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AOT/isooctane reverse micelles with a microaqueous core act as protective shells for enhancing the thermal stability of *Chromobacterium viscosum* lipase

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ABSTRACT

According to the different environmental systems for lipase reactions, changes in thermal stability were investigated by employing the *Chromobacterium viscosum* lipase and a two-step series-type deactivation model. The half-life (6.81 h) of the lipase entrapped in reverse micelles at 70 °C was 9.87- and 14.80-fold longer than that in glycerol pool or in aqueous buffer. The deactivation constants for the first and second step (k_1 and k_2) at all temperatures drastically decreased when the lipase was entrapped in reverse micelles. In particular, k_1 (3.84 h^{-1}) at 70 °C in reverse micelles was 1.57-fold lower than that in aqueous buffer (6.03 h^{-1}). Based on the fluorescence spectrometry, the amount of excited forms of tryptophan and tyrosine increased markedly during the thermal-treatment in aqueous buffer, whereas no significant fluctuation was noted in the reversed micellar system. These results indicated that the encapsulation in reverse micelles could be favorable for preventing the enzyme from heat-induced denaturation.

1. Introduction

Lipase (triacylglycerol hydrolase, E.C. 3.1.1.3) is one of the enzymes that have been widely applied in industrial bioconversions, such as the production of emulsifiers and functional esters, through hydrolysis, glycerolysis and esterification (Houde, Kademi, & Leblanc, 2004; Park, Lee, Sung, Lee, & Chang, 2011). For decades, many studies related to the enzymatic characterization of lipase, immobilization of the lipase and optimization of the enzyme–substrate reaction conditions have been extensively reported to enhance the performance of lipase-catalyzed bioconversions (Lee, Park, Choi, Shim, & Chang, 2013; Moniruzzaman, Hayashi, Talukder, & Kawanishi, 2007; Ognjanovic, Bezbradica, & Knezevic-Jugovic, 2009). Particularly, because of the unique characteristics of lipase-catalyzed reactions between hydrophilic enzymes and hydrophobic substrates, studies focused on the construction of highly efficient reaction systems have been considered to be more important (Chen et al., 2012; Klibanov, 2001).

The structure of reverse micelles consists of an aqueous microdomain (polar phase, core) facing the polar heads of the surfactant that surrounds this core and interacts with the bulk organic solvent (non-polar phase), which is supported by hydrophobic interactions (Carvalho & Cabral, 2000). In a reversed micellar system, lipase is encapsulated in the inner aqueous phase, and enzymatic reactions occur at the interfacial area between the enzymes and the substrates that are solubilized in the external non-polar phase (Correa, Silber, Riter, & Levinger, 2012). Excluding the characteristics that the reactant displays as a homogeneous, monophasic media, the encapsulation of lipases in reverse micelles has been preferred as a novel approach because of its various advantages, including its enormous interfacial area, simple control of the reaction variables and easy monitoring of water content (Park, Kim, Choi, & Chang, 2012; Park, Kwon, Ahn, Lee, & Chang, 2010; Sereti, Zoumpanioti, Papadimitriou, Pispas, & Xenakis, 2014).

Recently, the activation energy of lipase catalyzed reaction in reverse micelles was reported to be lower than that in biphasic

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media, which resulted in a reasonable advantage in the aspect of bioconversion efficiency. Moreover, increased stability (i.e., retention of the catalytic activity for a relatively long term) has been reported in many cases, despite the fact that a complete understanding of the parameters that affect enzyme deactivation in reverse micelles has not yet been revealed (Zaman, Hayashi, Talukder, & Kawanishi, 2006). Several notable studies demonstrated that the catalytic activity of lipase was maintained in anhydrous organic solvents under thermal treatment at 100 °C, which was contradictory to the fact that temperatures over 60 °C could cause the denaturation of enzymes (Zaks & Klibanov, 1984). Based on the information presented above, we have conducted an investigation on the changes in thermal-deactivation kinetics according to different reaction systems for the glycerolysis catalyzed by the lipase from *Pseudomonas fluorescens* (Park et al., 2013). As a result, the encapsulation of lipase in reverse micelles decreased the rate constant for the second deactivation-step (k_2) and increased the activation energy for denaturation (E_{de}), leading to the enhancement of resistance to heat-induced denaturation.

The primary purpose of the present study was to confirm the quantitative protection effect experienced after the encapsulation of the lipase in reverse micelles (i.e., the enzyme insulation by the encapsulation in reverse micelles defends its catalytic activity from thermal-deactivation) with a different reaction (hydrolysis catalyzed by *Chromobacterium viscosum* lipase). Additionally, the study aimed to elucidate the shielding effect against the structural denaturation caused by thermal-treatment. A further aim was to reveal that the water content in reverse micelles (referred to as the *R* value, [water]/[surfactant]) influences the thermostability through enzyme-deactivation kinetics by employing a two-step series-type equation model.

2. Materials and methods

2.1. Chemicals and reagents

Purified *C. viscosum* lipase, with a reported catalytic activity of 3400 units/mg solid, was purchased from Millipore (Billerica, MA, USA). Bis-(2-ethylhexyl) sulfosuccinate sodium salt (AOT) was purchased from Sigma–Aldrich (St. Louis, MO, USA) and was purified according to the method of Tamamushi and Watanabe (1980). Isooctane, spectrophotometric grade, was dehydrated over a 4 Å molecular sieve (Sigma–Aldrich) and filtered through a 0.45-μm membrane filter prior to use as the reaction medium in the reverse micelles. Triolein and glycerol (Sigma–Aldrich) were used as substrates in lipase-catalyzed hydrolysis and glycerolysis.

Cupric acetate reagent, used as a coloring reagent, was prepared according to the method of Lowry and Tinsley (1976). The cupric acetate solution was prepared by dissolving 5 g of cupric acetate in 100 ml of distilled water. The prepared cupric acetate solution was then filtered through Whatman No. 1 filter paper, and the pH was adjusted to 6.1 using pyridine. Oleic acid, 1-monoolein, 2-monoolein, 1,2-diolein, 1,3-diolein, and triolein with 99.9% purity were obtained from Sigma–Aldrich and used as the standard lipids in HPLC work.

2.2. Verification of enzyme purity by sodium dodecyl sulfate–polyacrylamide gel electrophoresis

In this study, the *C. viscosum* lipase, which has been widely applied to industrial bioconversions, was selected as a model enzyme. Although *C. viscosum* lipase was highly purified according to the manufacturer's information, the purity of the enzyme was confirmed by electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was conducted using the Laemmli method with a 12% polyacrylamide gel (Laemmli, Beguin, & Gujer-Kellenberger, 1970). Lipase was eluted (1:4, v/v) in sample buffer containing SDS and 2-mercaptoethanol. The eluted sample was boiled for 3 min before loading onto the gels. Electrophoresis was performed at a constant current of 20 mA per gel for 60 min at 25 °C in a Hoefer SE 250 mini-gel system (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). For protein staining, Coomassie Brilliant Blue R-250 (Sigma–Aldrich) was used.

2.3. Preparation of reverse micelles

Reverse micelles containing lipase were formed by adding appropriate amounts of glycerol, with predetermined amounts of water and lipase powder, to a solution of 50 mM AOT/isooctane. The solution was then mixed vigorously for 1 min to obtain a transparent micellar solution. The desired water content was defined as the *R* value, which indicates the molar ratio of water to surfactant ($[H_2O]$/[surfactant]) (Banerjee, Ghosh, & Datta, 2011), and the glycerol content was defined as the *G* value, which indicates the molar ratio of glycerol to surfactant ([glycerol]/[surfactant]) (Park et al., 2010). The *R* and *G* values were calibrated by considering the amount of water inherently present in the isooctane, glycerol, and AOT. In this study, experiments were conducted under optimized conditions using a *G* value of 4.0 and a *R* value of 10.0 (Park et al., 2013).

2.4. Analysis of glycerolysis activity

Glycerolysis activity was analyzed at 40 °C using triolein and glycerol as substrates. A screw-cap tube, the reactor, was filled with 10 ml of 50 mM AOT/isooctane solution containing triolein (6.67 mM). The desired amount of glycerol and water containing lipase was injected into the tube, and the reaction was initiated by vortex mixing until the mixture was clear. After incubation at 40 °C for a predetermined time, 0.2 ml of the sample was withdrawn from the reaction mixture with a small subcutaneous syringe. In total, 3.0 ml of chloroform was added to the sample, and the test tube was shaken vigorously for 2 min and then put aside for at least 1 h to inactivate the enzyme. Then, 0.5 ml of water was added, and the test tube was again shaken for 1.5 min. The mixture was centrifuged for 5 min at 2000×*g*. The lower chloroform layer was withdrawn and stored in a round-bottom flask. The upper water layer was re-extracted twice with 3.0 ml of chloroform. A blank was prepared by the identical procedure, as described above, except that a glycerol pool without enzyme was added (Chang, Rhee, & Kim, 1991). The content of triolein, 1,2-diolein, 1,3-diolein, 1-monoolein, 2-monoolein, and oleic acid in the condensed chloroform layer was determined by high performance liquid chromatography (Chang & Rhee, 1990). One unit of enzyme was defined as the amount of lipase that catalyzed the reaction of 1 μmol of triolein/min under the assay conditions. All data are averages of triplicate samples and are reproducible within ±10%.

2.5. Analysis of hydrolysis activity

The hydrolysis activity was analyzed according to the copper-soap colorimetric method. A screw-cap tube was filled with AOT/isooctane reversed-micellar solution that was pre-incubated in a water bath at 37 °C with magnetic stirring at 800 rpm. Lipase-catalyzed hydrolysis was initiated by adding triolein (10%, w/v) as a substrate for the reversed micellar solution. Each aliquot (400 μl) of reactant was collected at predetermined intervals and diluted with 4.6 ml of isooctane. Subsequently, 1 ml of cupric acetate reagent and 1 ml of acetonitrile (used to eliminate the turbidity caused by AOT) was added and mixed vigorously using a vortex