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Article

Investigation of Lipozyme TL IM-catalyzed transesterification using ultraviolet spectrophotometric assay

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ABSTRACT

A novel ultraviolet (UV) spectrophotometric method for evaluating lipase transesterification activity in organic solvents is described. The model reaction was lipase-catalyzed transesterification between *p*-nitrophenyl palmitate (pNPP) and *n*-butanol in *n*-hexane at 30 °C. The conversion of pNPP to *p*-nitrophenol was monitored by measuring UV absorbance changes at 310 nm. Seven commercial lipases were tested using this method, and the results were similar to those obtained using gas chromatography. The UV spectrophotometric method was used to investigate the kinetics of the Lipozyme TL IM-catalyzed reaction of pNPP, and the correlation between synthetic activity and biocatalyst concentration was determined. The method was also used to investigate the Lipozyme TL IM-catalyzed transesterification properties, namely solvent effect, acyl acceptor specificity, alcohol tolerance, optimum reaction temperature, and thermostability.

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1. Introduction

Lipases (also called triacylglycerol hydrolases, EC 3.1.1.3) catalyze a wide range of reactions, e.g., hydrolysis, esterification, transesterification, alcoholysis, and aminolysis [1]. Most lipases have a catalytic triad composed of Ser-His-Asp residues. Lipase molecules generally consist of hydrophilic and hydrophobic parts with interfacial activation, and the catalytic site is concealed under a short amphipathic helix, which acts as a “lid” in hydrophobic interactions [2,3]. Lipase-catalyzed reactions in non-aqueous media are increasingly finding applications in the fields of food additives, pharmaceuticals, flavors, chemical materials, and bioenergy [4–8].

Lipozyme TL IM is a versatile enzyme and has been widely used in industrial processes and laboratory tests, particularly in the interesterification of bulk fats and the production of frying fats, because it is inexpensive and commercially available

[9–11]. The development of a fast and reliable analytical method for characterizing the catalytic properties of Lipozyme TL IM is therefore imperative. At present, most methods for lipase activity determination are based on hydrolytic assays such as the routinely used titrimetric protocol for olive oil emulsions, ultraviolet (UV) spectrophotometry using *p*-nitrophenol (pNP) esters as substrates, and chromatographic methods [12–14]. However, the synthetic activities of the lipases in organic solvents do not correspond to the hydrolytic activities in aqueous solutions [15,16]. Enzymes identified using hydrolytic approaches are not necessarily suitable for the desired synthetic reactions. It is therefore important to establish an assay for the synthetic activities of lipases based on esterification or transesterification. Most of the reported approaches are based on gas chromatography (GC), high-performance liquid chromatography, or fluorometric detection [17–19], which are extremely time consuming, expensive, and

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In this study, we attempted to develop a novel lipase activity assay method for determining the properties of Lipozyme TL IM-catalyzed transesterification. pNPP, a well-known chromogenic substrate for lipase hydrolysis activity determination, was selected as the substrate in the present work. The lipase-catalyzed transesterification between pNPP and *n*-butanol in *n*-hexane was used as a model reaction (Scheme 1). The amount of released product was estimated spectrophotometrically at 310 nm after dilution of sample aliquots with ethanol instead of NaOH solution; this improved the detection accuracy. The method, which can be used for microorganism high-throughput screening and for identifying large libraries of mutants with lipase synthetic activity, uses a commercially available substrate, has a simple detection procedure, and gives accurate test results. The properties of Lipozyme TL IM-catalyzed transesterification, namely solvent effect, acyl acceptor specificity, alcohol tolerance, optimum reaction temperature, and thermostability, were also investigated using the UV spectrophotometric method. Characterization of the transesterification properties of Lipozyme TL IM will be helpful in industrial processes and laboratory tests.

2.1. Materials

pNPP was purchased from Sigma-Aldrich (Shanghai, China), and pNP was provided by the Aladdin Chemistry Co., Ltd.

2.4. Calculation of reaction conversion

3.1. UV spectrophotometric assay for lipase transesterification activity

Scheme 1. General reaction for transesterification between *p*-nitrophenyl palmitate (pNPP) and alcohol.

3.1.1. UV absorbances of pNP and pNPP in ethanol

To measure the maximum absorption wavelength of pNP in ethanol and investigate UV interference from the substrate (pNPP) and solvent (ethanol), scans at 200–400 nm of pNP (0.1 mmol/L in ethanol), pNPP (0.1 mmol/L in ethanol), and ethanol were carried out. The results (Fig. 1) showed that pNP had a maximum absorption peak at 310 nm (1.341); relatively low absorbances for pNPP (0.193) and ethanol (0.067) were detected at the same wavelength. Palmitate, the other product of transesterification between pNPP and an alcohol, made no significant contribution to the absorbance at 310 nm (data not shown). Based on the above results, 310 nm was considered to be the optimum wavelength.

3.1.2. Standard curves for UV spectrophotometric assay of transesterification activity

The standard calibration curves for pNP and pNPP are shown in Fig. 2. The absorbance at 310 nm versus concentration from 0.01 to 0.1 mmol/L for pNP ($R^2 = 0.9977$) and pNPP ($R^2 = 0.9988$) both showed good linear relationships. On the basis of the calibration curves, the molar extinction coefficient of pNP in ethanol at 310 nm was found to be $11.63 \times 10^3 \text{ mol}^{-1} \text{ L cm}^{-1}$, which is 11 times higher than that of pNPP ($1.06 \times 10^3 \text{ mol}^{-1} \text{ L cm}^{-1}$).

It was therefore possible to estimate the transesterification

Fig. 1. UV absorbance spectra of pNP (1), pNPP (2), and ethanol (3) between 200 and 400 nm. Experiments were carried out with 0.1 mmol/L pNP or pNPP in ethanol using a Multi-Mode microplate reader.

Fig. 2. Calibration curves of pNP (1) and pNPP (2) in ethanol.

activity for pNPP and a short-chain alcohol (*n*-butanol) by assessing the absorbance at 310 nm because of the good linear relationships shown by the standard curves and the difference between the molar extinction coefficients of pNP and pNPP.

3.1.3. Validation of UV spectrophotometric assay of lipase transesterification using GC analysis

To validate the effectiveness of the methodology, seven commercially available lipases with a wide range of activities were investigated using the UV spectrophotometric assay and GC-FID assay. Most of the data shown in Fig. 3 correlated well with the conversions determined using conventional GC analysis. Lipzyme TL IM gave the highest conversion (45%) of pNPP in the UV spectrophotometric assay. Lipzyme TL IM was therefore selected for the kinetic study.

3.1.4. Kinetics of Lipzyme TL IM-catalyzed transesterification

Figure 4 shows the kinetic curves for the Lipzyme TL IM-catalyzed transesterification reaction between pNPP and *n*-butanol at different biocatalyst concentrations. The biocatalyst loading was tested in the range 2–20 mg/mL; all other parameters were constant. The reaction was also carried out without Lipzyme TL IM, and the results showed that the absorbance at 310 nm was constant during the whole process (data not shown). As expected, with increasing enzyme concentration, the reaction rate increased, and a steady conversion was obtained when the reaction time was extended. Moreover, the initial rate increased proportionally to the lipase concentration (data not shown). These results confirmed the possibility of monitoring the synthetic activity through transesterification using the proposed UV spectrophotometric method.

A similar model reaction to that used by Teng et al. [20] was adopted in this study. However, the major breakthrough associated with this method is that the lipase transesterification activity is detected in ethanol instead of NaOH solution. The extinction coefficient of pNP is strongly pH dependent, and chemical hydrolysis of the remaining pNPP occurs in alkaline

Fig. 3. Comparison of various methods used for determination of synthetic activities of different lipases. Experiments were performed at 30 °C using samples of volume 1 mL containing 10 mmol/L pNPP and 1 mol/L *n*-butanol in *n*-hexane with a shaking speed of 200 r/min for 60 min. Samples (100 μL) were mixed with ethanol (3 mL) and assayed at 310 nm using UV spectrophotometry, and other 100 μL samples were diluted 10 times with ethyl acetate and analyzed using GC.

Fig. 4. Kinetics of transesterification of pNPP with *n*-butanol using different amounts of Lipozyme TL IM in *n*-hexane. (1) 2.0 mg/mL; (2) 5.0 mg/mL; (3) 10.0 mg/mL; (4) 15 mg/mL; (5) 20 mg/mL. Assays were carried out at 30 °C using 1 mL samples containing 10 mmol/L pNPP and 1 mol/L *n*-butanol in *n*-hexane with a shaking speed of 200 r/min, and then 30 μ L samples were mixed with ethanol (3 mL) and assayed at 310 nm at regular time intervals.

solution [22]. The measurement accuracy is therefore significantly improved using the proposed method.

3.2. Investigation of Lipozyme TL IM-catalyzed transesterification properties

3.2.1. Effect of solvent on Lipozyme TL IM-catalyzed transesterification

Organic solvents have a direct effect on enzymatic activity and stability in lipase-catalyzed transesterification. We investigated the influences of eight commonly used solvents on Lipozyme TL IM-catalyzed transesterification between pNPP and *n*-butanol. It has been reported that a minimum quantity of water surrounding the immobilized lipase is essential for maintaining enzymatic activity. Hydrophobic solvents are therefore preferable to hydrophilic solvents because the latter cause stripping of the essential water layer around the enzyme [1]. Among the tested solvents, *n*-hexane ($\log P = 3.76$) was found to be the optimum solvent for the Lipozyme TL IM-catalyzed transesterification reaction, giving the maximum conversion, 11.16% (Table 1). *n*-Hexane is a common hydrophobic reagent and is widely used as a solvent in biocatalysis, giving favorable enzymatic activities [23–25]. Soumanou et al.

Table 1
Solvent effect in Lipozyme TL IM-catalyzed transesterification reaction.

Solvent	Log P^a	Conversion (%)
Acetonitrile	−0.33	2.18±0.80
Tetrahydrofuran	0.47	0.24±0
<i>Tert</i> -Amyl alcohol	1.09	1.89±0
Methyl <i>tert</i> -butyl ether	1.29	6.62±0.47
Dichloromethane	1.41	0.38±0.13
Toluene	2.72	2.51±0.25
<i>n</i> -Hexane	3.76	11.16±0.74
Isooctane	4.37	8.44±0.11

The experiments were carried out in 1 mL different solvents containing 10 mmol/L pNPP, 1 mol/L *n*-butanol, and 0.01 g Lipozyme TL IM at 30 °C and 200 r/min for 10 min.

^a Data of Log P at 25 °C was cited from SciFinder Scholar.

[26] reported Lipozyme TL IM-catalyzed methanolysis of vegetable oil in *n*-hexane, achieving a high conversion, 97% in 24 h.

3.2.2. Acyl acceptor specificity of Lipozyme TL IM

Lipozyme TL IM-catalyzed transesterifications of pNPP with various alcohols, i.e., methanol, ethanol, *n*-propanol, isopropanol, and *n*-butanol were studied. As shown in Fig. 5, the highest conversion, approximately 9.36%, was achieved for transesterification between pNPP and *n*-butanol. The variations in the conversion as a result of using different alcohols can be attributed to the influences of various factors such as the molecular size of the alcohol, solubility in the reaction medium, and the affinity of the lipase for a particular alcohol [27]. Similar effects of the alcohol on conversion have been reported in the literature for lipase-catalyzed esterification and transesterification reactions [28]. *n*-Butanol was also used as the optimum acyl acceptor in the lipase-catalyzed transesterification of ethyl 3-phenylpropanoate [29]. Based on the above results, *n*-butanol was the optimum acyl receptor for lipase-catalyzed transesterification.

3.2.3. Alcohol tolerance of Lipozyme TL IM

The effect of *n*-butanol concentration on conversion was studied in the range 0.01–2 mol/L to determine the alcohol tolerance of Lipozyme TL IM. As shown in Fig. 6, the maximum conversion, 12.02%, was obtained using 1 mol/L *n*-butanol. The conversion increased rapidly as the *n*-butanol concentration increased from 0.01 to 0.2 mol/L. From 0.2 to 1 mol/L, a slight increase in the conversion was also obtained. When the concentration of *n*-butanol was further increased to 2 mol/L, the conversion decreased. This could be attributed to an inhibitory effect of the high *n*-butanol concentration on Lipozyme TL IM. The enzyme is hydrophobic and *n*-butanol contains a hydrophobic tail and a polar head. There may be hydrophobic-hydrophobic interactions between the enzyme and *n*-butanol, which would increase the *n*-butanol residence time. As a result of close contact with neighboring hydrophobic residues, the enzyme-*n*-butanol complex would be partially dehydrated, which may destabilize the native conformation of the enzyme. A large number of studies of the alcohol tolerance of Lipozyme

Fig. 5. Effects of alcohols on Lipozyme TL IM-catalyzed transesterification. Experiments were carried out at 30 °C using 1 mL of sample containing 10 mmol/L pNPP and 1 mol/L alcohol with a shaking speed of 200 r/min for 10 min.

Fig. 6. Effect of *n*-butanol on Lipozyme TL IM-catalyzed transesterification of pNPP. Experiments were carried out at 30 °C using a sample of volume of 1 mL containing 10 mmol/L pNPP and different concentrations of *n*-butanol in *n*-hexane, with a shaking speed of 200 r/min for 10 min.

435 have been reported, but there have been few reports on Lipozyme TL IM. Yadav et al. [30] reported Lipozyme 435-catalyzed transesterification of methyl acetoacetate with *n*-butanol; the optimum *n*-butanol concentration was 2.5 mol/L. Yadav et al. [31] and Talukder et al. [32] reported that the optimum alcohol concentrations in lipase-catalyzed transesterification were 0.75 and 0.1 mol/L, respectively. The effect of alcohol concentration on enzymatic activity varied depending on the reaction type.

3.2.4. Optimum reaction temperature for Lipozyme TL IM

In general, the reaction temperature influences the conversions and stabilities of biocatalysts. The optimum reaction temperature for Lipozyme TL IM-catalyzed transesterification between pNPP and *n*-butanol was evaluated by performing the reaction at different temperatures. As shown in Fig. 7, the highest enzymatic transesterification activity was obtained at 65 °C in *n*-hexane. The Lipozyme TL IM activity improved with increasing temperature from 30 to 65 °C in *n*-hexane (boiling point: 68.7 °C). With further increases in temperature in the range 65–80 °C in isooctane (boiling point: 99.2 °C), maximum

Fig. 7. Effect of temperature on Lipozyme TL IM-catalyzed transesterification of pNPP. Experiments were carried out using samples of volume 1 mL containing 10 mmol/L pNPP and 1 mol/L *n*-butanol in *n*-hexane (30–65 °C) or isooctane (65–80 °C, isooctane) with a shaking speed of 200 r/min for 10 min.

Fig. 8. Thermostability (based on residual activity) of Lipozyme TL IM at various incubation temperatures and times. The enzymatic activity was measured at 310 nm and 30 °C by following the formation of pNP in the presence of 10 mmol/L pNPP and 1 mol/L *n*-butanol in a total volume of 1 mL of *n*-hexane at 30 °C.

conversion was achieved at 75 °C, but the enzymatic activity was lower than at 65 °C in *n*-hexane. Hence, 65 °C was the optimum temperature for Lipozyme TL IM-catalyzed transesterification of pNPP in *n*-hexane. Zhang et al. [33] reported that the optimum reaction temperature for Lipozyme TL IM-catalyzed interesterification for the modification of margarine fats was 70 °C. Kohr et al. [34] reported the enzymatic production of biodiesel by Lipozyme TL IM-catalyzed transesterification between palm oil and methanol at 40 °C, with a conversion of 98%. The differences among the optimum reaction temperatures for Lipozyme TL IM-catalyzed transesterification have been reported to be associated with the lengths of the acyl donors [35].

3.2.5. Thermostability of Lipozyme TL IM

The thermostability is the length of time during which an enzyme remains active before undergoing conformational changes [36]. In other words, the useful lifetime of a biocatalyst is dictated by its thermostability. Thermostability is therefore a significant parameter in characterizing enzyme catalytic properties. However, a specific study of Lipozyme TL IM thermostability in transesterifications between pNPP and short-chain alcohols has not previously been reported. In this study, the thermostability of Lipozyme TL IM was investigated at various incubation temperatures. The residual activity at 65 °C (Fig. 8) demonstrated that Lipozyme TL IM is resistant to this temperature, but the residual activity at 75 °C decreased with increasing incubation time. This was probably the result of partial inactivation of the enzyme because elevated temperature is one of the most common causes of protein denaturation and deactivation [37].

4. Conclusions

A novel modified UV spectrophotometric method for the assay in organic solvents of lipases with transesterification activity was described. The major advantages of this assay were a simple detection method, few steps, accurate test re-

sults, and low instrument requirements. The method could be applied to microorganism screening and the identification of mutants with lipase synthetic activity. The kinetics of Lipozyme TL IM-catalyzed transesterification between pNPP and *n*-butanol in *n*-hexane was also investigated. The properties of Lipozyme TL IM-catalyzed transesterification were determined using this method. It was found that Lipozyme TL IM showed the highest catalytic activity in *n*-hexane at 65 °C using *n*-butanol as the acyl acceptor with favorable alcohol tolerance (1 mol/L) and excellent thermostability (65 °C in *n*-hexane).

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Graphical Abstract

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Investigation of Lipozyme TL IM-catalyzed transesterification using ultraviolet spectrophotometric assay

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A modified *p*-nitrophenyl palmitate assay for lipase transesterification activity determination in organic solvents was described. On the basis of the method, the transesterification properties of Lipozyme TL IM were determined.

紫外分光光度法表征Lipozyme TL IM脂肪酶转酯化活性

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摘要: 建立了一种新的有机相脂肪酶转酯化活性测定方法. 以正己烷为溶剂, 脂肪酶催化棕榈酸对硝基苯酯和正丁醇的转酯化反应为模型反应, 通过测定反应液中310 nm下吸光值的变化计算反应转化率. 以气相色谱法对新建的紫外分光光度法进行验证, 分别采用这两种方法测定了七种商品化脂肪酶的转酯化活性, 两种方法所得实验结果基本一致. 利用紫外分光光度检测法考察了Lipozyme TL IM脂肪酶催化转酯化的时间进程及合成活性与酶量的关系, 并对Lipozyme TL IM催化转酯化的性质(最适溶剂、酰基受体特异性、醇耐受性、最优反应温度和热力学稳定性)进行了表征.

关键词: 脂肪酶; Lipozyme TL IM; 转酯化活性; 紫外分光光度法; 棕榈酸对硝基苯酯; 有机溶剂

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