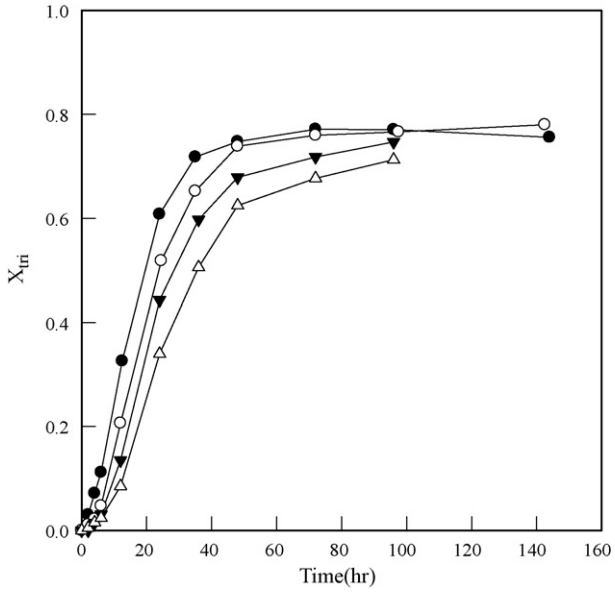


Fig. 1. Effect of the content of the Novozyme 435 on the time course of the yield of the triphenylbutyrin (X_{tri}) in an open solvent-free system. 2,2,2-trifluoroethyl 4-phenylbutyrate (3 mmol), glycerol (1 mmol), 65 °C. Novozyme 435 content (w/w): (●) 10%; (○) 3.6%; (▼) 2%; (△) 1%.

(X_{tri}) of 2,2,2-trifluoroethyl 4-phenylbutyrate by glycerolysis in an open solvent-free system reached a maximum of 80%, between 75 °C and 80 °C, as shown in Fig. 3. We also found that the degree of hydrolysis, a side-reaction of glycerolysis, decreased with an increase in reaction temperature, leading to the reduction of water level by free vaporization in the open solvent-free system.

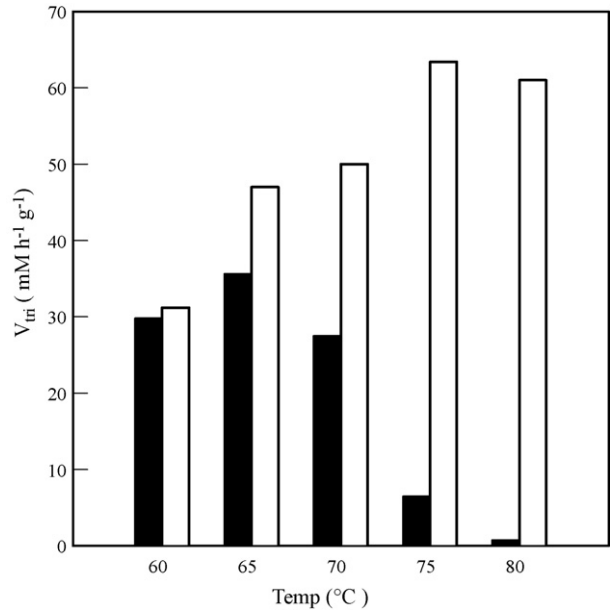


Fig. 2. Variation change in the synthetic rate of triglycerides (V_{tri}) with temperature in an open solvent-free system. (■) esterification: Novozym 435 (10%, w/w), 4-phenylbutyric acid (3 mmol), and glycerol (1 mmol); (□) glycerolysis: Novozym 435 (3.6%, w/w), 2,2,2-trifluoroethyl 4-phenylbutyrate (3 mmol), and glycerol (1 mmol). X_{mono}: the yield of monoglyceride, $X_{mono} = C_{mono}/(C_{PA} + C_{mono} + 2C_{di} + 3C_{tri})$; X_{di}: the yield of diglyceride, $X_{di} = 2C_{di}/(C_{PA} + C_{mono} + 2C_{di} + 3C_{tri})$; X_{tri}: the yield of triglyceride, $X_{tri} = 3C_{tri}/(C_{PA} + C_{mono} + 2C_{di} + 3C_{tri})$, where C_{PA}, C_{mono}, C_{di} and C_{tri} are the concentrations of phenylalkanoic acids, monoglyceride, diglyceride and triglyceride, respectively.

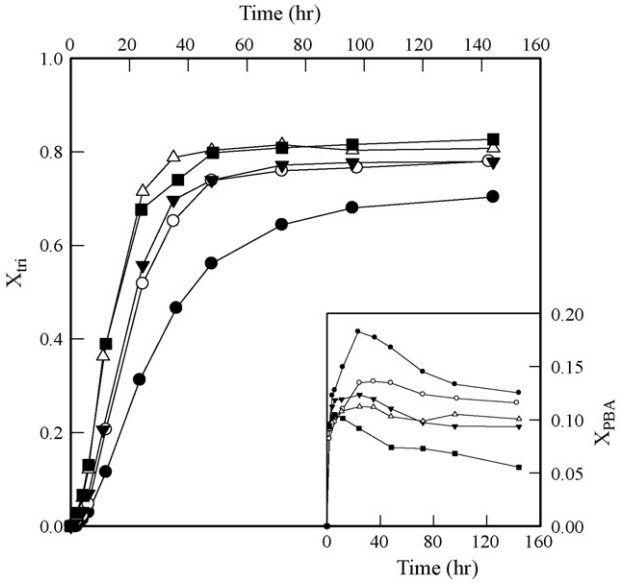


Fig. 3. Effect of reaction temperature on the time course of the conversion of triphenylbutyrin (X_{tri}) and the by-product yield of 4-phenylbutyric acid (X_{PBA}) in an open solvent-free system. Novozym 435 (3.6%, w/w), 2,2,2-trifluoroethyl 4-phenylbutyrate (3 mmol), and glycerol (1 mmol). (●) 60 °C; (○) 65 °C; (▼) 70 °C; (△) 75 °C; (■) 80 °C. $X_{tri} = 3C_{tri}/(C_{PA} + C_{mono} + 2C_{di} + 3C_{tri})$, where C_{PA}, C_{mono}, C_{di} and C_{tri} are the concentrations of phenylbutyric acid, monoglyceride, diglyceride and triglyceride, respectively.

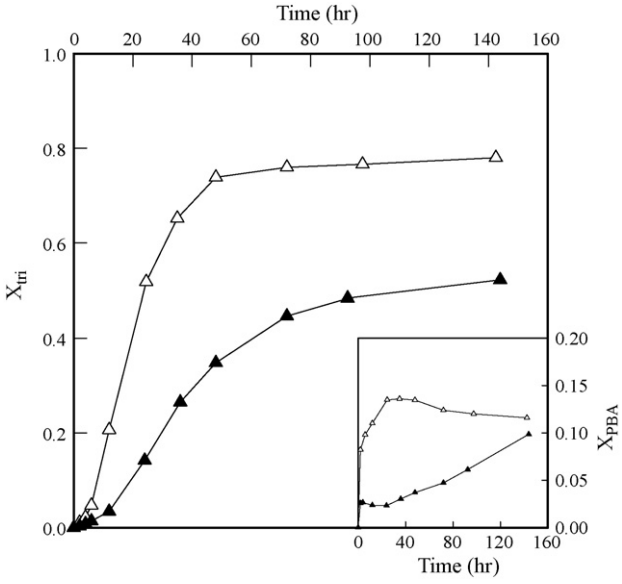


Fig. 5. Effect of different leaving alcohol moieties of 4-phenylbutyrate on the time course of the conversion of triphenylbutyrin (X_{tri}) and the by-product yield of 4-phenylbutyric acid (X_{PBA}) in an open solvent-free system. Novozym 435 (3.6%, w/w), 4-phenylbutyrate (3 mmol), glycerol (1 mmol). (△) 2,2,2-Trifluoroethyl 4-phenylbutyrate; (▲) ethyl 4-phenylbutyrate.

3.3. Effect of substrate molar ratio on the yield of triphenylbutyrin

Experiments were conducted at different molar ratios of 2,2,2-trifluoroethyl 4-phenylbutyrate to glycerol in an open solvent-free system at 75 °C to observe the change in the final yield of triphenylbutyrin, as shown in Fig. 4. As the substrate molar ratio was increased from 3:1 to 6:1 (the 2,2,2-trifluoroethyl 4-phenylbutyrate excess condition), the final yield of triphenylbutyrin declined from 81.5% to 52.1%. The highest final yield of triphenylbutyrin was produced by the substrate molar ratio of 3:1. We further found that when the substrate molar ratio deviated from the stoichiometric relationship resulted in an increase in the conversion of 4-phenylbutyric acid from the hydrolysis of 2,2,2-trifluoroethyl 4-phenylbutyrate (side reaction). In future experiments, we will keep the ratio of substrates constant at a stoichiometric relationship to reduce the degree of hydrolysis of 2,2,2-trifluoroethyl 4-phenylbutyrate (side reaction).

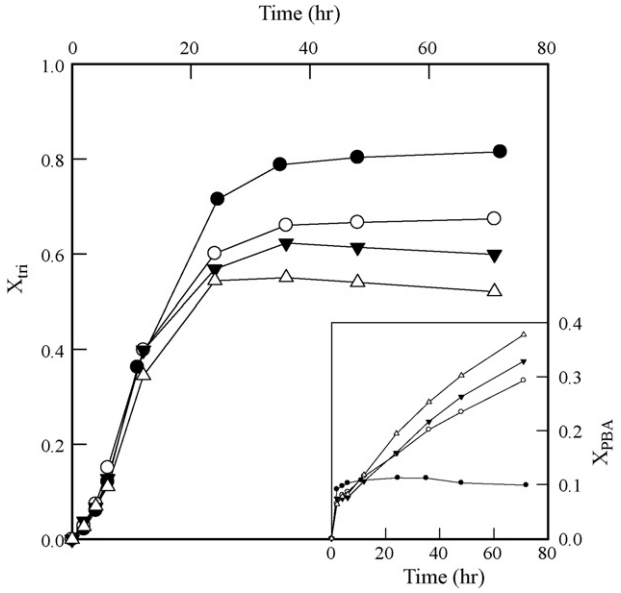


Fig. 4. Effect of the molar ratio of 2,2,2-trifluoroethyl 4-phenylbutyrate/glycerol on the yield of triphenylbutyrin in an open solvent-free system. Novozyme 435 (3.6%, w/w), 2,2,2-trifluoroethyl 4-phenylbutyrate = 3 mmol, glycerol = 1 mmol, 0.75 mmol, 0.6 mmol, 0.5 mmol, respectively, 75 °C. (●) Glycerol/2,2,2-trifluoroethyl 4-phenylbutyrate = 3/1; (○) 2,2,2-trifluoroethyl 4-phenylbutyrate/glycerol = 4/1; (▼) 2,2,2-trifluoroethyl 4-phenylbutyrate/glycerol = 5/1; (△) 2,2,2-trifluoroethyl 4-phenylbutyrate/glycerol = 6/1.

3.4. Comparison of 2,2,2-trifluoroethyl 4-phenylbutyrate and ethyl 4-phenylbutyrate as reactants in an open solvent-free system

As shown in Fig. 5, the synthetic rate of triphenylbutyrin from 2,2,2-trifluoroethyl 4-phenylbutyrate is apparently higher than that from ethyl 4-phenylbutyrate in an open solvent-free system. The hydrolytic rate of 2,2,2-trifluoroethyl 4-phenylbutyrate is also higher than the hydrolytic rate of ethyl 4-phenylbutyrate due to the side reaction of lipase-catalyzed glycerolysis. The final yield at 65 °C of triphenylbutyrin from 2,2,2-trifluoroethyl 4-phenylbutyrate (78%) is higher than that from ethyl 4-phenylbutyrate (52%). The phenomenon is caused by the presence of "activated" trifluoroethyl esters introduced by the electron-withdrawing trifluoroethyl group, which served as a leaving group on the alcohol moiety. The concept of an "activated" ester was proposed by Kirchner et al. [18]. Although acyl-transfer using an activated ester is reversible, we can create a "quasi-irreversible" reaction system by removing the alcohol by-product to shift the reaction equilibrium toward the product side.

3.5. Effects of mass transfer limitations on the immobilized lipase

The synthesis of triphenylbutyrin from 4-phenylbutyric acid by immobilized lipase-catalyzed esterification in an open solvent-free system has shown that the high viscosity of glycerol in the solvent-free system has important effects on the limitation of external mass transfer to the immobilized lipase [16]. As

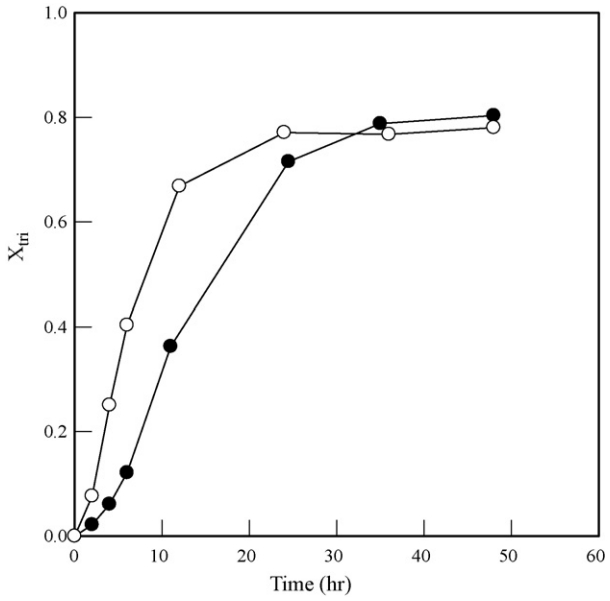


Fig. 6. Effect of agitation on the time course of the yield of the triphenylbutyrin (X_{tri}) in open solvent-free system. Novozyme 435 (3.6%, w/w), 2,2,2-trifluoroethyl 4-phenylbutyrate (3 mmol), glycerol (1 mmol), 75 °C. (●) No agitation; (○) agitated at 600 rpm.

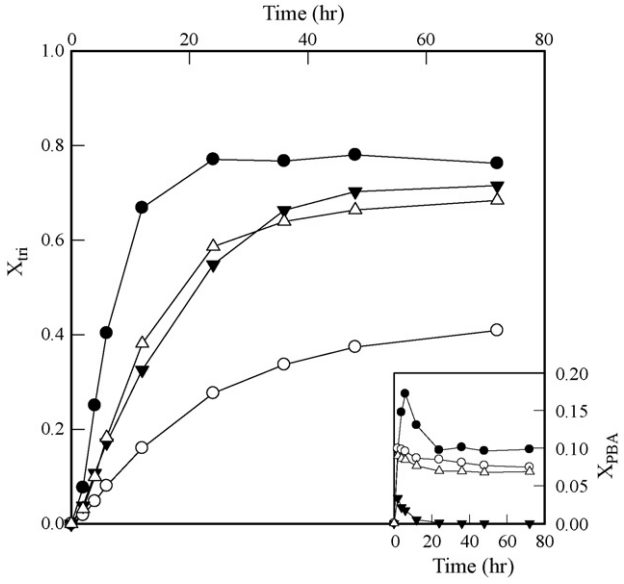


Fig. 7. Effect of different reaction systems on the final yield of triphenylbutyrin (X_{tri}) in a solvent-free system. Novozyme 435 (3.6%, w/w), 2,2,2-trifluoroethyl 4-phenylbutyrate (3 mmol), glycerol (1 mmol), 75 °C. (●) Open system; (○) closed system; (▼) closed system with molecular sieves; (△) closed system without molecular sieves.

shown in Fig. 6, the synthesis of triphenylbutyrin from 2,2,2-trifluoroethyl 4-phenylbutyrate by immobilized lipase-catalyzed glycerolysis in an open solvent-free system without agitation was limited by external mass transfer rates of reactants to the immobilized lipase apparently declined relative to the former with agitation.

3.6. Comparison of different systems for by-product removal

Previous studies have shown that the yield of triphenylbutyrin by esterification can reach 98% because of the strong ability of molecular sieves to adsorb water (the by-product of esterification) [15]. Although molecular sieves cannot adsorb the alcohol by-products from glycerolysis, they may be able to reduce the side hydrolysis reaction that results from the high water content in the reaction system. As shown in Fig. 7, the final yield of triphenylbutyrin in an open system was (76%) was higher than the final yields obtained using the small closed system (41%), the large closed system with molecular sieves (71%), or the large closed system without molecular sieves (68%). These results indicate that the air ventilation of the open system removes the CF_3CH_2OH by-product from the glycerolysis reaction, resulting in significant increases in synthetic rate and final yield of triphenylbutyrin. Meanwhile, 10% of the reactants were used up in the side reaction of hydrolysis. The closed system gave a lower final yield of triphenylbutyrin, apparently due to the accumulation of CF_3CH_2OH, which forces the equilibrium toward the synthesis of acylglycerols. Furthermore, the final yield of triphenylbutyrin in the large closed system was apparently higher than that of the small closed system because the former had a lower concentration of CF_3CH_2OH due to the larger enclosed volume.

In the large closed system, one method for reducing the rate of the hydrolysis reaction is to use a separate, larger vessel containing molecular sieves in contact with the reaction system to adsorb the excess water through vapor phase equilibrium while the lipase-catalyzed glycerolysis of 2,2,2-trifluoroethyl 4-phenylbutyrate is performed in a smaller vessel. Sufficient molecular sieves should be used to keep the water activity <0.01 by continuously adsorbing the water vapor. In a comparison between reactions in large closed systems with a separate, larger vessel containing molecular sieves and a separate, larger vessel without molecular sieves, we found the former effectively inhibited the formation of 4-phenylbutyric acid (X_{PBA}), a product of the hydrolysis side reaction.

3.7. Effect of molecular sieves and a vacuum system on glycerolysis

The initial glycerolysis of 2,2,2-trifluoroethyl 4-phenylbutyrate (or ethyl 4-phenylbutyrate) produced the side-product of 4-phenylbutric acid from the hydrolysis side reaction if the reactants were not dehydrated. As an operational strategy for the glycerolysis process, we expected that using molecular sieves to remove excess water would inhibit the side reaction of hydrolysis in the first stage of glycerolysis, and that gradually increasing the vacuum pressure would remove the volatile alcohol by-products during the last stage of glycerolysis. As shown in Fig. 8, the side reaction of hydrolysis during the glycerolysis of 2,2,2-trifluoroethyl 4-phenylbutyrate still reached a 6% yield. Because 2,2,2-trifluoroethyl 4-phenylbutyrate is an “activated” ester, the hydrolysis of ethyl 4-phenylbutyrate can be inhibited by the removal of water by molecular sieves. For the glycerolysis of 2,2,2-trifluoroethyl 4-phenylbutyrate and ethyl 4-phenylbutyrate, the X_{tri} of 2,2,2-trifluoroethyl 4-phenylbutyrate was lower than the X_{tri} of ethyl 4-phenylbutyrate, apparently because of the hydrolysis of the former. Comparison of Figs. 5 and 8 shows that an increase in the reaction temperature from 65 °C to 75 °C can abolish the difference in the glycerolysis reaction rates between a “nonactivated” and an “activated” ester.

Because the concentration of 4-phenylbutyric acid can be reduced by inhibiting hydrolysis with molecular sieves in the initial stage of glycerolysis, vacuum pressure was used to remove the alcohol by-products according to the following procedure. Over the reaction time period from 25 h to 75 h, the pressure of the reaction system was maintained at 200 Torr, increasing the yield of triphenylbutyrin from 80% to 93%; the final pressure of the reaction system was then reduced to 1 Torr until the equilibrium yield of triphenylbutyrin reached 97%.

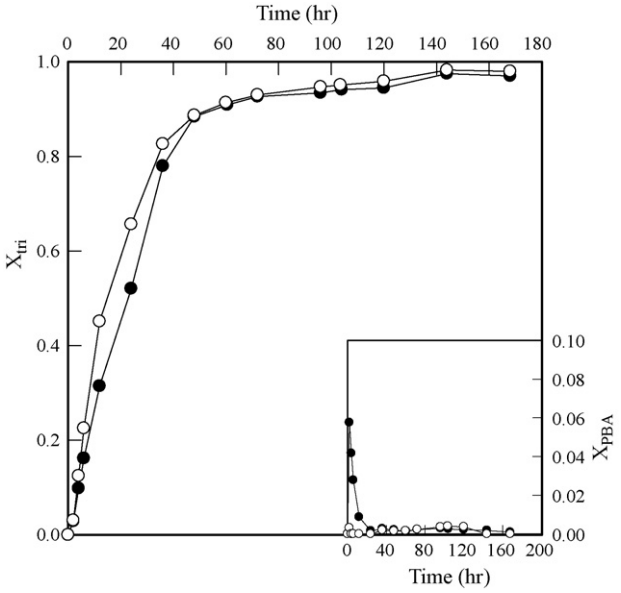


Fig. 8. The time course of the conversion of triphenylbutyrin (X_{tri}) and the by-product yield of 4-phenylbutyric acid (X_{PBA}) in a closed solvent-free system with molecular sieves under different vacuum pressures. Novozym 435 (3.6%, w/w), glycerol (1 mmol); 75 °C. (●) 2,2,2-Trifluoroethyl 4-phenylbutyrate (3 mmol); (○) ethyl 4-phenylbutyrate (3 mmol). 0–25 h: closed system with molecular sieves; 25–97 h: pressure maintained at 200 Torr; 97–121 h: pressure maintained at 100 Torr; 121–168 h: pressure maintained at 1 Torr.

4. Conclusion

An enzyme-catalyzed glycerolysis process for the synthesis of triglyceride prodrugs from phenylbutyrate was developed using Novozym 435 from *C. antarctica* lipase B as a biocatalyst. The incorporation of a procedure for the removal of alcohol by-products from the glycerolysis reaction was the main driving force for the shift in equilibrium toward enzymatically synthesized triglycerides. The volatility of the leaving alcohol moiety of 4-phenylbutyrate made the by-product of the glycerolysis easy to remove by vacuum pressure. In the solvent-free system, the degree of direct glycerolysis of glycerol with 4-phenylbutyrate was near 100% when the side reaction of hydrolysis was controlled by the removal of water from the solvent-free system with molecular sieves. The solvent-free system developed here effectively saved the consumption of organic solvent and simplified downstream processes.

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