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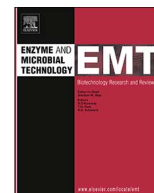

Hydration-aggregation pretreatment for drastically improving esterification activity of commercial lipases in non-aqueous media

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ABSTRACT

We investigated a novel, simple method for activating lipases in non-aqueous reaction media. Lipase powders were suspended in *n*-fatty alcohols and were then hydrated by adding a small amount of water. A paste-like aggregate was recovered from the mixture followed by lyophilization for obtaining activated lipases as dry powders. Lipase activity was evaluated for esterification between myristic acid and methanol in *n*-hexane. The activated lipases exhibited high esterification activity depending on the experiment conditions during hydration-aggregation pretreatment such as the amount of added water, the temperature, the pH of added buffer solutions, and the carbon chain length of the *n*-fatty alcohols used as pretreatment solvents. Various commercial lipases from different origins could be activated by this method. Changes in lipase conformation induced by the hydration-aggregation pretreatment were studied based on fluorescence and Fourier-transform infrared spectroscopy.

1. Introduction

Lipase (E.C.3.1.1.3, triacylglycerol hydrolase) can catalyze not only hydrolysis of esters such as fats and oils but also synthesis or modification of ester bonds between various alcohols and carboxylic acids. Lipase-catalyzed reactions have considerable advantages compared to chemical synthesis with their reaction ability with regioselectivity, chiral selectivity, environmentally friendly reaction process, and product safety. Therefore, lipase-assisted organic synthesis has been a promising alternative to conventional chemical synthesis for application in the fields of, for example, food, pharmaceuticals, fine chemicals, and renewable energy [1–6]. Generally, however, the catalytic reaction rate is low in unhydrous organic solvents that are utilized to shift the reaction equilibrium toward ester synthesis rather than hydrolysis.

Low catalytic activity in organic media is mainly due to the structural characteristics of lipase molecules: the catalytic centers of many lipases are covered with one or more polypeptide chains called lids and isolated from substrates dissolved in the reaction medium [7–9]. Lipase exhibits high catalytic activity when the lid moves to form an open-lid structure and the catalytic center is exposed to the reaction media. Such movement of the lid occurs when lipases are located on an oil-water interface, therefore this activation phenomenon on lipases is called interfacial activation [7,9,10–12].

Lipase activation based on the interfacial properties of lipases has been extensively investigated, including immobilization on hydrophobic supports [3,4,6,13–20], conjugation with synthetic polymers [21,22], modification with surfactants or lipids [23–28], entrapment in reversed micelles [29–31], and lyophilization from a hydrocarbon-water mixture [32,33]. These approaches succeeded in obtaining highly active lipases, although the activation procedures often involved using reactive reagents or co-existence with the chemicals used for lipase activation. In applications for foods and pharmaceuticals, the lipase preparations should not contain chemical additives used for lipase activation.

Recently we reported a novel immobilized lipase bioreactor developed using hydrated lipase aggregates [34]. This bioreactor achieved a high esterification rate in low-water organic media and repetitive reaction with good lipase stability for 2000 h. The key point of the technique was using hydrated lipase aggregates: the lipase showed high activity in the hydrated state, in which a paste-like aggregate was formed, while a dry powder of the same lipase exhibited quite low esterification activity. This motivated us to investigate the development of a novel pretreatment strategy for lipase activation by means of controlled hydration-aggregation of lipases.

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In this paper, we propose a novel activation method for lipase-catalyzed ester synthesis via hydration-aggregation pretreatment. Briefly, commercial lipase powders of diverse origin were first suspended in *n*-fatty alcohols and a small amount of water was added to the lipase suspension. Paste-like lipase aggregates were formed in *n*-fatty alcohols and were then lyophilized to obtain a dry solid. The advantages of this activation procedure are as follows. (1) Activation is carried out via fully physical processes, that is, the method involves no chemical reaction using reactive reagents. (2) Activated lipase does not contain any chemical contaminants derived from the activation processes. The final lipase preparation substantially consists of components of the original lipase powder (in this case, a small amount of salts from the added buffer solutions is included). (3) Activated lipases are insoluble in typical organic solvents, thus the recovery and repetitive use of lipase is possible. Here we evaluated the improved esterification activity using a model reaction, i.e., ester synthesis between methanol and myristic acid as substrates. The factors affecting esterification activity were investigated by reaction experiments. In addition, structural changes in lipase molecules induced by the pretreatment are discussed, based on spectroscopic measurement of the lipase preparations.

2. Materials and methods

2.1. Materials

Rhizopus japonicus lipase (Lipase “Saiken” 100: RJL) was purchased from Nagase ChemteX Corporation (formerly Nagase Biochemicals Ltd.), Osaka, Japan. *Chromobacterium viscosum* lipase (Lipase “Asahi”: CVL) was obtained from Asahi Chemical Industry Ltd., Tokyo, Japan. *Candida rugosa* lipase (CRL), porcine pancreas lipase (PPL), and *Mucor miehei* lipase (MML) were purchased from Sigma-Aldrich, St. Louis, MO, USA. *Candida antarctica* lipase fraction B (CALB) was purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. *Aspergillus niger* lipase (Lipase A “Amano” 6: ANL) and *Pseudomonas cepacia* lipase (Lipase P “Amano” 30: PCL) were obtained from Amano Enzyme Inc., Nagoya, Japan. These lipases were used for all experiments as obtained without further purification.

1-Butanol, 1-hexanol, 1-octanol, 1-decanol, *n*-hexane, myristic acid, 4-nitrophenyl acetate, and 2-methyl-2-butanol were obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Methyl myristate was a product of Tokyo Chemical Industry, Co., Ltd., Tokyo, Japan. Methanol was purchased from Kanto Chemical, Co., Ltd., Tokyo, Japan. All chemicals used were of extra-pure or analytical grade.

The water used in all experiments was prepared using a Direct-Q water purifier system (Merck Millipore Corporation, Billerica, MA, USA) and had 18.2 MΩ/cm resistivity.

2.2. Activation of lipases via hydration-aggregation pretreatment

Activated lipases were prepared via a modified hydration-aggregation procedure based on our previous report [34]. Lipase powder (20 mg) was suspended in 10 mL of an *n*-fatty alcohol (carbon number of 4–10) with magnetic stirring. A buffer solution (0.1 M acetate buffer solutions for pH 4.0 and 5.0; McIlvain buffer solutions for pH 6.0, 7.0, and 8.0; 0.1 M borate buffer solutions for pH 9.0 and 10.0) was added dropwise to the lipase suspension, and the mixture was further stirred for 2.5 h. After precipitation of lipase powders or aggregates, most of the supernatant was removed by sucking with a glass pipet. The paste-like residue containing hydrated lipase aggregates was pre-frozen at −80 °C for 30 min and then lyophilized to remove both water and *n*-fatty alcohols. Finally, the activated lipase was obtained as a dry residue.

In order to determine the water content of the hydrated-aggregated lipase pastes, the water concentration in the supernatant of the pretreatment mixture after precipitation of the lipase aggregates was measured using a volumetric Karl-Fischer titrator (870 KF Titrino plus, Metrohm Japan Ltd., Tokyo, Japan). The water content of the lipase aggregate was calculated based on the mass balance of water between solid phase (lipase aggregate) and liquid phase (*n*-fatty alcohol). At least triplicate measurements were carried out.

2.3. Evaluation of esterification activity of lipases in a non-aqueous system

Esterification reactions of myristic acid and methanol were performed in *n*-hexane. Typically, 4 mg of lipase powder was mixed with 2 mL of *n*-hexane containing both substrates (35 mM each) in a screw-capped test tube shaken with a thermoshaker (TS-100C, Biosan Ltd., Riga, Latvia) under controlled temperature. The concentration of synthesized methyl myristate was determined using a gas chromatograph (GC-2014, Shimadzu Corporation, Kyoto, Japan) equipped with a flame-ionization detector. N_2 gas was used as a carrier gas. A capillary column (URBON HR-TGC1, 0.25 mm i.d., 25 m column length, 0.1 µm film thickness, Shinwa Chemical Industries Ltd., Kyoto, Japan) was used. Injector and detector temperatures were maintained at 250 °C. The initial column temperature was set to 80 °C, the oven temperature was then raised at a rate of 10 °C/min to 230 °C, and finally maintained at this temperature for 5 min. Tetradecane was used as an internal standard for quantification of the concentration of methyl myristate.

2.4. Evaluation of hydrolytic activity of lipase in an aqueous system

Lipase-catalyzed hydrolysis reactions were carried out using 4-nitrophenylacetate as an ester substrate based on a reported method [16] with slight modification. The reaction mixture consisted of 2.95 mL of aqueous buffer solution containing lipases and 0.05 mL of 2-methyl-2-butanol containing 10 mM of 4-nitrophenylacetate. Release of 4-nitrophenol was monitored by absorbance measurement at 410 nm with a UV–vis spectrophotometer (UV-1800, Shimadzu Corporation, Kyoto, Japan).

2.5. Measurement of fluorescence spectra

The fluorescence spectra of lipases dissolved in water were measured with a spectrofluorometer (RF-5300PC, Shimadzu Corporation, Kyoto, Japan) at room temperature. The slit width of the excitation and the emission were set to 5 nm. Spectra were obtained in 1-cm quartz cells at an excitation wavelength of 280 nm.

2.6. Measurement of Fourier-transform infrared spectra

Fourier-transform infrared (FT-IR) spectra were obtained using a FT-IR spectrophotometer (Spectrum One, PerkinElmer, Waltham, MA, USA). Lipase powders (or lipase powder mixed with 1-octanol) were directly analyzed using an attenuated total reflection attachment with a diamond prism.

2.7. Statistical analysis

The data of enzymatic activity were obtained as the mean values of at least three measurements from at least two experiment replications unless otherwise noted. Typically the standard deviation values for the mean values are represented as error bars in graphs. A Student’s *t*-test was used to compare the experiment data statistically. A *P*-value less than 0.05 was considered to be statistically significant. Statistical calculations were performed with KaleidaGraph software (Synergy Software, Reading, PA).

3. Results and discussion

3.1. Activation of lipases by hydration-aggregation pretreatment

The effects of hydration-aggregation pretreatment on the synthetic

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