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A convenient test for lipase activity in aqueous-based solutions

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ABSTRACT

We proposed a convenient and accurate method for the measurement of lipase activity in a uniform aqueous-based substrate solution. In this work, lipase from *Candida rugosa* was used as the model lipase to test its catalytic ability toward *p*-nitrophenyl palmitate (*p*-NPP), which was suspended in a mixture of *p*-NPP ethanol solution and buffer. An ultraviolet–visible spectrophotometer was used to efficiently measure the liberated *p*-nitrophenol without extraction or centrifugation. Several factors that affected lipase activity were investigated, such as the ratio of *p*-NPP ethanol solution to buffer, the concentrations of *p*-NPP and lipase, as well as the temperature, reaction time, pH and agitation rate. Additionally, enzyme catalytic parameters such as K_m, V_m and "activation energy" were also assessed. We determined the optimal conditions for lipase in this homogeneous system and demonstrated lipase's catalytic performance in this condition followed Michealis–Menten kinetics.

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1. Introduction

Lipases, or triacylglycerol acyl ester hydrolases (EC 3.1.1.3), are enzymes possessing an intrinsic capacity to catalyze the cleavage of carboxyl ester bonds in tri-, di-, and monoacylglycerols (the major constituents of animal, plant, and microbial fats and oils) [1]. Generally, lipase can catalyze a wide range of reactions, such as hydrolysis, transesterification, alcoholysis, acidolysis and esterification [2–5]. More than 50 lipases have been identified and they have tremendous application potential in food industry, medicine, hygiene, chemistry, chemical engineering, environmental protection and energy development [6–10]. For lipases practical applications in industrial, their catalytic activity must be understood. Thus, establishing convenient and accurate methods for measuring lipase activity is of vital importance.

The catalysis systems of lipase may be divided into two categories: aqueous-based mediums and organic mediums [11–13]. Commonly, lipase is a water-soluble enzyme, with good solubility and stability in the aqueous phase. However, the substrates of lipase are often insoluble in water. Thus, substrates should first be dissolved in organic solvents and subsequently mixed with buffer. According to previous reports, the substrate mixtures prepared by this method are always two-phase systems, and additives are included in order to improve the uniformity and stability of these metastable mixtures [14,15]. However, many studies have reported that surfactants influence lipase activity, with various activation or inhibition effects [16–18]. In organic mediums, lipases are usually directly added to an organic solvent that also contains dissolved substrate [19]. This method is advantageous because the substrate is soluble and the mixture is stable. Unfortunately, protein molecules have both denature feature and conformational flexibility, which lead to their activity reduce in the presence of organic solvents [20,21]. Based on the discussion above, it is necessary to design a suitable method for the measurement of lipase activity.

Our study focused on the design of an aqueous-based substrate solution without additives for the determination of lipase catalytic properties. We test several solvents and ethanol was chosen as an appropriate solvent to dissolve *p*-NPP for the following reasons: first, it is miscible with water at any volume fraction. Second, *p*-NPP dissolves well in ethanol [14]. Finally, ethanol is a relative high polarity organic solvent and has less influence on lipase conformation compared with 2-propanol, which is a solvent of the substrate in previously reported methods [22]. In this work, a uniform solution was prepared by mixing a *p*-NPP ethanol solution with a certain volume of buffer at 65 °C. Then, this solution was equilibrated at a certain temperature and subsequently used for

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the measurement of enzymatic activity. Moreover, lipase from *Candida rugosa* was used to investigate the hydrolysis of *p*-NPP in this particular aqueous-based solution. After the enzyme-catalytic reaction, the amount of liberated product in the uniform solution was directly measured with an ultraviolet–visible (UV) spectrophotometer. Factors that affect enzyme activity, such as the volume proportion of water/ethanol, reaction time, *p*-NPP and lipase concentrations, temperature, pH and agitation rate were all investigated in this work. In addition, we also determined the catalytic parameters in this aqueous-based solution.

2. Materials and methods

2.1. Materials

Lipase powder from *C. rugosa* (1150 units/mg solid), Bradford reagent, bovine serum albumin (BSA, molecular mass: 67,000 Da) and *p*-NPP were obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA) and used as received. KH_2PO_4, Na_2HPO_4, NaCl and KCl were obtained from Chemsynlab Pharmaceutical Science & Technology (Beijing, China). All other chemicals were of analytical grade and used without further purification. The water used in all experiments was deionized and ultrafiltered in order to obtain a resistance of 18 MΩ cm with a TKA MicroPure water system. The phosphate buffer solution (PBS, 0.05 M) was prepared with NaCl (8.5 mg/mL), Na_2HPO_4 (2.2 mg/mL) and NaH_2PO_4 (0.4 mg/mL), and NaOH/HCl was used to adjust the pH.

2.2. Preparation of the homogeneous aqueous-based solution

Following standard protocols, *p*-NPP was dissolved in ethanol in a 50-mL Erlenmeyer flask and heated to 65 °C to form the *p*-NPP solution at the concentrations of 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0 mg/mL. Then, PBS (0.05 M) of 65 °C was cautiously and dropwise added. The resulting mixture was in a metastable state and the soluble *p*-NPP molecular may aggregate with the storage time and the change of temperature. The prepared mixture should be used timely in order to avoid the phase separation. Dynamic light scattering (DLS) was used to characterize the substrate states in this media stored at room temperature at several different equilibration times.

2.3. Analysis of lipase secondary structure via Circular Dichroism (CD) spectroscopy

Circular Dichroism (CD) spectroscopy was used for the examination of lipase structures in different solutions, which were recorded on a spectropolarimeter (MOS-450 AF/AF-CD, Bio-Logic, France) using a 0.2 cm quartz cuvette. For those three lipase different solution, lipase powder (3 mg/mL) was diluted in PBS (pH 7.0, 0.05 M), ethanol/PBS (pH 7.0, 0.05 M) (v:v, 1:1) and 2-propanol/PBS (pH 7.0, 0.05 M) (v:v, 1:1), respectively. Then, those solutions were centrifuged at 400 rpm for 5 min to remove insoluble impurities. The cell holder compartment was maintained in a nitrogen atmosphere at room temperature. At least five spectra were measured and the average was recorded.

2.4. Lipase activity measurement with a UV-spectrophotometer

An aqueous enzyme solution (PBS, 0.05 M, pH 7.0) of lipase (3 mg/mL) was centrifuged at 400 rpm for 5 min to remove any insoluble impurities. The protein concentration in the solution was determined using Coomassie Brilliant Blue reagent, following Bradford's method [23]. BSA was used as a standard to construct a calibration curve. The prepared homogeneous solution was pre-equilibrated at a determined temperature for a certain time. The catalytic reaction was initiated by adding an enzyme solution. Sodium carbonate solution (0.5 M) of the same volume was added to terminate the reaction (the pH value for this solution was 11.8) and enhance the detection sensitivity after the catalytic reacted for a certain time. Finally, the lipase catalytic activity was calculated from the absorbance of this mixture at an appropriate dilution in phosphate buffer against the blank without enzyme using a UV spectrophotometer (UV-1610, Shimadzu. Japan) at 410 nm. This was measured as one enzyme unit was the amount of protein liberating 1.0 μmol *p*-NP/min under these conditions. Each treatment was measured in at least three parallel experiments and the average was recorded.

3. Results and discussion

3.1. Solubility of p-NPP in ethanol

In this study the hydrolysis of *p*-NPP by *C. rugosa* lipase was used as a test reaction. *p*-NPP was dissolved in ethanol and the solubility of *p*-NPP was investigated. As shown in Fig. 1, the solubility of *p*-NPP in ethanol conformed to Eq. (1). "S" indicates the highest weight (g)

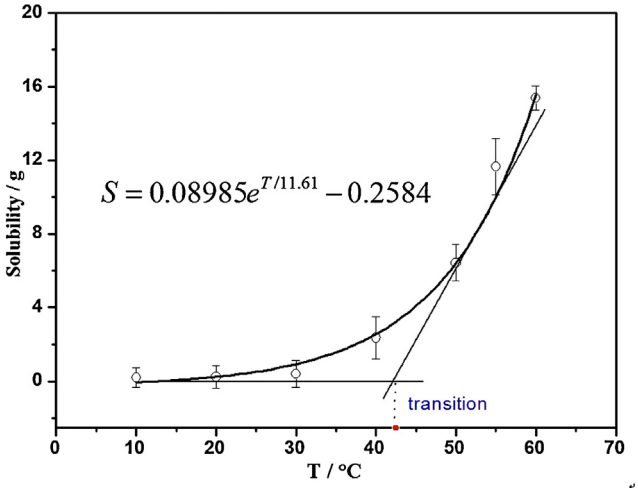

Fig. 1. Solubility of *p*-NPP in ethanol, measured by change temperature.

of *p*-NPP dissolved in 100 g ethanol, which increased as the temperature increased. The intersection of the tangent of the curve indicated that the solubility mutation temperature was nearly 41 °C. At room temperature *p*-NPP solubility in 100 g ethanol solvent was approximately 0.52 g (2.44 mg/mL) at room temperature.

$$S = 0.08985 \exp\left(\frac{T}{11.61}\right) - 0.2584 \quad (1)$$

We found that ethanol was a satisfactory organic solvent for lipase. However, organic molecules may have an effect on lipase secondary structures, CD spectroscopy was used to examine changes of lipase secondary structure in different organic solvent/buffer (v:v = 1:1) mixtures (Fig. S1). The molecules spectra in the far ultraviolet regions were dominated by the $n \rightarrow p^*$ and $p \rightarrow p^*$ transitions of amide groups, which was influenced by the geometries of the polypeptide backbones, their spectra were reflective of the different types of secondary structures present [24]. Obviously, the secondary structures of lipase in ethanol/PBS and 2-propanol/PBS mixture differed from the structure in PBS. Lipase underwent less structural change in ethanol/PBS than in the isopropanol/PBS mixture. DLS measurements revealed that this substrate remains clear and uniform for 1 h, which was enough to measure lipase activity. We concluded that ethanol is a suitable organic solvent for the measurement of lipase activity in aqueous-based solutions.

3.2. Effect of PBS volume on lipase reaction rate

The purpose of this research was to design a uniform aqueous solution without additives that would be optimal for the measurement of lipase activity. As described above, we dissolved a certain amount of substrate (*p*-NPP) in ethanol and then mixed it with PBS. The optimum ratio of *p*-NPP ethanol solution and PBS was dependent on many factors, including solvent and substrate properties and reaction temperature.

We also investigated the catalytic properties of lipase in different volume percentages of PBS (0.05 M, pH 7.0). A bell-shaped curve of enzyme activity was obtained as PBS volume increased (Fig. S2). Lipase catalytic activity firstly increased until the peak point at 50% with the maximal reaction rate of 1.337 ± 0.081 U mg^{-1}. Then, it reduced in high water content because of the substrate state transformed to small solid particles by aggregation, which might cause *p*-NPP diffusion limitation [25]. It was obvious that lipase showed low activity in neat ethanol, which was consistent with previous reports [21]. In most cases, lipase enzyme catalytic efficiency in

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